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Cancer and inflammatory diseases research: from the basics to the precision medicine

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Cancer and inflammatory diseases research: from the basics to the precision medicine

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Editorial: Cancer and inflammatory diseases research: from the basics to the precision medicine

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Editorial on the Research Topic
Cancer and inflammatory diseases research: from the basics to the precision medicine

1 Introduction

The field of pharmacology is rapidly evolving, with significant advancements in our understanding of cancer and inflammatory diseases. This editorial highlights nine groundbreaking studies exemplifying the transition from basic research to precision medicine, offering novel insights and potential therapeutic strategies for these challenging conditions.

1.1 Exosomes and colorectal cancer

Exosomes, extracellular vesicles rich in bioactive substances such as DNA, RNA, lipids, and proteins, play a crucial role in cell-to-cell communication and the pathogenesis of colorectal cancer (CRC). A comprehensive review focuses on the relatively unexplored area of exosomal genomic DNA (gDNA) and its significance in CRC. Exosome gDNA, which includes clinically relevant tumor-specific mutation genes, is pivotal in liquid biopsy applications for early diagnosis and treatment. Additionally, exosome gDNA influences immune and metabolic functions in CRC, positioning it as a critical target for future research and clinical interventions (Li et al.).

1.2 PARP inhibitors in cutaneous squamous cell carcinoma

Poly ADP-ribose polymerase inhibitors (PARPis) have been effective in treating BRCA1/2 mutation-related cancers. A case study of a 40-year-old man with metastatic cutaneous squamous cell carcinoma (cSCC) with a BRCA2 mutation demonstrated the efficacy of PARPi fluzoparib. This treatment resulted in tumor stability and progression-free survival of 5 months, suggesting that PARPis can be a viable option for cSCC patients with BRCA mutations, thereby expanding the therapeutic options for this patient population (Sun et al.).

1.3 Mortality analysis in breast cancer patients

An extensive study on breast cancer patients highlights the importance of understanding mortality from various causes to improve healthcare planning and clinical predictions. The research, involving 12,742 women, revealed that breast cancer is the leading cause of death, followed by cardiovascular disease. Notably, the contribution of breast cancer to overall mortality varied significantly by age and disease stage, emphasizing the need for age-specific and stage-specific survivorship care models that incorporate multidisciplinary approaches (Contiero et al.).

1.4 Foam-based intraperitoneal chemotherapy

Innovative approaches in drug delivery, such as foam-based intraperitoneal chemotherapy (FBIC), are being explored to improve treatment efficacy and safety. A study on swine models demonstrated the feasibility and safety of using doxorubicin in FBIC. The results showed promising intraoperative and postoperative outcomes without significant complications, suggesting that FBIC could be a viable alternative to traditional liquid solutions for intraperitoneal chemotherapy, pending further long-term studies (Khosrawipour et al.).

1.5 Monitoring cellular immunotherapy with synthetic notch receptors

Cellular immunotherapy, particularly CAR T cell therapy, has revolutionized cancer treatment. However, monitoring these therapies remains challenging. Researchers have developed a synthetic Notch (synNotch) receptor system that links immune-cancer cell interactions to a simple blood test. This system allows for the detection of intratumoral activity via a secreted reporter, providing a convenient and effective method to monitor the efficacy of immunotherapies and potentially improving patient-specific treatment strategies (Fu et al.).

1.6 Janus kinase inhibitors: from autoimmune diseases to cancer therapy

Janus kinase (JAK) inhibitors, initially developed for autoimmune diseases, have shown promise in cancer therapy due to their role in cytokine signaling pathways involved in tumorigenesis. A systematic review of JAK inhibitors highlighted their anti-tumor potential and clinical applicability. The review underscores the importance of further studies to optimize their use in oncology, potentially offering new therapeutic avenues for cancer patients (Wei and Liu).

1.7 Therapeutic drug monitoring of adalimumab

Adalimumab, a widely used biologic for inflammatory diseases, benefits from therapeutic drug monitoring (TDM) to optimize treatment outcomes. A systematic review assessed the impact of TDM on adalimumab therapy, finding that proactive TDM can guide dose adjustments and improve clinical responses. However, the evidence was not statistically significant, highlighting the need for more robust studies to validate the role of TDM in managing adalimumab therapy effectively (Li et al.).

1.8 Ferroptosis and the ubiquitin-proteasome system in cancer

Ferroptosis, an iron-dependent form of cell death, has emerged as a potential target for cancer therapy. Recent research explores the interplay between ferroptosis and the ubiquitin-proteasome system (UPS), which regulates protein stability. The study highlights key regulators of ferroptosis, such as GPX4 and NRF2, and their modulation by the UPS. Understanding this relationship could lead to novel therapeutic strategies that harness ferroptosis to combat cancer (Din et al.).

1.9 Clobenpropit and the CXCL12/CXCR4 axis

Clobenpropit, a histamine H3 receptor antagonist, has shown potential in treating autoimmune diseases and cancer by inhibiting the CXCR4 receptor. The CXCL12/CXCR4 axis is crucial in various biological processes, including cell proliferation, migration, and inflammation. A review of Clobenpropit's effects on this pathway suggests that it could be an effective therapeutic agent for managing diseases like juvenile idiopathic arthritis and certain cancers, providing a new avenue for targeted therapy (Abbasifard et al.).

2 Conclusion

The articles featured in this Research Topic highlight the diverse and innovative approaches being explored in cancer and inflammatory disease research. From the molecular mechanisms of exosomes and ferroptosis to the clinical applications of PARPis and JAK inhibitors, these studies underscore the importance of integrating basic research with clinical practice. As precision medicine continues

to evolve, these advancements will play a crucial role in developing more effective and personalized treatments, ultimately improving patient outcomes and transforming healthcare.

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Roles of Exosome Genomic DNA in Colorectal Cancer

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Exosomes are extracellular vesicles that mediate cell-to-cell communication. Bioactive substances such as DNA, RNA, lipids, and proteins are present in it, and they play an essential role in the pathogenesis of colorectal cancer (CRC). The role of RNA and protein in exosomes has been extensively studied. Exosome DNA has recently attracted the attention of a great deal of scientists. According to studies, exosome DNA mainly contains genomic DNA (gDNA) and mitochondrial DNA (mtDNA), of which exosome gDNA is widely used in liquid biopsy of CRC. It includes a variety of clinically relevant tumor-specific mutation genes. In addition to liquid biopsy, researchers find that exosome gDNA regulates immune and metabolic functions in CRC, making it an important research object. However, the primary research on exosome gDNA is still limited. Here, we describe the occurrence and composition of exosomes. Summarize the essential characteristics and mode of action of exosome gDNA. Remarkably, this paper constitutes a comprehensive summary on the role of exosome gDNA on CRC with the intent of providing a theoretical basis and reference for early diagnosis and clinical treatment of cancer.

Keywords: colorectal cancer, exosome, genomic DNA, liquid biopsy, tumor immunity

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INTRODUCTION

CRC is the third most common malignant tumor in the world. Every year one million people worldwide will develop CRC (Franke et al., 2019; Keum and Giovannucci, 2019). It is a heterogeneous intestinal epithelial disease characterized by the accumulation of mutations and disordered immune responses (Janney et al., 2020; Lichtenstern et al., 2020; Stoffel and Murphy, 2020). Currently, the choice of treatment scheme for CRC is mostly determined by tissue biopsy. Despite tissue biopsy being the gold standard for diagnosis, classification, and treatment decision-making, it is not always available. Tumor tissue also exhibits solid spatial heterogeneity due to the uneven distribution of tumor subclones. Tumor molecular compositions may change dynamically in response to micro-environmental stimuli and therapeutic pressure, making tissue biopsy an unreliable method of diagnosis. Therefore, liquid biopsy is becoming increasingly popular (Dekker and Rex, 2018). A liquid biopsy is a non-invasive procedure in which samples of blood or other body fluids are collected to analyze exosomes, circulating tumor cells, and ctDNA (Marcuello et al., 2019). In the early stages of CRC, there are no obvious symptoms. Most patients with CRC are found in the advanced stage. Liquid biopsy provides a more accurate picture of tumor details in real time, which is particularly important for early detection of cancer and reduction of mortality from it (Marcuello et al., 2019; Kolencik et al., 2020; Martini et al., 2020; Rodriguez-Casanova et al., 2021). Nevertheless, the key

to prolonging the survival time of CRC patients is not only to make early diagnosis of the disease, but also to understand the tumor progression (Ogunwobi et al., 2020; Sveen et al., 2020). The identification of a sensitive biomarker is important for observing the immune response and metabolism during the treatment of CRC and assessing the drug resistance and heterogeneity further (Marcuello et al., 2019; Kolencik et al., 2020; Martini et al., 2020; Rodriguez-Casanova et al., 2021).

Exosomes are extracellular nano-sized vesicles containing DNA, RNA, lipids, and protein (Pegtel and Gould, 2019; Jafari et al., 2020; Kalluri and Lebleu, 2020; Ahmadi and Rezaie, 2021; Liu et al., 2021; Yu et al., 2021; Rezaie et al., 2022; Vahabi et al., 2022). CRC exosomes have the ability to transport their contents to recipient cells in the tumor microenvironment, thereby playing an important role in cell-to-cell communication. It plays a vital role in tumor immunity, tumor survival, tumor chemotherapy resistance, and metastasis (Xiao et al., 2020; Liu et al., 2021; Chang et al., 2021). In CRC exosomes, there are a variety of active substances. DNA is one of the most stable substances (Jeppesen et al., 2019). However, there is little research on exosome DNA compared to proteins and RNA. Essentially, exosome DNA is derived from normal DNA metabolism or damage induction, primarily from gDNA from the nucleus and mtDNA from the mitochondria (Kahlert et al., 2014; Thakur et al., 2014; Kalluri and Lebleu, 2016). The gDNA of exosomes contains DNA fragments from multiple chromosomes, including mutant DNA fragments, and is mainly found in body fluids or the genome of some immune cells (Thakur et al., 2014; Waldenstrom et al., 2012) (**Table 1**). gDNA from exosomes is widely used in liquid biopsy, it also affects tumor immunity and metabolism (Lian et al., 2017; Vanpouille-Box et al., 2017; Zhang et al., 2018; Sharma and Johnson, 2020; Zhao et al., 2021). In this review, we discuss the relevance of exosome gDNA in the early detection, development and prognosis, immune response, and drug resistance of CRC.

OVERVIEW OF EXOSOMES

Exosomes are extracellular nano-sized bilayer membrane vesicles with an average diameter of 30–150 nm (Pegtel and Gould, 2019; Kalluri and Lebleu, 2020; Yu et al., 2021). It is produced in the cytoplasm by the classical endosomal sorting complex. It is formed by the inward budding of cell membranes containing ubiquitinated surface receptors, leading to the formation of early endosomes. With the help of the Golgi apparatus, these early endocorpuscles become late endocorpuscles and intracavitary vesicles. Intracavitary vesicles accumulate in endosomes, leading to the formation of multivesicular bodies (MVB). MVB is ultimately transported into lysosomes for degradation or fuses with the cytoplasmic membrane, releasing its contents (including exosomes) into the extracellular space (He et al., 2018; Doyle and Wang, 2019; Zhang and Yu, 2019; Rezaie et al., 2021) (**Figure 2**). Exosomes are identified by classical molecular markers, such as tetrapeptide (CD63, CD9, CD81), FLOTILIN-1, and heat shock 70 protein (Liang et al., 2021). Numerous literature has been extensively studied to reveal the detailed mechanism of exosome biogenesis.

Exosomes can be found in almost all cells and body fluids, including blood, sweat, tears, urine, saliva, breast milk, ascites, and cerebrospinal fluid (Liang et al., 2021; Lin et al., 2021). Exosomes carry various macromolecules from different tissues and organs, loaded with nucleic acids (DNA and RNA), structural components of cells (protein and lipids), and cell metabolites. It is a part of the intercellular communication system, which carries and transmits signal molecules that regulate the physiological state of cells, participates in antigen presentation, cell differentiation, growth, and tumor immunity, and is closely related to the occurrence and development of various diseases (Pegtel and Gould, 2019; Liu et al., 2021). After different active substances in exosomes are thrown out of cells, functional protein, RNA, and DNA fragments can be transferred to recipient cells, resulting in cascade changes in the genome and non-genome (Liu et al., 2021; Kalluri and Lebleu, 2020) (**Table 2**).

TABLE 1 | Exosome gDNA as biomarkers for diagnosis of diseases.

Disease	Mutantion	Detection mode	Biofluid	Reference
Pancreatic Cancer	KRAS TP53	ddPCR	Plasma	(Kahlert et al., 2014; Yang et al., 2017)
Glioma	EGFR	Conventional PCR	Cerebrospinal fluid	Vaidya and Sugaya, (2020)
non-small cell lung cancer	EGFR	PNA-PCR	Alveolar lavage fluid and Plasma	Hur et al. (2018)
Lung Adenocarcinoma	EGFR	PNA-PCR	Pleural effusion	Lee et al. (2018)
Pancreatic cancer	KRAS	ddPCR	Plasma	Allenson et al. (2017)
Pulmonary adenocarcinoma	EGFR	ddPCR	Bronchial Washing	Park et al. (2020)
Glioma	RET HIF2A VHL SDHB	Conventional PCR	Plasma	Wang et al. (2018)
non-small cell lung cancer	EGFR	ddPCR	Plasma	Castellanos-Rizaldos et al. (2018)
non-small cell lung cancer	EGFR	ddPCR	plasma and pleural fluid	Kim et al. (2021)
Lung Adenocarcinoma	EGFR	Quantitative PCR	Pleural Effusions	Qu et al. (2019)
Neuroblastoma	BRAF	Quantitative PCR	Plasma	Degli et al. (2021)
Bladder cancer	KRAS	ddPCR	Urine	Zhou et al. (2021)
prostate cancer	PTEN TP53	Quantitative PCR	Plasma	Lazaro-Ibanez et al. (2014)
Colorectal cancer	KRAS	ddPCR	Plasma	Lucchetti et al. (2021)
non-small cell lung cancer	EGFR	PNA-PCR	bronchoalveolar lavage fluid	Hur et al. (2019)
non-small cell lung cancer	EGFR	ddPCR	Plasma	Krug et al. (2018)
Pancreatic cancer	KRAS	ddPCR	Plasma	Bernard et al. (2019)
Glioma	IDH1	ddPCR	Plasma	Garcia-Romero et al. (2017)

TABLE 2 | Exosome gDNA transfer between cells.

Donor cell	Receptor cell	Exosome gDNA	Integrated into the genome	Reference
Cardiomyocyte	Fibroblast	Non specicial	Not sure	Waldenstrom et al. (2012)
Colon cancer cell	Fibroblast and epithelial cells	HRAS	No	Lee et al. (2016)
Glioma cells	Fibroblast	c-Myc	Yes	Balaj et al. (2011)
K562	HEK293	AT1	Yes	Cai et al. (2013)
SW480	*In vivo*	KRAS RAB	Not sure	Trejo-Becerril et al. (2012)
hBMSC	hMSC	A.t.-plasmid	Yes	Fischer et al. (2016)
H-ras-driven Intestinal epithelial cells	RAT-1	HRAS	No	Lee et al. (2014)
K562	Neutrophils	BCR/ABL	Yes	Cai et al. (2014)

Various bioactive molecular substances in exosomes are cell signaling effectors and valuable tools for tumor diagnosis. Exosomes can monitor the occurrence and development of tumors and provide new targets and strategies for tumor treatment and diagnosis (Liang et al., 2021; Lin et al., 2021).

ORIGIN AND CHARACTERISTICS OF EXOSOME GENOMIC DNA

Exosomes contain many bioactive substances. However, compared with proteins and RNA in exosomes, there is little research on exosome DNA. Essentially, mammalian cells can excrete harmful cytoplasmic DNA through exosomes, thus avoiding cell aging and death (Takahashi et al., 2017). However, tumor cells often accumulate damaged DNA fragments of the cytoplasm because of their aging or abnormal leakage of DNA during division, autophagy, oxidative stress (Riches et al., 2014; Yokoi et al., 2019). For these reasons, tumor cells can secrete more exosomes to avoid the accumulation of nuclear and mitochondrial DNA fragments in the cytoplasm induced by metabolic stress, thus releasing more soluble DNA fragments (Parolini et al., 2009; Whiteside, 2016; Yokoi et al., 2019). There are single-stranded and double-stranded DNA in tumor exosomes (Balaj et al., 2011; Cai et al., 2013; Hur and Lee, 2021), and gDNA, mtDNA, and plasmid DNA all have been found in tumor exosomes (Erdmann et al., 2017; Sansone et al., 2017; Lazaro-Ibanez et al., 2019; Elzanowska et al., 2021).

After analyzing the exosomes in the supernatants of various tumor cell lines and non-tumor-related fibroblasts, scientists found that the exosome DNA in tumor cells are more abundant (Kahlert et al., 2014; Thakur et al., 2014; Kalluri and Lebleu, 2016). Genome-wide sequencing showed that tumor exosomes contained large double-stranded gDNA fragments ranging from 100bp to 20kbp, which could cover the whole chromosome range and reflect the mutation state of tumor parent cells (Kahlert et al., 2014; Thakur et al., 2014; Kalluri and Lebleu, 2016). This gDNA is packed in exosomes in the form of nucleosomes or supercoils. As exosomes contain larger gDNA fragments and originate from living cells, which is more conducive to mutation detection by PCR, they may have advantages over circulating cfDNA (Kahlert et al., 2014; Thakur et al., 2014; Kalluri and Lebleu, 2016). Some of its tumor-related mutations can reflect the progress and prognosis of many kinds of tumors (**Table 1**), used for molecular map analysis of tumors. As the double-stranded gDNA is very stable. The membrane of the exosome can protect the nucleic acid substances inside it from degradation induced by nuclease. It is also reported that exosome gDNA in serum can remain stable for 1 week at 4°C and 1 day at room temperature, even after repeated freezing and thawing cycles (Jin et al., 2016), so exosome gDNA is more stable. Based on these advantages, scientists can do more in-depth research on it, whether it is screening for early diseases, monitoring drug resistance, or evaluating prognosis, which has its significance.

EXTRACTION, DETECTION, AND FUNCTIONAL MECHANISM OF EXOSOME GENOMIC DNA

As the study of exosome gDNA has attracted extensive attention from scientists, the exosome separation and subsequent exosome gDNA detection still require more specialized studies to ensure optimal performance. When analyzing the gDNA inside exosomes, digestion outside of exosomes by DNase I should be the first procedure. DNase I can reduce the residue of cfDNA in samples and the pollution of DNA outside of exosomes (Spada et al., 2020; Wang et al., 2020) (**Figure 1**). It has been reported that the number of mutant DNA will be increased if DNase I is not pretreatment. Next, gDNA was extracted from exosomes which were treated with DNase I. The genomic DNA kit for tissues, cells, and blood can be used to extract gDNA from exosomes. Scientists using the Qiagen kit could get a higher concentration of exosome gDNA, but the Qiagen kit may be a bit expensive (Thakur et al., 2014; Kalluri and Lebleu, 2016; Spada et al., 2020; Wang et al., 2020). Our laboratory found that applying the inexpensive Tiangen kit can also successfully obtain a higher concentration of exosome gDNA. Typically, extracting 200 μl exosome according to the instructions will yield at least 20 ng/μl gDNA. After removing gDNA from exosomes, mutation detection of exosome gDNA is carried out. Most detection methods are digital PCR and fluorescence PCR to ensure more accurate mutation detection (**Table 1**). However, when the mutation to be detected is a point mutation, Sanger sequencing should be adopted as far as

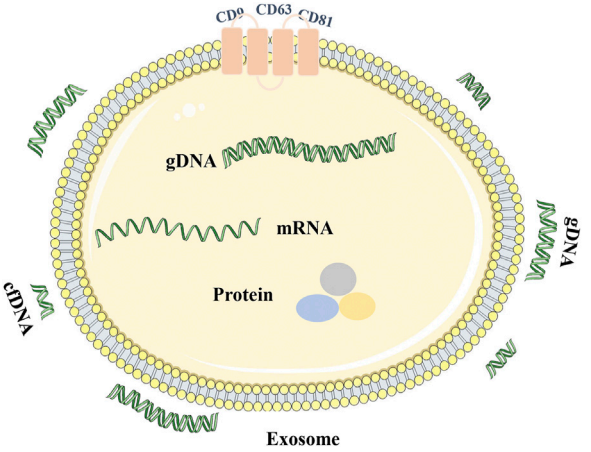

FIGURE 1 | Characterization of exosome gDNA. The short double-stranded DNA is cfDNA and the long double-stranded DNA is gDNA. DNA can be enclosed within exosome, attached to the outer surface of exosome or enclosed and attached to the outer surface of exosome.

possible, and then T-A clones obtained a large number of clones for follow-up detection.

Exosomes are an essential form of intercellular communication. Exosomes can deliver both RNA and protein as well as DNA (Kalluri and Lebleu, 2020) (**Table 2**). The transferred exosome gDNA affects the function of recipient cells by increasing mRNA transcription and protein translation (Cai et al., 2013; Cai et al., 2014; Lee et al., 2014; Fischer et al., 2016). The schematic diagram of the release of exosome gDNA is displayed in **Figure 2**. In addition to the open reading frame, exosomes should also contain the 5′ promoter region and 3′ untranslated region elements necessary for the transcription mechanism so that the delivered DNA can perform its function. It has been reported that there are DNA fragments in exosomes, including the 5′ promoter region, 3′ untranslated region, and active retrotransposon, which indicates that exosome gDNA may play a role in the genetic instability of receptor cells (Balaj et al., 2011; Cai et al., 2013). It has also been confirmed that exosome gDNA can be located in the recipient nucleus through late endosomal transport related to nuclear membrane invagination (Waldenstrom et al., 2012; Cai et al., 2013). According to these viewpoints, it shows that the function of oncogenes in tumor cells is not only to accumulate in the genome of tumor cells but also to transfer exosome gDNA and spread in tumor and normal cells (**Table 2**), which may lead to the tumorigenic transformation of normal cells and accelerate the progress of diseases. However, whether the tumor exosome gDNA can be horizontally transmitted to the recipient cells or the changes in the function of the recipient cells caused by the horizontal transmission are still controversial. In terms of the types of recipient cells, it has been reported that not all recipient cells can be horizontally transmitted. When the recipient cells are tumor cells, fibroblasts, or endothelial cells, the exosome gDNA can be easily shared, but it cannot be transmitted when the recipient cells are dormant cells such as epithelial cells (Lee et al., 2016); In the aspect of functional changes after horizontal transmission, some reports suggest that the gDNA transmitted to the recipient cells can be integrated into the genome of the recipient cells for a long time (Balaj et al., 2011; Cai et al., 2013; Cai et al., 2014; Fischer et al., 2016). Some reports suggest that the transmitted gDNA enters the recipient cells only for an instant function and loses its position after 1 month (Trejo-Becerril et al., 2012; Lee et al., 2014; Lee et al., 2016). There is also a report suggests that the transmitted gDNA is a driving factor rather than an initial factor, and only obtaining this driving factor can make the recipient cells deteriorate (Stefanius et al., 2019). The specific mechanism needs further study. From these perspectives, exosome gDNA horizontally may represent a new method of gene transfer and signal transduction between cells, which may be an essential mechanism of tumor occurrence, development, and metastasis, and provide a new direction for discovering new disease mechanisms and developing new treatment strategies.

COLORECTAL CANCER MICROENVIRONMENT PLAYS AN ESSENTIAL ROLE ON EXOSOME GENOMIC DNA PACKAGING AND TRANSMISSION

Hypoxia and acidification stimulate the tumor microenvironment, further activate the cell stress response mechanism, and increase the production of exosomes (Hoshino et al., 2013; Khaksar et al., 2018; Panigrahi et al., 2018; Soraya et al., 2021). At the same time, it may promote the package of gDNA into exosomes, and transfer it to the recipient cells, thus enhancing the ability of exosomes to induce malignant transformation of the recipient cells (Nemeth et al., 2017).

It has been reported that TP53 mutation in the recipient cells increased after the treatment factors affected the tumor microenvironment, and the transcription in colon epithelial cells and liver cells increased. The difference in mutant TP53 between colon epithelial cells and liver cells was also observed. It seems that liver cells are less likely to absorb mutant genes carried by exosomes than colon epithelial cells. The author also proved that LPS activated TLR4 of colon cancer cell SW480 and promoted the packaging of mutant TP53 in exosomes but could not promote the selective packaging of the KRAS gene. It indicates that the mechanism of gDNA packaging to exosomes is not random (Domenis et al., 2021). More and more experiments proved that exosomes derived from primary tumors could be loaded with specific molecules (Stobiecka et al., 2019; Baris et al., 2021), and the expression or lack of these molecules is helpful to the phenotype transformation of recipient cells. These results indicate that the tumor microenvironment increases the possibility of tumor gene integration by promoting exosome metastasis. Although the integration of mutant genes is still considered a rare event, it

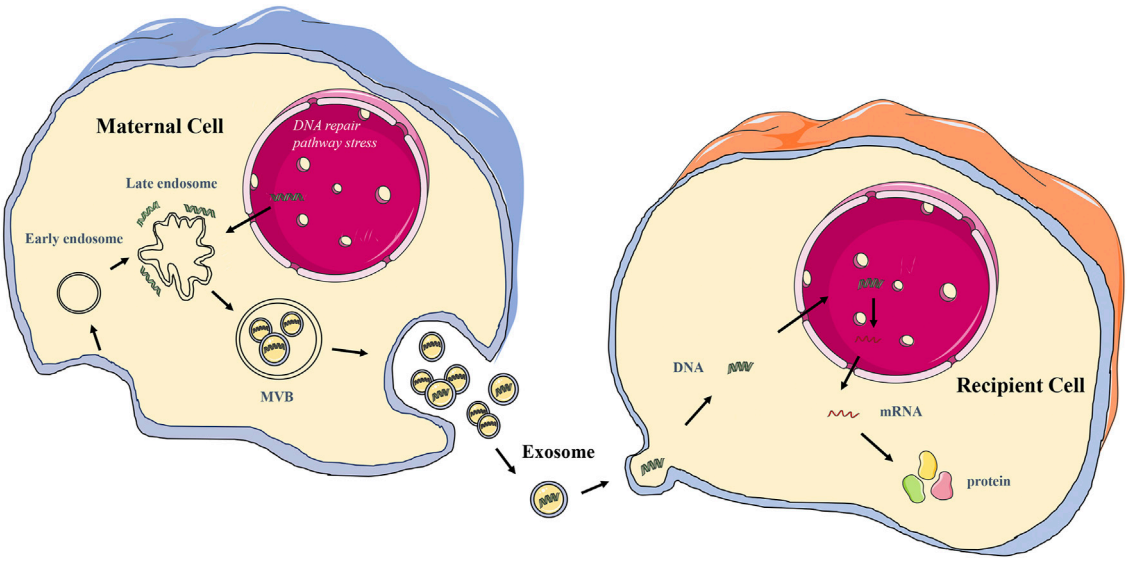

FIGURE 2 | The schematic diagram of exosome gDNA transfer between cells.

may be a related phenomenon *in vivo*. Further studies are needed to evaluate the effect of tumor-driven genes transferred through the exosome on malignant transformation and their role in tumor progression of microenvironmental stimulation.

EXOSOME GENOMIC DNA AS A TOOL FOR COLORECTAL CANCER LIQUID BIOPSY

Due to the lack of mismatch repair function in CRC, genome instability often leads to the accumulation of frameshift mutation in the microsatellite region. And CRC in an early stage can be prevented and cured, so it is necessary to explore non-invasive tools to help with early detection and treatment monitoring (Janney et al., 2020; Stoffel and Murphy, 2020). A liquid biopsy is a dynamic tool for the non-invasive detection of tumor heterogeneity and mutation over time. Studies have shown that compared with the healthy group, the circulating exosomes in the body fluid of tumor patients are more, and exosome gDNA may provide information about tumor-specific mutations. The relationship between mutated gDNA and types of body fluids is summarized below (**Table 1**). Therefore, detecting exosome gDNA mutations in tumor progression can help diagnosis early-stage, drug selection, and prognosis analysis.

EGFR mutation was seen in exosomes of malignant pleural effusion (Lee et al., 2018), and T790M mutation was also detected in exosomes of bronchoalveolar lavage fluid of patients with non-small cell lung cancer (Park et al., 2020). The exosome gDNA isolated from ascites of ovarian cancer patients reflects the copy number variation of primary tumors (Crow et al., 2017; Giannopoulou et al., 2019). KRAS mutation is also confirmed in exosomes of pancreatic ductal adenocarcinoma in the early stage and the late phase (Allenson et al., 2017). The serum exosomes of glioma patients also carry the gDNA sequence

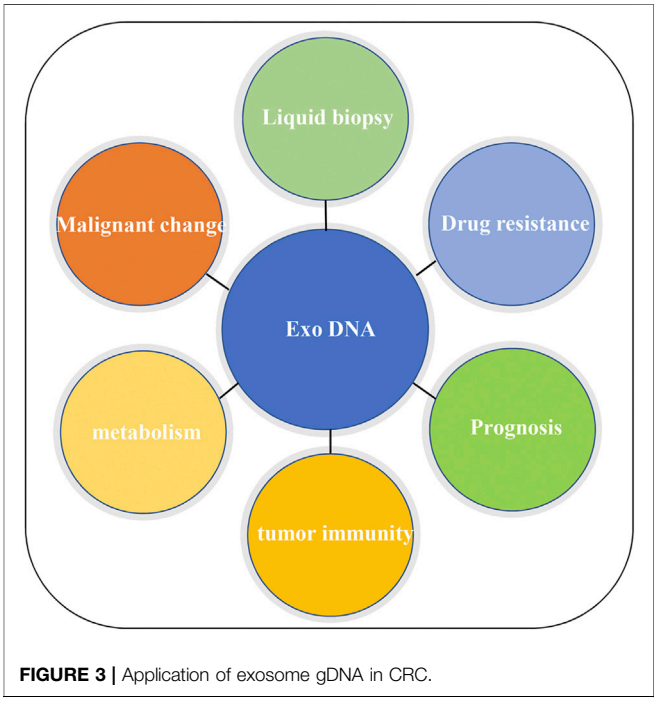

FIGURE 3 | Application of exosome gDNA in CRC.

with the same biological mutation as glioblastoma (Wang et al., 2018; Vaidya and Sugaya, 2020). In addition, the corresponding driving mutation was also found in the exosomes of prostate cancer (Lazaro-Ibanez et al., 2014). The genome-wide sequencing of exosomes of these tumor patients may provide information for diagnosis and prediction results.

In CRC, KRAS and BRAF mutation was detected from the serum exosomes of CRC patients with higher sensitivity and specificity (Hao et al., 2017; Lucchetti et al., 2021). In addition, it

has been reported that transforming growth factor receptor 2 (TGFR2) is a part of the key signal pathway in colon epithelial cells. Its double allele frameshift mutation occurs repeatedly in most colorectal tumors, which is thought to drive colorectal cancer. It has been found that the frameshift mutation in the microsatellite region of TGFR2 is wrapped in the exosome derived from CRC cells. Although it can be detected at the DNA level, the author has not observed any frameshift protein in the exosome protein group. And then, acting on the receptor cells can up-regulate the expression of cytokines (Fricke et al., 2017). It has also been reported that the level of exosome gDNA of patients with KRAS mutation in CRC increases significantly in the course of the disease and shows more changes after treatment. Moreover, in CRC patients who meet the surgical conditions, the tumor size is related to the copy number of KRAS mutation. The copy number and abundance score of KRAS mutation in exosomes of patients with liver metastasis is significantly increased. The author also confirmed that KRAS mutation disappeared rapidly after the first chemotherapy cycle. However, it is necessary to closely monitor secondary drug resistance after anti-EGFR treatment to stop this treatment as soon as possible (Lucchetti et al., 2021). Therefore, exosome gDNA can be used as an innovative tool to monitor the diagnosis, drug resistance, and prognosis of CRC patients during treatment.

EXOSOME GENOMIC DNA REGULATES IMMUNITY AND METABOLISM OF COLORECTAL CANCER

Tumor immunosuppression is a sign of cancer progression, signaled by immune checkpoints on immune cell subsets (Nikfarjam et al., 2020). In recent years, targeted checkpoint immunotherapy has therapeutic effects on cancer patients (Li et al., 2016; Li et al., 2018). Exosomes play a significant role in tumor immunity by paracrine signal regulators. Among many components of exosomes, exosome gDNA can be used as a potent regulator of the STING pathway, which can regulate tumor immunity. During radiotherapy, gDNA fragments accumulated in the cytoplasm of tumor cells can induce an IFN-1 response and then activate dendritic cells through paracrine to trigger an anti-tumor response. This process is regulated by the feedback loop that DNA exonuclease Trex1 degrades cytosolic DNA fragments to prevent anti-tumor reactions. However, to avoid this phenomenon, tumor cells can export gDNA pieces to the exosome. Once the exosome gDNA is internalized by tumor-infiltrated dendritic cells, it will activate the STING signal path in dendritic cells. Once dendritic cells are activated by exosome gDNA, they will produce IFN-1 and recruit more CD8+T lymphocytes to prevent tumor growth further (Vanpouille-Box et al., 2017). Crohn's disease is related to the risk of colorectal cancer. Exosome gDNA internalized into macrophages can activate the STING signal path. The research also shows that exosome gDNA can't work after inhibiting STING, which further confirms the importance of the STING pathway in exosome gDNA. It shows that exosome gDNA plays a vital role in the immunity of CRC and can be used as a potential biomarker and therapeutic target for Crohn's disease (Zhao et al., 2021). Reports suggest CRC will produce intestinal syndrome after being treated with irinotecan and fluorouracil, and the severity of diarrhea is closely related to the content of exosome gDNA. It is suggested that drug therapy can trigger the release of gDNA by intestinal epithelial cells through exosomes. Then the AIM2 inflammasome in immune cells will be activated, which will promote the body's inflammatory response. Therefore, exosome gDNA is not only a direct tumor immunomodulatory but also can affect the inflammatory reaction related to chemotherapy (Lian et al., 2017). Exosomes gDNA can indeed destroy the influence of checkpoint inhibitors. Therefore, in drug development, we should pay more attention to the signal of cell autonomic paracrine. In CRC, KRAS mutation will participate in metabolism *in vitro* and *vivo* by inducing glucose transporter 1 (Zhang et al., 2018). However, it is not sure if the exosome gDNA works, which needs further study in tumor metabolism.

FURTHER PERSPECTIVE

The discovery of high-frequency tumor mutant genes in exosome gDNA has attracted the attention of clinicians and basic researchers. Exosome gDNA is long and stable so that it can be used as excellent biomaterials for liquid biopsy (Jin et al., 2016) (**Table 1**). Many experts predict that in the near future, liquid biopsy based on exosome gDNA may improve the individualized treatment and prognosis of patients, especially for CRC patients, as the essential feature of CRC patients is the accumulation of mutations (Lichtenstern et al., 2020). Exosome gDNA can also be transferred from one cell to another by endocytosis or fusion. The transferred exosome gDNA can increase mRNA and protein expression in recipient cells and affect the function of recipient cells (**Table 2**). This may explain new pathogenesis of CRC and provide a new method for diagnosis or treatment in the future. However, it is necessary to conduct a larger-scale clinical study on these markers and targeted substances to verify the transformation of exosome gDNA into high-throughput practical solutions in the clinical environment.

Before exosome gDNA is officially applied in the clinic, it needs to meet the repeatable and verifiable conditions. However, due to the small amount of gDNA in exosomes and the different extraction methods, there is no uniform extraction and detection method, so its application is limited. Some hospitals have set up some large-scale analysis platforms to analyze it, which can provide helpful information for doctors' diagnoses and make it possible to specialize in the analysis of exosome gDNA.

However, if we don't know the mechanism of exosome gDNA, we can't get the clinical effect. Therefore, the primary research on the packaging of exosome gDNA is the premise of developing the clinical treatment of exosome gDNA. Finally,

further studies on the length, base composition, and stability of exosome gDNA under different physiological conditions will open up a new field for the diagnosis and treatment of colorectal cancer.

CONCLUSION

This article systematically reviewed the origin, mode of action of exosome gDNA, and its relationship with diagnosis, drug resistance, prognosis, immunity, and metabolism of CRC. The schematic diagram of the function of exosome gDNA is displayed in **Figure 3**. So far, we may have only seen the tip of the iceberg, and most of it is unknown. Therefore, more research is needed to reveal anonymous information about the application of exosome gDNA in the diagnosis and treatment of CRC.

AUTHOR CONTRIBUTIONS

RW contributed to the text and tables, QW contributed to revise the review manuscript, and XL contributed to the figures in the review manuscript.

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Case Report: Fluzoparib for multiple lines of chemotherapy refractory in metastatic cutaneous squamous cell carcinoma with BRCA2 pathogenic mutation

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Background: Poly ADP-ribose polymerase inhibitors (PARPis) are widely used for patients with BRCA1/2 mutations. However, until now, there is no available evidence reported for the efficiency of PARPis in cutaneous squamous cell carcinoma (cSCC).

Case presentation: We presented a case of a 40-year-old man diagnosed with metastatic cSCC, relapsing after multiple lines of chemotherapy. Liquid biopsy detected a BRCA2 pathogenic germline mutation (c.3109C > T), indicating PARPis might be effective for this patient. The patient achieved tumor stability, and progression-free survival was five months without severe adverse effects after taking fluzoparib.

Conclusion: This result confirmed that PARPis were effective for metastatic cSCC patients with germline BRCA2 pathogenic mutations and provided a new treatment option for this group of patients.

KEYWORDS

cutaneous squamous cell carcinoma, PARPi, fluzoparib, BRCA2 germline mutation, case report

Introduction

The main types of skin cancers include basal cell carcinoma, squamous cell carcinoma, melanoma, and Merkel cell carcinoma (Garbe et al., 2022; Lebbe et al., 2015; Leiter et al., 2020). Among these, cutaneous squamous cell carcinoma (cSCC) accounts for 20% of nonmelanoma skin cancer cases and is the second most common type of skin cancer (Leiter et al., 2020). It may originate from the keratinocytes of the epidermis or its appendages and is a local invasion and likely to metastasize to other organs (Motley

et al., 2002). Surgical excision is the treatment of choice for cSCC (Leiter et al., 2020). Ten-year survival after surgery exceeds 90% for cSCC but drops significantly when metastases occur (Varra et al., 2018). Chemotherapy, targeted therapy such as cetuximab, and immunotherapy have been adopted to treat cSCC patients, but the response rates of targeted therapy and chemotherapy were relatively low, and the duration of response was short (Weis et al., 2022).

Evidence from studies indicated that pathogenic or likely pathogenic BRCA1/2 germline mutations occurred in many cancers, including breast cancer (Borg et al., 2010; Kwong et al., 2012), ovarian cancer (Azribi et al., 2021), urothelial cancer (Carlo et al., 2020; Nassar et al., 2020), prostate cancer (Ghose et al., 2021), and so on. In prostate cancer, a study has shown that BRCA2 pathogenic mutations are connected with some clinicopathological parameters and indicated a poor prognosis (Ghose et al., 2021). In skin cancers, recent studies showed that BRCA1/2 mutations could be found in cutaneous melanoma (Johansson et al., 2019). Few studies reported BRCA1/2 mutations in cSCC. Thus, although poly ADP-ribose polymerase inhibitors (PARPis) have been successfully applied to treat ovarian cancer (Mirza et al., 2016), hereditary breast cancer (Han et al., 2018), and prostate cancer (Boussios et al., 2021b) with BRCA1/2 germline mutations, whether PARPis are effective in cSCC patients carrying BRCA1/2 germline mutations remains unclear, as no study has been conducted in cSCC.

In this case, we first reported the treatment of fluzoparib for metastatic cSCC patients with a BRCA2 pathogenic germline mutation and found remission to fluzoparib. This study first provided evidence for the efficiency of PARPis in treating metastatic cSCC.

Case description

A 40-year-old man first presented to the hospital with a mass in the left inguinal area in July 2018. It was about 2.0 cm * 3.0 cm, hard, and unpainful. The boundary of the mass was clear to some extent; however, the degree of the activity was poor. Extended resection was performed. The pathology demonstrated carcinosarcoma, which was mainly squamous cell carcinoma, and vascular tumor thrombus was positive. The patient denied any medical history and family history of cancer. The treatment after surgery was standard chemotherapy regimens combined with ifosfamide (2.5 g, every 21 days) with pirarubicin (30 mg, every 21 days) for seven cycles and combined cis-platinum (60 mg, every 21 days) with pirarubicin (30 mg, every 21 days) for the final cycle from August 2018 to May 2019. No adverse reaction occurred. In October 2018, the patient happened to find a mass in the palm of the right hand, which was shrunk during subsequent chemotherapy. However, in November 2019, the mass was suddenly enlarged to about 5.5 cm * 5.3 cm, painful, swollen, itchy, and accompanied by skin ulceration. The pathology after mass resection suggested well and moderately differentiated squamous cell carcinoma in the palm of the right hand, and it was difficult to identify the tumor tissue and normal tissue clearly. Immunohistochemistry (IHC) showed positive for P40, P63, and CK5/6; Ki67 was 50% positive. In February 2020, a mass appeared in the right forearm, and it was about 4.0 cm * 4.0 cm and ulcerated. After a series of examinations, excisional surgery for the right forearm and axillary lymph node extirpation was performed. The pathology suggested well and moderately differentiated squamous cell carcinoma, as found in relation to the mass in the palm. Furthermore, muscle tissue invasion, neural invasion, and lymph node metastasis (5/7) were also found upon pathological inspection. IHC showed positive for P40, P63, S-100, D2-40, CD34, and CK5/6; Ki67 was 40% positive. To get promising anti-tumor activity with less toxicity, postoperative chemotherapy on this occasion was combined with docetaxel (100 mg, every 21 days) and lobaplatin (50 mg, every 21 days) for six cycles from March 2020 to July 2020. During the treatment, grade 2 to 4 adverse effects were not observed. In January 2021, the patient began to cough with gradually increasing sputum and blood. Enhanced computed tomography (CT) in March 2021 revealed multiple pulmonary nodules in the right middle lobe and subpleural region of the left upper lobe (the largest one was about 1.9 cm), which were partially larger than those of a month before. The pathological consultation of the right palm and right forearm indicated squamous cell carcinoma (Figure 1). The patient was finally diagnosed with cSCC with inguinal area and pulmonary metastasis (stage IV) after a multi-disciplinary team discussion. Additional IHC of tumor in the left inguinal area showed negative expression of PD-L1 (combined positive score, CPS <1, by 22C3 pharmDx assay, Dako, Carpinteria, CA, United States), indicating immunotherapy was less effective for this patient. Then, the patient underwent a chemotherapy regimen consisting of albumin-bound paclitaxel (200 mg, d1/d8 of 3 weeks) and gemcitabine (2,000 mg, d1/d8 of 3 weeks) for eight cycles from March 2021 to September 2021. During treatment, enhanced CT showed significant tumor shrinkage of pulmonary nodules, and the best clinical efficacy reached partial remission. However, the patient presented grade 3 neutropenia, which was improved by pegylated recombinant human granulocyte colony-stimulating factor (PEG-rhG-CSF). In October 2021, enhanced CT implied disease progression. Her-2 was negative. Detection of BRCA1/2 germline mutations and other homologous recombination repair related genes for the patient in blood identified BRCA2 germline mutation (c.3109C > T) (Table 1), and all relative genes detected are listed in Supplementary Table S1. Considering there was no standard treatment for the patient, the patient was advised to take fluzoparib. The patient agreed and regularly took fluzoparib (150 mg orally twice a day) from November 2021. A month later, the patient achieved a stable state of tumor in enhanced CT. Three months later, pulmonary nodules were in regression again

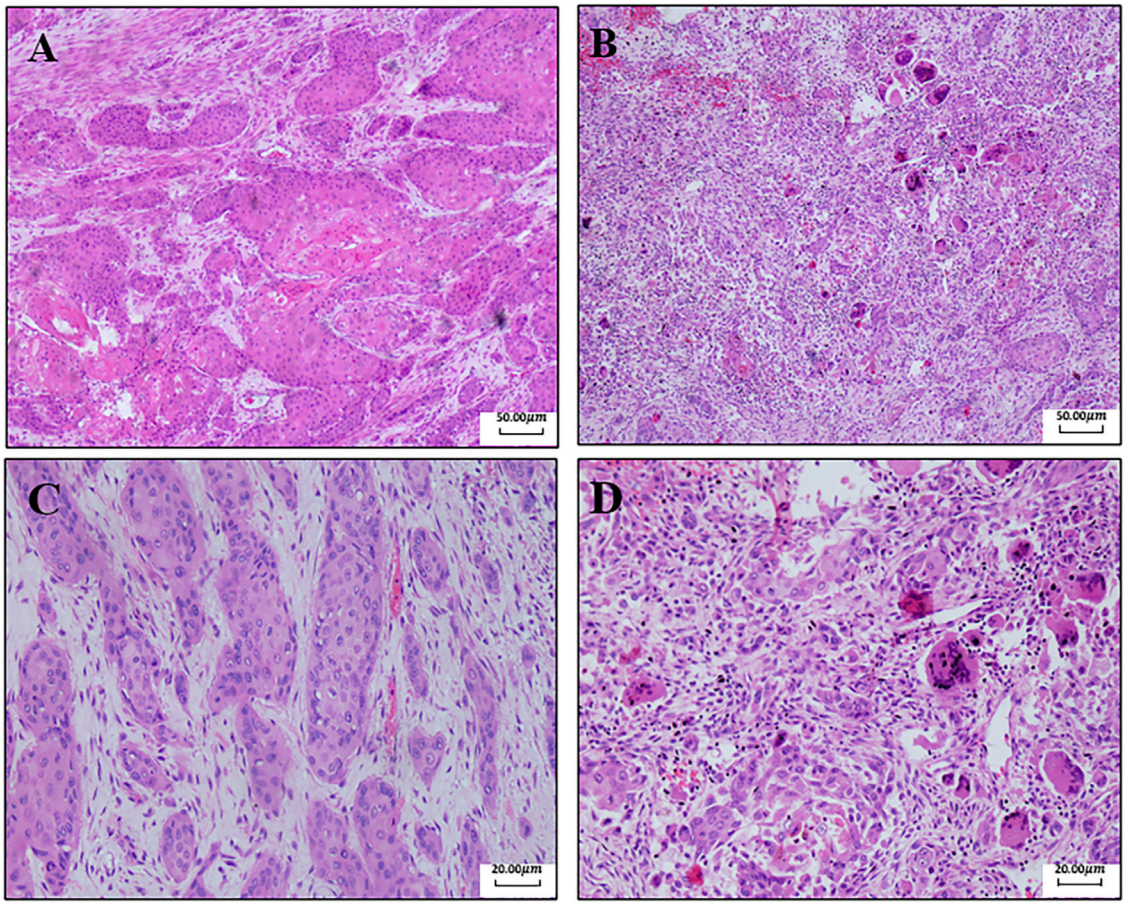

FIGURE 1
Pathological results of the right palm and right forearm. **(A)** Pathology of the right palm (40x): there are a lot of multinucleated giant cells and inflammatory cells in the tissue. Fibrosis occurs, and cells are swelling and deformed. These are the changes after chemotherapy. **(B)** Pathology of the right forearm (40x). **(C)** Pathology of the right palm (100x). **(D)** Pathology of the right forearm (100x).

TABLE 1 Detection result of BRCA1/2 germline mutations in a blood sample.

Tumor-specific mutations

Gene	Nucleotide variation	Amino acid variation	Mutation type	Germline/somatic mutation
BRCA2(NM_000059.3)	c.3109C>T	p.(Gin 1037Ter)	Heterozygous mutation	Germline mutations

(Figure 2). Also, no severe adverse effects occurred. Tumor markers remained normal during the patient's treatment. The patient has been progression-free for five months after fluzoparib treatment at the last follow-up in April 2022 (Figure 3).

Discussion

To our best knowledge, this case first proved successful treatment by PARPis in a patient with cSCC subjected to multi-line treatments.

One of the highlights in this case was that the initial symptom of the patient was atypical until the diagnosis of the cSCC in the right palm. It has been reported that approximately 3% of melanomas lack an identifiable primary, otherwise known as melanoma of unknown primary (Boussios et al., 2021a). However, for SCC, it usually shows a hard nodular keratinizing tumor or crusted tumor which could ulcerate. Sometimes, it could show as a non-keratinized ulcer. For this patient, he first showed a mass in the left inguinal area without any other symptoms. Examinations and pathology demonstrated SCC of the left inguinal area without other abnormalities.

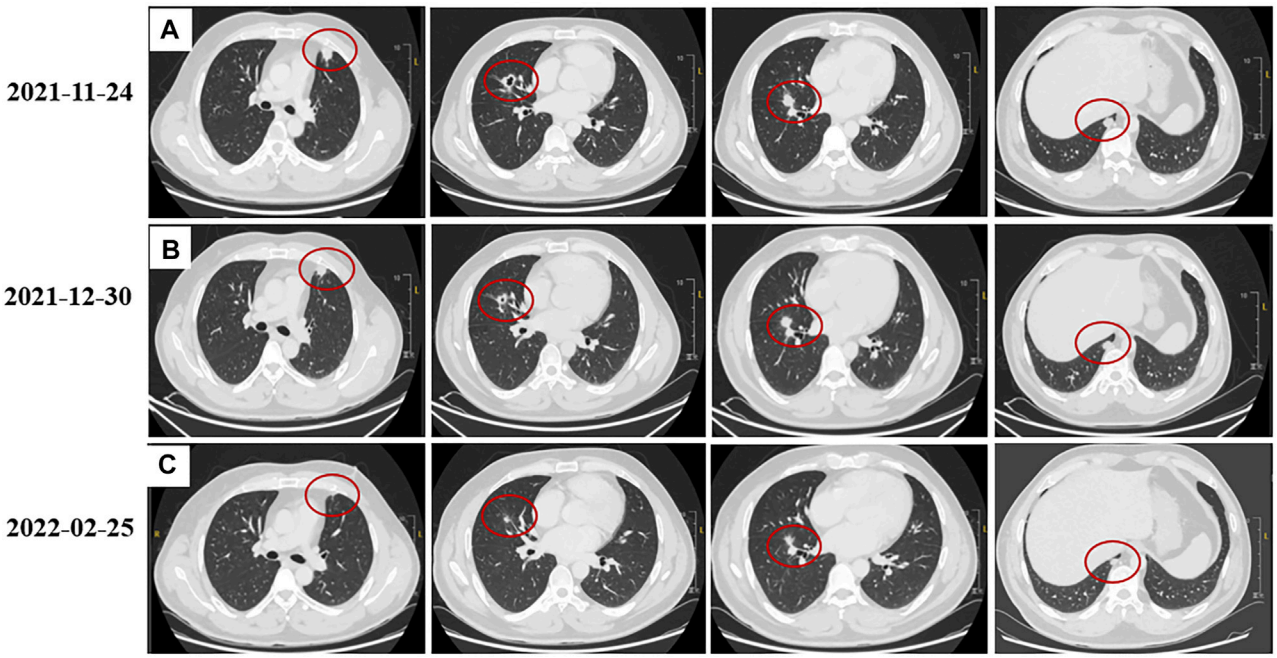

FIGURE 2
Tumor response to fluzoparib. **(A)**: Lung lesions before using fluzoparib. **(B)**: Lung lesions a month after using fluzoparib. **(C)**: Lung lesions three months after using fluzoparib. The red circles represent the target lesion.

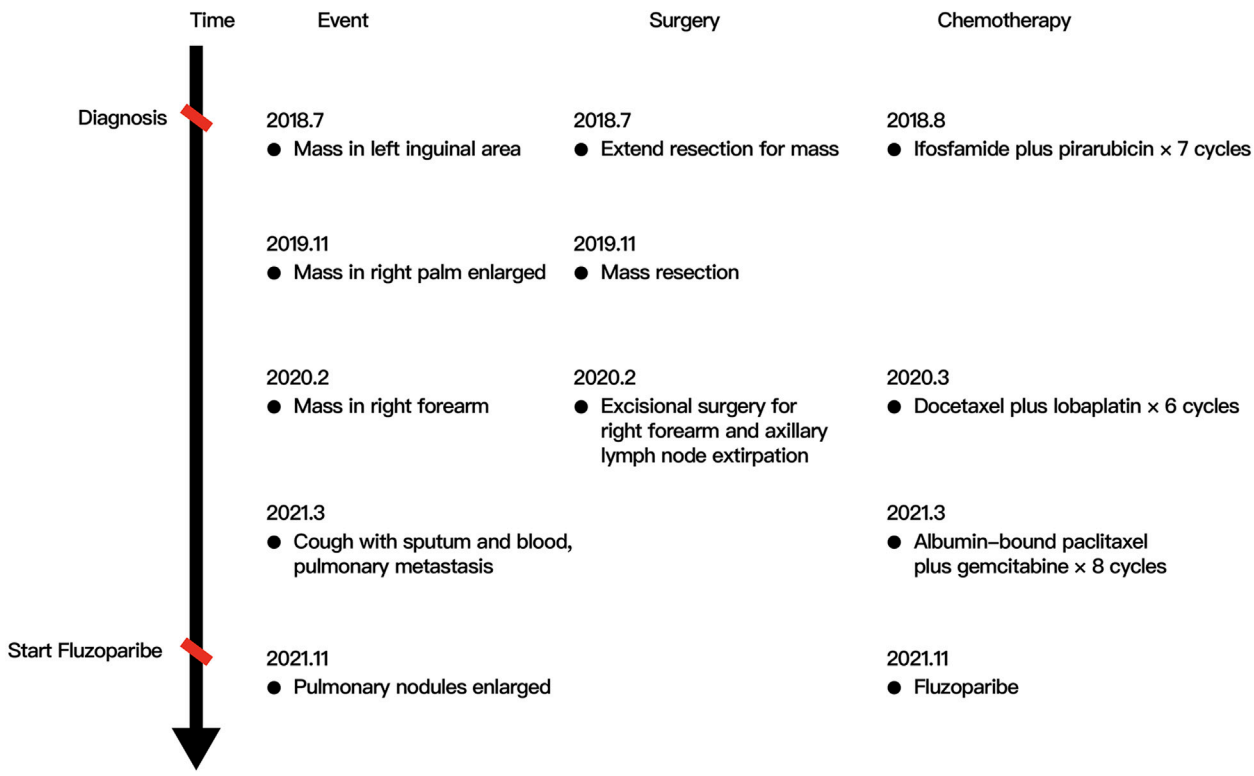

FIGURE 3
Case of a metastatic cutaneous squamous cell carcinoma patient.

Considering that cancer may derive from either primary or secondary tumors, standard chemotherapy was used for the patient. During treatment, the development of cSCC in the right palm was observed, proving that cSCC might be the primary tumor for this patient and the mass in the left inguinal area arose from metastasis. A clear diagnosis provided evidence for subsequent therapy.

Another valuable finding in the case was that a pathogenic BRCA2 germline mutation (c.3109C>T) was observed in this patient, which is rarely reported in cSCC. This mutation occurs at 3109 bp of BRCA2, with a base "C″ changing to "T", transforming glutamine to a stop codon. This mutation terminates the protein coding in advance, further affecting the degradation of mRNA and tumor development. Except for BRCA1/2, other genomic alterations related to homologous recombination deficiency have been recognized, including Fanconi anemia genes (BRIP1 and PALB2), the core RAD genes (RAD51C and RAD51D), and genes involved in homologous recombination pathways either directly (BARD1, NBN, and ATM) or indirectly (CDK12) (Ramus et al., 2015; Shah et al., 2022). Recent studies have reported that PAPRi therapy shows good efficiency in patients with homologous recombination deficiency (mainly BRCA1/2 mutations), and some targeted drugs have been approved by The United States Food and Drug Administration (FDA) to treat malignant cancers. For example, olaparib for treating ovarian and breast cancer was approved (Kim et al., 2015), and the III-phase NOVA trial showed niraparib could be used for ovarian patients with recurrence through treatment with platinum-based chemotherapy (Mirza et al., 2016). In high-grade ovarian cancer, the III-phase ARIEL3 trial showed patients who had ever responded to platinum-based chemotherapy could benefit from the maintenance of rucaparib (Coleman et al., 2017). In prostate cancer, olaparib was approved for metastatic castration-resistant prostate cancer patients with deleterious BRCA1/2 mutations with disease progression following androgen receptor signaling inhibitor treatment (Boussios et al., 2021b). PARPis for gastrointestinal cancers are also underway (Pilie et al., 2019). Some clinical studies have shown that PARPis could provide important benefits with acceptable toxicities when they were used for maintenance therapy after responding to platinum-based chemotherapy (Pilie et al., 2019). For this patient, he has suffered from multi-line treatments, but the disease was in progression. Expression of PD-L1 was low, suggesting he was unlikely to benefit from immunotherapy. BRCA2 was a pathologic mutation that was likely to benefit from PARPis. The patient exhibited platinum sensitivity because the metastasis occurred around six months after platinum-based chemotherapy. These suggest that the patient might benefit from PARPis in this scenario.

Fluzoparib is a type of novel PARPi on the basis of olaparib. It could inhibit the PARP1 enzyme and further could induce DNA double-strand breaks, G2/M arrest, and apoptosis in homologous recombination repair (HR)-deficient cells (Wang et al., 2019). It also showed good pharmacokinetics and stable anti-tumor activity, as well as a favorable toxicity profile for the treatment of many cancers (Wang et al., 2019; Li et al., 2022). A phase-II trial about ovarian cancer observed that patients with platinum-sensitive and BRCA1/2-mutant showed a 69.9% for objective response rate, The median follow-up duration was 15.9 months by 21 March 2020 (Lee, 2021; Li et al., 2021); fuzuloparib is also used to treat cancers of the pancreas, prostate, breast, and lungs in some phase-II or phase-III clinical trials (Lee, 2021). Moreover, fuzuloparib, used for treating ovarian cancer (including fallopian tube cancer or primary peritoneal cancer), has been approved in China. Although clinical trials by using PARPis in cSCC have not been conducted in China, the patient was willing to take an investigational therapy with strict informed consent, providing strong evidence for PARPi prescription.

Liquid biopsy of blood has been widely used to diagnose and treat lung cancer (Paweletz et al., 2016), gastric cancer (Shoda et al., 2017; Wu et al., 2006), breast cancer (Wallwiener et al., 2015; Mayor et al., 2017), and urothelial cancer (Agarwal et al., 2018). Studies also found that liquid biopsy could be used in oropharyngeal SCC (Haring et al., 2022) and cSCC (Haring et al., 2022). In this case, a liquid biopsy of blood detected the alterations in mutational status without invasion, providing evidence for the use of fluzoparib.

The strength of the case was that we presented a rare case in which the symptom of the patient was uncommon in cutaneous squamous cell carcinoma (cSCC). Moreover, we first showed a case using fluzoparib to treat cSCC patients with BRCA2 germline mutations, and the patient achieved a stable state. Our patient also thought he was benefiting from the regime in therapeutic effect, economy, and convenience. This might provide clues for similar patients. However, this is just a case report, and there are individual differences in response. The efficacy of PARPis in cSCC treatment remains to be confirmed by clinical trials.

In conclusion, our study first proved that cSCC patients, with BRCA2 pathogenic germline mutations and sensitivity to platinum-based chemotherapy, responded well to fluzoparib, suggesting that PARPis may be used for treating cSCC patients carrying BRCA1/2 germline or somatic mutations; detecting mutations is necessary for identifying the correct therapeutic regime.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XS and WC contributed to collecting the data and drafting the manuscript. YC and XQ helped design and revise the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.968060/full#supplementary-material

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Causes of death in women with breast cancer: a risks and rates study on a population-based cohort

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Introduction: The increasing survival of patients with breast cancer has prompted the assessment of mortality due to all causes of death in these patients. We estimated the absolute risks of death from different causes, useful for health-care planning and clinical prediction, as well as cause-specific hazards, useful for hypothesis generation on etiology and risk factors.

Materials and methods: Using data from population-based cancer registries we performed a retrospective study on a cohort of women diagnosed with primary breast cancer. We carried out a competing-cause analysis computing cumulative incidence functions (CIFs) and cause-specific hazards (CSHs) in the whole cohort, separately by age, stage and registry area.

Results: The study cohort comprised 12,742 women followed up for six years. Breast cancer showed the highest CIF, 13.71%, and cardiovascular disease was the second leading cause of death with a CIF of 3.60%. The contribution of breast cancer deaths to the CIF for all causes varied widely by age class: 89.25% in women diagnosed at age <50 years, 72.94% in women diagnosed at age 50–69 and 48.25% in women diagnosed at age ≥70. Greater CIF variations were

observed according to stage: the contribution of causes other than breast cancer to CIF for all causes was 73.4% in women with stage I disease, 42.9% in stage II–III and only 13.2% in stage IV. CSH computation revealed temporal variations: in women diagnosed at age ≥70 the CSH for breast cancer was equaled by that for cardiovascular disease and "other diseases" in the sixth year following diagnosis, and an early peak for breast cancer was identified in the first year following diagnosis. Among women aged 50–69 we identified an early peak for breast cancer followed by a further peak near the second year of follow-up. Comparison by geographic area highlighted conspicuous variations: the highest CIF for cardiovascular disease was more than 70% higher than the lowest, while for breast cancer the highest CIF doubled the lowest.

Conclusion: The integrated interpretation of absolute risks and hazards suggests the need for multidisciplinary surveillance and prevention using community-based, holistic and well-coordinated survivorship care models.

KEYWORDS

cardio-oncology, geographic variation, breast cancer, cardiovascular disease, respiratory disease, cause-specific death, competing-risk model, population-based cancer registries

1 Introduction

In 2020 an estimated 2.26 million new cases of breast cancer were diagnosed across the globe (1). For women diagnosed during 2010–2014, the five-year survival for breast cancer was reported to range from 89.5% in Australia and 90.2% in the USA to 66.1% in India (2). The combination of high incidence and high survival has led to an increased interest in non-breast-cancer deaths following a breast cancer diagnosis. Cardiovascular disease (CVD) is an important cause of death following breast cancer: 1.6% to 10.4% of all women with breast cancer died of CVD (3), not only due to the high prevalence of CVD in the general population but also because of the overlapping risk factors between breast cancer and CVD and the adverse cardiovascular effects of cancer treatments.

A systematic review (3) showed that only a limited number of studies investigated the risk of CVD mortality following breast cancer, and these studies were heterogeneous in design, study populations and study periods and selection by age and stage of the women included in the analysis (4–11). Specific causes of cancer-related death other than breast cancer included ovarian and endometrial cancer, while pulmonary and gastrointestinal diseases were prominent among non-cancer causes (12–14).

The analysis of cause-specific deaths requires specific attention because of the competition between different causes of death. In breast cancer patients the mortality from a specific cause of death, for example CVD, is strongly influenced by breast cancer mortality, as the occurrence of breast cancer death precludes the possibility for a person to die from other diseases.

The analysis of competing mortality is useful in two main settings. The first is clinical resource allocation and predictive research; in this case the need is to estimate the absolute risk of dying in patients with a specific disease. The second is the setting of etiology studies, where it is important to study the instantaneous rate of occurrence of the event of interest in subjects still at risk of the event in every instant (hazard). The recommendation from the literature is to use cumulative incidence functions (CIFs) and risk ratios for the estimation of absolute risks, and cause-specific hazards (CSHs) and cause-specific hazard ratios (CSHRs) for the investigation of instantaneous rates (15–20). CIF describes the proportion of patients with a certain event over the course of time, and in the simplest case refers to the number of individuals initially enrolled in the study. For example, it could be the proportion of all patients in a breast cancer cohort who develop breast cancer recurrences in 10 years from diagnosis, e.g., 12% of the total of women initially included in the observation. CSH is a function of time and describes the instantaneous rate of occurrence of the event of interest in subjects who are still at risk of the event. For example, in a breast cancer cohort it is the rate at which patients develop recurrences during the second year of observation considering patients who survived until the beginning of the second year, for example one woman in 50 in one year. While many studies have investigated CIFs, very few have studied CSHs, and not a single paper has been published presenting the results of both metrics together. We therefore considered that a step forward in the knowledge and interpretation of the causes of death in women affected by breast cancer could be a study that analyzed hazards (CSHs) and absolute risks (CIFs) side by side, following the indications of leading authors according to whom this is the most rigorous scientific approach to evaluate competing risk data such as cause-specific deaths (21). By adopting this approach we were able

to study the cumulative proportion of patients dying from a certain disease during the entire follow-up time along with the variations in the risk of death in different periods.

As a further study objective we decided to analyze the geographic variations of CIFs and CSHs. To our knowledge only the paper by Ho et al. (22) reported on this topic, concluding that other studies with both individual and county-level information are needed to inform public health interventions in this field.

We designed a competing-mortality study taking into account breast cancer deaths, other cancer-related deaths, CVD deaths, respiratory-disease deaths, and deaths from other causes than the above listed. We included all women with breast cancer regardless of age and disease stage. We decided to analyze the data in a relatively recent time span to be able to provide an up-to-date picture of the causes of death following breast cancer. We used a retrospective cohort design based on population-based registries, thereby avoiding any selection bias.

2 Materials and methods

2.1 Breast cancer cases

This was a retrospective study on a cohort of women diagnosed with primary breast cancer. The cases were archived in the following population-based cancer registries: Aosta Valley (2010–2013), Brindisi (2010–2013), Pavia (2010–2013), Modena (2014–2017), Ragusa and Caltanissetta (2010–2013), Sondrio (2010–2016), South Tyrol (2010–2016) and Trapani (2010–2013). Selection using the site code C50 and malignant epithelial morphology codes M8010–M8575 of the International Classification of Diseases for Oncology (ICD-O-3) (23) retrieved a total of 12,742 primary breast cancer cases diagnosed in the predetermined study period and meeting our selection criteria. All 12,742 cases were used in the analysis. Causes of death were categorized by the following ICD-10 codes (24): C50 (breast cancer), I00–I99 (CVD), C00–C97 except C50 (cancers other than breast), J00–J99 (respiratory diseases), and other causes than the ones listed. Disease stage was specified according to the sixth edition of the TNM classification of malignant tumors (25).

2.2 Statistical analysis

This study was based on the computation of two functions: CIF and CSH. CIF is generally used to estimate the absolute risk of the occurrence of an event of interest up to a follow-up time point *t* and refers to the number of individuals enrolled in the study. In a simplified case – observation without censoring – CIF at time *t* can be computed as the fraction D/N, where N is the number of individuals under study at time 0 and D is the number of persons who die or develop a disease in a specified time period, from 0 to *t*. CSH estimates the instantaneous probability at time *t* of an event, considering as denominator the population surviving up to time point *t*, thereby providing a picture of the instantaneous modifications in the risk under study. While CIF refers to the initial number of patients, CSH refers to patients surviving at time *t*. The CSH analysis results are useful to intercept variations in rates, taking into account at every time point the number of people surviving up to that moment and thereby making it possible to analyze determinants in the causes of disease development or progression rates. In survival analysis, risk takes the denomination of CIF and rate is substituted by CSH. We estimated CIFs and CSHs, taking all cases of the cohort together and separately by registry to investigate the extent of geographic variation. We also analyzed CIFs and CSHs separately by age (0–50, 50–69 and ≥70 years), stage (local, regional and metastatic) and registry. Differences in CSH were determined by log-rank test and those in CIF with Gray's test (26). We used cubic splines to estimate CSH curves, exploring possible changes over time since the breast cancer diagnosis. We ran multivariate Cox proportional hazards models to estimate CSHRs with 95% confidence intervals (CI) of cause-specific deaths to analyze differences by registry, introducing into the model a variable for each of the eight cancer registries under analysis and stratifying the model for age as a potential confounding factor. Time to event or end of follow-up was calculated from the date of diagnosis. The hazard proportionality was tested by analysis of scaled Schoenfeld residuals. To explore the effect of competing causes of death on CIFs we estimated sub-distribution hazard ratios (SHRs) with the Fine-Gray model, which adjusts for the influence of other causes of death that may prevent cause-specific deaths from being observed. The analyses were performed according to the methods described in the competing risk literature (15–21), using the R statistical package (version 4.2.2) and the add-on packages Epi, cmprsk, crr-addson and splines (27–29). Differences were considered significant at $P<0.05$.

3 Results

3.1 Cohort characteristics

The median age at diagnosis of the cohort of 12,742 patients was 63 years (interquartile range 51–74). Stage information was available for 9131 (71.66%) women; the breakdown by stage I, II–III (grouped together for the study) and IV was 4024 (31.58%), 4418 (34.67%) and 689 (5.41%), respectively. A total of 2760 women died during the first six years, 1630 (12.79% of cohort, 59.06% of deaths) of breast cancer, 288 (2.3% of cohort, 10.43% of deaths) of cancers other than breast, 415 (3.2% of cohort, 15.04% of deaths) of CVD, 76 (0.59% of cohort, 2.75% of deaths) of respiratory diseases, and 351 (2.75% of cohort, 12.72% of deaths) of causes other than the above listed.

3.2 Analysis of cumulative incidence

To illustrate the absolute risk of mortality in this breast cancer cohort, Figure 1 and Table 1 show the CIFs (%) for every cause under analysis by the year from diagnosis to six years of follow-up. Breast cancer presented the highest CIF (13.71%) and CVD ranked second (3.60%). CIF amounted to 3.10% for other diseases, 2.50%

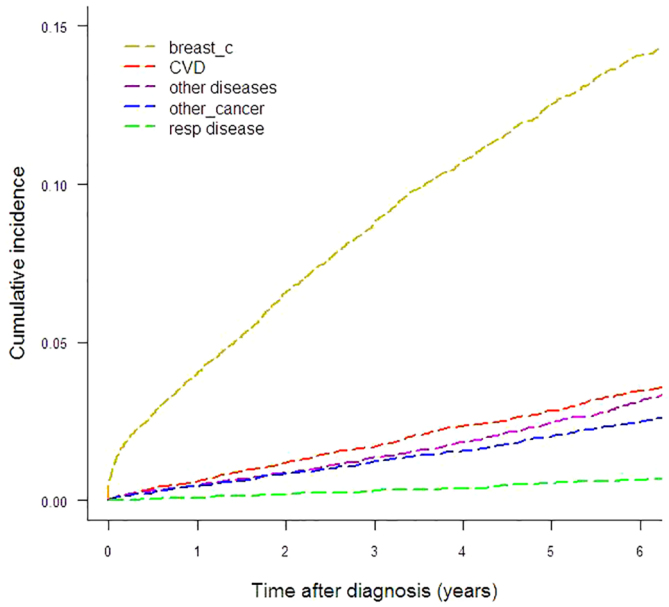

FIGURE 1
Cumulative incidence of mortality by different causes-of-death groups.

for other cancers and 0.66% for respiratory diseases. For CVD the most represented ICD-10 categories were cerebrovascular disease (n=117), chronic ischemic heart disease (n=61), hypertensive disease (n=58) and acute myocardial infarction (n=35). The main contributors to the "other diseases" category of deaths were diseases of the digestive system (n=55), mental and behavioral disorders (n=34), certain infectious and parasitic diseases (n=31), endocrine, nutritional and metabolic diseases (n=30, of which n=25 diabetes mellitus) and diseases of the nervous system (n=29). Malignant neoplasms of the bronchus and lung (n=35), colon (n=34), pancreas (n=27), liver and intrahepatic bile ducts (n=26), stomach (n=21) and ovary (n=16), multiple myeloma and malignant plasma cell neoplasm (n=11) were the largest contributing causes of death for the "other cancers" category. Among respiratory disease deaths pneumonia (n=37) was the most common cause.

Gray's test reported statistically significant differences of CIFs by age and stage for all five causes-of-death groups. This finding led us to explore CIFs separately for age and stage. CIFs by age are presented in Table 1 and Figures 2A–C and CIFs by stage in Table S1 (Additional file 1) and Figures 2D–F. The proportion for every specified cause of death with respect to the total CIF (all causes) showed variations according to age. In women diagnosed at age <50 years the CIF for breast cancer mortality was predominant, accounting for 89.25% of the total CIF (resulting from the ratio between the two CIFs, 9.22% and 10.33%, respectively). Likewise, the CIF for CVD accounted for only 0.77% of the total CIF. In women diagnosed at age 50–69, the CIF for breast cancer mortality was 72.94% and the CIF for CVD mortality was 7.36% of the total CIF, a marked increase with respect to the CIF in the <50-year age class. For women diagnosed at age ≥70 the CIF for breast cancer mortality diminished with respect to that of the younger age classes, accounting for only 48.25% of the total CIF, while the CIF for CVD mortality increased, accounting for 20.92% of the total CIF. Also the CIFs for other diseases varied according to age: the CIFs for death from respiratory diseases went from 0.66% (age <50) to 1.79% (age ≥70), for death from other cancers from 0.42% (age <50) to 4.89% (age ≥70), and for death from other diseases from 0.57% (age <50) to 6.85% (age ≥70). The CIFs for each disease group were markedly different in each stage group. The CIF for CVD mortality at six years from diagnosis was similar to that of breast cancer (1.59% and 1.90%, respectively) in women with stage I disease, while in stage II–III the CIF for breast cancer mortality was much higher (12.90%) than that for CVD mortality (3.54%). In women with stage IV disease breast cancer became by far the leading cause of death (71.64%), while the CIF for CVD deaths was 2.94%. The CIF for causes of death other than breast cancer accounted for 73.4% of the total CIF in women with stage I disease, while in stage II–III it was 42.9% and in stage IV only 13.2%.

We also performed specific analyses by different combinations of the staging variables T (primary tumor), N (regional lymph nodes) and M (distant metastases), and age (data not shown). The most interesting result was that in older women (≥70 years at diagnosis) with small-size cancers (T1) and no metastases at presentation the CIF for CVD mortality at six years from diagnosis was higher than that for breast cancer, 6.03% versus 5.68%, respectively.

3.3 Analysis of cause-specific hazards

Table 2 and Figure 3 describe how CSHs in women with a breast cancer diagnosis varied by time since diagnosis for different age and stage groups. We observed a peak in breast cancer CSH in women diagnosed at age <50 (Figure 3A) occurring two to three years since

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TABLE 1 Cumulative incidence of cause-specific deaths by years of follow-up for all ages and by age class.

Cumulative incidence (%)							
Deaths	1y	2y	3y	4y	5y	6y	95% CI at 6y
Cardiovascular disease							
All ages	0.66	1.29	1.78	2.43	2.91	3.60	3.26-3.95
<50	0.00	0.00	0.04	0.04	0.08	0.08	0.02-0.28
50-69	0.16	0.20	0.33	0.53	0.62	0.76	0.54-1.04
≥70	1.66	3.39	4.60	6.17	7.39	9.17	8.30-10.08
Respiratory disease							
All ages	0.10	0.24	0.32	0.39	0.56	0.66	0.53-0.82
<50	0.00	0.00	0.00	0.00	0.04	0.04	0.01-0.24
50-69	0.00	0.02	0.02	0.04	0.04	0.04	0.01-0.13
≥70	0.29	0.64	0.89	1.06	1.51	1.79	1.42-2.24
Breast cancer							
All ages	3.84	6.31	8.54	10.50	12.29	13.71	13.09-14.34
<50	1.18	2.76	4.59	6.13	7.92	9.22	8.11-10.42
50-70	1.88	3.71	5.54	7.25	8.63	9.81	9.00-10.66
≥70	7.82	11.61	14.54	17.08	19.37	21.13	19.90-22.38
Other cancers							
All ages	0.48	0.87	1.28	1.59	2.05	2.50	2.23-2.81
<50	0.11	0.11	0.19	0.23	0.27	0.42	0.21-0.77
50-69	0.16	0.42	0.72	0.86	1.21	1.57	1.24-1.95
≥70	1.09	1.88	2.62	3.29	4.14	4.89	4.26-5.59
Other diseases							
All ages	0.47	0.92	1.38	1.87	2.47	3.10	2.79-3.43
<50	0.07	0.18	0.30	0.37	0.46	0.57	0.33-0.94
50-69	0.13	0.27	0.48	0.66	1.01	1.27	0.98-1.62
≥70	1.13	2.15	3.14	4.24	5.44	6.85	6.09-7.67
All causes							
All ages	5.55	9.64	13.31	16.79	20.29	23.57	22.8-24.35
<50	1.36	3.05	5.12	6.77	8.77	10.33	9.16-11.59
50-69	2.33	4.62	7.09	9.34	11.51	13.45	12.51-14.42
≥70	11.98	19.68	25.79	31.85	37.85	43.83	42.28-45.38

the diagnosis. The CSH for CVD was very low during the whole observation period of six years. Among women diagnosed at age 50–69 (Figure 3B), we identified an early peak for breast cancer followed by a further peak between the second and third years of follow-up, almost at the same time as the peak observed in younger women. We identified a peak in the CSH of CVD in the first year after breast cancer diagnosis. The CSH for CVD ranked second near the diagnosis, while near the end of the follow-up period the CSH for the diagnostic group of other diseases ranked second. Among women diagnosed at age ≥70 (Figure 3C) we identified a strong early peak (first year after diagnosis) for breast cancer. Interestingly, by the sixth year following diagnosis the CSH for breast cancer deaths was matched by those for death from CVD and from the "other diseases" group.

CSH estimates by stage are presented in Figures 3D–F. The pattern of the diseases under analysis varied greatly by stage. In women with stage I breast cancer the contribution of breast cancer deaths was comparable to that of the group of "other diseases" at six

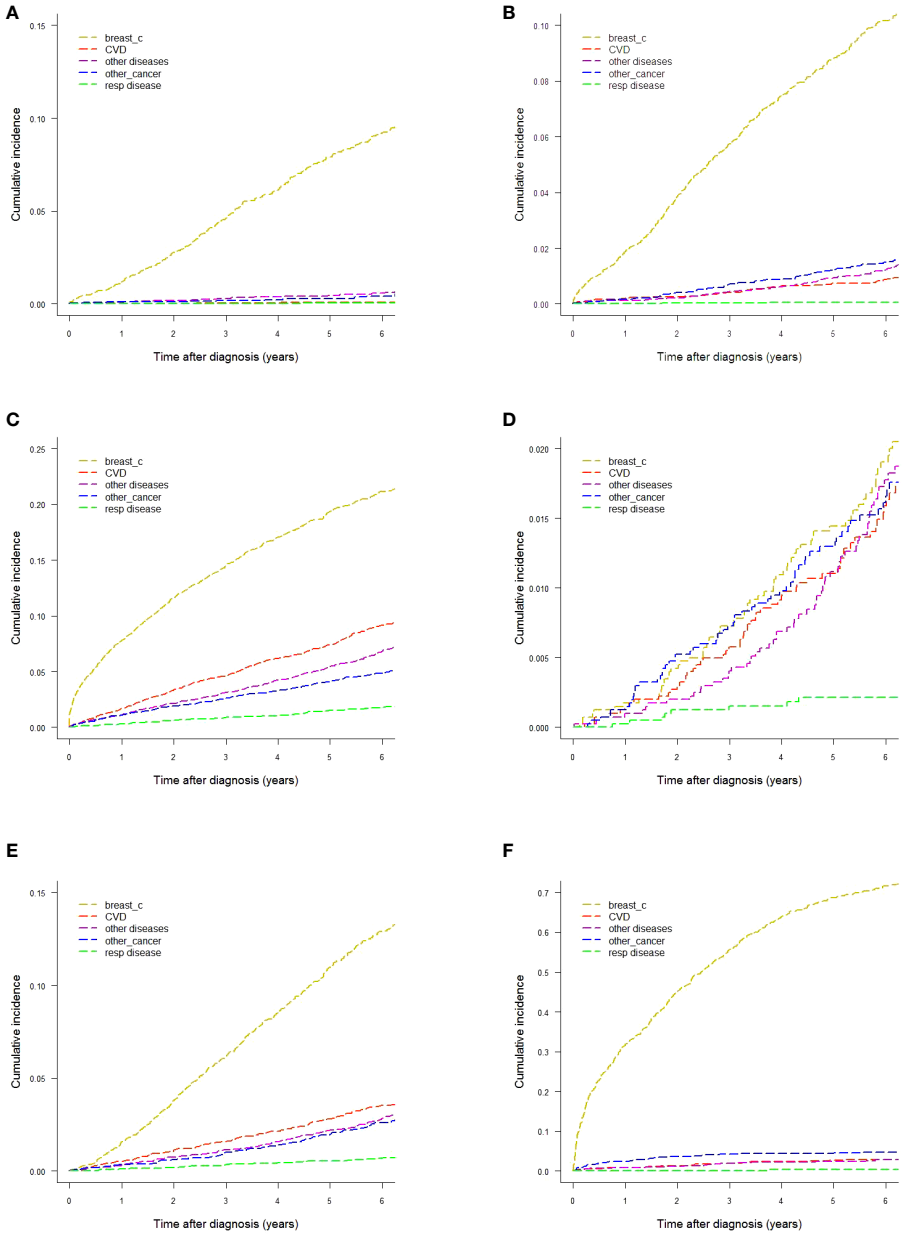

FIGURE 2
Cumulative incidence of mortality by different causes-of-death groups. **(A)** Age <50 years. **(B)** Age 50–69 years. **(C)** Age ≥70 years. **(D)** Women with stage I breast cancer. **(E)** Women with stage II–III breast cancer. **(F)** Women with stage IV breast cancer.

years from diagnosis. We performed a further analysis splitting CVD into cerebrovascular and other cardiovascular diseases for women diagnosed at age 50–69 and at age ≥70. (We excluded women <50 years because of the low CVD CSH in this age class.) The results are shown in Figures 3G, H. Differences by age were as follows: in women diagnosed at age 50–69, the CSH for other cardiovascular diseases peaked in the first year after diagnosis and both CVD subgroups showed a peak at the third year of follow-up and an increase in the last years of observation; in women diagnosed at age ≥70 the CSH for cerebrovascular disease showed a peak at the end of the first year after diagnosis, while the CSHs for the two CVD subgroups increased from the fifth year onward.

3.4 Comparison of CIFs and CSHs between registries

Gray's test for subdistribution hazards revealed statistically significant differences in CIF by registry for all five causes-of-death groups, prompting us to compute CIFs by registry (Supplementary Table S2). Interestingly, the highest CIF value among registries for all-cause deaths (26.49%) was almost 25% higher than the lowest (21.22%). The two highest CIFs for CVD (4.24% and 4.19%) were more than 70% higher than the lowest (2.43%). The highest CIF at six years for breast cancer (18.50%) was 2.2 times the lowest (8.16%). The CIFs for other cancers ranged from 1.46% to 3.60% and those for

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TABLE 2 Cause-specific hazards by years of follow-up for all ages and by age class.

Cause-specific hazards per 10,000 person years							
Deaths	1y	2y	3y	4y	5y	6y	95% CI at 6y
Cardiovascular disease							
All ages	65.18	68.21	56.68	75.46	58.87	87.75	68.42-112.55
<50	0.00	0.00	3.89	0.00	4.78	0.00	NA
50-69	16.65	3.78	13.75	21.68	9.76	14.58	6.07-35.03
≥70	170.33	205.61	159.18	218.96	186.01	302.01	232.96-391.54
Respiratory disease							
All ages	9.78	14.50	9.90	8.05	20.78	11.32	5.66-22.64
<50	0.00	0.00	0.00	0.00	4.78	0.00	NA
50-69	0.00	1.89	0.00	2.17	0.00	0.00	NA
≥70	28.79	42.18	31.84	23.58	68.74	42.39	21.20-84.76
Breast cancer							
All ages	351.18	267.74	252.81	230.40	219.31	179.75	151.06-213.90
<50	111.13	162.26	190.81	165.32	196.05	137.21	91.96-204.70
50-69	186.86	189.24	194.43	186.49	153.69	137.07	102.99-182.44
≥ 70	719.71	450.76	384.93	350.34	347.75	296.71	228.34-385.56
Other cancers							
All ages	47.26	42.63	45.88	36.22	56.56	58.03	42.73-78.81
<50	7.41	0.00	7.79	4.24	4.78	17.15	5.53-53.18
50-69	14.80	26.49	31.42	15.18	39.03	40.83	24.18-68.94
≥70	115.15	94.90	95.51	94.32	129.40	127.16	85.23-189.72
Other causes							
All ages	44.81	48.60	52.18	57.35	72.72	79.26	61.00-102.99
<50	7.41	11.32	11.68	8.48	9.56	11.43	2.86-45.72
50-69	11.10	15.14	21.60	19.52	39.03	29.16	15.69-54.20
≥70	112.75	121.26	127.34	154.96	181.96	233.13	173.49-313.28
All causes							
All ages	518.21	441.69	417.45	407.48	428.23	416.11	371.17-466.51
<50	125.94	173.58	214.17	178.04	219.96	165.79	115.21-238.58
50-69	229.42	236.55	261.20	245.03	241.51	221.65	177.02-277.53
≥70	1146.74	914.70	798.79	842.17	913.86	1001.41	868.35-1154.86

respiratory diseases from 0.14% to 2.28%. CIFs for the "other diseases" category ranged from 1.73% to 6.63%.

Similarly to the analysis of CIFs, computation of CSHs at the sixth year for CVD by registry (Supplementary Table S3) showed highest values (143.15 and 132.47) that were markedly higher than the lowest (21.70 and 68.18). For breast cancer the highest CSH was 294.54 and the lowest 90.90. For the category "other cancers" the highest CSH was 66.11 and the lowest 33.66. The highest CSH for respiratory diseases was 67.33, while the CSH for this disease category was 0 in three registries. Fine-Gray SHR models were run with the inclusion of a covariate for age and taking the cancer registry of Trapani as the reference (Supplementary Table S4). The risk excesses identified by the computation of SHRs for CVD, with highest values of 1.65 (1.06–2.58) and 1.63 (1.01–2.62), match the comparison of CIFs by registry, where the highest CIFs were more than 70% higher than the lowest. Computation of the other SHRs confirms the results obtained in the comparison between CIFs. Cox models were run including a covariate for age (Supplementary Table S5) and taking the cancer registry of Trapani as the reference. The highest CSHRs for CVD, 1.69 (1.08–2.63) and 1.64 (1.02–2.63),

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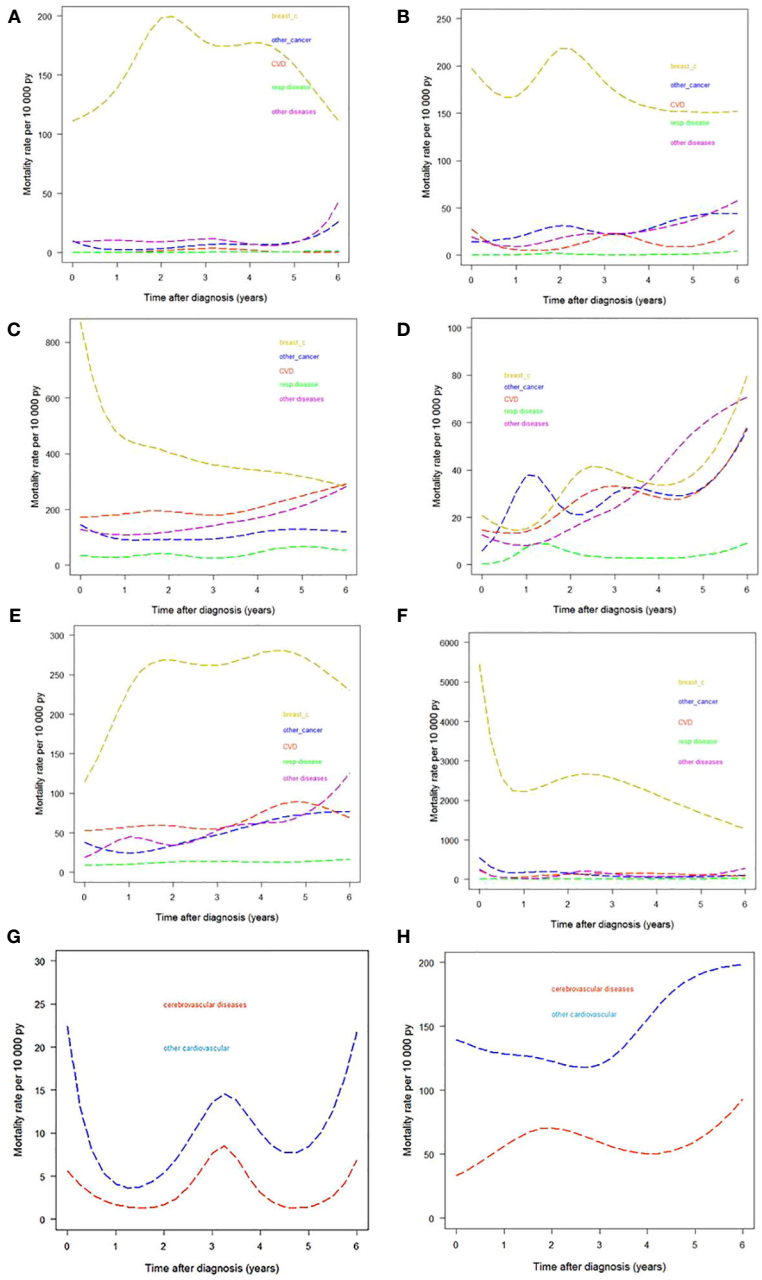

FIGURE 3
Cause-specific hazards by different causes-of-death groups. **(A)** Age <50 years. **(B)** Age 50–69 years. **(C)** Age ≥70 years. **(D)** Women with stage I breast cancer. **(E)** Women with stage II–III breast cancer. **(F)** Women with stage IV breast cancer. **(G)** CVD divided into cerebrovascular and other CVDs in women diagnosed at age 50–69. **(H)** CVD divided into cerebrovascular and other CVDs in women diagnosed at age ≥70. py, person years.

confirm the differences in CSHs by registry, where the highest CSHs were more than 70% higher than the lowest.

4 Discussion

This study was designed to give a complete epidemiologic picture of the event dynamics of causes of death in a competing-mortality setting analyzing absolute risks (CIFs) and hazards (CSHs) side by side. Important CIF variations for different causes of death were identified by age class and stage, and the relevance of causes of death other than breast cancer was demonstrated. Analysis of CSHs revealed marked variations during the follow-up period, identifying peaks that were useful to understand the development of all diseases that may affect women with a diagnosis of breast cancer. This information completes the analysis of CIFs, because it shows that diseases other than breast cancer that contribute significantly to the absolute risk of dying (CIF) do not act uniformly during the follow-up period but are more likely to present in specific moments in time.

These results support our hypothesis that side-by-side computation of CIFs and CSHs may provide a more complete epidemiologic picture of causes of death following a breast cancer

diagnosis. With regard to CIF (a measure of absolute risks), our results pointed in the same direction as studies using similar metrics. Clèries et al. (14), despite differences in the structure of their study with respect to ours, obtained similar results. For example, in women with stage I breast cancer we both found that the non-cancer mortality surpassed breast cancer deaths after some years from diagnosis. We are also in agreement with the main result of the study by Afifi et al. (12), according to which causes of death other that breast cancer (mainly heart and cerebrovascular diseases) account for a significant number of deaths among patients with a breast cancer diagnosis.

The computation of absolute risks allowed us to identify CVD as the second leading cause of death over a six-year follow-up with a CIF of 3.60%, amounting to 15.27% of CIFs for all causes. These results are similar to what Gernaat et al. reported in their review (3), according to which deaths from CVD ranged from 1.6% to 10.4%. Comparing our results with those of the studies included in the review is difficult due to differences in the composition of the cohorts; for example, the studies by Hooning et al. (6) and Solanki et al. (30) selected women with stage I–III breast cancer, while our cohort consisted of women with infiltrating breast cancer regardless of stage. In addition, the studies included in the review were performed in earlier periods than ours. Nevertheless, all of them identified CVD as the leading cause of competing mortality with respect to breast cancer. The study by Abdel-Qadir et al. (11) on women with a diagnosis of early-stage breast cancer concluded that CVD death is an important competing risk in older women with early-stage breast cancer, which is in agreement with our results.

The other principal metric we computed was CSH, analyzing it over time since diagnosis. To our knowledge only a paper by Colzani et al. (31) reported similar CSH computations. Like Colzani et al. we observed a breast cancer CSH peak in women aged <50 and 50–69 two to three years from the breast cancer diagnosis, but we failed to see such a peak in women aged ≥70 years. This difference may be due to the different selection criteria of the Colzani study, which included only women aged ≤75 years without metastases at diagnosis. We identified a peak in CSH for CVD in women aged 50–69 years, while Colzani et al. observed a peak in the 65–74-year age class. Demicheli et al. (32) identified peaks similar to ours when analyzing disease progression in breast cancer.

The results of this paper may also be useful to generate hypotheses about disease progression following a breast cancer diagnosis. Breast cancer metastases have been shown to appear at variable intervals (33), and two hypotheses have been proposed to explain this. The first postulated uninterrupted tumor growth, while the second hypothesized tumor progression as a discontinuous process alternating states of dormancy followed by rapid growth. In our study we did not analyze metastatic spread but mortality, which depends also on treatments after the occurrence of metastases. However, some common inferences might be drawn. Although our study was not designed to test these two hypotheses on progression, the presence of multiple peaks in the time trend of some CSHs and the discontinuities we observed favor the dormancy hypothesis. To analyze which factors prompt the onset of tumor growth, *ad hoc* studies will be needed.

The differences we observed in CIFs and CSHs could be explored by taking into account exposure to different diets and environmental pollutants. However, these aspects are beyond the scope of this paper, which aims to present the first population-based analysis in Europe using CSHs and CIFs. Further analysis will be performed on the dataset to explain risk factors.

A further aim of our study was to investigate the geographic variations of CIF and CSH. CIFs and CSHs showed remarkable differences. For example, the CIF for CVD in the cancer registry with the highest value was more than 70% higher than the CIF for CVD in the cancer registry with the lowest value. To our knowledge the only study that reported variations in cause-specific deaths according to geographic region was the one by Ho et al. (22). They drew similar conclusions to ours, identifying an association of geographic factors at breast cancer diagnosis with an increased CVD mortality risk. Since no studies have been published about the geographic distribution of causes of death other than breast cancer and CVD following a breast cancer diagnosis, we cannot compare our study to others in this respect. From a methodological point of view, this study confirms the notion, shared with many experts on prevention, that cancer is a disease showing marked geographical heterogeneity. The implications are relevant from a public health point of view because they highlight the need for nuanced and geographically specific rather than generalized policies, a belief we share with other authors (34). In the area we observed, further investigations are needed to address, for example, the death hazards for CDV in South Tyrol and Ragusa-Caltanissetta with respect to those of the Trapani province, and the breast cancer death rates of Trapani with respect to those of other provinces.

A limitation of our study is the presence of "R99" (Ill-defined and unknown cause of mortality) (n=115) among the ICD-10 mortality codes; however, its percentage, 0.9% of the whole cohort, can hardly be considered to invalidate the results. The analysis of cause-specific mortality may be challenging because of the possible misclassification of causes of death. A study in Italy (35) on a population-based cohort re-evaluated causes of death as classified by death certificates, revealing only a slight overestimate of deaths attributed to breast cancer. A study in Belgium (36) reported a fair agreement (84.7%) between death certificates and medical files for women with a breast cancer diagnosis treated at University Hospitals Leuven. A paper by Schaffar et al. (37) reported a high overall agreement comparing the causes of death by official death certificates and those revised by personnel of the population-based cancer registry of Genève (Switzerland). Considering the results of these studies we can assume that the bias related to misclassification of specific causes of death in our study was minimal.

A further limitation of the study is that information on breast cancer stage was available for only 71.7% of cases.

We decided to observe the follow-up until the sixth year from diagnosis in order to use the most up-to-date data available, which allowed us to describe the most recent epidemiology of the causes of death. However, this choice precluded us from observing what happened from the sixth year onward.

A major strength of our study is that for the first time, to our knowledge, CIFs and CSHs were analyzed side by side, enabling us to measure absolute risks and rate variations at the same time. A further strength is the population-based design, which allowed us to describe what happened in the general population of a vast area, taking into account women at whichever age and whichever breast

cancer stage. This prevented the introduction of selection bias and enhanced the generalizability of our results. Likewise, we analyzed for the first time in Europe and to our knowledge the second time worldwide geographic variations in the risks and rates of cause-specific mortality. Geographic variations in risks are very important to identify because they may point to a different distribution of modifiable risk factors. The availability of such information may prompt changes in the clinical follow-up of patients and indicate regional differences in the pathologic features of breast cancer. It can also direct etiologic research and be used in the planning of geographically tailored preventive and clinical strategies and resource allocation.

The results of this study also suggest considerations in the setting of tertiary prevention because of the weight of non-breast-cancer deaths among women with a breast cancer diagnosis. The first two causes of death we observed were breast cancer and CVD. The risk of disease progression in breast cancer is associated with modifiable factors such as cigarette smoking, obesity, metabolic syndrome and diabetes, high blood pressure and a sedentary lifestyle, with some studies also identifying a link with atmospheric particulate matter exposure (38–40). Breast cancer progression shares these risk factors with CVD and other cancers such as colorectal cancer, suggesting common prevention pathways. For example, in the setting of physical activity and exercise it has been proposed to extend the Cardiac Rehabilitation model to a Cardio-Oncology Rehabilitation (CORE) model (40), defined as "an exercise-based multicomponent intervention to improve the care and prognosis of a patient's cancer". The model encourages the use of existing, ready-to-use resources, including a network of professionals dedicated to cardiac rehabilitation.

The need to take into account causes of death other than breast cancer in women with a breast cancer diagnosis is also related to survivorship care models. Some authors (41–44) proposed the use of innovative models for effective clinical governance of survivorship care based on a possible role of the "community oncologist", defined as a trained health professional acting as a link between hospital specialists, who are frequently overburdened, and general practitioners. The results of our study encourage the application of new methods for the management of survivorship.

5 Conclusions

The results of our competing-cause analysis show that causes of death other than breast cancer are important in women with a diagnosis of breast cancer, especially women aged ≥50 years and those with stage I–III cancer, and that causes of death vary with time and also between registry areas. These results underscore the need for oncologists to balance the types and intensity of breast cancer treatment, taking into account possible cardiovascular and other side effects and the application of differential follow-up pathways. Furthermore, the observed geographic differences warrant research into the association of such differences with risk factors. The integrated interpretation of absolute risks and hazards highlights the necessity of surveillance and prevention by a multidisciplinary approach, and the need for community-based, holistic and well-coordinated survivorship care models.

Data availability statement

The datasets presented in this article are not readily available because In respect to privacy legislation the dataset could not be accessed. Requests to access the datasets should be directed to alessandro.borgini@istitutotumori.mi.it.

Ethics statement

The study was approved by the Ethics Committee of Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy. Record number 155/19.

Author contributions

AB: Data curation, Investigation, Validation, Writing – original draft. PC: Conceptualization, Formal Analysis, Methodology, Supervision, Writing – original draft. RB: Conceptualization, Writing – review & editing. SF: Data curation, Investigation, Validation, Writing – review & editing. AT: Data curation, Investigation, Writing – original draft. MM: Data curation, Validation, Writing – review & editing. FV: Data curation, Validation, Writing – review & editing. SE: Data curation, Validation, Writing – review & editing. AA: Data curation, Validation, Writing – review & editing. CC: Data curation, Validation, Writing – review & editing. LB: Data curation, Validation, Writing – review & editing. SM: Data curation, Validation, Writing – review & editing. GCas: Data curation, Validation, Writing – review & editing. RT: Data curation, Validation, Writing – review & editing. AF: Data curation, Validation, Writing – review & editing. PG: Data curation, Validation, Writing – review & editing. GCan: Data curation, Validation, Writing – review & editing. TS: Data curation, Validation, Writing – review & editing. MC: Data curation, Validation, Writing – review & editing. SB: Data curation, Validation, Writing – review & editing. GB: Investigation, Software, Writing – review & editing. VP: Validation, Writing – review & editing. CV: Validation, Writing – review & editing. FT: Validation, Writing – review & editing. MC: Validation, Writing – review & editing. GT: Conceptualization, Methodology, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1270877/full#supplementary-material

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Intraoperative parameters and postoperative follow-up of foam-based intraperitoneal chemotherapy (FBIC)

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Background: For decades, intraperitoneal chemotherapy (IPC) has been delivered into the abdominal cavity as a liquid solution. Recently the concept of foam as a carrier-solution for IPC was suggested. This *in-vivo* swine study aims to evaluate the safety, intraoperative parameters, limitations and postoperative complications of foam-based intraperitoneal chemotherapy (FBIC).

Methods: Three 65-day-old swine received FBIC with doxorubicin in a laparoscopy setting. Intraoperative parameters were monitored throughout the procedure and an extensive postoperative laboratory monitoring was conducted for 7 days. At day seven an autopsy was performed for further evaluation.

Results: The insufflation of FBIC caused a temporary rise in blood pressure and a simultaneous drop in heart rate. Capnography detected a continuous increase in end-tital CO_2 levels. A temporary drop of intraabdominal temperature was noted. Postoperative blood and serum laboratory results did not indicate any organ failure. No indication of intraperitoneal infections was noted and no structural tissue changes were visible in the autopsy.

Discussion: The application of FBIC appears to be a feasible approach regarding intraoperative anesthesiology and postoperative surgical management. A lack of postoperative structural changes on the seventh day were a promising sign of

safety and biocompatibility. Surgical reintervention would have been possible. To discuss a possible clinical application, further studies are required to investigate long-term safety, pharmacodynamics and the antitumoral potential of FBIC.

KEYWORDS

foam-based intraperitoneal chemotherapy, chemotherapy, doxorubicin, peritoneal metastasis, laparoscopy

1 Introduction

Decades of clinical and experimental research on the management of Peritoneal Metastasis (PM) have not significantly changed the overall poor prognosis rate. Due to the rapid progression of PM median survival rates are only about 3–4 months (Sadeghi et al., 2000; Goéré et al., 2017; Gelli et al., 2018). Patients suffering from PM endure a lot of morbidities. Significant contributions have been made in an attempt to improve the outcome of peritoneal surface malignancies. The current research focuses on a wide variety of topics such as molecular biology (Cheng et al., 2019; Shao et al., 2023), pharmacological enhancements (Schubert et al., 2019; Khosrawipour T. et al., 2020; Mikolajczyk et al., 2022) and clinically established concepts like HIPEC and cytoreductive surgery (Souadka et al., 2022; Patel et al., 2023). One of the main areas of PM research is the improvement and the better understanding of locoregional and intraperitoneal chemotherapy (IPC).

In the previous decades IPC has been delivered into the abdominal cavity as a liquid solution. However, a range of limitations were witnessed when "classic" liquid chemo solutions were used. Therefore, Pressurized intraperitoneal aerosol chemotherapy (PIPAC) had been proposed as a new and improved concept (Khosrawipour et al., 2018; Khosrawipour V. et al., 2020). Since then, the delivery of aerosolized chemotherapy has been used for a selected groups of patients who would otherwise not qualify for Hyperthermic intraperitoneal chemotherapy (HIPEC) and Cytoreductive surgery (CRS) (Khosrawipour et al., 2017). The advantage of PIPAC delivery is due to the physics behind its aerosolized application method (Göhler et al., 2017; Mikolajczyk et al., 2018a; Khosrawipour et al., 2019). Furthermore, a large amount of research has been conducted to try to further improve this technology (Lau et al., 2020; Diakun et al., 2022a; Diakun et al., 2022b; Diakun et al., 2022c; Hwang et al., 2022).

Foam has some unique characteristics. Some possible advantages of foam are slow degradation which allows for an extended drug contact time in the peritoneum. The expansion of foam is multidirectional and more homogenous. This means that the contact of a concentrated drug with peritoneal tissue can be expanded without diluting drug volume. Foam-based intraperitoneal chemotherapy (FBIC) could be a feasible option for the treatment of PM. Therefore, it has been proposed as a drug carrier for intraperitoneal chemo applications (Schubert et al., 2020). Our *in-vivo* study aims to evaluate FBIC as a new vehicle for IPC delivery. The focus of this study is about the consequences on the cardiovascular system, pulmonary system and the postoperative laboratory parameters. The effects of FBIC will be evaluated by postoperative data which will also be helpful for further clinical application.

2 Methods

2.1 The laparoscopic *in-vivo* swine model

The study includes three 65-day-old swine. A laparoscopy was planned, all swine were prepared for anesthesia and ventilation. The swine received an intramuscular injection of midazolam (0.3 mg/kg, WZF Polfa S.A., Warsaw, Poland), medetomidine (0.02 mg/kg, Cepetor 1 mg/mL, CP-Pharma Handelsgesellschaft, Burgdorf, Germany) and ketamine (9 mg/kg, Ketamine 100 mg/mL, Biowet Puławy sp. z o.o., Warsaw, Poland). Additionally, all swine received analgesia with propofol at 1 mg/kg. The swine were intubated, and anesthesia was continued with isoflurane 1%. Additional analgesia was provided with fentanyl 2 μg/kg. A continuous intravenous fluid line was established, and crystalloid fluid was given at 0,2–0,3 μg/kg/min. Before surgery all swine were placed in a supine position. An infra-umbilical mini laparotomy was performed. A 10 mm trocar (Kii®Balloon Blunt Tip System, Applied Medical, Rancho Santa Margarita, CA, USA) was inserted through the mini laparotomy. Under visual guidance A 5 mm trocar was placed at a distance to the first trocar. The abdominal cavity was insufflated with CO_2 to maintain a capnoperitoneum (Olympus UHI-3 insufflator, Olympus medical life science and industrial divisions, Olympus, Shinjuku, Tokio Japan). A full diagnostic laparoscopy was performed using a 5 mm camera system (Karl Storz 5mm/30° Laparoscope/Tuttlingen, Germany) (Figures 1A–C). After visual confirmation of no pathologies the "foam-insufflation" tube was introduced into the 10 mm trocar. After the tube was inserted and its positioning was confirmed via visual imaging. Afterwards the laparoscope was removed. Then a temperature probe was inserted through the trocar into the abdomen. The CO_2 from the capnoperitoneum was evacuated. For the measurement of the central body temperature, a temperature probe was placed in the esophagus. Another probe was placed outside the abdomen fixed with adhesive tape. An invasive arterial pressure line was placed and ECG electrodes monitored the heart rate.

2.2 Postoperative monitoring

One operative procedure was performed per day. All swine were kept together and were monitored for the next 7 days for behavior changes, feeding habits, indication of pain and surgical site infection. At postoperative days 1, 3 and 7 (1 d, 3 d, 7 d) blood was taken for a blood count and serological measurements. On the last postoperative day (7 d) an autopsy was performed (Figure 1D).

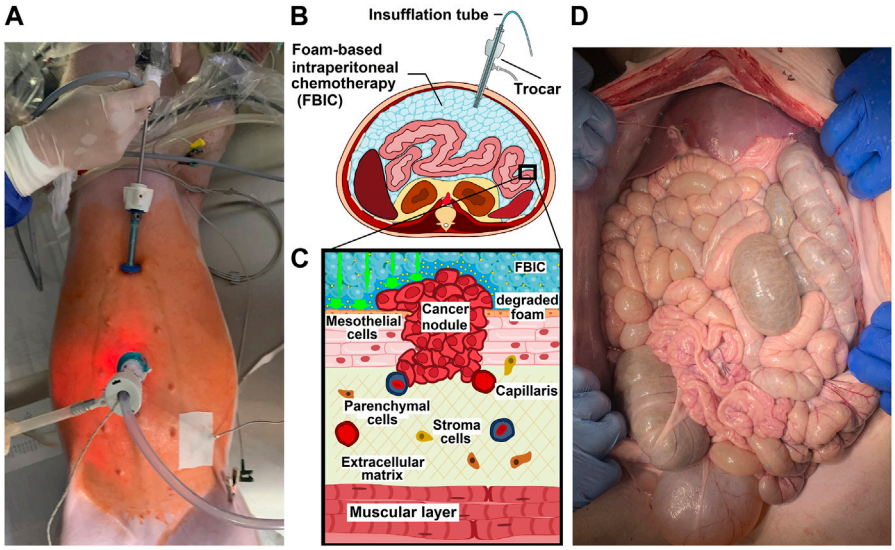

FIGURE 1
(A). Birds eye view onto a *in-vivo* laparoscopy while foam based intraperitoneal chemotherapy (FBIC) is applied. Disinfected operative field is colored red (iodine). Surgical entrance site is visible: a large 10mm trocar is placed periumbilical and a small 5 mm trocar is placed epigastral. Additional temperature sensors are placed to monitor temperature development during the procedure. **(B)**. Transversal section of FBIC delivery. **(C)**. Model of surface interaction of FBIC in the peritoneum. FBIC degradation on the peritoneal surface with highly concentrated doxorubicin fluid-film (large green-arrow). **(D)**. Birds eye view: laparotomy and exploration after autopsy at postoperative day 7.

2.3 Euthanization

The swine were premedicated with an intramuscular injection of midazolam (0.1 mg/kg, Midanium 5 mg/mL), medetomidine (0.02 mg/kg, Cepetor 1 mg/mL) and ketamine (8 mg/kg, Ketamine 100 mg/mL) mixture. After that, they were euthanized according to recommendations (Garber et al., 2011) with an intravenous injection by Sodium Pentobarbital with Pentobarbital (50 mg/kg with 12 mg/kg, Morbital 133,3 mg/mL + 26,7 mg/mL).

2.4 The bicarbonate-based foam carrier

The ratio of foam ingredients for the bicarbonate-foam were mathematically and experimentally predetermined. The basic chemical reactions were analyzed and quantified in order to determine the most suitable molar combination. The major components of foam are citric acid (Sigma-Aldrich, St. Louis, USA) and sodium bicarbonate (Sigma-Aldrich, St. Louis, USA). Doxorubicin hydrochloride (PFS®, 2 mg/mL, Pfizer, Sandwich, United Kingdom) was added as a chemotherapeutic component (1,5 mg/meter^2 body surface). Furthermore, iodide-based contrast media was used for CT (AccupaqueTM 350 mg J/ml, GE Healthcare, Chicago USA).

2.5 Statistical analyses

Experiments were independently performed. The statistical analyses were done with GraphPad Prism [GraphPad Software Inc., version 8.0.2 (263)]. Descriptive statistics included mean, median and percentiles. Probability (p) values were calculated via one factorial ANOVA (parametric) test results include: $^*p < 0.05$ and $^{**}p < 0.005$, and $\#p > 0.05$, with p-value <0.05 considered to be statistically significant.

2.6 Ethical approval and regulations

An Approval of the Local Board on Animal Care was obtained for the *in-vivo* swine experiments (UCHWALA NR 029/2021/P1) according to Polish regulations and European Union law. The data in the study displays a multi-stage study on *in-vivo* FBIC.

3 Results

The *in-vivo* experiments on the three swine were successfully conducted. No intraoperative or postoperative complications were detected. No major anesthesiologic complications were observed. There was no indication of anaphylaxis or cardiovascular collapse and no problems concerning intubation or extubation of the swine occurred. No (general) hypothermia or respiratory depression was noted. 30 min into the procedure all swine were successfully extubated. 20 min after insufflation a camera was inserted into the peritoneum in order to visualize the abdominal cavity. The intraabdominal foam did not interfere with the diagnostic overview. After a total of 30 min the trocars were removed and the mini laparotomy was sutured. No surgical problems or complications were observed during and after surgery. All animals survived the following postsurgical recovery. After the procedure all swine drank and ate adequately. No pain or behavioral changes were observed during the days of recovery.

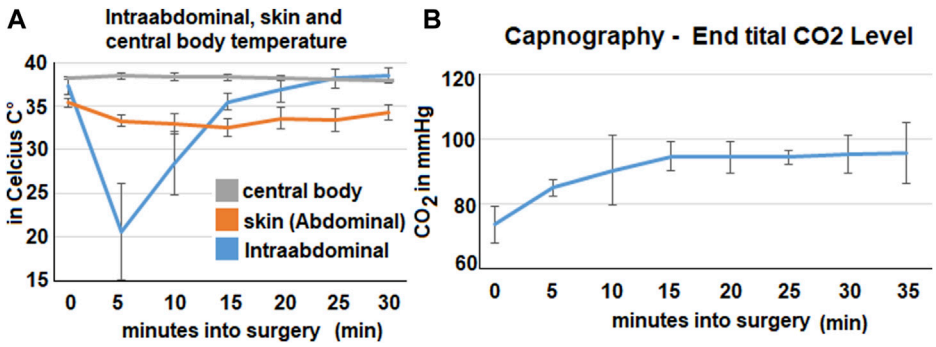

FIGURE 2
Intraoperative data from temperature probes, capnometry and respiratory rate. The mean values calculated from all three swine are represented by the data points. **(A)**. Data from the three temperature probes at various locations: skin probe on the abdomen, intraabdominal probe and central body temperature (esophageal probe). **(B)**. Expiratory CO_2 -levels in the capnometry in mmHg.

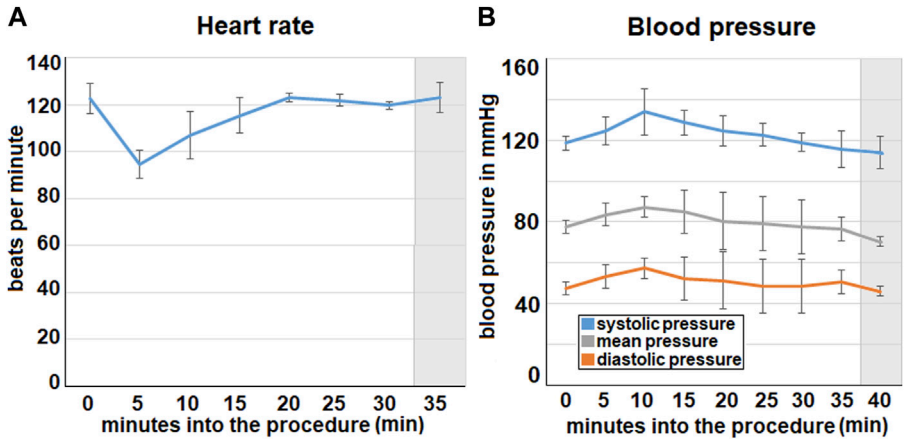

FIGURE 3
Intraoperative data concerning **(A)**. heart rate and **(B)**. blood pressure (via invasive measurement). The mean values calculated from all three swine are represented by the data points. **(A)**. Mean heart rate from three swine with the standard deviation. **(B)**. Mean blood pressure from all three swine with standard deviation. Extubating phase and postoperative monitoring are marked in grey.

3.1 Development of central body temperature, abdominal cavity and skin temperature

Within the cavity a rapid reduction of temperature can be observed during the insufflation of the bicarbonate foam. Within the first 5 min the medium temperature drops down to 20,6°Celsius and then steadily increases again (Figure 2A). After 15 min, it is above 35°Celsius. During the procedure the skin temperature on the abdomen decreases. It reaches its lowest point 15 min into the procedure when it drops to 32,5°Celcius (mean). After that it increases slowly and reaches around 35,4°Celsius (mean). During the whole procedure the central body temperature remains stable and does not change significantly.

The capnography shows an increase in mean end-tital CO_2 (Figure 2B). The initial level of 74 mmHg CO_2 increases to around 94–95 mmHg. This increase happens within the first 15 min and plateaus afterwards.

3.2 Results on heart rate and blood pressure

In the first 5 min of the procedure the mean heart rate decreases from a baseline of 123 ± 6 beats per minutes down to 95 ± 6. 20 min into the procedure the heart rate then increases again and reaches its initial level (Figure 3). After this the heart rate remains stable.

The blood pressure increases from the beginning of the foam insufflation and reaches its peak at about 10 min into the procedure where it is 134 ± 12 mmHg. After this the pressure declines continuously. This can be observed for the systolic, diastolic and the mean blood pressure.

3.3 Development of intraoperative and postoperative blood count

The red blood count could be gathered form the intraoperative measurements at 0 min and at 30 min into the procedure.

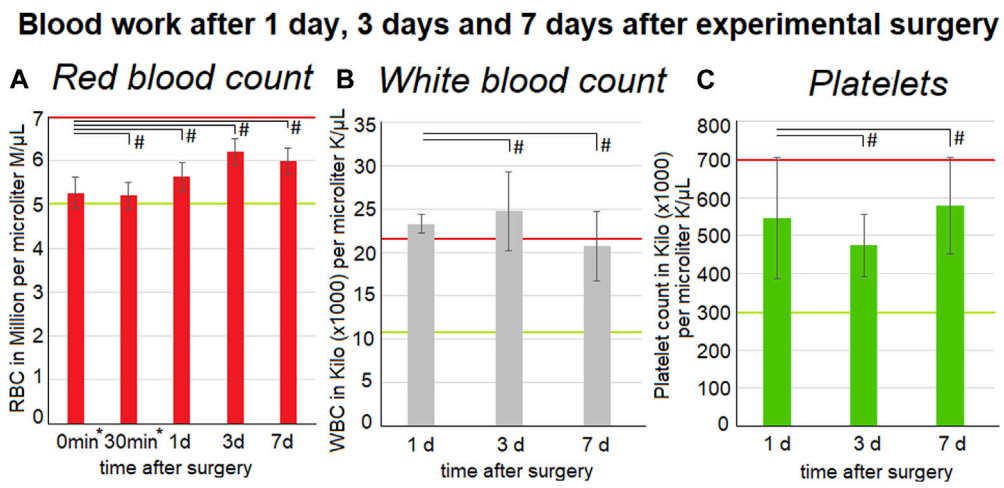

FIGURE 4
Blood work-up one, three and 7 days after surgery. The mean values calculated from all three swine are represented by the data points. Listed below are red blood cell count (left) **(A)** inclusive intraoperative measurements at 0* and 30* minutes into surgery and also white blood cell count (middle) **(B)** and platelet count (right) **(C)**. Medium and standard deviations are presented. Red and green lines indicate the normal reference levels (95% Interval - red: upper limited and green: lower limit) for the parameters. The *p* values are indicated as $*p < 0.05$, $**p < 0.005$, and $\#p > 0.05$, with *p*-value <0.05 considered to be statistically significant.

Furthermore, data was gathered on the first, third and seventh postoperative day. The red blood count did not change and remained within the reference levels of above 5 million per microliter. The white blood count remains at the upper reference level of around 22.000 per microliter. Although no significant changes could be observed there was a peak within the values on the third(3 d) postoperative day (Figures 4A–C). The postoperative platelet count varied a lot on the first (1 d) (5,5 ± 1,6) ×106/µl, 3 d (4,7 ±0,8) ×106/µl and seventh day (7d) (5,8 ± 1,3) ×106/µl. The platelet count did not exceed the upper reference which is around 3-7 ×106/µl.

3.4 Development of postoperative serum parameters

The kidney related parameters remained mostly stable. The creatinine level did not change significantly ($p < 0.05$) from the first day 0,7 ± 0,1 mg/dL to 7d 0,77 ± 0,15 mg/dL although a slight mean increase was noted. The maximum level for creatinine at 2,1 mg/dL was not reached (Figures 5A–C). The blood urea levels peaked on the third day with 13,7 ± 5,1 mg/dL but decreased again on the seventh day with 7 ± 1 mg/dL. The maximum physiological level for blood urea 30 mg/dl was not reached. While the white blood count was slightly above the upper reference range, the levels of C-reactive protein remained below the upper reference limit of ≤ 0,4 mg/dL. Therefore, no indications of an extensive tissue infection were present.

3.5 Development of postoperative liver parameters

The blood levels of two liver enzymes were measured. One was Alanine Aminotransferase (ALT) and the other one was Alkaline Phosphatase (ALP) (Figures 5D, E). The mean ALT level was at 81,7 ± 13,3 U/L for the first day, 85,7 ± 23,6 U/L for the third day and 87,7 ± 28,1 U/L for the seventh day. During these 3 days the ALT level was stable but still higher than the refence range of 9–43 U/L. No levels of ALT were available from before the procedure, so no comparison was possible.

The ALP level was at 284,7 ± 89,2 U/L for the first day, 302 ± 43,2 U/L for the third day and 260,7 ± 51,7 U/L for the seventh day. During all 3 days the levels of ALP were close to the upper reference level of 294 U/L. There is no indication of an increase or decrease of ALP levels during the observed interval. For the ALP no preoperative levels were available, so a comparison was not possible.

4 Discussion

The intraperitoneal administration of chemotherapeutic solution is an established treatment of PM. The local instillation of chemotherapeutic solution increases cell toxicity when it is in direct contact with the cancer nodules of the peritoneal cavity. Even though extensive attempts have been made to improve IPC delivery (Mikolajczyk et al., 2020; Buggisch et al., 2022; Khosrawipour et al., 2023a) limitations still exist in both liquid instillations as well as aerosol-based systems (Dedrick and Flessner, 1997; Sugarbaker and Ryan, 2012; Khosrawipour et al., 2016a; Bellendorf et al., 2018). These limitations include inhomogeneous drug distribution and limited penetration into PM. Novel substances have also been used for intraperitoneal delivery however some show limited drug tissue penetration (Mikolajczyk et al., 2018b). Therefore, new delivery methods have been presented to improve IPC (Buggisch et al., 2022; Hwang et al., 2022). For the patients to have a better outcome, new physical methods of drug delivery have been used to enhance the antitumoral effect of IPC (Khosrawipour et al., 2016b; Khosrawipour et al., 2023a). One of these new physical methods is FBIC (Schubert et al., 2020).

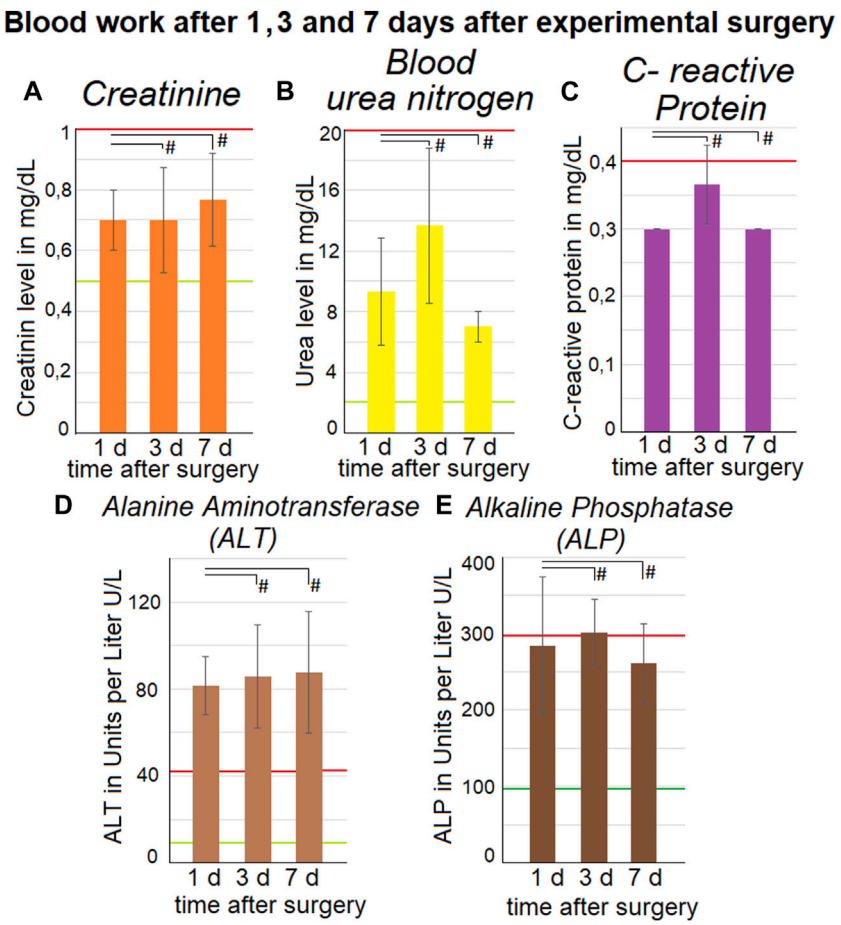

FIGURE 5
Blood work-up one, three and 7 days after surgery. The mean values calculated from all three swine are represented by the data points. Upper-section: listed are **(A)**. creatinine, **(B)**. blood urea level and **(C)**. C-reactive protein. Medium and standard deviations are presented. Liver parameters are listed in the lower section with **(D)**. alanine aminotransferase (ALT) and **(E)**. Alkaline Phosphatase (ALP). Mean and standard deviations are presented. Green lines indicate the lower limit and red lines indicate the upper limit for the parameters. The *p* values are indicated as $*p < 0.05$, $**p < 0.005$, and $\#p > 0.05$, with *p*-value <0.05 considered to be statistically significant.

In this study we were able to demonstrate that FBIC is a feasible concept. The data on intraoperative vital parameters, capnography, body temperature and respiratory rate indicate that the insufflation of FBIC is compatible with total anesthesia under external ventilation. Analyses of the postoperative blood work reveal no major changes on the red and white blood cell count and no changes on the platelet cell count. The postoperative time was limited to 7 days which shortened the observation of pathologies in the swine. However, within this period the large exposure to citric acid did not cause liver tissue necrosis.

Although the ALT was slightly above the upper reference range, ALP levels were not increased. This observation is relevant for FBIC because it shows that there is only limited damage to the liver. Citric acid at higher dosages can cause oxidative damage of hepatocytes by decreasing the activity of antioxidative enzymes. This has been studied on *in-vivo* mice models after intraperitoneal delivery with exposure to large amounts of citric acid (120–480 mg/kg-body weight) (Chen et al., 2014). However, no final conclusions regarding liver toxicity could be made because preoperative levels of ALT and ALP were not available. This needs to be reexamined in follow up studies.

CRP and white blood count show no signs of infection or inflammation. This is highly relevant as FBIC could have induced local inflammation, perforation or necrosis on large surfaces such as the peritoneum. This is important because of the effects that barium sulfate can have when it gets into contact with the peritoneal cavity. Although barium sulfate is a safe and non-toxic substance for oral intake it causes severe inflammation (Williams and Harned, 1991) upon entering the peritoneal cavity.

At the beginning, it was not possible to exclude a similar reaction on the peritoneal surface with the intraperitoneal delivery of FBIC. Due to this, it was important to evaluate the interaction of FBIC with the peritoneal surface. Therefore, on the seventh day the swine were autopsied. A thorough examination revealed no intraabdominal macroscopic pathologies nor signs of "changes" in the peritoneum (Figure 1D). This was also supported by histopathological analyses of the peritoneal tissue at various locations which is currently studied (Khosrawipour et al., 2023b).

The core body temperature did not change even though the intraabdominal temperature decreased. At this point it is important to mention that the chemical reaction of bicarbonate and citric acid is endotherm. Looking forward we want to establish a better understanding of the temperature and distribution profile of FBIC. These aspects have been studied for the HIPEC in more detail (Cianc et al., 2018).

However, based on the presented data we can assume that the bicarbonate carrier system does not cause significant local toxicity and inflammation.

Intraoperative and early postoperative systemic effects of FBIC could potentially be more critical than concerns over local tissue biocompatibility.

The limited number of swine for the pilot project were set by the animal ethic commission. We hope that the current results will enable us to receive approval for a larger study series where more parameters can be analyzed. The postoperative observation was limited to 7 days. Increasing the overall observation time to 30 days or even 6 weeks would be helpful for a more substantial evaluation of the procedure. However, the combination of FBIC with a surgical procedure such as partial intestinal resection with anastomosis is feasible and could be considered in a follow-up study.

After further studies potential systemic effects could be addressed. However, it is very promising that we do not observe any adverse reactions 7 days into the procedure. Although we only had three swine in this study no postoperative complications and kidney, liver and pulmonary failures could be observed 7 days postoperatively.

The safety of this method needs to be confirmed by other studies involving more subjects. The evaluation of toxicity in an *in-vivo* setting and the pharmacological and antitumoral potentials of FBIC must be studied as well.

5 Conclusion

The unique characteristics of foam might be significantly advantageous for IPC treatment in PM. From an anesthesiologic perspective, if intraperitoneal foam is applied the challenges involving abdominal expansion and intraperitoneal degradation are manageable. The early postoperative organ functions do not seem to be critically impaired by the particular carrier-system. Although the current results are encouraging further research needs to be conducted to evaluate pharmacological and antitumoral potentials of FBIC as well as safety beyond the postoperative period of 7 days.

Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Ethic Committee Name: Bioethics Committee of the Hirszfeld Institute of Immunology and Experimental Therapy Approval Code: 029/2021/P1 Approval Date: 19.05.2021r. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CK: Investigation, Methodology, Writing–original draft. JN: Conceptualization, Project administration, Writing–original draft. ZK: Conceptualization, Project administration, Writing–review and editing. PP: Writing–review and editing, Investigation, Methodology. BL: Investigation, Methodology, Writing–review and editing. SA-J: Conceptualization, Formal Analysis, Writing–review and editing. VK: Conceptualization, Methodology, Writing–original draft. SL: Conceptualization, Formal Analysis, Writing–review and editing. HL: Conceptualization, Formal Analysis, Writing–review and editing. JK: Software, Visualization, Writing–review and editing. AD: Investigation, Validation, Writing–review and editing. WK: Conceptualization, Supervision, Writing–review and editing. MC: Conceptualization, Methodology, Writing–review and editing. AM-M: Conceptualization, Investigation, Supervision, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision

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A synthetic notch (synNotch) system linking intratumoral immune-cancer cell communication to a synthetic blood biomarker assay

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Introduction: Cellular immunotherapy has greatly improved cancer treatment in recent years. For instance, chimeric antigen receptor (CAR) T cell therapy has been proven highly effective in treating hematological malignancies, and many CAR cell designs are being explored for solid tumors. However, many questions remain why responses differ across patients and some tumor types are resistant. Improved and relatively inexpensive ways to monitor these cells could provide some answers. Clinically, blood tests are regularly used to monitor these therapies, but blood signals often do not reflect the activity of immune cells within the tumor(s). Here, using the synthetic Notch (synNotch) receptor that tethers antigen binding to customized transgene expression, we linked intratumoral immune-cancer cell communication to a simple secreted reporter blood test. Specifically, we engineered immune cells with a CD19-targeted synNotch receptor and demonstrated that binding to CD19 on cancer cells *in vivo* resulted in the production of secreted embryonic alkaline phosphatase (SEAP) at levels that are readily detected in the blood.

Methods and Results: Jurkat T cells were engineered via sequential lentiviral transduction of two components: an anti-CD19 synNotch receptor and a synNotch response element encoding SEAP. Co-culture of engineered cells with $CD19^+$, but not $CD19^-$, Nalm6 cells, resulted in significantly elevated SEAP in media. Nod-scid-gamma (NSG) mice were subcutaneously injected with either $CD19^+$ or $CD19^-$ Nalm6 cells. Intratumoral injection of engineered T cells ($1x10^7$) resulted in significantly elevated blood SEAP activity in mice bearing $CD19^+$ tumors ($n = 7$), but not $CD19^-$ tumors ($n = 5$).

Discussion: Our synNotch reporter system allows for the monitoring of antigen-dependent intratumoral immune-cancer cell interactions through a simple and convenient blood test. Continued development of this system for different target antigens of interest should provide a broadly applicable platform for improved monitoring of many cell-based immunotherapies during their initial development and clinical translation, ultimately improving our understanding of design considerations and patient-specific responses.

KEYWORDS

cancer, immunotherapy, blood test, synthetic biology, synthetic notch receptor, secreted reporter gene

1 Introduction

Cell-cell communication plays a vital role in human development, homeostasis, and pathogenesis (Schultz, 1985; Wilson et al., 2000; Trosko and Ruch, 2003). The advent of therapeutic cells specifically designed to interact and communicate with diseased cells has ushered in a new era in the treatment of numerous medical conditions. This approach has already shown promising results for infectious diseases (Zumla et al., 2016), immunologic deficiency syndromes (Arnold and Heimall, 2017; Schlabe and Rockstroh, 2018), neurodegenerative and movement disorders (Lunn et al., 2011), as well as cancer (June et al., 2018; Mehta et al., 2018).

T cells have been extensively used in cell-based cancer immunotherapies due to their cytotoxic capabilities, and the ability to home to and proliferate within tumors upon adoptive transfer (Sadelain et al., 2003; Porter et al., 2011; Maude et al., 2014; Weber et al., 2020). Specifically, T cells can elicit cytotoxic effects through interactions between endogenous or engineered receptors with molecular targets on cancer cells (Grakoui et al., 1999; Huppa and Davis, 2003). Although these therapies have been transformative for the treatment of numerous malignancies, their ineffectiveness in some individuals often stem from poor tumor homing, a hostile tumor microenvironment, tumor heterogeneity, and antigen loss or escape (El-Sayes et al., 2021; Miao et al., 2021). Moreover, on-target/off-tumor toxicities can sometimes result in detrimental and even life-threatening side effects (Zhang et al., 2016; Graham et al., 2018; Cao et al., 2019; Penack and Koenecke, 2020; Akhoundi et al., 2021). To better understand the effects of these therapies in individual patients, assays to monitor therapeutic cells over time would be of great benefit to develop safer and more robust immunotherapies.

Affordable and minimally invasive blood assays offer convenient ways to monitor *in vivo* biological events. For instance, blood tests using quantitative PCR or flow cytometry have been used to directly monitor the levels and persistence of adoptively transferred immune cells over time (Demaret et al., 2021). However, there is often a disconnect between circulating blood measures of immune cell numbers and how many there are in tumors, the site of action. For instance, quantitative measures of intratumoral chimeric antigen receptor T (CAR-T) cells using PET reporter gene imaging was shown to not correlate with blood levels of CAR-T cells in mice (Minn et al., 2019). Moreover, current blood assays do not easily reflect the activity and behavior of intratumoral T cells. Here we sought to develop an assay that links antigen-specific T cell interactions with cancer cells within tumors to an easily measurable synthetic biomarker in the blood. Specifically, we leveraged an activatable synthetic biology system called the synthetic Notch (synNotch) receptor to relay intratumoral immune cell-cancer cell communications into the blood activity levels of a secreted reporter gene.

The synNotch system was described in a series of papers in 2016 as a general platform for building novel cell-cell contact signaling pathways (Roybal et al., 2016a; Morsut et al., 2016; Cho et al., 2018). This system is composed of both the synNotch receptor and the synNotch response element (RE), which are co-engineered into the same cell. Traditionally, like clinically-used CARs, the extracellular domain of the synNotch receptor is comprised of the single-chain variable fragment (scFv). Upon binding to the target antigen, this interaction triggers the cleavage of the GAL4-VP64 intracellular domain of the receptor. Subsequently, the GAL4-VP64 fusion protein binds to the upstream activation sequence (UAS) located within a minimal promoter. This binding event leads to the activation of transcription, thereby driving the expression of transgenes of interest encoded in the RE. By maintaining the Notch core regulatory region but appending a customized extracellular input recognition and intracellular output transcription activator module, novel cell-to-cell contact signaling pathways that carry out user-defined functionalities can be established (Roybal et al., 2016a). We have recently linked synNotch antigen binding with the transcriptional activation of imaging reporter genes to monitor immune cell-cancer cell communication *in vivo* with both bioluminescence and clinically relevant magnetic resonance imaging (Wang et al., 2023). Similarly, a next-gen synNotch system has been linked to positron emission tomography (PET) reporter gene expression and antigen-activation has been detected using PET (Shin et al., 2023). However, while imaging provides important spatial information, the ability to perform repetitive imaging can be expensive, especially in patients. To complement our imaging system, we developed a synNotch system where the output is a convenient blood assay using a human-derived secreted reporter gene called secreted embryonic alkaline phosphatase (SEAP) (Figure 1A).

SEAP stands out as the most widely utilized secretable reporter protein due to its distinct characteristics. It is a truncated form of human placental alkaline phosphatase that is expressed specifically during embryogenesis, with minimal post-natal expression (Berger et al., 1988; Tannous and Teng, 2011; Ronald et al., 2015), rendering it a highly specific blood reporter. SEAP exhibits heat stability, specifically, the heating of serum samples to 65°C enables its selective detection in assays without detecting other phosphatases that may be present in the blood (Bronstein et al., 1994; Wang et al., 2001). Commercial SEAP detection assays are also extremely sensitive over at least a 4-log order concentration range, with detection limits in the picogram per milliliter range. Additionally, murine SEAP showed no immunogenic potential in mice, implying that human-derived SEAP is safe for clinical translation, and has already been successfully used in the clinic (Wang et al., 2001; Kemp et al., 2008). For the antigen target of our synNotch receptor we chose CD19 as a proof-of-concept due to its effectiveness as a target for CAR-T cell therapies against B cell leukemia and lymphomas (Davila et al., 2015; Hay and Turtle, 2017).

In this study we developed and validated a novel synNotch system to provide specific readouts of intratumoral CD19-triggered immune-cancer cell communication by measuring blood SEAP activity levels. Continued development of this cell-cell communication blood assay, and merger with other readouts such as imaging, has the potential to provide extended insight on the efficacy of T cell immunotherapy during both its development and clinical integration.

2 Materials and methods

2.1 Lentiviral design and production

A lentiviral transfer plasmid encoding encoding an anti-CD19 synNotch receptor driven by pPGK was acquired from Addgene (pHR_PGK_antiCD19_synNotch_Gal4VP64 was a gift from Wendell Lim; Addgene plasmid # 79125; http://n2t.net/addgene:79125; RRID:Addgene_79125).

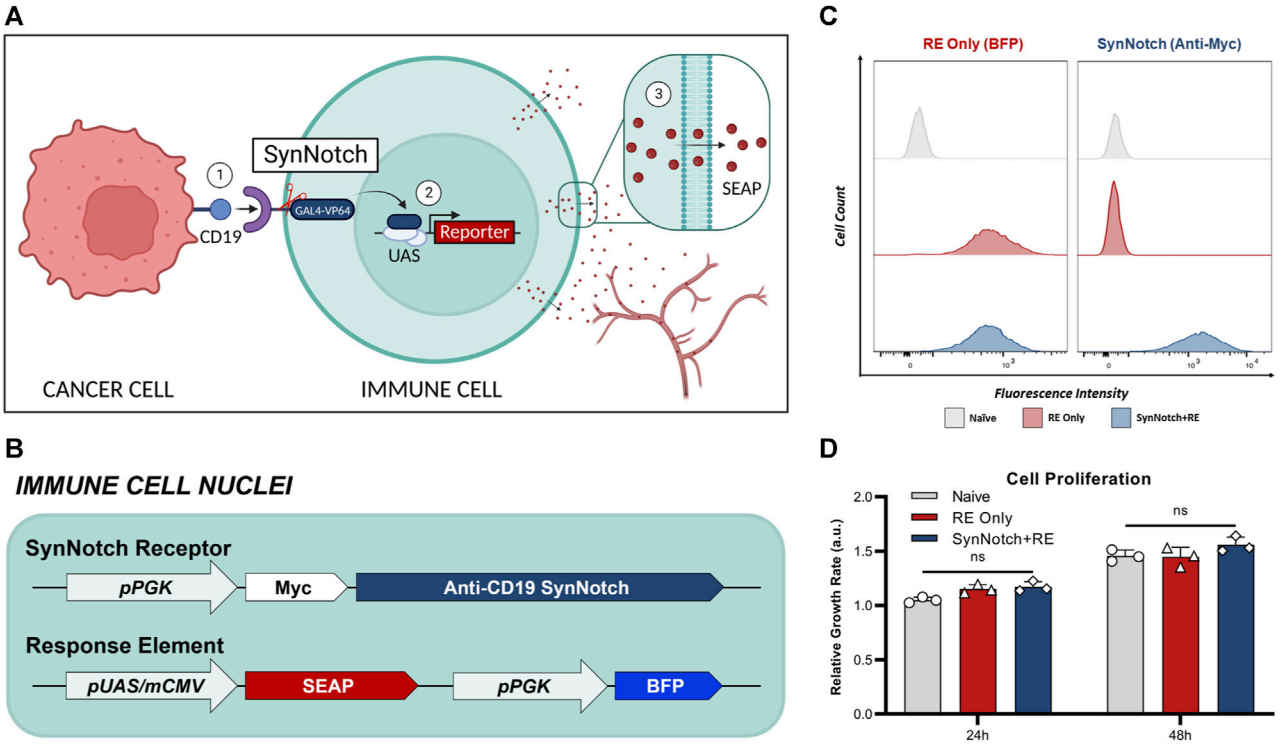

FIGURE 1
Engineering of T cells with blood-based reporter system for cell-cell communication. **(A)** Schematic of synNotch system. The synNotch receptor has three components: the antigen-targeting extracellular domain; a core regulatory region that controls cleavage of the receptor upon antigen interaction; and an intracellular domain comprised of a GAL4-VP64 DNA-binding/transcriptional activator fusion protein[26]. Upon binding of the anti-CD19 synNotch receptor in T cells to CD19 on an adjacent cancer cell, GAL4-VP64 is cleaved from the synNotch receptor and can bind the upstream activation sequence (UAS) of a minimal promoter in the response element (RE) to drive expression of transgenes of interest[53]—such as secreted embryonic alkaline phosphatase (SEAP). *In vivo*, SEAP can diffuse into the bloodstream and measured using a luminescence-based assay in blood samples. **(B)** T cells were first transduced with a response element (RE) containing SEAP and a constitutively expressed blue fluorescence protien (BFP) driven by the phosphoglycerate kinase 1 promoter (pPGK) for cell sorting. **(C)** Flow cytometry of naïve and twice transduced T cells post-sorting, assessing the expression of RE (BEP^+) and synNotch (Myc^+). **(D)** Relative growth rates of T cell populations at 24- and 48-hours post-seeding (n = 3). Data presented as meam ± SD.

A response element (RE) construct was made by also acquiring a lentiviral transfer plasmid containing the GAL4-VP64 inducible upstream activation sequence from Addgene (pHR_5x Gal4 UAS was a gift from Wendell Lim (Addgene plasmid # 79119; http://n2t.net/addgene:79119; RRID:Addgene_79119). The SEAP transgene from our previously developed plasmid pSurvivin-SEAP-WPRE (Wang et al., 2021) was cloned into pHR_5x GAL4 UAS using In-Fusion HD cloning (Takara Bio, CA, United States) to make pHR_5x Gal4 UAS SEAP. A constitutively expressed blue fluorescence protein (BFP) driven by the phosphoglycerate kinase promoter (pPGK) was inserted further downstream of the same reporter cassette via the same cloning kit to make pHR_5x Gal4 UAS SEAP pPGK BFP.

A second-generation lentiviral packaging plasmid (pCMV delta R8.2, #12263), and envelope plasmid (pMD2. G, #12259) were acquired from Addgene (pCMV delta R8.2 and pMD2. G were gifts from Didier Trono (Addgene plasmid # 12263; http://n2t.net/addgene:12263; RRID: Addgene_12263 and Addgene plasmid # 12259; http://n2t.net/addgene:12259; RRID:Addgene_12259). Lentiviral production involved co-transfection of transfer, packaging, and envelope plasmids into human embryonic kidney (HEK 293T) cells using Lipofectamine 3,000 according to the manufacturer's instructions (ThermoFisher Scientific, MA, United States). Cell supernatant containing lentivirus was collected at 24 and 48 h, filtered through a 0.45 µm filter, concentrated using a Lenti-X Concentrator (TakaraBio), and stored at −80°C prior to transduction.

2.2 Cell culture and engineering

Human Jurkat T cells (clone E6-1) and human $CD19^+$ Nalm6 lymphoblastic leukemia cells (clone G5) were purchased from ATCC (VA, United States). Cells were grown in RPMI-1640 medium (Wisent Bioproducts, QC, Canada) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 5% (v/v) Antibiotic-Antimycotic at 37°C in 5% CO2. The absence of *mycoplasma* contamination in cell cultures was frequently validated using the MycoAlert *Mycoplasma* Detection Kit (Lonza, NY, United States).

The generation of engineered T cells involved the initial transduction of naïve (non-engineered) Jurkat cells with the pHR_5x Gal4 UAS SEAP pPGK BFP lentivirus with 8 µg/mL polybrene for 6 h. Cells that expressed BFP were deemed "RE only" cells and were sorted using a FACSAria III fluorescence-activated cell sorter (BD Biosciences, CA, United States; RRID:SCR_016695). Sequentially, RE only cells were transduced with pHR_PGK_antiCD19_synNotch_Gal4VP64

lentivirus, again with 8 μg/mL polybrene for 6 h. Cells that were both Myc- and BFP-positive were sorted to obtain "SynNotch + RE" cells. The expression of each engineered component was validated pre- and post-sort by staining Jurkat cells with an anti-Myc antibody (#2233S, NEB, MA, United States; RRID:AB_823474), followed by performing flow cytometry on a FACSCanto (BD Biosciences; RRID:SCR_018055). All flow cytometry results were analyzed using FlowJo v10 software (FlowJo LLC, BD Biosciences; RRID:SCR_008520).

As SEAP expression is activatable, T cells were also engineered with lentivirus to constitutively express zsGreen and Gaussia Luciferase (GLuc), the latter of which is also a secreted reporter protein detectable in culture media. This allowed the relative number of live cells in culture to be assayed at the same time as SEAP.

We used CRISPR/Cas9 to generate CD19$^-$ Nalm6 cells, as previously described (Wang et al., 2023).

2.3 *In vitro* validation

CD19-targeted SynNotch + RE, RE only, and naïve Jurkat cells were co-cultured with either CD19$^-$ or CD19$^+$ Nalm6 cells. Within each well of a 96-well round bottom plate, 10^5 T cells were seeded with equal number of Nalm6 cells in a total volume of 125 μL media per well. Co-cultures were then centrifuged for 5 min at 400 × g to encourage cells to come into proximity. Prior to collecting media on each day, plates were spun down at 400×g for 5 min, then 120 μL of media was collected from each well, fresh media was added, and plates were centrifuged again. The collected supernatant was centrifuged at 10,000×g for 10 min and stored at −20°C until assayed. SEAP activity in supernatant (25 μL) was measured using the Great EscAPe SEAP Chemiluminescence Assay kit 2.0 (Clontech, Fremonet, CA). The GLuc activity in supernatant (20 μL) was measured using the Gaussia Luciferase Assay reagent (Targeting Systems, San Diego, United States). The luminescence signal from both assays was measured using the Glomax 20/20 luminometer from Promega (Madison, WI).

2.4 *In vivo* evaluation in tumor model

All animal procedures were performed as approved by the University Council on Animal Care at the University of Western Ontario (Protocol #2020–025) and follow the Canadian Council on Animal Care (CCAC) and Ontario Ministry of Agricultural, Food and Rural Affairs (OMAFRA) guidelines. For all animal work, 4–6 weeks old female NOD. Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice were obtained from an in-house breeding colony at Western University. These immunodeficient mice were chosen to establish tumors expressing either CD19$^+$ or CD19$^-$ phenotypes. Tumors were initiated by subcutaneously injecting 10^6 CD19$^+$ (n = 7) or CD19$^-$ (n = 5) Nalm6 cells in 50 μL of PBS and 50 μL of Matrigel (Corning, NY, United States) into the right flank of each mouse. Tumor volumes were periodically calculated using caliper measurements and the following formula:

$$V = \frac{\pi}{6}(l \times w \times h)$$

Once tumors reached ~100 mm^3 (~3–5 weeks post inoculation), mice received an intratumoral injection of 10^7 SynNotch + RE T cells suspended in 100 μL of PBS (Day 0). On days −1, 2, 4, and 7 post-delivery of immune cells, 70 μL of blood was collected from the saphenous vein from each mouse and stored in blood-collection tubes containing heparin and gel-barrier for anticoagulation and plasma separation (Becton Dickinson, ON, CA). Immediately following the blood collection, each blood sample was centrifuged at 10,000 × g for 10 min to isolate blood plasma. SEAP assays were performed on 25 μL of the isolated plasma for each mouse, following the SEAP assay protocol described above.

2.5 Histology and immunostaining

At the study endpoint, mice were euthanized using an overdose of isoflurane. Each mouse was pressure perfused via the left ventricle using a solution of 4% paraformaldehyde (PFA). Tumors were carefully excised, submerged in 4% PFA for a period of 24 h, and subsequently stored in PBS at 4°C before being prepared for sectioning and staining. To facilitate sectioning, the tumors underwent a series of sucrose gradients ranging from 10% to 30% and were then frozen using optimal cutting temperature medium (Sakura Finetek). Ten-micron sections were obtained, fixed in 4% PFA for 10 min at room temperature, and stained with DAPI. Fluorescence images of zsGreen (T cells) and DAPI were acquired using an EVOS FL Auto 2 microscope (ThermoFisher).

2.6 Statistics

All statistical analysis was performed using GraphPad Prism 9.0 software (GraphPad Software Inc., CA, United States; RRID: SCR_002798). For *in vitro* cell proliferation assay and SEAP activity, a two-way ANOVA followed by Tukey's multiple comparisons test was used. For quantifying total *in vitro* SEAP activity over 4 days, area under the curve calculations were performed. A one-way ANOVA followed by Tukey's multiple comparisons test was used to assess GLuc activity across samples. In the case of the *in vivo* SEAP assay, a two-way ANOVA followed by Tukey's multiple comparisons test was used for analysis. Differences between groups in the *in vivo* SEAP assay data, represented as area under the curve, were measured using an unpaired *t*-test. A nominal *p*-value of less than 0.05 was considered significant for all statistical analyses.

3 Results

3.1 Engineering of immune cells with synNotch blood reporter system

Naïve T cells were first engineered with a response element (RE) encoding the secreted reporter gene SEAP (RE only cells), and then a subset of these were sequentially engineered with a synNotch receptor targeted to CD19 (SynNotch + RE cells) (Figures 1B, C). Following cell sorting, 98% of engineered cells expressed their intended components, no notable differences in BFP was observed between RE only and synNotch + RE populations (Figure 1C). No significant difference in proliferation rates over 48 h were found when comparing naïve, RE and SynNotch + RE T cells (Figure 1D).

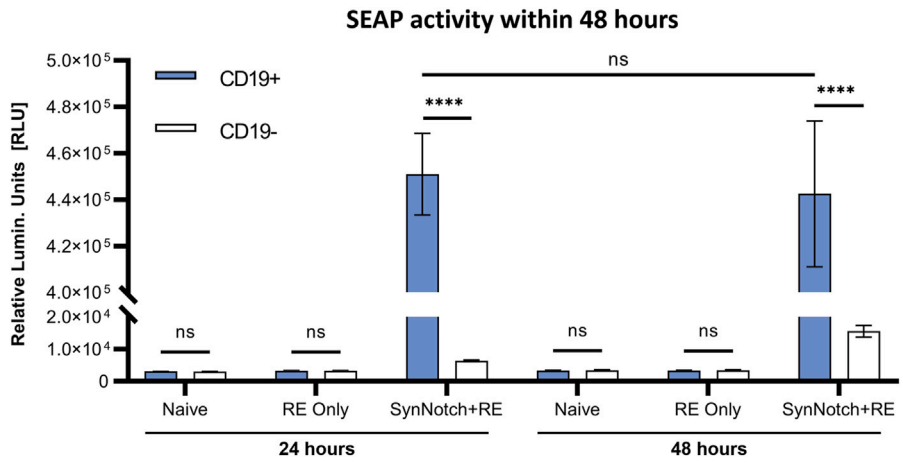

FIGURE 2
In vitro assessment antigen-specificity of synNotch secreted reporter system. SEAP activity values obtained from co-culturing naive, RE only, and SynNotch + RE T cells with CD19$^+$ and CD19-cells at 1:1 ratio (n = 3) at 24 h and 48 h respectively, **** represent $p < 0.0005$, ns represent No Significant difference. Data are presented as mean ± SD.

3.2 SEAP activation via synNotch receptor based on antigen-dependent cell-cell interactions

To first evaluate our system, SEAP activity in media over time was measured in co-cultures of SynNotch + RE, RE only, or naïve T cells with CD19$^+$ or CD19$^-$ Nalm6 leukemia cells at an effector: target (E:T; T cell:cancer cell) ratio of 1:1 (Figure 2). SEAP activity was significantly higher in CD19$^+$ *versus* CD19$^-$ co-cultures at both 24 and 48 h ($p < 0.0005$), and SEAP was elevated to the same degree at both time points (Figure 2C). Co-cultures of CD19$^+$ or CD19$^-$ Nalm6 cells with RE Only or naïve T cells did not show any significant increases in SEAP activity (Figures 2A, B). Compared to these other control conditions, a marginal but non-significant increase in SEAP activity was seen when SynNotch + RE cells were co-cultured with CD19$^-$ Nalm6 cells.

Next, to model the sensitivity of this system, we varied the E:T ratio for co-cultures of SynNotch + RE cells with CD19$^+$ Nalm6 cells and assessed SEAP activity over 4 days (Figure 3). First, we kept a constant number of target Nalm6 cells (10 (Arnold and Heimall, 2017) cells) and looked at the impact of increasing the number of effector cells in the co-cultures on SEAP activity in media (Figure 3A). T cells constitutively expressed GLuc, and the increases in effector cell number were confirmed via increasing GLuc activity measurements in media. Cumulative SEAP activity also increased linearly with the number of effector T cells ($R^2 = 1.0$), with SEAP activity plateauing beyond 5×10^4 effector cells (an E:T of 0.5:1; Figure 3A). In opposition, when increasing the number of target Nalm6 cells while keeping effector numbers constant (10 (Arnold and Heimall, 2017) cells; the GLuc activity remained constant), the total SEAP activity increases with greater target number, however this time plateauing at 10^4 target cells (an E:T of 1:1; Figure 3B).

3.2 Antigen-dependent synNotch activation in tumors results in increased blood SEAP activity

To evaluate the synNotch system *in vivo*, subcutaneous CD19$^+$ or CD19$^-$ Nalm6 tumors were formed on the right flank of 4–6 weeks old female nod-scid-gamma mice. Once tumors were ~100 mm^3, 10^7 SynNotch + RE T cells were injected intratumorally, blood samples were collected, and SEAP activity in blood was measured over time (Figure 4A). Prior to T cell delivery, SEAP activity was at assay background levels for both CD19$^+$ and CD19$^-$ groups (Figure 4B). At 48 h post-T cell injection, mice carrying CD19$^+$ tumors showed significantly elevated blood SEAP activity compared to their CD19$^-$ counterparts ($p < 0.05$). SEAP activity at 96 and 168 h in the CD19$^+$ cohort decreased significantly from 48 h but remained significantly higher that from the CD19$^-$ cohort (Figure 4B). TO measure the total SEAP production over 7 days, area under the curve measurements of SEAP activity over time was calculated from the longitudinal data in Figure 4B and was significantly higher for the mice bearing CD19$^+$ *versus* CD19$^-$ tumors ($p < 0.01$; Figure 4C).

At endpoint, fluorescence microscopy was utilized to visualize zsGreen-positive T cells. Qualitatively, a similar number of zsGreen cells were observed in both tumor cohorts (Figure 5). This finding suggests that differences in SEAP expression were not attributable to insufficient delivery or survival of the engineered T cells across tumor types. Instead, it provides evidence supporting the persistence of the T cells post-delivery for up to 7 days.

4 Discussion

It is attractive to utilize and customize a cell's ability to translate extracellular triggers into the intracellular signals, as this would allow

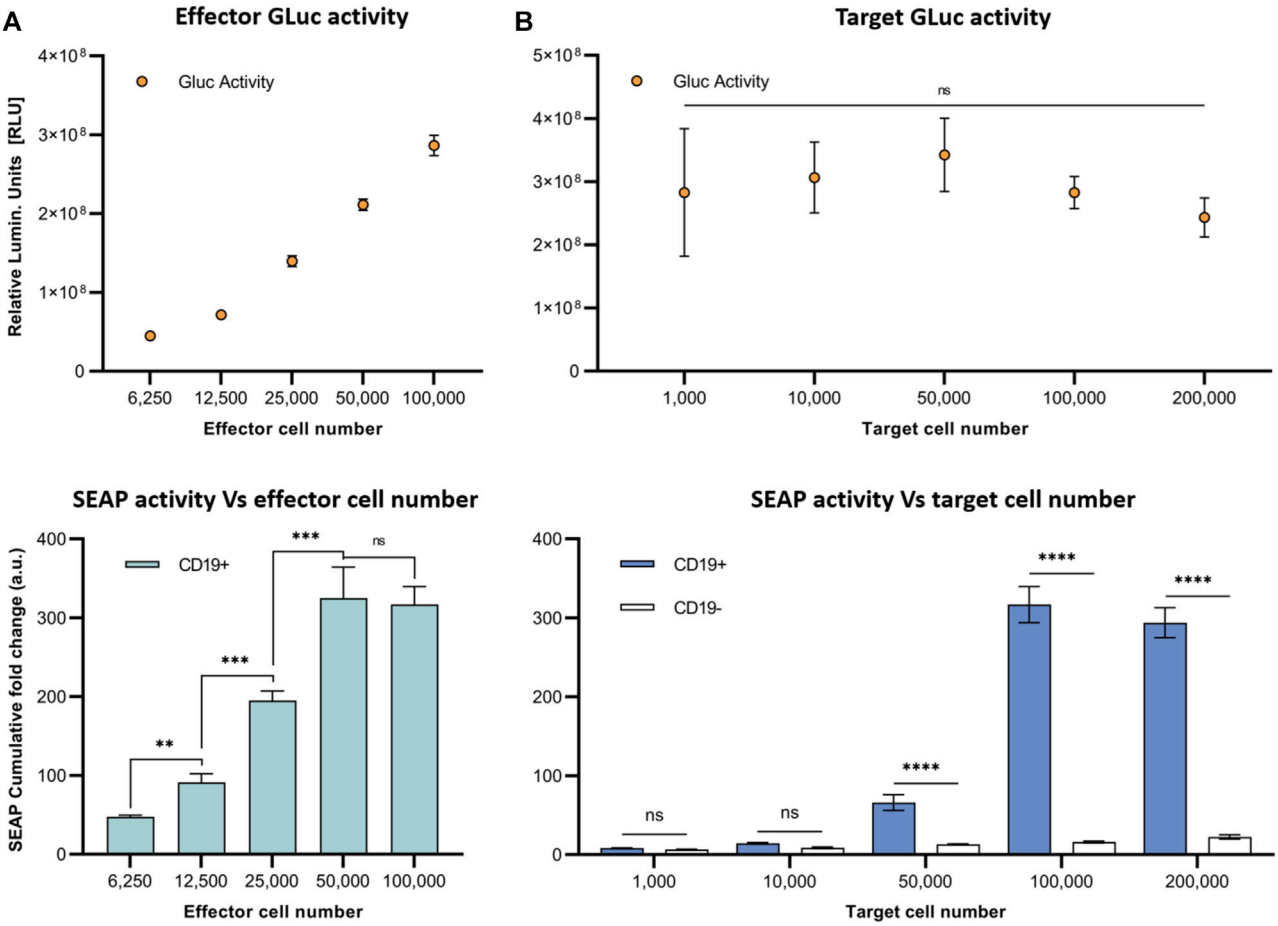

FIGURE 3
In vitro assessment of synNotch secreted reporter system at varying effector and target cell ratios. **(A)** Respective GLuc and SEAP activity at varying effector cell numbers. **(B)** Respective GLuc and SEAP activity at varying target cell numbers. Data presented as cumulative GLuc activity measured as the relative luminescence units (Top). SEAP activity obtained from SynNotch + RE T cells (effector cells) co-cultured with CD19$^+$ cells (target cells) at varying effector cell numbers. Data presented as cumulative SEAP activity fold changes over 4 days (Bottom). Data are presented as mean ± SD (n = 3). (****$p < 0.0005$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns for No Significant difference).

for the development of better diagnostics, therapeutics, and theranostics in patients (Cho et al., 2018; P Teixeira and Fussenegger, 2019; Zhao et al., 2023). Cell-cell communication plays a critical role in cellular immunotherapies, such as CAR-T cells, which rely heavily on interactions of the CAR with target antigens to carry out their therapeutic effects. Unfortunately, unintended interactions between immune cells and antigens on normal tissues can result in unwanted side effects and reduce treatment efficacy (Gargett and Brown, 2014; Flugel et al., 2023). Moreover, heterogeneity in antigen density exists, where some cancer cells express the target antigens at a density that is lower than required to induce T cell cytotoxicity (Chen et al., 2018; Kailayangiri et al., 2020). To address these issues and improve cellular therapies, it is crucial to understand adoptive cell behavior in a minimally-invasive manner. In this study, we developed an antigen-activatable secreted reporter system that allows for the detection of direct immune-cancer cell interactions within tumors via detectable signals in the blood.

We engineered human T cells with an activatable blood reporter system, utilizing the synNotch receptor system expressing SEAP in the response element. This system consists of two essential components: the CD19-directed receptor and the secreted reporter SEAP, with the latter being activated by the release of the transcription activator Gal4-VP64 upon antigen binding. To evaluate the engineered system, we tested its leakiness and target-dependent activatable nature. Through co-culturing naïve, RE only, and SynNotch + RE T cells with CD19$^+$ or CD19$^-$ cancer cells, we were able to observe baseline SEAP activity in the RE only group. Even in the presence of CD19, T cells did not express detectable SEAP without the synNotch receptor. In contrast, the SynNotch + RE group demonstrated significantly elevated SEAP activity in CD19$^+$ co-cultures, while CD19$^-$ co-cultures exhibited minimally elevated SEAP activity. This minimal activity may be from residual CD19 from incomplete knockout of our Nalm6 cells. These results demonstrated the dependence on both the engineered receptor and target to express SEAP, and overall shows high target specificity with little system leakiness (Figure 2).

We next aimed to investigate the dynamics of SEAP activity after antigen binding in our closed *in vitro* system and how varying the number of target or effector cells influenced SEAP expression. First, we maintained CD19$^+$ Nalm6 and SynNotch + RE T cell co-cultures using the same number of each cell type over 2 days and observed

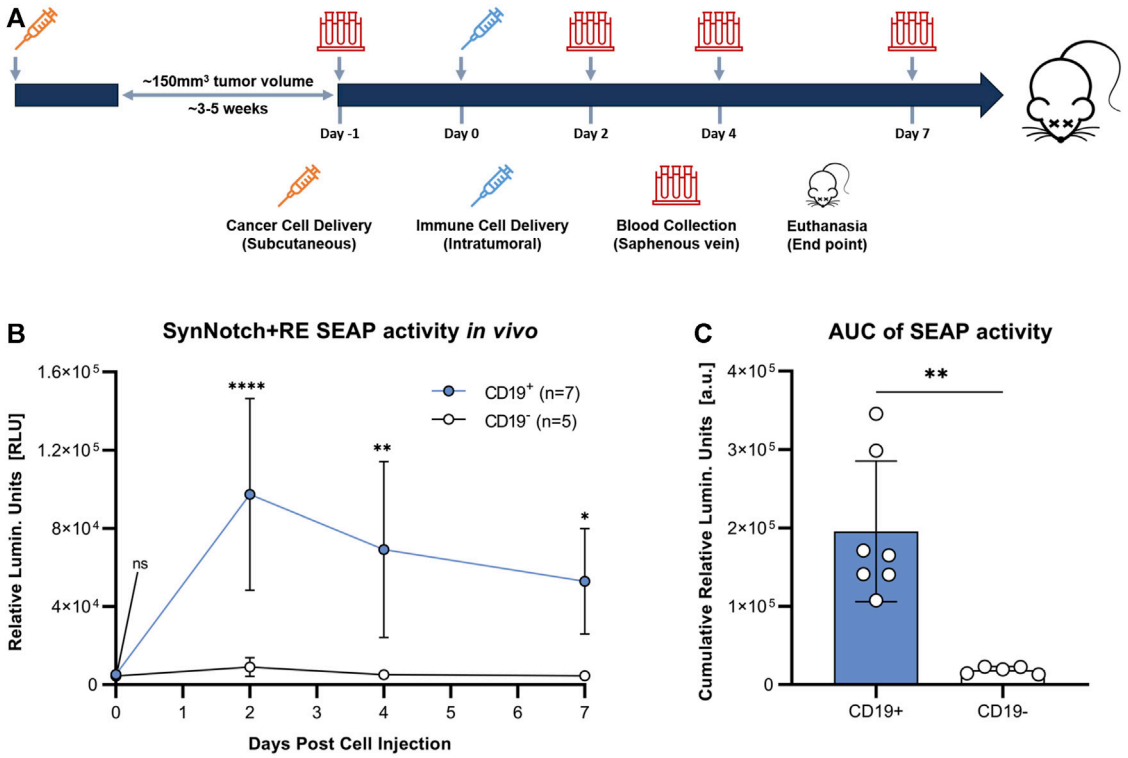

FIGURE 4
Evaluation of synNotch blood reporter system in mouse cancer model. **(A)** Experimental timeline for tumor establishment, T cell injection, and blood collection. **(B)** Average blood SEAP activity measured from mice bearing CD19⁺ (n = 7) or CDI 9- (n = 5) tumors before and after the delivery of SynNotch + RE T cells. **(C)** Cumulative blood SEAP activity over 7 days (area under curve: AUC) between CD19⁺ and CDI 9- cohorts. Data are presented as mean ± SD (****$p < 0.0005$, **$p < 0.01$, *$p < 0.05$, ns for No Significant difference).

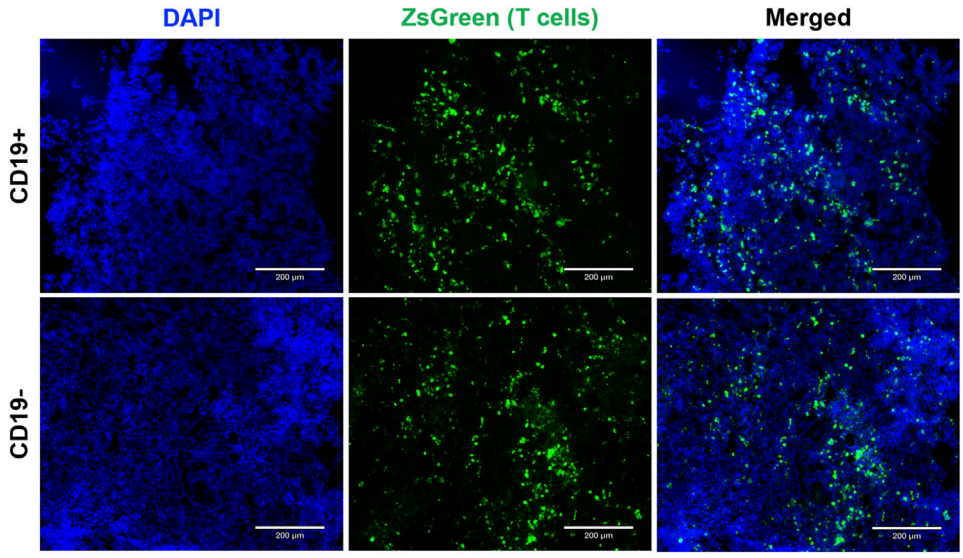

FIGURE 5
Endpoint tumor histology to visualize reporter-expressing cells. Fluorescence microscopy images (10x magnification) showcase CD19+ and CDI 9- Nalm6 tumors containing SynNotch + RE T cells expressing ZsGreen. Sections were counterstained with DAPI to visualize cell nuclei. The scale bar in the images corresponds to 200 µm.

similar elevated levels of SEAP activity at both the 24- and 48-h mark. Due to high cell density in the 96-well plates, we observed cell death due to over confluency beyond the 48 h time point. These results provided valuable insights into the optimal SEAP activation period for our *in vivo* experiments. Given that the activation of the synNotch system depends on physical cell-cell contact, it is important to explore the impact of varying the engineered T cell and cancer cell ratio on SEAP activity. When the number of T cells were increased at a constant cancer cell number, SEAP activity rose linearly until the number of T cells was 50% the number of cancer cells (an E:T of 0.5:1). Even when more T cells were added (an E:T of 1:1) this did not increase SEAP further, suggesting that some T cells did not interact with the cancer cells due to the possible restrictions in the proximity between them. However, having double the number of target cells (an E:T of 1:2) in our co-cultures did not increase SEAP activity further. These results raise important parameters for interpreting SEAP blood levels *in vivo* that are discussed further below and will need to be explored in future studies.

We next evaluated our synNotch blood reporter system in subcutaneous Nalm6 tumors in NSG mice. Due to the influence of E:T ratio on the amount of SEAP produced *in vitro*, we chose to inject a standard number of T cells (10 (Lunn et al., 2011)) into tumors of a standard volume (~100 mm^3). Significantly elevated blood SEAP activity was only observed in mice carrying the $CD19^+$ tumor following intratumoral delivery of SynNotch + RE T cells. SEAP activity peaked at 48 h and marginally declined thereafter up to 7 days. As the blood half-life of SEAP is 3 h (Tannous and Teng, 2011), the continued elevation of SEAP over the 7 days is likely due to continued GAL4-VP64 binding to the response element after synNotch cleavage and/or additional synNotch interactions with CD19 over time. The length of RE activation after synNotch-antigen binding is unknown and could provide a better understanding of RE transgene expression over time. Fluorescence microscopy for T cells was used to confirm that the persisting SEAP signal was due to continued T cell presence in the tumor. Qualitative assessment of $CD19^+$ and $CD19^-$ tumors showed similar numbers of immune cells, indicating that the difference in SEAP expression between the two groups was due to the presence of the target CD19, not variations in T cell survival.

Measurements of SEAP activity were ended at 7 days due to reaching heavy tumor burden (endpoint >1.6 cm tumor diameter), however literature has reported persisting synNotch-driven CAR and luciferase expression in mice for up to 11 days following intravenous injection of T cells (Roybal et al., 2016b). Compared to our findings, this could be attributed to differences in T cell persistence within the tumor between intratumoral and intravenous delivery methods (Sridhar and Petrocca, 2017). We hypothesis that intratumoral delivery results in immediate activation of the synNotch receptor in the target-expressing tumor, while systemic delivery involves T cell traffic to the tumor, resulting in gradual and sustained reporter expression (Fu et al., 2021). To fully understand the dynamics of synNotch expression and optimize its *in vivo* potential, it will be valuable to investigate the rate of replenishment of new synNotch receptors on the cell surface. Additionally, in future studies, it will be valuable to understand whether intratumoral delivery causes proteolytic cleavage of synNotch receptors at a rate that outpaces their replenishment.

SEAP is a useful reporter for monitoring immune cell activity with minimal invasiveness and can potentially provide a single measure of all the immune-cancer interactions in the whole body. However, in the context of multi-organ metastatic diseases, it is likely also important to obtain more spatial intertumoral information on the activity of immune cells. Non-invasive imaging tools like MRI and PET-based reporter genes can be used for this purpose as we and another group have recently demonstrated (Shin et al., 2023; Wang et al., 2023). Future work to build response elements that encode both imaging and secreted reporter genes would allow both spatial information to be acquired with infrequent and relatively expensive imaging, and more frequent whole-body monitoring with relatively low-cost blood tests.

A significant limitation of the detection system described in this study is the challenge of accurately reporting the live T cell and cancer cell numbers *in vivo*. While the system demonstrated its ability to specifically report the amount of local immune-cancer cell communications, translating the use of GLuc as a measurement of live T cells *in vivo* was a major obstacle. Several reasons contribute to this limitation, particularly the reliability of detecting GLuc within blood samples due to its short half-life, and the clearance rate of GLuc through urine, making it difficult to establish consistent output (Tannous, 2009). Additionally, it would be valuable to monitor cancer cell numbers simultaneously with T cell numbers. Although the Jurkat T cells used in this study lacked cytotoxic abilities to focus on establishing a detection system, monitoring cancer cell numbers would be crucial in future studies where this system is combined with other cell-based therapies. Estimating cancer cell numbers through tumor volume measurement may not be accurate in future studies involving immunocompetent mice due to the infiltration of various immune cells (Vera et al., 2018).

An alternative and more optimal preclinical approach would be to incorporate different imaging reporter genes into both T cells and cancer cells during the engineering process. Imaging reporters like bioluminescence imaging (BLI), MRI-contrast, and PET reporter genes have been previously applied in the study of many cell- or gene-based therapies (Liu et al., 2021; Shalaby et al., 2022a; Shalaby et al., 2022b). Among these options, BLI is frequently applied in pre-clinical small animal studies of cell-based therapies (Liu et al., 2021). By using different imaging reporters, we can overcome the limitations associated with using GLuc and improve our ability to accurately monitor and quantify live T cells and cancer cells *in vivo*. This advancement will significantly enhance the utility and effectiveness of the detection system for future studies and potential therapeutic applications.

A potential hurdle in adapting our synNotch system from Jurkat cells to primary immune cells for clinical applications is the limited capacity of lentiviral vectors. While sequential transduction of cells might be feasible, using separate vectors for the synNotch receptor and the response element (RE), is suboptimal due to the potential to increase cellular stress, toxicity, and the complexity and time required for the process. Currently, the aggregate size of these elements surpasses the capacity of standard lentiviral vectors, making a single-step transduction unfeasible. To circumvent these issues, we propose employing transposon systems, which have a higher capacity for larger genetic payloads, potentially allowing for the integration of the complete synNotch + RE system in a single vector (Li et al., 2011). Further, recent innovations like the development of more compact synNotch receptors, exemplified by SNIPR, offer promising avenues for primary cell applications through either viral or transposon systems (I et al., 2022). These advances highlight the opportunity

for future research to refine and adapt these methods, establishing our study with Jurkat T cells as a critical foundational work that lays the groundwork for the clinical translation of these sophisticated cellular engineering approaches. The activatable secreted reporter system described in the study offers a novel approach to measure local immune-cancer cell interactions beyond conventional methods *in vivo*. It can potentially aid in the development and monitoring of novel cell therapies by allowing minimally-invasive and rapid interrogation of cell behavior. Moreover, when coupled with other imaging systems, it has the potential to provide insights into the reasons for the failure of certain cell-based immunotherapies in patients. Additionally, this system can be broadly applicable in preclinical research, enabling the study of cellular behavior during development, normal physiology, and disease progression. Overall, it represents a promising tool that can advance cell-based therapies and contribute to more effective and targeted treatments in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below [figshare repository]: https://doi.org/10.6084/m9.figshare.24213249.v1.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by the University Council on Animal Care at the University of Western Ontario and follow the Canadian Council on Animal Care (CCAC) and Ontario Ministry of Agricultural, Food and Rural Affairs (OMAFRA) guidelines. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YF: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing–original draft, Writing–review and editing. TW: Conceptualization, Data curation, Methodology, Project administration, Writing–review and editing. JR: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Potential applications of JAK inhibitors, clinically approved drugs against autoimmune diseases, in cancer therapy

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Disturbances in immunoregulation may lead to both cancer and autoimmune diseases. Many therapeutic drugs for autoimmune diseases also display anti-tumor efficacy. The Janus kinase/signal transducer and activator of transcription signaling pathways are involved in the secretion of more than 50 distinct cytokines, which have critical roles in inducing autoimmune diseases and tumorigenesis. Thus, Janus kinases have become classical immunotherapeutic targets for immune disease. More than 70 Janus kinase inhibitors have been approved as immunomodulatory drugs for clinical use, of which 12 are used in the treatment of autoimmune diseases. This systematic review aims to elucidate the anti-tumor role of clinically approved Janus kinase inhibitors that were primarily designed for the treatment of autoimmune diseases and their potential for clinical translation as cancer treatments.

KEYWORDS

autoimmune diseases, JAK inhibitors, clinical translation, anti-cancer, immunotherapeutic therapy

1 Introduction

Chronic inflammation is often associated with autoimmune diseases (AIDs) and cancer. Only 5%–10% of cancers are caused by an inherited gene defect, with the remaining 90%–95% resulting from environment- or lifestyle-induced chronic inflammation (Hirano, 2021). In general, immune cells target and kill cancer cells in the early stage of tumorigenesis. However, during crosstalk with the tumor microenvironment, they are "domesticated," thereby losing their ability to eliminate cancer cells and possibly even facilitating the progression of tumors (Lei et al., 2020). Harnessing immune cells has been widely explored as a powerful strategy to inhibit cancer in both clinical and pre-clinical studies (Yang, 2015). Various drugs targeting immune cells that are used in the treatment of immunological diseases have also been explored with respect to their potential role in anti-cancer therapy. In this review, we summarize the effects on tumorigenesis and potential use in cancer therapy of clinically approved JAK inhibitors that were primarily designed for treatment of AIDs.

The immune system plays a critical part in the maintenance of individuals' health. Immune deficiency may lead to an inability to activate the necessary response to protect against pathogen invasion, whereas immune overactivation may cause AIDs. There are nearly 100 distinct AIDs, affecting approximately 3% of the population (Youinou et al., 2010). In general, these can be divided into two main categories: organ-specific AIDs, which

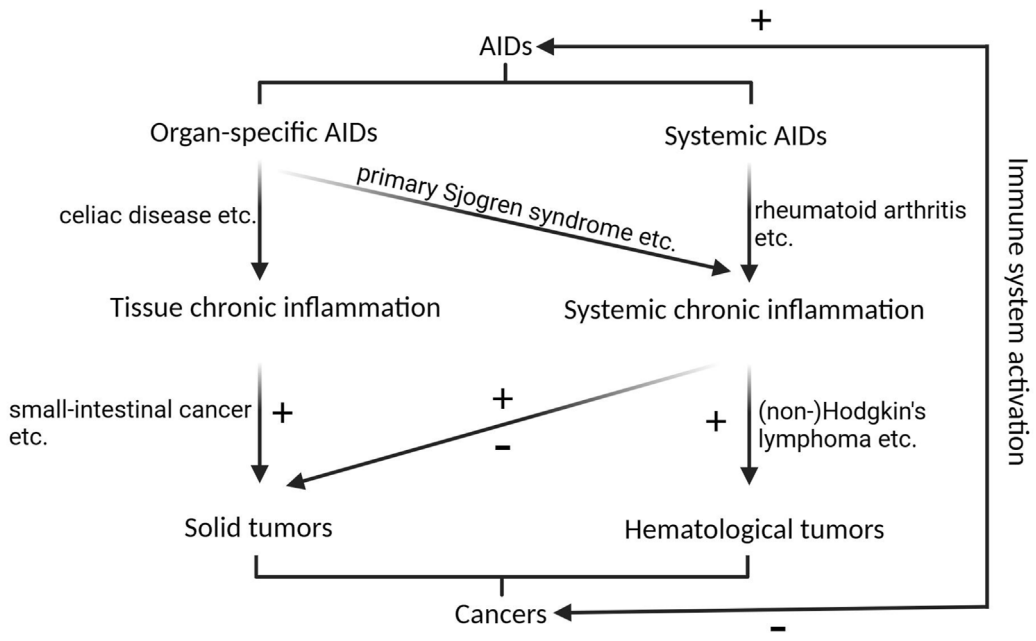

FIGURE 1
The correlation between AIDs and cancers. Organ-specific AIDs primarily contribute to solid tumorigenesis by inducing chronic inflammation in the affected tissues, while skin-specific AIDs, like primary Sjogren syndrome, can trigger systemic chronic inflammation, elevating the susceptibility to both overall solid tumors and lymphomas. Systemic AIDs increase the risk of hematological malignancies, and their impact on solid malignancies is multifaceted. Activation of immune system inhibits cancers, while promotes AIDs. + indicates promotional role on tumorigenesis or AIDs, - indicates inhibitory role on tumorigenesis.

affect organs including the inner ear, skin, thyroid and parathyroid gland, heart, liver, adrenal gland, pancreas, gastrointestinal system, reproductive system, and connective tissue; and systemic AIDs, which may affect the cardiovascular, hematopoietic, and neurological systems (Wang et al., 2015).

The synthetic drugs approved to date for the treatment of AIDs are dominated by JAK inhibitors (Schwartz et al., 2017). This is because the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is involved in the mediation of more than 50 distinct cytokines, and elevated cytokine expression caused by excessive activation of the JAK/STAT signaling pathway is a decisive factor in the occurrence of AIDs (O'Shea et al., 2002). Cytokines are also critical for malignant cell growth (Kontzias et al., 2012); thus, almost of those drugs have also been investigated with respect to their ability to treat cancer.

2 Relationship between AIDs and cancer

The association between AIDs and cancer is well established and has been systematically summarized in previous reviews (Giat et al., 2017), and we briefly summarized the association between AIDs and cancer as indicated in Figure 1. In brief, organ-specific AIDs tend to induce tissue tumors, possibly owing to chronic inflammation around the tissues. For example, celiac disease is a gastrointestinal system AID that leads to difficulty in digesting food and carries a high risk of small-intestinal cancer (Green et al., 2003); and a skin-specific AIDs, primary Sjogren syndrome, increases the risk of overall solid tumors and lymphomas, especially non-Hodgkin's lymphoma (NHL), for which the risk may well exceed a ten-fold increase (Smedby et al., 2006), possibly owing to systemic chronic inflammation.

Systemic AIDs tend to affect both hematological malignancies and organ-specific solid tumors. For example, systemic lupus erythematosus increases the risk of cancer in various organs, while decreasing the risk of others (Mao et al., 2016). Patients with rheumatoid arthritis (RA) have an increased risk of hematological malignancies, including Hodgkin's lymphoma and NHL; however, they are at decreased risk of most solid malignancies, including kidney, liver, prostate, gynecological, and gastric cancers (Giat et al., 2017).

The associations of other AIDs, such as polymyalgia rheumatica and giant cell arteritis, with cancer are not clear. Some studies have reported an increased risk of cancer in patients with polymyalgia rheumatica and giant cell arteritis (Ji et al., 2010; Muller et al., 2014), whereas others report no association or even a reduced risk (Kermani et al., 2010a; Kermani et al., 2010b).

Studies have also illustrated the phenomenon of autoimmunity secondary to malignancy and the co-occurrence of cancer and AIDs. Treatment of cancer patients by activating an immune reaction could lead to AIDs, especially in patients with pre-existing AIDs. There have been reports that more than 30% of such patients experience AIDs relapses or develop new autoimmune manifestations (Coureau et al., 2020).

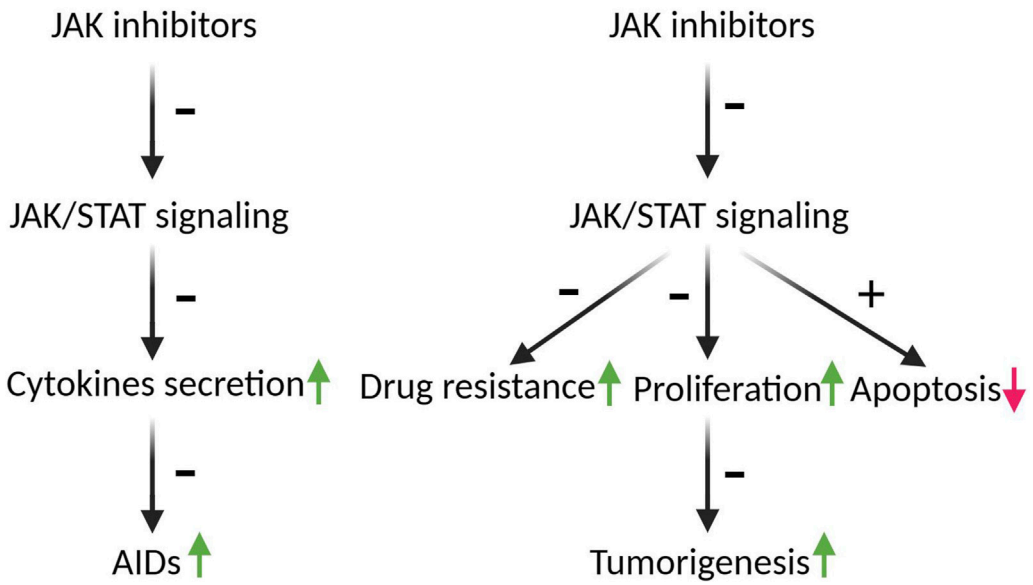

FIGURE 2
The schematic depicting the mechanism of action of JAK inhibitors in the progression of AIDs and cancers. The JAK/STAT pathways play a crucial role in the secretion of more than 50 distinct cytokines, which play pivotal roles in the development of AIDS and tumorigenesis. Inhibition of JAK/STAT pathways by JAK inhibitors mitigates AIDs by decreasing cytokines secretion. Moreover, hyperactivation of JAK/STAT is implicated in drug resistance and the survival of tumor cells. JAK inhibitors have the potential to counteract tumorigenesis by reversing drug resistance, inducing G2 arrest, and augmenting apoptosis. Arrows indicate the roles of JAK/STAT in AIDs and tumorigenesis, green arrows indicate promotion, red arrow indicates inhibition; ± indicate the roles of JAK inhibitors in AIDs and tumorigenesis, +means promotion, - means inhibition.

3 Potential anti-tumor role of JAK inhibitor drugs approved for AIDs treatment

Cancer and AIDs are closely related, as discussed above; thus, many drugs that have been clinically approved for treatment of AIDs have also been investigated with respect to their potential roles in the treatment of cancer. JAK/STAT signaling pathways are the classical immunotherapeutic targets. There are four JAKs, JAK1-3 and TYK2 (tyrosine kinase 2), and seven STATs, STAT1/2/3/4/6 and STAT5A/5B, in humans (Roskoski, 2023a). The regulatory role of the JAK/STAT signaling pathway in AIDs has been extensively summarized in a previous review (Xue et al., 2023). Thus, inhibition the of JAK/STAT pathway is widely used to treat AIDs.

The first generation of JAK inhibitors, known as jakinibs, bind to the kinase domain of JAKs. To date, 72 small-molecule protein kinase inhibitors have been approved by the US Food and Drug Administration (FDA) (Roskoski, 2023b), of which 12 JAK inhibitors have been approved for clinical use against AIDs; these comprise ruxolitinib, pacritinib, fedratinib, tofacitinib, baricitinib, abrocitinib, filgotinib, oclacitinib, peficitinib, upadacitinib, deucravacitinib, and delgocitinib (Shawky et al., 2022; Roskoski, 2023a; Li et al., 2023). Various mutations of JAKs or overactivation of JAK/STAT signaling pathways also been reported in various malignant tumors, including hematological and solid tumors; thus, most of the jakinibs also been demonstrated have anti-tumor efficacy, and scheme with JAK inhibitors mechanism of action in the course of AIDs and cancers was shown in Figure 2.

3.1 Ruxolitinib

Ruxolitinib, formerly known as INCB018424 or INC424, a selective oral inhibitor of JAK1 and JAK2 (Ajayi et al., 2018), was approved for treatment of myelofibrosis (MF) by the FDA in 2011 and by the European Medicines Agency (EMA) in 2012 (Mascarenhas and Hoffman, 2012) for polycythemia vera (PV) (2014) (Raedler, 2015) and acute and chronic graft *versus* host disease (GVHD) (2021) (Yang et al., 2021). Clinical research has demonstrated that it benefits some patients with pancreatic cancer (American Association for Cancer Research, 2015). Ruxolitinib has also resulted in positive response in patients with head and neck cancer with STAT3 overactivation (Qureshy et al., 2022). Systematic screening of T cell acute lymphoblastic leukemia (T-ALL) genomes revealed activating mutations in JAK1, JAK3, and STAT5 in 20%–30% of T-ALL cases (Belver and Ferrando, 2016), and ruxolitinib has displayed significant anti-tumor efficacy against T-ALL in both primary xenograft models and clinical trials (Lato et al., 2021; Moskowitz et al., 2021; Kolodrubiec et al., 2022). Patients with chronic neutrophilic leukemia (CNL) and atypical chronic myeloid leukemia (aCML) have also shown positive responses to ruxolitinib (Dao et al., 2020). In T-cell prolymphocytic leukemia, ruxolitinib promotes apoptosis and shows synergistical efficacy in combination with venetoclax, a B-cell lymphoma-2 inhibitor (Herbaux et al., 2021). Ruxolitinib combined with other mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitors have been shown to overcome therapeutic resistance and promote immune checkpoint therapy in pancreatic ductal adenocarcinoma (PDAC) (Datta et al., 2022). Ruxolitinib and calcitriol combination treatment showed synergistic anti-cancer effects on some breast cancer cell

lines (Schneider et al., 2022), and combinations of ruxolitinib with SMO-GLI1/tGLI1 pathway inhibitors synergistically inhibited growth of triple-negative breast cancer and human epidermal growth factor receptor-2 (HER2)-positive breast cancer both *in vitro* and *in vivo* (Doheny et al., 2020). Other studies have also reported anti-solid-tumor effects of ruxolitinib alone or in combination against various cancers, including metastatic lung cancer (Taverna et al., 2020), non-small-cell lung cancer (Patel et al., 2019), hepatocellular carcinoma (Wilson et al., 2013), and myeloproliferative neoplasms (MPN) (Eliacik et al., 2015; Wang et al., 2023).

3.2 Pacritinib

Pacritinib (SB1518), an inhibitor against JAK2 and mutationally activated JAK2 (JAK2V617F), was approved for treatment of MF by the FDA in 2022 (Shawky et al., 2022). Oral administration of pacritinib in murine models of acute myeloid leukemia (AML) led to significant inhibition of primary tumor growth and lung metastasis (Hart et al., 2011). Pacritinib also decreased the viability of patient-derived initiating cells of glioblastoma multiforme (GBM) *in vitro* at low micromolar doses, as well as improving response to tumozolomide in tumozolomide-resistant glioblastoma multiforme and thus improving overall median survival of mice in an orthotopical xenograft model (Jensen et al., 2017). Furthermore, pacritinib suppresses the expression of checkpoint proteins; a preclinical trial and a pilot phase I study of pacritinib and chemotherapy in FLT3-ITD-positive AML found that combination therapy was well tolerated, and preliminary results indicated anti-leukemic activity in patients with FLT3 mutations (Jeon et al., 2020). Clinical trials have also been performed to explore the benefits of pacritinib treatment for patients with metastatic refractory colorectal adenocarcinoma (CRC); however, no objective response was achieved (Chen et al., 1997). Elevated glucose consumption has a critical role in maintaining the growth of squamous cell lung cancer; pacritinib reduced glucose consumption in squamous cell lung cancer by inhibiting hexokinase activity (Ghezzi et al., 2023). Synergistic effects of pacritinib with other treatments have also been widely investigated. A combination of pacritinib with histone deacetylase (HDAC) inhibitor pracinostat (SB939) led to synergistic effects on tumor growth and a reduction of metastasis in AML (Novotny-Diermayr et al., 2012). In PC3/ER3 xenografts, a combination of pacritinib with erlotinib (ELTN), an epidermal growth factor receptor tyrosine kinase inhibitor, showed synergistic effects on tumor shrinkage by suppressing MET (Ochi et al., 2016). Combined treatment with pacritinib and SMO inhibitors (vismodegib and sonidegib) synergistically inhibited growth of triple-negative breast cancer and HER2-positve trastuzumab-resistant BT474-TtzmR cells both *in vitro* and *in vivo*. The combination therapy also synergistically inhibited breast cancer stem cells and suppressed lung metastasis in an orthotopic BT474-TtzmR xenograft model (Doheny et al., 2020). Overexpression of P-glycoprotein (P-gp) in cancer cells leads to multidrug resistance. Co-treatment with low-dose pacritinib induced G2 arrest, reduced cell viability, and greatly increased apoptosis of P-gp overexpressing cancer cells with multidrug resistance, indicating sensitization of P-gp-overexpressing drug-resistant cancer cells (Oh et al., 2022a). Pacritinib treatment overcame resistance to paclitaxel in nasopharyngeal carcinoma (NPC) by blocking IRAK1; and combination treatment with pacritinib and paclitaxel exhibited a superior anti-tumor effect (Liu et al., 2021).

3.3 Fedratinib

Fedratinib (INREBIC®), an oral selective kinase inhibitor against both wild-type JAK2 and JAK2V617F, was approved for the treatment of adult patients with intermediate-1 or high-risk primary or secondary MF in 2019 by the FDA (Blair, 2019). Kirsten rat sarcoma 2 viral oncogene homolog (KRAS) is the major driver mutation gene for PDAC tumorigenesis. Liu et al. predicted that fedratinib would exhibit KRAS-dependent anti-cancer activity in PDAC cells based on mining of bioinformatics data (Liu et al., 2020). Activation of the JAK2/STAT3 signaling pathway induces acquired ELTN resistance; fedratinib reversed this ELTN resistance by downregulation of JAK2/STAT3 signaling, thereby ameliorating the anti-cancer effects of ELTN in non-small cell lung cancer (NSCLC) (Chen et al., 2018). Furthermore, the JAK/STAT pathway leads to HDAC inhibitor resistance in cancers; novel JAK inhibitors based on a fedratinib moiety also suppressed tumor growth of acute erythroid leukemia (AEL) and NSCLC with HDAC inhibitor resistance (Qiu et al., 2023). Fedratinib further sensitizes P-gp-overexpression-induced drug resistance, and co-treatment with anti-mitotic drugs has been shown to increase the cytotoxicity of fedratinib to KBV20C oral cancer cells by reducing cell viability, increasing G2 arrest, and upregulating apoptosis (Oh et al., 2022b). In brief, the functions of fedratinib, including the inhibition of cell activity and drug resistance in cancer therapy, have been investigated to a certain extent.

3.4 Tofacitinib

Tofacitinib, an oral selective inhibitor of JAK1, JAK3, and (to a certain extent) JAK2 has been approved for treatment of RA (2012) (Coricello et al., 2020), psoriasis arthritis (PsA) (2017) (Ayala-Aguilera et al., 2022), ulcerative colitis (UC) (2018) (Ayala-Aguilera et al., 2022), juvenile idiopathic arthritis (JIA) (2020) (Kostik et al., 2022), and ankylosing spondylitis (AS) (2021) (Mohanakrishnan et al., 2022). Preclinical studies have reported that tofacitinib is effective in T-ALL patients with JAK1/JAK3 mutations (Girardi et al., 2017). However, AIDs are treated by decreasing the immune response, whereas tumorigenesis may occur during immune suppression conditions; thus drugs that inhibit immune response may also lead to tumorigenesis. There have been reports that therapy with tofacitinib increases the risk of tumorigenesis compared with tumor necrosis factor (TNF) inhibitor therapy (Ytterberg et al., 2022; Curtis et al., 2023). However, a meta-analysis of observational studies found no increased risk of malignancy in patients with RA treated with tofacitinib therapy

TABLE 1 Summary of JAK inhibitor drug for AIDs explored in cancer therapy.

Name	Targets	Approved for anti-AIDs	Explored anti-cancer efficacy
Ruxolitinib	JAK1, 2	MF, PV, GVHD	pancreatic cancer, head and neck cancer, breast cancer, lung cancer, hepatocellular cancer, T-ALL, CNL, aCML, MPN
Pacritinib	JAK2, JAK2V617F	MF	AML, GBM,CRC, lung cancer, prostate cancer, breast cancer, NPC
Fedratinib	JAK2, JAK2V617F	MF	PDAC, NSCLC, AEL, oral cancer cell
Tofacitinib	JAK1, 2, 3	RA, PsA, UC, JIA, AS	T-ALL
Baricitinib	JAK1, 2	RA	T-ALL, prostate cancer
Abrocitinib	JAK1, 2	AD	N.D. (no data)
Filgotinib	JAK1	RA	NSCLC
Oclacitinib	JAK1	CAD	pulmonary metastasis
Peficitinib	Pan-JAK	RA	epithelial ovarian cancer
Upadacitinib	JAK1	RA, PsA, AD, UC	NSCLC, lung cancer cells, breast cancer
Deucravacitinib	TYK2	PP	MPNST
Delgocitinib	JAK1, 2, 3, TYK2	AD	N.D.

compared with those receiving conventional synthetic disease-modifying anti-rheumatic drugs or TNF inhibitor therapy (Benucci et al., 2022). Thus, the association of tofacitinib with tumorigenesis is controversial.

3.5 Baricitinib

Baricitinib, an oral selective JAK1/2 inhibitor, was approved as a monotherapy for the treatment of RA in 2017 by the EMA and in 2018 by the FDA (Markham, 2017; Coricello et al., 2020), as well as in combination with methotrexate (Taylor et al., 2023). Baricitinib was also approved as a treatment for COVID-19 in 2020 by the EUA and in 2022 by the FDA (Shawky et al., 2022). The efficacy of baricitinib as a cancer therapy has rarely been considered so far, although one has study reported that baricitinib did not induce apoptosis of T-ALL (Akahane et al., 2017), whereas synergistic effects for its combination with docetaxel were observed in androgen-receptor-negative prostate cancer cells (Nalairndran et al., 2021). Potential effects on the risk of tumorigenesis have also been evaluated; one study reported that the malignancy rate in RA patients treated with baricitinib was high but not significantly different from that of the general population or of patients treated with TNF inhibitors (Uchida et al., 2023). Another study has evaluated the long-term safety of baricitinib in RA patients; at present, the data do not show an increased risk of malignancy (Taylor et al., 2022).

3.6 Abrocitinib

Abrocitinib, an inhibitor of JAK1 and JAK2, was approved for the treatment of adult patients with refractory and moderate-to-severe atopic dermatitis (AD) in 2022 by the FDA; it was also approved by the European Commission in 2021 (Deeks and Duggan, 2021; De, 2023). The anti-tumor efficacy of abrocitinib has not yet been investigated. Its safety has been evaluated by a long-term observation study, during which three patients (0.3%) treated with 100 mg abrocitinib and four patients (0.2%) treated with 200 mg abrocitinib developed nonmelanoma skin cancer, and there were three events of adjudicated malignancies, including two of prostate cancer and one of gastric adenocarcinoma, indicating a slight increase in cancer risk among patients receiving abrocitinib treatment (Simpson et al., 2021).

3.7 Filgotinib

Filgotinib, a specific inhibitor targeting JAK1, was approved for the treatment of RA by the EMA in 2020 (Dhillon and Keam, 2020). JAK1/STAT3 activation leads to targeted drug resistance of NSCLC, and inhibition of the JAK1/STAT3 signaling pathway by filgotinib reversed resistance to targeted drugs (Shien et al., 2017). No malignancies (solid tumor or lymphoma) were observed in clinical trials of filgotinib treatment (Biggioggero et al., 2019). During a long-term safety clinical trial for up to 4 years, only one case of NHL was considered to be related to filgotinib treatment among 739 enrolled patients (Kavanaugh et al., 2021).

3.8 Oclacitinib

Oclacitinib (Apoquel®), a specific inhibitor targeting JAK1, was approved for the treatment of canine allergic dermatitis (CAD) in

2013 (Gonzales et al., 2014). To achieve synergistic anti-cancer activity, combination therapies of oclacitinib with cytotoxic chemotherapy, including carboplatin and doxorubicin, have been investigated in multi-pulmonary metastasis models; results indicate that oclacitinib is well tolerated in combination with carboplatin or doxorubicin, although whether anti-tumor efficacy is enhanced has not been determined (Barrett et al., 2019). Long-term treatment with oclacitinib did not appear to increase the risk of malignancy in dogs (Cosgrove et al., 2015; Lancellotti et al., 2020).

3.9 Peficitinib

Peficitinib, a pan-JAK inhibitor, was approved for treatment of RA in Japan in 2019 (Markham and Keam, 2019). Peficitinib has been proved to have an inhibitory effect on cancer cells that is dependent on JAK/STAT signaling. Chromatin assembly factor 1 subunit A was shown to promote the proliferation and growth of epithelial ovarian cancer cells by activating the JAK2/STAT3 signaling pathway, and peficitinib decreased cancer growth by inhibition of this pathway (Xia et al., 2023). Treatment with peficitinib inhibited octamer-binding transcription factor 4-induced promotion of viability, invasion, and tumorigenesis of ovarian cancer side-population cells (Ruan et al., 2019).

3.10 Upadacitinib

Upadacitinib is an oral JAK1 inhibitor that has been approved for treatment of RA (2019) (Duggan and Keam, 2019), PsA (2021) (Muensterman et al., 2022), AD (2022) (Shawky et al., 2022), and UC (2022) (Shawky et al., 2022). Induction of inflammation is a factor leading to cisplatin-induced renal and hepatic dysfunction. It has been reported that upadacitinib protects against renal and hepatic dysfunction induced by cisplatin without impairing its efficacy against breast cancer and NSCLC. Moreover, upadacitinib promoted the potency of cisplatin against lung cancer cells (Anbar et al., 2022). No malignancies (hematoma or solid tumor) were observed in clinical trials to assess the safety of filgotinib therapy, whereas seven cases (*versus* three in the placebo group) were described in patients treated with upadacitinib, indicating that upadacitinib may increase the risk of tumorigenesis (Genovese et al., 2018; Biggioggero et al., 2019).

3.11 Deucravacitinib

Deucravacitinib (Sotyktu™), a specific inhibitor against TYK2, was approved for treatment of moderate-to-severe plaque psoriasis (PP) in 2022 by the FDA (Papp et al., 2018). Overexpression of TYK2 occurs in the majority of malignant peripheral nerve sheath tumors (MPNST); inhibition of TYK2 by deucravacitinib decreased proliferation and induced apoptosis of these tumors through decreased expression of proteins involved in the cell cycle, mitotic, and glycolysis pathways (Borcherding et al., 2023). It has been reported that therapeutic TYK2 inhibition may increase the risk of lung cancer and NHL; however, the safety profile of deucravacitinib has not yet been determined (Yarmolinsky et al., 2022).

3.12 Delgocitinib

Delgocitinib (Corectim®), a nonselective inhibitor that inhibits all members of the JAK family, including JAK1, JAK2, JAK3, and TYK2, was approved for treatment of AD in 2020 in Japan (Dhillon, 2020). However, the anti-tumor efficacy and the risk of tumorigenesis in AIDs patient treated with delgocitinib have not yet been established.

4 Summary

Both cancer and AIDs are related to immune diseases (Yasunaga, 2020). Based on these associations, drugs that were designed for the treatment of AIDs have also been extensively explored with respect to their potential to treat cancers. The summary of approved JAK inhibitors for AIDs and their exploration in understanding anti-cancer functions was provided in Table 1. JAK/STAT signaling is known as the double-edged sword of cancer progression (Owen et al., 2019). Numerous studies summarized in this review illustrate that inhibition of JAK/STAT signaling by approved JAK inhibitors has potential anti-tumor effects, although those drugs were primarily designed for against AIDs. While the JAK inhibitors approved for AIDs treatment have not yet received approval for cancer therapy. Certain drugs, such as Ruxolitinib, have undergone clinical trials to assess their anti-tumor efficacy against various solid tumors. These trials have included pancreatic, head and neck cancers, as well as hematological tumors, and have shown positive responses. Pacritinib has also been investigated in clinical trials for its anti-tumor effectiveness against AML and CRC. Additionally, combining these JAK inhibitors with other drugs to achieve synergistic anti-tumor efficacy also been explored in clinical trials. More and more JAK inhibitors have undergone extensive research to assess their potential efficacy against tumors in animal models and clinical trials. These findings are paving the way for their future clinical applications in cancer treatment.

Author contributions

X-HW: Investigation, Writing–original draft. Y-YL: Conceptualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ferroptosis and the ubiquitin-proteasome system: exploring treatment targets in cancer

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Ferroptosis is an emerging mode of programmed cell death fueled by iron buildup and lipid peroxidation. Recent evidence points to the function of ferroptosis in the aetiology and development of cancer and other disorders. Consequently, harnessing iron death for disease treatment has diverted the interest of the researchers in the field of basic and clinical research. The ubiquitin-proteasome system (UPS) represents a primary protein degradation pathway in eukaryotes. It involves labelling proteins to be degraded by ubiquitin (Ub), followed by recognition and degradation by the proteasome. Dysfunction of the UPS can contribute to diverse pathological processes, emphasizing the importance of maintaining organismal homeostasis. The regulation of protein stability is a critical component of the intricate molecular mechanism underlying iron death. Moreover, the intricate involvement of the UPS in regulating iron death-related molecules and signaling pathways, providing valuable insights for targeted treatment strategies. Besides, it highlights the potential of ferroptosis as a promising target for cancer therapy, emphasizing the combination between ferroptosis and the UPS. The molecular mechanisms underlying ferroptosis, including key regulators such as glutathione peroxidase 4 (GPX4), cysteine/glutamate transporter (system XC-), and iron metabolism, are thoroughly examined, alongside the role of the UPS in modulating the abundance and activity of crucial proteins for ferroptotic cell death, such as GPX4, and nuclear factor erythroid 2–related factor 2 (NRF2). As a pivotal regulatory system for macromolecular homeostasis, the UPS substantially impacts ferroptosis by directly or indirectly modulating iron death-related molecules or associated signaling pathways. This review explores the involvement of the UPS in regulating iron death-related molecules and signaling pathways, providing valuable insights for the targeted treatment of diseases associated with ferroptosis.

KEYWORDS

ferroptosis, ubiquitin-proteasome pathway, degradation, cancer, treatment

1 Introduction

The primary goal of cancer treatment research has been to eliminate malignant cells while protecting healthy cells (Cassetta and Pollard, 2023). To prevent the uncontrolled growth of cancer cells and other abnormal tissues, the body relies on a process known as "regulated cell death" (RCD) to keep things in check (Coradduzza et al., 2023; Huang et al., 2023). Apoptosis, paraptosis, necroptosis, and ferroptosis are among the distinct modes of RCD that have been characterized so far (Tong et al., 2022). The tumor's development and the treatment response are impacted differently by each RCD subtype. In contrast to necrosis, apoptosis, and paraptosis, ferroptosis is distinguished by ferrous ions' buildup and lipids' peroxidation (Su et al., 2019; Zhi et al., 2022; Wu et al., 2023). Ferroptosis has been acknowledged as a promising cellular mechanism with potential anti-tumor benefits since its discovery. Furthermore, it has been connected to several other diseases, such as neurological disorders and harm caused by strokes (Dixon et al., 2012; Gao et al., 2022; Liu et al., 2022). By controlling the metabolism of iron and lipids, ferroptosis regulates the resistance of tumor to chemotherapy or targeted medicines (Qi et al., 2022). The metabolic adaptability of tumor cells, notably their sensitivity to ferroptosis, sheds light on the mechanisms underpinning tumor persistence (Lin et al., 2022). Significant strides have been achieved in increasing cancer cells' sensitivity to ferroptosis by changing their metabolism or activating oncogenic pathways (Deng et al., 2023). Additionally, it has been found that immunotherapy modifies the efficacy of ferroptosis by increasing the levels of lipid peroxide and iron in tumor cells, which facilitates ferroptosis (Yao et al., 2021).

Among the many molecular mechanisms affecting ferroptosis, the ubiquitin-proteasome pathway is widely involved and is closely related to the occurrence and development of tumors and other diseases (Tang and Kroemer, 2020). The exogenous pathway is initiated by the inhibition of system XC- or activation of transferrin (TF) and lactotransferrin (LTF) on the cell membrane. The endogenous mechanism is triggered by the depletion of intracellular glutathione (GSH) and inactivation of GSH peroxidase 4 (GPX4) (Ingold et al., 2018). Ferroptosis plays a crucial role in the development and diseases (Distéfano et al., 2017; Jiang et al., 2021), and has become a hot spot in biological research in recent years. Therefore, understanding the relationship between the UPS and ferroptosis can help understand the complex mechanism of ferroptosis and provide new strategies for treating human diseases, including tumors (Chen et al., 2021), neurodegenerative diseases (Masaldan et al., 2019), cardiomyopathy (Fang et al., 2019) ischemia-reperfusion injury (Li et al., 2020), stroke (Alim et al., 2019), traumatic brain injury of cerebral hemorrhage (Bao et al., 2021), ageing (Timmers et al., 2020), atherosclerosis (Ouyang et al., 2021), liver injury (Chen et al., 2022), and kidney injury (Tonnus et al., 2021), or related pathological cell death. With the development of molecular biology, various regulatory factors and related mechanisms mediating ferroptosis are being revealed, and the basic research of ferroptosis is gradually deepening. The ubiquitin-proteasome pathway plays a significant role in ferroptosis and has been linked to the development of cancer and other diseases. Therefore, comprehending the connection between the UPS and ferroptosis will aid in understanding the intricate process of ferroptosis and offer fresh approaches for treating human disorders, such as tumor.

2 Distinctive features of ferroptosis

Ferroptosis is defined by mitochondrial dysfunction and cell death due to iron-dependent lipid peroxidation. It is characterized by several morphological changes, including the blistering of the plasma membrane, the reduction or absence of the mitochondrial ridges, and the preservation of the usual size of the nucleus without chromatin condensation (Rodriguez et al., 2022). The delicate lipid peroxide balance within the cell plays a critical role in the incidence of ferroptosis. Metabolic pathways tightly control ferroptosis, including lipids, iron, and amino acids Figure 1.

Ferroptosis is induced by the iron-mediated oxidation of polyunsaturated fatty acid (PUFA). Regarding biological properties, ferroptosis cell death is associated with the formation of reactive oxygen species (ROS), evidenced mainly by iron and lipid peroxidation buildup. When the metabolism of the antioxidant system in cells is disturbed, it causes an increase in the accumulation of Fe^{2+} within the cell, which in turn mediate the Fenton reaction to produce hydroxyl (-OH) or alkoxy (R-O) free radicals with different peroxides (Su et al., 2019) which can peroxidize with unsaturated fatty acids on the cell membrane. It causes the destruction of the stability of the lipid bilayer and the disintegration of the cell membrane, thus promoting ferroptosis in cells. The mitochondrial morphology of cells undergoing ferroptosis changes significantly, including mitochondrial volume reduction, membrane density increases and ridge reduction or even disappearance. Compared with apoptosis, the nuclear structure of cells undergoing ferroptosis is complete, without nuclear membrane rupture and chromatin marginalization. It is also accompanied by cell shedding and aggregation (Li and Li, 2020).

The occurrence or inhibition of ferroptosis is associated with various molecular mechanisms closely related to the metabolism of amino acids, lipids, iron and other metabolic modes in the cell. The regulatory mechanisms of ferroptosis can be divided into positive and negative ones. Iron metabolism is the essential positive regulatory mechanism of ferroptosis. As mentioned above, excessive accumulation of iron ions in cells can promote the occurrence of ferroptosis. Extracellular Fe^{3+} is endocytosed into the cell body by transferrin receptor protein 1 (TFR1). After the reduction of Fe^{3+} into ferrous ion Fe^{2+} in the cell, some Fe^{2+} is further stored in the unstable iron pool mediated by DMT1 (divalent metal transporter 1) or zinc-regulated, iron-regulated transporter-like proteins 8 ZIP8/14 (ZRT/IRT-like proteins 8/14). The other part is stored in an iron storage protein complex composed of FTL (ferritin light chain) and FTH1 (ferritin heavy chain). Excessive accumulation of Fe^{2+} triggers the Fenton reaction, which promotes cell ferroptosis.

The SLC7A11-GSH-GPX4 pathway is considered to be the primary negative regulatory mechanism in cells (Lei et al., 2022). In mammal cells, SLC7A11, a member of system XC- on the cell membrane, can take cystine into the cell to synthesize GSH, which is a necessary component for the activity of GPX4, a vital lipid peroxidase in the cell, so that GPX4 can directly reduce phospholipid hydrogen peroxide to hydroxy phospholipid. It

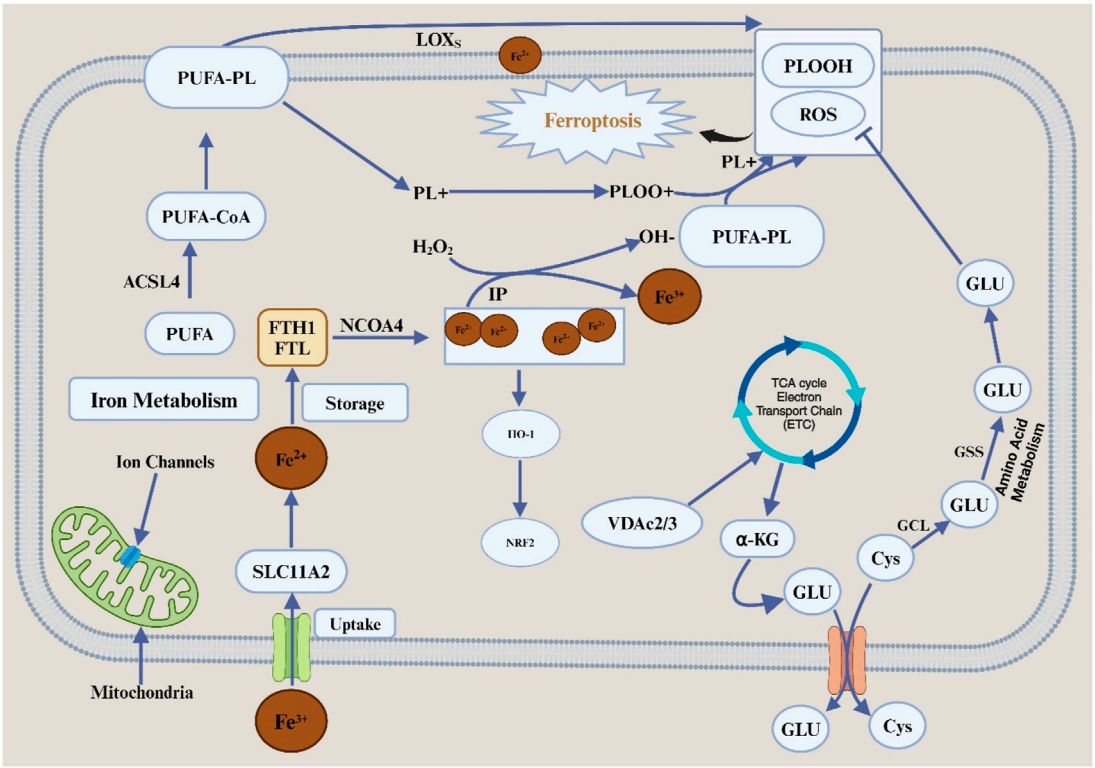

FIGURE 1
Features and mechanisms of ferroptosis. Abbreviation: TFRC, transferrin receptor; SLC11A, solute carrier family 11 member 2; FTL, ferritin light chain; FTH1, ferratin heavy chain 1; NCOA4, nuclear receptor coactivator 4; HO-1, heme oxygenase 1; NRF2, nuclear factor erythroid2-related factor 2; IP, iron pool; PUFA, polyunsaturated fatty acid; CoA, coenzyme A; PL, phospholipids; LOXs, lipid oxygenase; PLOOH, phospholipid hydroperoxides; Cys, cystine; Glu, glutamate; SLC7A11, solute carrier family 7 member 11; SLC3A2, solute carrier family 3 member 2; GCL, glutamate-cystine ligase; GSS, glutathione synthetase; GSH, glutathione; GPX4, glutathione peroxidase 4; ROS, reactive oxygen species; TCA, tricarboxylic acid; VDAC2/3, voltage-dependent anion channel 2/3; α-KG, alpha ketoglutaric acid; ETC, electron transfer chain.

prevents the peroxidation of PUFA on the cell membrane and thus inhibits ferroptosis in cells (Koppula et al., 2021). At the same time, more studies have shown that there are non-GPX4-dependent harmful regulatory mechanisms of ferroptosis in cells, such as the ferroptosis suppressor protein (FSP1-CoQH2 system), human dihydroorotate dehydrogenase (DHODH-CoQH2 system) and GCH1-BH4 system. Among them, FSP1, as a nicotinamide adenine dinucleotide phosphate [NAD(P) H-dependent oxidoreductase], can reduce CoQ (CoQ) to panthenol (CoQH2), thereby trapping lipid peroxy radicals and inhibiting lipid peroxidation and ferroptosis (Bersuker et al., 2019; Doll et al., 2019). Additionally, one study discovered the first ferroptosis defense mechanism operating within mitochondria: dihydroorotate dehydrogenase (DHODH), an enzyme involved in pyrimidine synthesis, converts CoQ to CoQH2 in the inner mitochondrial membrane, neutralizing the lipid peroxidation defense against ferroptosis in mitochondria (Mao et al., 2021). Through the synthesis of BH4 (tetrahydrobiopterin), a free radical-scavenging antioxidant, CoQH2, and phospholipids (FLs) with two PUFA tails, GCH1 (GTP cyclohydrolase 1) prevents ferroptosis in the GCH1-BH4 system (Soula et al., 2020). Additionally, several transcription factors involved in ferroptosis, including tumor protein 53 (TP53), NFE2L2/NRF2, and activating transcription factor (ATF3), have diverse functions in modulating ferroptosis sensitivity using transcription-dependent or transcription-independent techniques (Dai et al., 2020).

2.1 Ferroptosis and iron metabolism

Ferroptosis, the process of acquiring, using, storing, and eventually excreting iron, relies heavily on iron metabolism (Hu et al., 2021). During digestion, the duodenum's epithelial cells absorb iron from meals. DMT1 then transports Fe^{2+} into the cells after iron reductase converts Fe^{3+} in the intestinal epithelium (Richardson and Ponka, 1997). Iron homeostasis in the body is crucial for maintaining the balance of this essential element. Transferrin and ferritin play vital roles in this process. Transferrin is a glycoprotein that binds to Fe^{3+} in the blood, forming a complex. This complex is taken up by cells through endocytosis mediated by the TfR (Anderson and Frazer, 2017; Yanatori and Kishi, 2019; Shamsi et al., 2020; Qiu et al., 2022). Once inside the cell, the iron is released from transferrin in the acidic environment of the endosome. This release is facilitated by a combination of low pH, a conformational change in transferrin upon binding to its receptor, and reduction of transferrin-bound Fe^{3+}. The released iron can then be used for metabolic functions, stored within cytosolic ferritin, or exported from the cell via ferroportin (FPN1) (Anderson and Frazer,

2017). Ferritin, an intracellular protein, serves as the primary storage site for excess iron. It accumulates and sequesters iron, preventing its potential neurotoxic effects. Ferritin molecules can accumulate excess iron and are engulfed by lysosomes in a process called "autophagy" (Arosio et al., 2017). Together, transferrin and ferritin play critical roles in maintaining iron homeostasis. Transferrin transports iron to cells, while ferritin stores excess iron, preventing its toxicity. This intricate interplay ensures the proper distribution and storage of iron, supporting various physiological processes in the body (Anderson and Frazer, 2017). An iron pool (IP) that is non-toxic to cells can be formed when iron is safely stored in ferritin. Compared to the IP, the cell's free Fe^{2+} concentration is substantially lower (Lin et al., 2020; Philpott et al., 2020). Free Fe^{2+} is highly reactive and readily forms hydroxyl free radicals when combined with intracellular hydrogen peroxide (H_2O_2). This causes oxidative damage to DNA, proteins, and membrane lipids. This pathway promotes lipid oxidation, which harms cell membranes and eventually results in cell death (Hong et al., 2021; Qin et al., 2023). Additionally, by lowering the amount of redox-active iron available, several mitochondrial proteins, such as NFS1-ISCU26, CISD1, and CISD2, can negatively control ferroptosis (Chen et al., 2021). There are two parts of ferritin, the FTH1 and the FTL (Zhang et al., 2021). Ferritinophagy is the process whereby ferritin degrades and releases Fe^{2+} (Sun et al., 2022). To create a ferritin complex, the nuclear receptor coactivator 4 (NCOA4) can attach to FTH1 directly. An increase in intracellular free Fe^{2+} hastens ferroptotic cell death, an increase in the concentration of ferritin phagosomes, and an increase in NCOA4 (Jin et al., 2022; Mi et al., 2023).

Another typical mechanism for ferritin breakdown in cardiomyocytes is heme catalysis via heme oxygenase-1 (HO-1). NRF2 and BAY have been suggested to activate this pathway (Wang et al., 2022; Xu et al., 2023). Researchers have found that increasing HO-1 expression accelerates elastin-induced ferroptosis (Guan et al., 2022). Most cells do not have an efficient method to remove iron when it accumulates beyond storage capacity, which results in elevated levels of the labile IP. A higher concentration of free iron within the cell stimulates the production of labile IP, which speeds up the onset of ferroptosis (Ou et al., 2022).

2.2 Role of lipid metabolism in ferroptosis

PUFAs are essential to the fluidity of cell membranes because of their location in the phospholipid bilayer. An overabundance of PUFAs can cause the Fenton reaction to convert them into hydroxyl radicals, which in turn can cause an overabundance of lipid peroxide and, in turn, cause cells to enter ferroptosis (Dierge et al., 2021; Mishima and Conrad, 2022). Phosphatidyl ethanolamine (PE) makes up roughly 15%–25% of the phospholipids in the membranes of other organelles compared to about 40%–45% of the total phospholipids in the inner membrane of mitochondria (Feng and Stockwell, 2018; Yi et al., 2021). Data indicates that PE contributes to the ferroptosis triggered by arachidonic acid (AA) and its analogues. Acyl-CoA synthetase long-chain family member 4 (ACSL4) catalyzes the reaction in which PUFAs attach to coenzyme A (CoA) to form Acyl-CoA, a key intermediate in the non-enzymatic process of lipid peroxidation (Bouchaoui et al., 2023). Acyl-CoA then undergoes re-esterification to create phospholipids, a process performed by lysophosphatidylcholine acyltransferase 3 (LPCAT3) (Xu et al., 2022). The membrane remodeling enzymes ACSL4 and LPCAT3 are crucial for promoting ferroptosis (Feng et al., 2022). They control the levels of PUFAs in phospholipids.

PE and LPCAT3 boost ferroptosis by inducing membrane phospholipid peroxidation via lipid oxygenase (LOX) activity. Therefore, protecting cells against elastin-induced ferroptosis can be achieved by silencing LOXs (Lee et al., 2022). Multiple mechanisms exist by which lipid peroxides cause damage to cells, including the generation of ROS through the amplification of lipid peroxidation processes, alterations to the membrane's physical structure, and lipid peroxidation byproducts (Zhan et al., 2022).

2.3 Connection of ferroptosis to amino acid metabolism

One of the most effective antioxidants is GSH (Yang et al., 2022). It helps repair damaged cell membranes by acting as a GPX4 substrate, part of the body's natural defense mechanism. An essential transporter for GSH production is the cystine/glutamate antiporter (XC-) (Wu et al., 2021; Du and Guo, 2022). The system XC- and GPX4 are both essential for the amino acid metabolism associated with ferroptosis. There is only one cellular component, selenocysteine, that can convert lipid peroxide to the equivalent alcohol, and it is located in the center of GPX4. Ferroptosis can occur when GPX4 cannot remove hydrogen peroxide from the environment (Luo et al., 2022; Ma et al., 2022). The system XC- allows cystine and glutamate to be imported and expelled from cells, respectively. To aid in the production of GSH, cystine is converted to cysteine by the internal enzymes glutamate-cysteine ligase (GCL) and glutathione synthase (GSS). Heterodimers of the solute carrier family 3 member 2 (SLC3A2) and the system XC- are responsible for transporting cystine and glutamic acid, respectively (Li et al., 2022). Inducing ferroptosis is possible either by blocking the system XC- directly or indirectly via elevated extracellular glutamate levels.

Additionally, recent research suggests that tryptophan metabolites play a role in the resistance of tumor cells to ferroptosis, a type of cell death distinct from cysteine-mediated ferroptosis. These metabolites include 3-hydroxycyananilic acid (3-HA) and 5-hydroxytryptamine (5-HT). As a result, blocking tryptophan metabolism may be an effective new strategy in the fight against cancer (Liu et al., 2023).

3 The ubiquitin-proteasome system (UPS) pathway

Eukaryotic cells rely heavily on the UPS to regulate various cellular processes, including protein homeostasis, cell cycle progression, and response to stress, signal transmission, and transcriptional activation (Myung et al., 2001; Lan et al., 2018). In eukaryotic cells, intracellular proteins that are oxidized, damaged, or misfolded are broken down by UPS in around 80% of cases (Yang et al., 2021). Although UPS and autophagy are essential mechanisms for protein degradation, the size of the degraded materials strongly

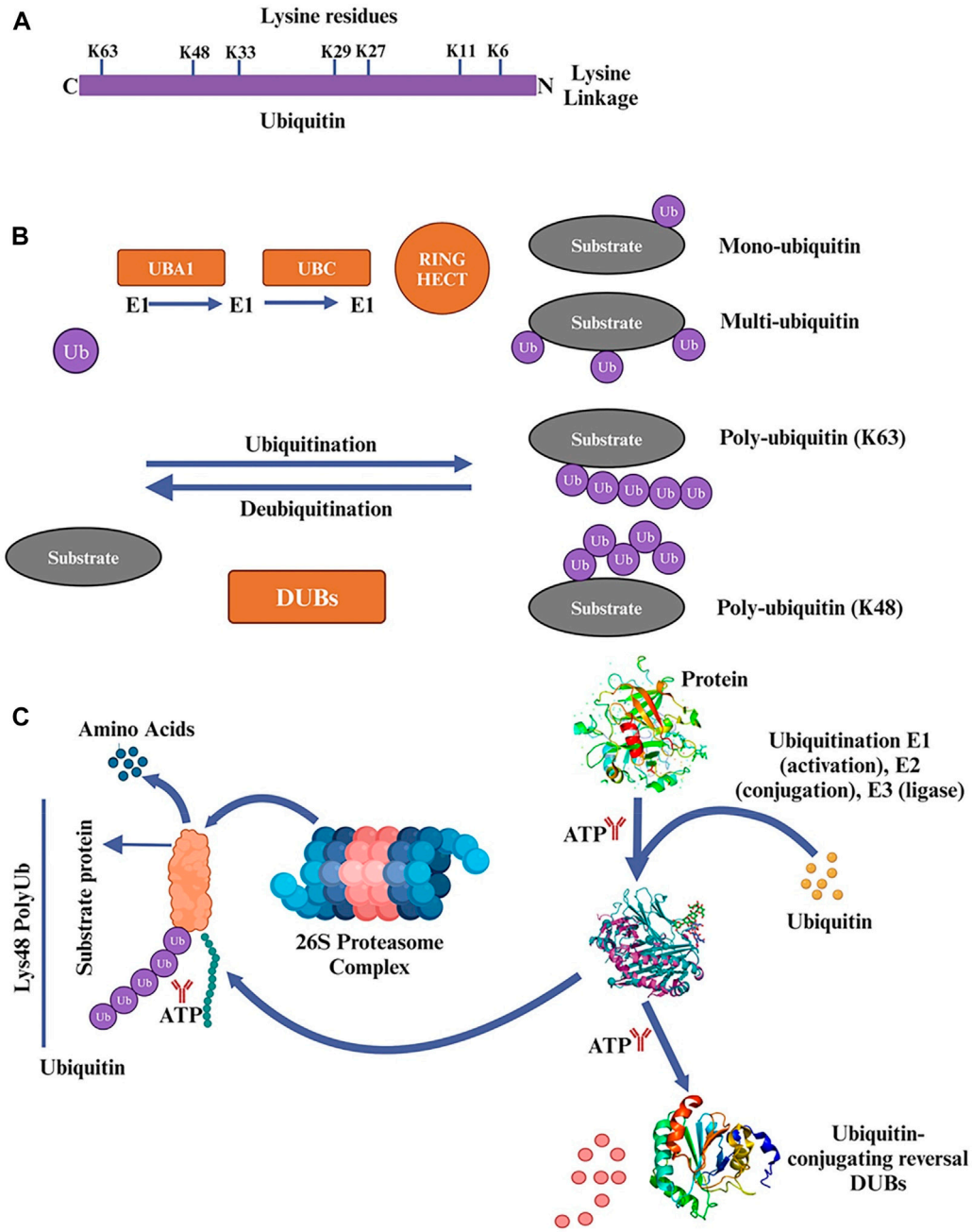

FIGURE 2
(A) Ubiquitin 76 amino acids that is found in various organisms and is highly conserved throughout evolution. It plays a crucial role in ubiquitination, which is involved in regulating protein degradation, cellular signaling, and other important cellular functions. Ubiquitin contains lysine residues (K6, K11, K27, K29, K33, K48, and K63) that are crucial for forming different types of ubiquitin chains and determining the fate of the target protein. **(B)** In the ubiquitination process, ubiquitin is attached to lysine residues of substrate proteins through a three-step enzymatic reaction. First, ubiquitin is activated by a protein called ubiquitin-activating enzyme (E1). Then, the activated ubiquitin is transferred to another protein called ubiquitin-conjugating enzyme (E2). Finally, a protein called ubiquitin ligase (E3) transfers the ubiquitin from E2 to a lysine residue on the target protein. Mono- and polyubiquitin serve as signals in cellular processes such as endocytosis, DNA repair, apoptosis, and transcriptional regulation. Ubiquitin conjugation plays a crucial role in multiple pathways and is not limited to protein degradation alone. **(C)** The ubiquitin-proteasome system (UPS) is a process in which substrate proteins are ubiquitinated through the action of three enzymes: E1, E2, and E3. E1 binds to activated ubiquitin and transfers it to E2, which carries the ubiquitin to E3. E3 then facilitates the transfer of ubiquitin from E2 to a lysine residue on the target protein. Proteins can undergo modification with a single mono-ubiquitin molecule or with ubiquitin chains of varying lengths and linkage types. Substrate proteins modified with specific chains are recognized and degraded by the 26S proteasome. Deubiquitinating enzymes (DUBs) play a role in removing ubiquitin from substrate proteins. DUBs can remove mono-ubiquitination or trim/remove ubiquitin chains. Poly-ubiquitination is typically associated with protein clearance through proteasomal degradation, while mono-ubiquitination affects cellular processes by adding a single ubiquitin moiety to the substrate protein.

influences the degradation pathway employed (Dikic, 2017). The UPS typically breaks down unfolded polypeptides, whereas more enormous cytosolic complexes, cellular aggregates, and organelles are handled by autophagy. A group of small peptides with 76 amino acids known as ubiquitin molecules contains lysine residues (K6, K11, K27, K29, K33, K48, and K63) Figure 2A, which are activated by the enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin-protein ligase), are used in covalent bonding to the target protein for alterations during the catalytic cascade (Glickman and Ciechanover, 2002). Monoubiquitination and polyubiquitination are the two basic types of protein ubiquitination. A single ubiquitin molecule is covalently joined to a lysine residue in the target protein during monoubiquitination, the most basic type of ubiquitination (Tonnus et al., 2021). K6, K11, K33, K29, K27, K48, and K63 are polyubiquitination alterations. K48 and K63 changes have received the most research attention of them Figure 2B. The 26S proteasome, which controls protein stability, can identify and eliminate the polyubiquitination of K48.

In contrast, the role of K63 is mainly involved in DNA repair, mediating signal transduction and regulating protein activity (Walczak et al., 2012; Yau and Rape, 2016). In addition to the above ubiquitin modifications, there is also a type of ubiquitin chain used to modify proteins, the linear ubiquitin chain (M1 type), which is covalently linked to the N-terminal methionine residue of one ubiquitin and the C-terminal glycine residue of another. Linear ubiquitin chain synthesis and degradation are distinct processes (Shabek and Ciechanover, 2010). Only the E3 ubiquitin ligase HOIP, along with its regulatory subunits HOIL1L and SHARPIN, can facilitate the synthesis of linear ubiquitin chains, hence the name "linear ubiquitin chain assembly complex" (LUBAC). Deubiquitinating enzymes (DUBs) such as OTULIN (OTU deubiquitinase with linear linkage specificity) remove ubiquitin chains from their targets (Jahan et al., 2021).

The covalent binding of ubiquitin, a tiny protein with 76 amino acids, to the protein substrate, is a crucial step in the complex and well-coordinated process of targeted protein destruction (Nandi et al., 2006). Three enzymes work together in sequence to accomplish this. The E1 requires the energy released by the breakdown of ATP to activate itself, which then causes the ubiquitin molecule to establish a thioester bond with E1. Once again, creating a thioester bond like the first, ubiquitin is transported from E1 to the E2. E3 finally catalyzes the covalent attachment of ubiquitin to the target protein's lysine residues (Fang and Weissman, 2004).

This process is greatly aided by the 26S proteasome complex, which consists of a core 20S proteasome and one or two regulatory 19S proteasome units. When a target protein is modified with a polyubiquitin chain, the 19S proteasome recognizes it, digests the polyubiquitin chain, unfolds the protein, and then translocates it to the 20S proteasome where it is broken down into short peptides (Adams, 2003). In contrast to polyubiquitination, which often results in the proteasomal destruction of proteins, monoubiquitination, in which a single ubiquitin unit is added to the substrate protein, has been shown to affect several cellular functions (Miranda and Sorkin, 2007; Wang et al., 2012). Some examples are protein translocation, DNA damage signaling, epigenetic regulation, and kinase activity as shown in Figure 2C.

Protein ubiquitination is often reversible, and proteins modified by ubiquitination can also be removed by DUBs (Lange et al., 2022). Current studies have shown that the human genome can encode more than 100 deubiquitination enzymes, which are divided into two categories: cysteine proteinases and metalloproteinases. Cysteine proteases can be subdivided into USPs (ubiquitin-specific proteases), MJDs (machadoJoseph disease domain superfamily), OTUs (otubain/ovarian tumor-domain containing proteins), UCHs (ubiquitin carboxyl-terminal hydrolases), MINDYs (motif interacting with each other) Ub-containing novel DUB family), ZUP1 (zinc finger-containing ubiquitin peptidase 1) has six families (Clague et al., 2019). In addition to reversing the ubiquitin modification of the substrate protein, DUBs also participate in the editing of the ubiquitin chain, the recovery of ubiquitin molecules, and the processing and maturation of ubiquitin precursors (Haq and Ramakrishna, 2017). Therefore, the abnormal expression or activation of deubiquitination enzymes in cells will directly or indirectly disrupt the regulation of the corresponding signaling pathways, leading to various diseases such as tumors.

The UPS is made up of the 26S proteasome, ubiquitin, enzymes that activate, bind, link, deubiquitinate, and deubiquitinate ubiquitin, as well proteins broken down by 26S proteasome. Research has shown that the UPS is crucial for protein degradation in cells and that it can destroy up to 80%–85% of proteins in eukaryotic cells (Amm et al., 2014). When a ubiquitin tag is added to a substrate, it can alter the substrate's function, localization, protein interaction, or stability and then regulate many different cellular life activities (Lu et al., 2013; Streich Jr and Lima, 2014). These activities are crucial to cell growth. Diseases including cancer, Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis (ALS), spongiform encephalopathy (SLE), and arrhythmia can all be effectively treated by inhibiting the proteasome system (Pohl and Dikic, 2019).

4 The UPS and ferroptosis

4.1 The UPS and iron transport system

Factors related to iron metabolism play an essential role in ferroptosis and are potential targets to induce ferroptosis in cells. Studies have shown that in colon cancer, OTUD1 can bind and promote the deubiquitination modification of IREB2 (iron-responsive element-binding protein2), a primary regulator of iron metabolism. Promote the stability of IREB2 protein and activate the expression of its downstream TFRC gene, resulting in intracellular Fe^{2+} aggregation and increased ROS level, and eventually raise colon cancer cells' susceptibility to ferroptosis. It also encourages the release of DAMPs (DAM-associated molecular patterns) to draw in white blood cells and strengthen the host's immune response (Song et al., 2021). Lactoferrin (LF) is a glycoprotein transferrin with the unique ability to bind and transport iron. LTF, like transferrin, increases iron uptake, encouraging ferroptosis in ovarian and pancreatic cancer cells. When RSL3 and Erastin activate ferroptosis *in vitro*, iron buildup and oxidative damage are decreased because the E3 ubiquitin ligase NEDD4L mediates the protein degradation of LTF (Wang et al., 2020). Ferroportin is an essential transferrin for the balance of intracellular iron metabolism,

responsible for the outward transport of iron, and it is also the only iron transporter found in mammals (Ganz, 2005; Kuang and Wang, 2019). Studies have shown that USP35 is significantly overexpressed in human lung cancer cells and tissues, and USP35 can maintain the stability of FPN protein by targeting FPN. Functionally, knocking down USP35 in lung cancer cells can directly promote ferroptosis by down-regulating FPN protein levels and inhibiting cell growth and clone formation. In addition, under ferroptosis activator Erastin or RSL3 stimulation, knocking down USP35 can cause iron metabolism disorder and pig ferroptosis of lung cancer cells, thus promoting the growth of lung cancer cells and tumor progression (Tang et al., 2021).

4.2 The UPS and SLC7A11

Studies have shown that the deubiquitinating enzyme OTUB1, which can bind the CD44 (cluster of differentiation-44), is recruited under the effect of removing the ubiquitin chain on SLC7A11 to prevent its degradation through the proteasome and improve its stability, thereby inhibiting the occurrence of ferroptosis in tumors (Liu et al., 2019). However, the essential E3 ligase that causes SLC7A11 degradation in human tumors is unknown. SLC7A11 is regulated by TP53, NFE2L2/NRF2, ATF3, ATF4, BACH1 and other transcription factors and epigenetic regulation. Therefore, in addition to being directly regulated by the proteasome pathway at the protein level, SLC7A11 is also indirectly affected by the proteasome pathway through transcription factors and regulates ferroptosis. Studies have shown that in glioma cells, activation of the KEAP1-NRF2 signaling axis upregulates SLC7A11 and promotes glutamate secretion, thereby affecting the tumor microenvironment and promoting cell proliferation and resistance to ferroptosis (Fan et al., 2017). In lung cancer cells, p53 can promote the nuclear translocation of the deubiquitinating enzyme USP7, which further removes the ubiquitination of histone H2B from the SLC7A11 gene's regulatory area, thus inhibiting the transcription of SLC7A11 and inactivating the expression of SCL7A11, thereby encouraging ferroptosis in cells (Wang et al., 2019). In addition, the deubiquitinating enzyme BAP1 can remove monoubiquitination at the lysine 119 site of histone H2A on the SLC7A11 promoter, thereby inhibiting SLC7A11 transcription, further reducing cystine uptake of cells and increasing ferroptosis sensitivity. This process is independent of p53 (Wang et al., 2004; Zhang et al., 2018).

Further studies have shown that PRC1 (Polycomb Repressive Complex 1) can ubiquitinate histone H2A using Ring1B, Bmi1, and an E2 ubiquitin-binding enzyme UbcH5c. The SLC7A11 promoter's H2A is ubiquitinated, which inhibits SLC7A11's transcription. BAP1 and PCR1 can work together to simultaneously control the promoter's level of ubiquitination, which in turn controls SLC7A11 production (Zhang et al., 2019a). It is not ruled out that BAP1 and PRC1 may be involved in regulating ferroptosis through synergistic regulation of SLC7A11.

4.3 The UPS and GPX4

Studies have shown that PdPT, a broad-spectrum inhibitor of deubiquitination enzyme, induces ferroptosis by promoting the degradation of GPX4 protein in non-small cell lung cancer cells and further inhibits tumor growth (Yang et al., 2020). The specific mechanism is that PdPT can inhibit the deubiquitination of GPX4 by a variety of deubiquitination enzymes, including USP family members (USP14, USP15, USP10, USP7, and USP25) and UCH family members UCHL5, resulting in ubiquitination degradation of GPX4. However, the proteasome inhibitor bortezomide can reverse PDPT-induced GPX4 degradation, suggesting that the proteasome plays an essential role in the ferroptosis induced by PDPT-induced GPX4 degradation (Dong et al., 2022). In addition, by analyzing the changes in different types of ubiquitination modifications in cell ferroptosis, it has been found that cell ferroptosis promotes the increase of intracellular linear ubiquitination levels, and linear ubiquitination can regulate cell ferroptosis. When stimulated by the ferroptosis activator RSL3, GPX4 is ubiquitinated in large quantities, thus recruiting a more LUBAC. LUBAC enhances the protein stability of GPX4 by regulating its linear ubiquitination level and delaying the occurrence of cell ferroptosis, thereby inhibiting cell ferroptosis. The *in vitro* deubiquitination reaction system was used to analyze the ubiquitination status of GPX4 during ferroptosis. It was found that there were various types of ubiquitination modifications in GPX4, among which K63 ubiquitination was the primary type, and M1 and K48 ubiquitin chains could be linked to K63 ubiquitin chains to form hybrid ubiquitin chains. OTUD5 can remove the K63 ubiquitin chain of GPX4, reduce LUBAC recruitment, decrease linear ubiquitination level, destroy the protein stability of GPX4, and further affect ferroptosis of cells.

4.4 The UPS and NRF2

A transcription factor called NRF2 is essential for regulating antioxidant genes and managing cellular REDOX equilibrium. Under normal physiological conditions, cells undergo a process where NRF2 is kept in a liquid state. This occurs when NRF2 binds to its regulatory molecule known as KEAP1 (Kelch-like ECH-associated protein 1) within the cytoplasm. Following this binding, NRF2 is transported to the proteasome, a cellular structure responsible for protein degradation. However, when the cells are subjected to oxidative stress and electrophilic stimulation, NRF2 can be activated. The cysteine residues on the structure of KEAP1 will bind to intracellular ROS or electrophiles, causing conformational changes, during which NRF2 dissociates from KEAP1. Stable NRF2 undergoes nuclear translocation and interacts with antioxidant response elements (ARE) in the target gene promoter region to activate the expression of many cell protection genes, affecting various cell biological functions, such as apoptosis and senescence (Yamamoto et al., 2018). The role of the KEAP1-NRF2 signaling pathway in maintaining appropriate REDOX homeostasis has been well established, and there is growing proof that KEAP1-NRF2 is closely related to the regulation of ferroptosis. For example, recently, researchers using CRISPR screening found that KEAP1 is a key regulator of ferroptosis and showed that the lack of KEAP1 can enhance the resistance of cells to ferroptosis (Cao et al., 2019). This mechanism may be through the p62-KEAP1-NRF2 pathway to upregulate the expression of multip

le genes involved in iron and ROS metabolism (such as SLC7A11, NQO1, HO1 and FTH1), thereby inhibiting cell ferroptosis (Sun et al., 2016b). When NRF2 is strictly regulated by the E3 enzyme KEAP1, it has a corresponding cell stabilization

TABLE 1 Deubiquitinating enzymes' regulation of ferroptosis-associated proteins.

Deubiquitinating enzyme type	Deubiquitinating enzyme	Impact on ferroptosis	Target	Reference
USP	USP7	Promote	p53, TFRC	Tang et al. (2021a)
USP	USP7	Inhibit	hnRNPA1	Zhang et al. (2020)
USP	USP7	Promote	SLC7A11	Wang et al. (2019)
USP	USP11	Promote	Beclin1	Rong et al. (2022)
USP	USP14	Promote	IL-6, NRF2	Zhu et al. (2022)
USP	USP11	Inhibit	NRF2	Meng et al. (2021)
USP	USP14	Inhibit	Beclin1	Tsai et al. (2020)
USP	USP35	Inhibit	Ferroportin	Tang et al. (2021b)
OTU	OTUD1	Promote	IRP2, TFRC	Song et al. (2021)
USP	USP14	Promote	NCOA4	Li et al. (2021)
OTU	OTUB1	Inhibit	SLC7A11	Zhao et al. (2021)
USP	USP22	Inhibit	SLC7A11	Ma et al. (2020)
UCH	BAP1	Promote	SLC7A11	Zhang et al. (2019b)
UCH	BAP1	Promote	IP3R	Ye et al. (2022)
OTU	A20	Promote	ACSL4, SLC7A11	Gao et al. (2022a)

mechanism. Studies have shown that by maintaining the stability of the NRF2 protein, the deubiquitination enzyme USP11 controls ferroptosis and cell division in non-small cell lung cancer (Meng et al., 2021). With the increasing research on applying the ferroptosis mechanism in tumors, NRF2 is also an essential target for tumor treatment. Studies have shown that blocking NRF2 enhances the *in vitro* and *in vivo* anti-cancer activity of ferroptosis inducers, including Erastin and sorafenib (Meng et al., 2021). A further benefit of NRF2 activation is stopping ferroptosis from damaging renal tissue (Hu et al., 2022). In addition to inducing ferroptosis *in vitro* when stimulated by RSL3, Erastin, and ML162 (Dodson et al., 2019), NRF2 also suppresses the expression of genes that regulate GSH release, such as ABCC1/MRP1. These results suggest that most of NRF2's actions in ferroptosis are transcription-dependent; through its transcription factors, it controls the transcription of genes involved in ferroptosis before impacting ferroptosis. However, investigations have yet to be done on its transcription-independent roles in ferroptosis. NRF2 target genes can regulate cells' antioxidant, iron, and intermediate metabolic states in the context of disease therapy.

Additionally, it has been established that NRF2 regulates GPX4 and SLC7A11, the two critical targets for suppressing ferroptosis (Shin et al., 2018). These two molecules also serve to regulate ferroptosis further. Therefore, it is still possible to target NRF2 in disorders marked mainly by ferroptosis and lipid peroxidation.

4.5 The UPS and KEAP1-NFE2L2

In ferroptosis, the NFE2L2/NRF2 system functions as a transcriptional defense system (Dai et al., 2020). Because it binds to Kelch-like ECH-associated protein 1 (KEAP1), the transcription factor NFE2L2 is constitutively degraded by the UPS during quiescent conditions. When electrophilic stress and oxidative stress occur, KEAP1 is inactivated, stabilizing NFE2L2 and triggering the transcriptional activation of several cytoprotective genes. The autophagy receptor Sequestosome 1 (SQSTM1/p62) can interact with KEAP1's NFE2L2-binding site to stabilize NFE2L2 (Komatsu et al., 2010). Eratin and sorafenib, two ferroptosis activators, cause an increase in the interaction between SQSTM1 and KEAP1, which in turn causes an accumulation of NFE2L2 and enhanced nuclear transcription of NFE2L2 target genes, including FTH1, SLC7A11, NAD(P)H quinone dehydrogenase 1 (NQO1), and heme oxygenase 1 (HMOX1) (Sun et al., 2016b; Anandhan et al., 2020). Blocking NFE2L2 consistently increases the anticancer activity of ferroptosis inducers *in vitro* and *in vivo*, including sorafenib, a first-line medication for patients with liver cancer (Sun et al., 2016a; Sun et al., 2016b). NFE2L2 activation, on the other hand, might stop ferroptotic tissue damage. Moreover, NFE2L2 suppresses GSH release-related genes [such as ATP-binding cassette sub family C member 1 (ABCC1/MRP1)] to enhance ferroptosis *in vitro* brought on by RSL3, erastin, and ML162 (Cao et al., 2019). While nothing is known about NFE2L2's transcription-independent role in ferroptosis, all of these studies point to a transcription-dependent role for the protein in the process.

5 The promise of directing therapies towards ubiquitin enzymes and ferroptosis

Ferroptosis-based treatments have produced intriguing results in cancer experimentation. Increasing amounts of evidence point to the possibility that cancer cells' production of ferroptosis could

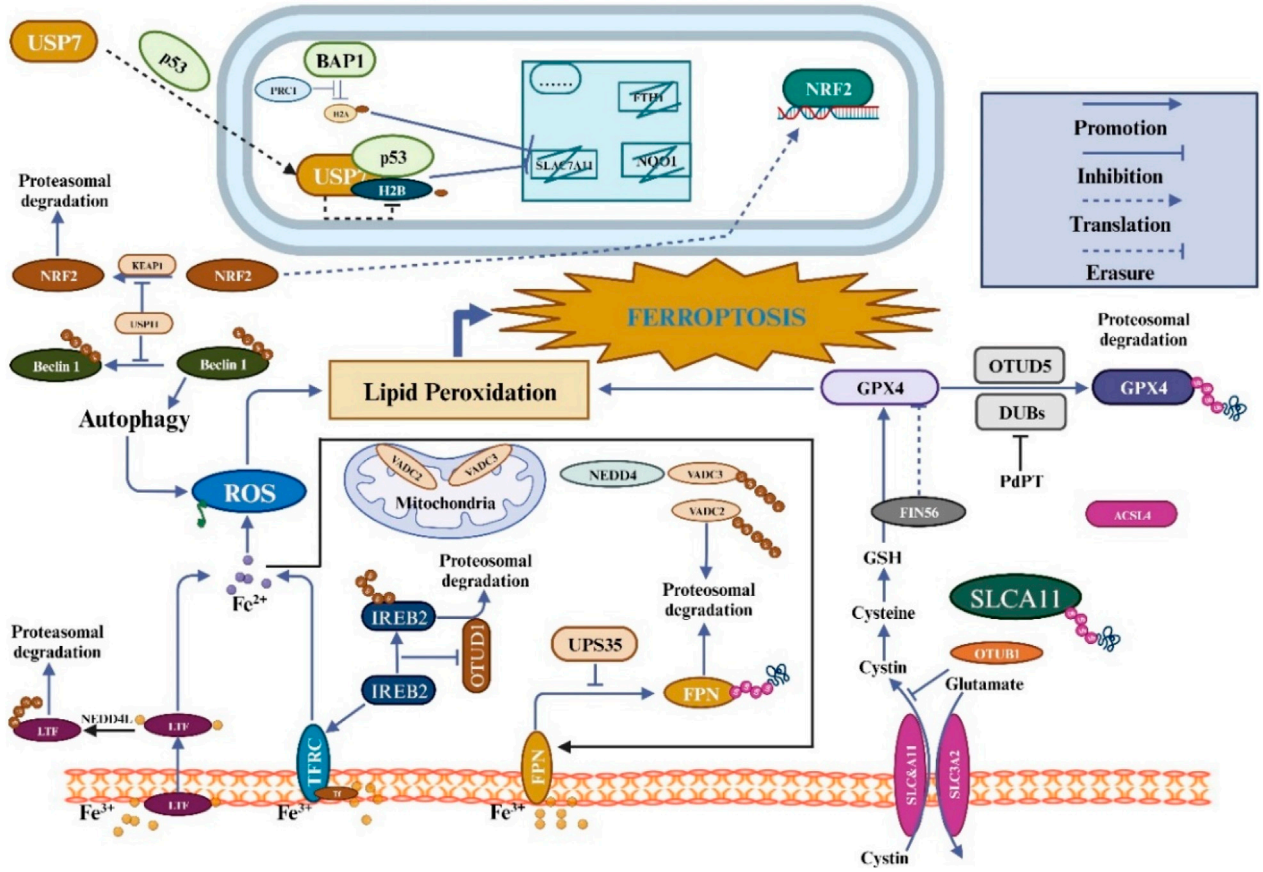

FIGURE 3
The regulation of ferroptosis involves ubiquitination and is closely linked to amino acid, iron, and lipid metabolism. Ferroptosis is induced by intracellular labile iron, which triggers lipid peroxidation. Together with GPX4 activity, cystine uptake via the System XC-transporter aids in the inhibition of ferroptosis and reduction of harmful lipid peroxides. Additionally, FSP1 and DHODH can independently inhibit ferroptosis without the involvement of GPX4. Ferroptosis regulation involves the control of these pathways via ubiquitination.

represent a brand-new avenue for clinical intervention (Wang et al., 2021). Unfortunately, the cancer patient community has not yet fully realized the potential of medicines producing ferroptosis because of a lack of effective treatment candidates and the varied sensitivity of different tumor types to ferroptosis (Chen et al., 2021). DUBs and E3 ligases are essential in controlling cells' susceptibility to ferroptosis. They have potential as both prognostic indicators and ubiquitination pathway targets for novel small compounds, which could significantly speed up the clinical implementation of ferroptosis-based therapy. Many types of anti-cancer medications affect ubiquitin enzymes. These include inhibitors, proteolysis-targeting chimaeras (PROTACs), agonists, and molecular glues. Over the past 2 decades, we have seen substantial progress in creating E3 and DUB inhibitors, with several small compounds that target E3 or DUBs being used in preclinical trials and being improved by commercial companies. To promote the ubiquitination and proteasomal degradation of the protein of interest (POI), PROTAC, a tripartite molecule made up of an E3 ligand, a POI ligand, and a linker, binds to both E3 and POI (Schauer et al., 2019; Yang et al., 2020; Ye et al., 2021). Small compounds known as "molecular glues" can re-align ubiquitin enzymes with their substrates, triggering the breakdown of the substrates (Dale et al., 2021).

However, putting ubiquitin-based pro-ferroptosis medicines into practical use will take much work due to the complexity of ubiquitination regulation. Ubiquitin enzymes have a wide range of enzymatic activity. Therefore, it is essential to consider issues like selectivity and potential side effects. Moreover, it is essential to remember that ferroptosis is not limited to cancer cells; it also happens in other cells in the tumor microenvironment, like tumor-associated macrophages (TAMs) and T cells + CD8. These immune cells' ability to ferroptosis can occasionally promote the growth of tumors (Xu et al., 2021). To find the most effective ubiquitin-based pro-ferroptosis therapy, it may be helpful to have a better understanding of how E3s or DUBs regulate ferroptosis.

6 The function of deubiquitinating enzymes (DUBs) in modulating ferroptosis

DUBs, are essential controllers of protein activity. They accomplish this by selectively eliminating ubiquitin chains from particular proteins. There are seven major groups into which these enzymes can be divided. The others are cysteine-based superfamilies, including the ubiquitin-specific proteases (USPs),

TABLE 2 Ferroptosis control is accomplished by the ubiquitin-proteasome system in two ways.

Name	Molecular mechanism	Promote or inhibit ferroptosis	References
OTUD5	Remove the K63 ubiquitin chain of GPX4	+	Dong et al. (2022)
OTUB1	Deubiquitination and stabilization of SLC7A11	–	Liu et al. (2019)
KEAP1	The E3 ligase adaptor of NRF2	–	Sun et al. (2016b)
USP7	p53 promotes the nuclear translocation of USP7, which Deubiq uit and stabilize of H2B	+	Wang et al. (2019)
	deubiquitination and stabilization of hnRNPA	–	Zhang et al. (2020)
BAP1	Deubiquitination and stabilization of H2A	+	Wang et al. (2004)
PRC1	Ubiquitination of H2A	–	Zhang et al. (2019a)
UbcH5c		–	Song et al. (2021)
OTUD1	Deubiquitination and stabilization of IREB2	+	Wang et al. (2020)
NEDD4L	The E3 ligase adaptor of LTF, ubiquitination of LTF	–	
USP35	Deubiquitination and stabilization of FPN	+	Tang et al. (2021b)
USP11	Deubiquitination and stabilization of NRF2	–	Meng et al. (2021)
	Deubiquitination and stabilization of Beclin 1	+	Rong et al. (2022)
VHL	The E3 ligase adaptor of HIFs	–	Zou et al. (2019)
NEDD4	The E3 ligase adaptor of VDAC2 and VDAC3	–	Yang et al. (2020b)

Note: +: promotion; –: inhibition.

Ub C-terminal hydrolases (UCHs), Machado-Joseph disease domain proteases (MJDs), motif interacting with Ub containing novel DUB family (MINDYs), ovarian tumour proteases (OTUs) and the zinc-finger and UFSP domain protein (ZUFSP) (Lange et al., 2022).

These DUBs can play various biological roles, thanks to their unique structural characteristics. The processing of ubiquitin precursors and the cleavage of poly-ubiquitin chains are two examples; trimming ubiquitin chains to protect proteins from degradation and modifying ubiquitin chains to alter the signals they transmit are two more (Cheng et al., 2019).

Recent studies have shown that DUBs regulate ferroptosis's apoptotic process by altering substrate stability and signaling. The ways that DUBs regulate ferroptosis will be discussed in this text, along with how they might be used to establish a relationship between ferroptosis and tumor suppression. We'll also examine the possible upsides of targeting DUBs in cancer therapies (Table 1). Shows the deubiquitinating enzymes' regulation of ferroptosis-associated proteins.

7 Additional interactions of the UPS in ferroptosis

Recent research has shown that ubiquitin-specific peptidase 11 (USP11) regulates autophagy-dependent ferroptosis in response to spinal cord ischemia-reperfusion injury by deubiquitinating Beclin 1. This connection enhances ferritin breakdown during autophagy and activates autophagy resulting in further ferroptosis (Rong et al., 2022). The process of a cell's ferroptosis is also significantly influenced by a low-oxygen environment. Specific transcription factors susceptible to cellular hypoxia are known as hypoxia-inducible factors (HIFs), which include three subunits (HIF1A/HIF1, EPAS1/HIF2, HIF3A/HIF3, and one ARNT/HIF1 subunit). According to the findings, HIFs may play a dual role in controlling ferroptosis in various tumor types. First, HIF1A promotes the absorption as well as storage of fatty acids in human fibrosarcoma cells by inducing the development of two fatty acid-binding proteins by transcription, consequently suppressing ferroptosis triggered by RSL3 and ferroptosis activator FIN56. In contrast, EPAS1 selectively enriched polyunsaturated lipids by transactivating genes encoding hypoxia-induced lipid droplets in renal cancer cells *in vitro*, thereby promoting ferroptosis induced by ferroptosis activators RSL3, ML162, or ML210 (Miess et al., 2018; Zou et al., 2019). The VHL, a particular E3 enzyme of HIFs, acts as a direct indicator of HIF stability, which makes it an indirect regulator of ferroptosis.

Ferroptosis depends on the stability of the functioning of mitochondria and activity because mitochondria are the primary source of RO synthesis. The volt-dependent anion channel (VDAC), a member of the multifunctional channel protein family, is essential for facilitating the passage of ions and metabolites through the outer membrane of mitochondria in eukaryotic cells (Shoshan-Barmatz et al., 2010). Studies have shown that Erastin can bind directly to VDAC2 and induce ferroptosis. When Erastin induced melanoma cells, VDAC2 and VDAC3 formed a negative feedback regulatory mechanism through proteasome-dependent degradation regulated by E3 NEDD4 (Yang et al., 2020). But more research will be needed to determine whether particular mitochondrial E3 ligases are responsible for the destruction of VDAC during ferroptosis. Recent research has shown that tumor-associated fibroblasts (CAFs) contain higher levels of Ubiquitin-specific protease-7 (USP7), which promotes the deubiquitination of miRNA sort-related protein hnRNPA1. Encourage the release of CAFs

exosome miR-522, and this miR-522-secreted exosome further prevents gastric cancer cells from ferroptosizing by focusing on and suppressing the expression of ALOX15 in these cells (Zhang et al., 2020). As the understanding of ferroptosis and its mechanisms continues to improve, there is increasing evidence that members of the ubiquitin-proteasome system regulate ferroptosis and play an essential role (Figure 3). From the above, it can be found that the ubiquitin-proteasome system has a dual function in regulating ferroptosis (Table 2).

8 Conclusion and future prospects

A unique form of cell death called ferroptosis is brought on by iron-mediated lipid peroxidation. This recently found method offers hope for eliminating cancer cells but raises an intriguing problem in developing the disease. The need for a thorough understanding of the regulatory components driving ferroptosis is further highlighted because it operates through a dualistic process, impacting distinct cells in the tumor microenvironment (TME) in various ways.

In this scenario, the post-transcriptional modification mechanism known as ubiquitination is crucial. Through substrate ubiquitination and deubiquitination regulation, this route exerts influence over a wide range of cellular processes. Cancer development and therapy both involve ubiquitin enzymes and their regulation. These enzymes interact with proteins involved in ferroptosis, which mediates cells' sensitivity to ferroptosis and elucidates their relationship. Recent studies have started to outline how E3 ligases and DUBs collectively affect ferroptosis. However, there is still a significant gap in our knowledge of the precise mechanisms involved, such as the specifics of polyubiquitination, the specifics of ubiquitination sites, or additional procedures that are independent of ligase activity, due to the complex nature of the regulation of ubiquitin enzymes.

We currently have a limited understanding of how developing ferroptosis-regulating proteins like FSP1, DHODH, and GCH1 are regulated by E3s and DUBs. Drug development efforts to suppress ferroptosis with an E3 or DUB inhibitor will advance more quickly if these open questions are resolved. E3s and DUBs, among other ubiquitin system enzymes, are critical regulators of the ferroptosis process. Therefore, deepening our comprehension of this complex regulatory network may reveal novel cancer therapy strategies, adding a potent weapon to our therapeutic toolbox. As we get a deeper understanding of these mechanisms and their interdependencies, we may be able to shed light on hitherto uncharted avenues leading to more effective cancer therapy.

Author contributions

MD: Writing–review and editing. YL: Conceptualization, Writing–review and editing. NW: Writing–review and editing. BW: Writing–original draft. FM: Conceptualization, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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What are the benefits of therapeutic drug monitoring in the optimization of adalimumab therapy? a systematic review and meta-analysis up to 2022

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Aims: Persistent uncertainties exist surrounding the therapeutic drug monitoring (TDM) of adalimumab in clinical settings. To address these issues, we conducted a systematic review to assess the current evidence regarding the benefits of TDM for adalimumab.

Methods: PubMed, EMBASE, and Cochrane Databases were searched from inception to October 2022. The trials regarding to the list three key questions were considered: 1) Could routine proactive TDM assist in improving outcomes in patients receiving adalimumab? 2) Could reactive TDM assist in guiding subsequent treatment strategies for patients with treatment failure to adalimumab? 3) Could TDM assist in informing dose reduction or discontinuation in patients with low disease activity or in remission treated with adalimumab? Two reviewers independently selected the studies and extracted the data. Meta-analysis was performed to calculate the relative risk (RR) and 95% confidence interval (CI).

Results: A total of 9 studies was included in this review. For proactive TDM, meta-analysis indicated that proactive TDM (n = 163/257, 63.42%) showed no significant superiority over reactive TDM and/or conventional management (n = 336/606, 55.44%) in achieving and/or maintaining clinical remission by random effects model (RR: 1.24, 95% CI 0.98–1.58, I^2 = 73%). There were three studies that supporting the reactive TDM, low drug levels in the absence of anti-drug antibodies (ADA) strongly indicate the need for dose intensification, and infliximab is a feasible choice for patients with low drug levels and ADA positivity. While swapping to another class should be considered in patients with adequate drug levels. In addition, TDM can help clinicians optimize dosing schedules and prevent overtreatment in patients who have achieved low disease activity and sufficient drug concentrations, with no predictive value for successful adalimumab discontinuation.

Conclusion: Current evidence suggests that proactive TDM is numerically but not statistically significant superiority over reactive TDM and/or conventional management. Reactive TDM can aid in understanding treatment failure and developing subsequent therapy. For patients reaching low disease activity and

remission, TDM can help successful dose reduction, while it cannot inform the successful drug discontinuation. However, existing trials are limited, and more well-designed trials are necessary to clarify the role of TDM in adalimumab treatment.

KEYWORDS

adalimumab, biologics, therapeutic drug monitoring, benefits, optimization

1 Introduction

Adalimumab (ADM), a fully human monoclonal antibody that neutralizes tumor necrosis factor-α (TNF-α), was initially approved for the treatment of moderate to severe rheumatoid arthritis (RA) in 2002 (Rau, 2002). Since then, it has been found to be effective in treating a variety of other conditions, such as ankylosing spondylitis, psoriasis, Crohn's disease (CD), ulcerative colitis (UC), uveitis, and juvenile idiopathic arthritis, making it the most widely used agent.

Therapeutic drug monitoring (TDM) is a practical method used to monitor the drug concentration and their metabolites in the blood, which can help guide clinical medication decisions, enhance drug effectiveness, prevent drug toxicity, and establish personalized treatment schedules. Recently, TDM has become essential in biological therapy due to the impact of drug concentrations of TNF-α inhibitors on clinical outcomes (Pouw et al., 2015; Rinawi et al., 2021). Anti-drug antibodies (ADA) play a significant role in the inter-individual variability of drug clearance, leading to insufficient drug exposure and treatment failure, such as primary non-response (PNR) and loss-of-response (LOR) (Bartelds et al., 2011; Baert et al., 2016; Ding et al., 2020). Reactive TDM refers to measure biological concentration and ADA in patients experiencing treatment failure. This approach is endorsed by the American Gastroenterological Association and expert consensus statements to understand treatment failure (Feuerstein et al., 2017; Mitrev et al., 2017; Cheifetz et al., 2021; Krieckaert et al., 2023), despite the limited quality of evidence. The supported evidence comes primary from studies involving infliximab therapy. It is not yet clear how many benefits of TDM can bring to the clinical application of ADM. However, the use of proactive TDM, which involves scheduled testing and adjusting dosages to achieve predefined target concentrations, lacks consistent recommendations (Feuerstein et al., 2017; Cheifetz et al., 2021). There are persistent uncertainties surrounding the most effective use of TDM in clinical settings. Specifically, the evidence supporting the use of TDM to guide dose reduction or discontinuation in patients achieving deep remission has not been reviewed.

To systematically review the value of TDM in optimizing ADM therapy, three key questions throughout the entire drug treatment process were considered: 1) Could routine proactive TDM assist in improving outcomes in patients receiving ADM? 2) Could reactive TDM assist in guiding subsequent treatment strategies for patients PNR or LOR to ADM? 3) Could TDM assist in informing dose reduction or discontinuation in patients with low disease activity or in remission treated with ADM?

2 Methods

2.1 Search strategy

This systematic review and meta-analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Page et al., 2021). We systematically searched PubMed, EMBASE and Cochrane Database from inception to October 2022 to identify applicable studies. A search strategy was created based on the PICO (Population, Intervention, Comparison, Outcomes) questions. The search terms used were combinations of text-free terms and Medical Subject Headings (MeSH) terms as follows: ADM, therapeutic drug monitoring, therapeutic monitoring, serum concentration monitoring. There were no language or publication date restrictions. The full search terminology was included in the Supplementary Table S1. We also hand-searched trial registries such as ClinicalTrials.gov (https://clinicaltrials.gov) and the World Health Organization (WHO) International Clinical Trials Registry Platform (http://apps.who.int/trialsearch) and reference lists of included trials for completeness.

2.2 Selection criteria

Studies published as full manuscripts related to the PICO questions were included. These involved studies assessing: 1) Could routine proactive TDM assist in improving outcomes in patients receiving ADM? 2) Could reactive TDM assist in guiding subsequent treatment strategies for patients PNR or LOR to ADM? 3) Could TDM assist in informing dose reduction or discontinuation in patients with low disease activity or in remission treated with ADM? There were no restrictions on disease types or TDM measurements. Reviews, editorials, guidelines, case reports, and studies that focused only on pharmacokinetics and pharmacodynamics were excluded.

2.3 Data extraction

Two reviewers (Yun Li and Cheng Xie) independently assessed studies for possible inclusion by reading titles and/or abstracts, then viewed the full texts of the remaining publications to pick up the ultimately available studies. Data extraction was done by one reviewer (Yun Li), and subsequently cross-checked by the other reviewer (Cheng Xie). Any divergences were discussed or determined by a third investigator (Xiaoliang Ding). Following information was abstracted: the first author and publication year,

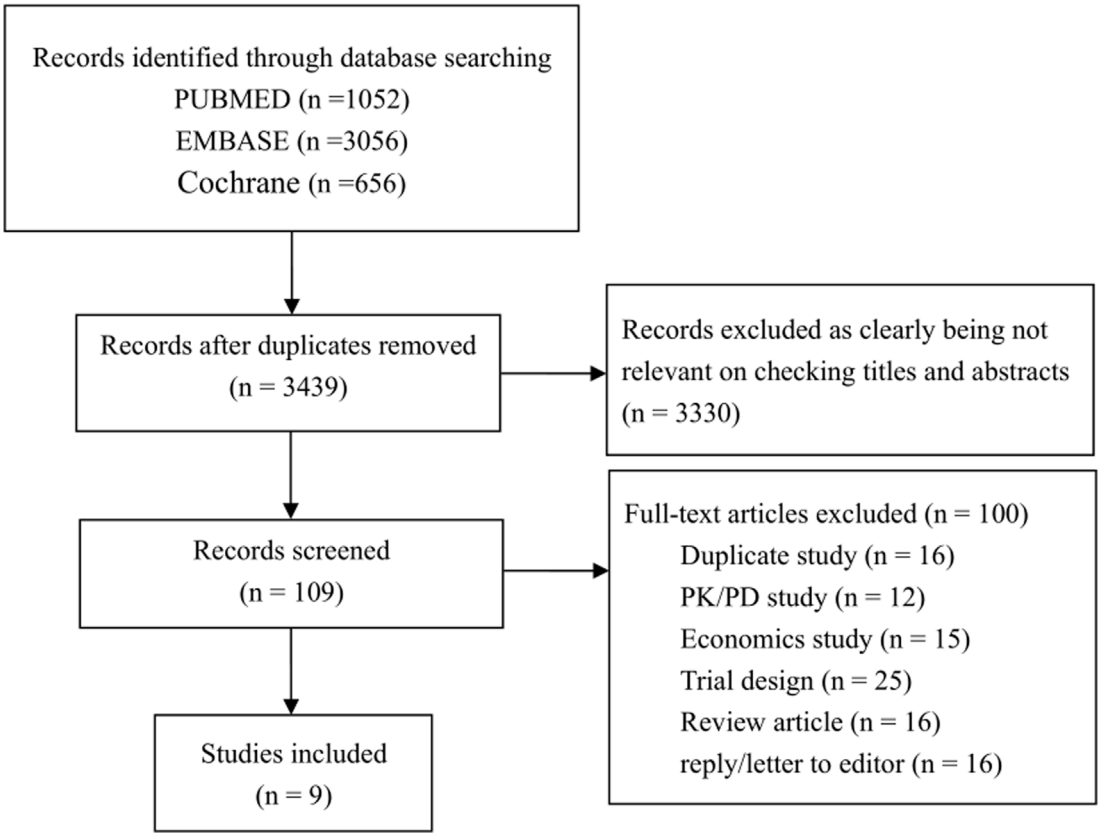

FIGURE 1
Flow chart depicting the process of selecting the studies included.

country, study type, sample size, baseline, patients feature, treatment feature, follow-up time, the clinical outcomes and their definitions.

2.4 Quality appraisal

Two reviewers (Yun Li and Cheng Xie) independently evaluated the quality of the studies. Disagreements were resolved through discussion and consultation with the third investigator (Xiaoliang Ding). The risk of bias in the randomized controlled trial (RCT) was evaluated according to the standards developed by the Cochrane Bias Risk Tool (Sterne et al., 2019). The quality of the observational studies was assessed using the Newcastle–Ottawa scale (NOS) (Stang, 2010).

2.5 Data analysis

In this systematic review, we conducted a narrative review and utilized meta-analysis when dichotomous outcomes were sufficiently similar across studies, considering the diversity of these focused questions. Both fixed-effect and random-effects model were employed to calculate the relative risk (RR) and 95% confidence interval (CI). Heterogeneity of effect size across the studies was tested using Q statistics at the $p < 0.10$ level of significance. We also calculated the I^2 statistic with a quantitative measure of inconsistency across the studies. The data were pooled by random-effects model in case significant heterogeneity (Cochran test with $p < 0.10$ or $I^2 > 50\%$) was found. Otherwise, the fixed-effects model was used. Subgroup analysis, sensitivity analysis, and publication bias analysis were not conducted due to the limited number of included studies. The analysis was carried out using the "meta" package in R (version 4.3.2).

3 Results

3.1 Search results and characteristics of the included studies

Figure 1 shows the research selection process for inclusion in the systematic review. The initial search generated 4764 references. After deleting 1325 duplicate articles titles and abstracts of all the articles were reviewed. A total of 109 studies were reviewed in full, while 100 studies were excluded because of not meet the inclusion criteria. The main reasons for excluding full articles were the inability to extract data related to ADM alone, noncompliance with research objectives, review articles and editorials/letters to editors. The final 9 studies were included (Papamichael et al., 2019; Assa et al., 2019; D'Haens et al., 2022; Panes et al., 2022; Roblin et al., 2014; Roblin et al., 2022; Ulijn et al., 2020; Chen et al., 2016; Lamers-Karnebeek et al., 2019) and the details are shown in Table 1.

TABLE 1 Summary of studies.

Trial, author name, year	Study type	Patient population, research duration	Sample size; age (median/ range or mean/SD); % males	Phase of study; disease score at inclusion (median/ range or mean/SD)	Disease duration; duration of ADA therapy (median/ range or mean/SD)	Immunosuppressive treatment (N, %); glucocorticoid treatment (N, %)
Roblin, 2014 (Roblin et al., 2014)	Prospective, cohort, single center (France)	Adult IBD (55% CD), 29 months	82, 43 ± 12, 50	Maintenance phase; CDAI: 340 (110); Mayo: 9 2)	7.4 (3.2) years; 17 9) months	10 (12%); NR
Ulijn, 2020 (Ulijn et al., 2020)	Retrospective, cohort, single center (Netherlands)	Adult RA, 6 months	137, 64.4 ± 13.2, 31.4	Induction phase and maintenance phase; NR	8.7 (12.7) years; 0.75 (3.2) years	Azathioprine: 20 (14.6%); Methotrexate: 60 (43.8%); Leflunomide: 23 (16.8%); Glucocorticoid:24 (17.5%)
Roblin, 2022 (Roblin et al., 2022)	Nonrandomized comparative study, multicenter (2 sites in France)	Adult IBD (70.2% CD), 38 months	131, 36.5 ± 14.6, 50.3	Maintenance phase; CDAI: 300 (240–365); Mayo: 8 (6–10)	80 (32–108) months; 43 (12–68) months	76 (58.0); NR
Chen, 2016 (Chen et al., 2016)	Prospective, cohort, single center (Taiwan)	Adult RA, 24 weeks	64, 55, 9.4	LDA or remission; DAS28: 2.7	9.1 years; 5.8 years	Methotrexate: 58 (90.6%); Salazopyridine: 19 (29.7%); Hydroxychloroquine sulfate: 14 (21.9)
POET, Lamers-Karnebeek 2018 (Lamers-Karnebeek et al., 2019)	Prospective, cohort, multicenter (13 sites in Netherlands)	Adult RA, 12 months	210, 59 ± 13, 31	Stop treatment; DAS28-ESR: 1.96 (0.76)	9 (2.2) years; NR	NR
Papamichael, 2019 (Papamichael et al., 2019)	Retrospective, cohort, multicenter (2 sites in United States)	Adult IBD (81% CD), 37.2 months	382, 25 (19–36), 49	Maintenance phase; NR	9 (3–19) years; NR	Thiopurines: 90 (83%); methotrexate: 19 (17%)
PAILOT, Assa, 2019 (Assa et al., 2019)	RCT, multicenter (9 sites in Israel)	Pediatric CD, 18 months	78, 14 (6–18), 71	Maintenance phase; PCDAI:3.1 (1.0-7.5)	6.0 (1.2-24.7) years; NR	Thiopurines: 28 (35.9%); methotrexate: 7 (9.0%)
SERENE–UC, Panés, 2022 (Panes et al., 2022)	RCT, multicenter (144 sites in 20 countries)	Adult UC, 44 weeks	219, 37 (19–63), 48.6	Maintenance phase; NR	NR	NR
SERENE–CD, D'Haens, 2022 (D'Haens et al., 2022)	RCT, multicenter (93 sites in 19 countries)	Adult CD, 44 weeks	184, 34 (18–73), 53.3	Maintenance phase; CDAI: 303.4 (56.3)	6.4 (8.2) years; NR	25 (27.2); 56 (60.9)

Inflammatory bowel disease, IBD; Crohn's disease, CD; Crohn's disease activity index, CDAI; ulcerative colitis, UC; rheumatoid arthritis, RA; not reported, NR; low disease activity, LDA; disease activity score 28, DAS28; erythrocyte sedimentation rate, ESR.

3.2 Quality of the included studies

A summary of the bias risk data is shown in Figure 2 and Table 2. The quality evaluation of the RCTs revealed that three trials were at high risk of bias across one domain (randomization domain, PAILOT; Other bias, SERENE UC and SERENE CD). PAILOT study is an Open-label study, and most outcomes likely to be influenced. There was no sample size calculation for the maintenance study in SERENE UC and SERENE CD studies. All six observational studies received 8–9 stars out of 9 on the NOS, indicating low risk of bias. Four studies did not fully meet the scoring criteria in terms of inter group comparability and population representativeness. In Roblin's study, they combined CD and UC together, which may affect the comparability of the results (Roblin et al., 2014; Roblin et al., 2022). In addition, therapeutic groups were not fully comparable at baseline, especially in terms of disease (Roblin et al., 2022). In Lamers Karnebeek's study, the included population had a longer duration of disease (average of 9 years), which may not fully represent the population of patients with RA (Lamers-Karnebeek et al., 2019). In Papamichael's research the control group received standard of care which was defined as empirical dose escalation and/or reactive TDM. Therefore, it is not possible to draw clear conclusions between proactive TDM and reactive TDM, as well as between proactive TDM and empirical dose escalation (Papamichael et al., 2019).

3.3 Benefits gained from TDM

3.3.1 Scenario A: value of target concentration intervention

Dosage adjustment to target and maintain a predefined drug concentration was the primary format of TDM, specifically referred

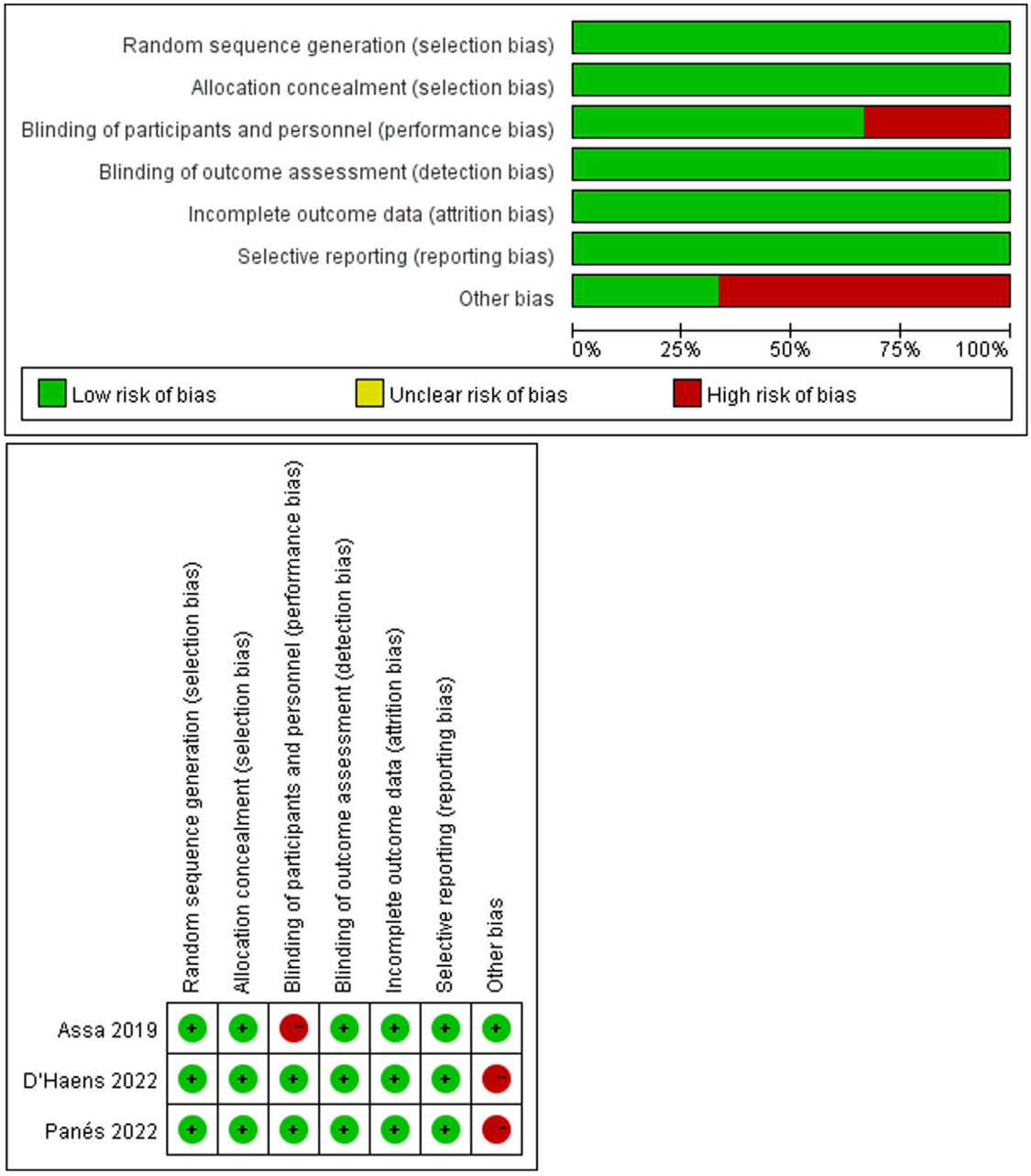

FIGURE 2
The risk of bias in randomized controlled trials was assessed using the Cochrane risk of bias tool.

to routine proactive TDM. This scenario included three RCTs (Assa et al., 2019; D'Haens et al., 2022; Panes et al., 2022) and one observational study (Papamichael et al., 2019), with detailed characteristics outlined in Table 3. Results from the meta-analysis indicated that proactive TDM (n = 163/257, 63.42%) showed no significant superiority over reactive TDM and/or conventional management (n = 336/606, 55.44%) in achieving and/or maintaining clinical remission by random effects model (RR: 1.24, 95% CI 0.98–1.58, I^2 = 73%; Figure 3).

3.3.2 Scenario B: value of guiding treatment strategy in patients experiencing treatment failure

Reactive TDM plays a crucial role in understanding and addressing treatment failure with ADM treatment. A total of three studies were included in this scenario and the detailed characteristics were shown in Table 3. Two retrospective cohorts (Roblin et al., 2014; Ulijn et al., 2020) were conducted to evaluate the predictive value of TDM in guiding subsequent strategies. Roblin et al. (2014) studied 82 patients with inflammatory bowel disease

TABLE 2 Risk of bias in nonrandomized studies using the Newcastle–Ottawa scale.

References	Quality indicators								Total number of stars (out of 9)
	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	
Roblin, 2014 (Roblin et al., 2014)	★	★	★	★	★	★	★	★	8
Ulijn, 2020 (Ulijn et al., 2020)	★	★	★	★	★★	★	★	★	9
Roblin, 2022 (Roblin et al., 2022)	★	★	★	★	★	★	★	★	8
Chen, 2016 (Chen et al., 2016)	★	★	★	★	★★	★	★	★	9
Lamers-Karnebeek 2018 (Lamers-Karnebeek et al., 2019)		★	★	★	★★	★	★	★	8
Papamichael, 2019 (Papamichael et al., 2019)	★	★	★	★	★	★	★	★	8

[a]Indicates exposed cohort truly representative.
[b]Non exposed cohort drawn from the same community.
[c]Ascertainment of exposure from a secure record.
[d]Outcome of interest not present at start of study.
[e]Study controls for important confounder 1 ± additional confounders.
[f]Assessment of outcome of record linkage or independent blind assessment.
[g]Follow-up long enough for outcomes to occur.
[h]Follow-up adequacy.

(IBD) who experienced disease relapse and were treated with ADM at a weekly dose of 40 mg. Results showed that after 6 months, patients with drug level <4.9 μg/mL and negative ADA tested at time of relapse had a higher clinical remission rate (67%, n = 16/24) compared to those with drug level >4.9 μg/mL (29.2%, n = 12/41) or drug level <4.9 μg/mL and ADA positive (12%, n = 2/17). Subsequently, the remaining fifty-two patients who did not respond to ADM were switched to infliximab treatment. Among these patients, those with drug level <4.9 μg/mL and ADA positive exhibited higher clinical response rate (80%, n = 12/15) than those with drug level >4.9 μg/mL (6.9%, n = 2/29) or drug level <4.9 μg/mL and ADA negative (25%, n = 2/8). Ulijn et al. (Ulijn et al., 2020) conducted a retrospectively study involving 137 RA patients who failed treatment with ADM. The study analyzed the predictive value of TDM results for the use of subsequent biological agents and did not find clear predictive value of ADM concentrations or ADA status in either the TNF-α inhibitors or non-TNF-α inhibitors groups. A nonrandomized controlled trial conducted by Roblin et al. (Roblin et al., 2022) compared dose intensification (n = 61) with swapping to different class (ustekinumab or vedolizumab, n = 70) in patients under ADM maintenance therapy who experienced LOR and had ADM concentration >4.9 μg/mL. The median time without discontinuation in the swapping group was significantly longer than that in the intensification group (24 months vs. 13.3 months, $p < 0.001$). In summary, reactive TDM may assist in understanding the mechanisms of treatment failure and making subsequent treatment strategies. Low drug levels in the absence of ADA strongly indicate the need for dose intensification, with infliximab being a viable option for patients with low drug levels and ADA positive. While swapping to another class should be considered in patients with adequate drug levels.

3.3.3 Scenario C: value of guiding dose reduction or discontinuation

TDM can help reduce overtreatment in patients with low disease activity or in remission by identifying higher drug concentrations. This approach allows for dose reduction or tapering while still maintaining efficacy. Two studies (Chen et al., 2016; Lamers-Karnebeek et al., 2019) were included in this scenario, and their characteristics were shown in Table 3. Chen et al. (2016) evaluated the predictive value of ADM concentrations for dose reduction. 64 RA patients who had already achieved low disease activity (LDA) or remission after receiving ADM full-dose therapy at least 2 years were included, and then received ADM dose-halving at a dose of 40 mg monthly. After 24-week follow-up, they found that ADM concentration above a cutoff of 6.4 μg/mL predicted a persistent remission (AUC: 0.998, 95% CI: 0.936-1.000, sensitivity: 100%, specificity: 93.4%), and a persistent LDA (AUC: 0.995, 95% CI: 0.931-1.000, sensitivity: 93.9%, specificity: 100%) after dose halving. ADM dose halving is feasible for patients who have achieved remission and adequate drug levels. Lamers-Karnebeek et al. (2019) investigated whether the ADM concentration and ADA status predict disease flares after ADM cessation in RA patients who received ADM therapy for more than 1 year and achieved LDA for at least 6 months. 210 RA patients with 1 year follow-up after ADM discontinuation were included and analyzed. 62 (53%) of 117 patients with ADM concentrations ≥5 μg/mL experienced a flare versus 44 (47%) of 93 patients with concentrations <5 μg/mL, with no cut-off of ADM concentration at stopping ADM clearly predicted disease flare. TDM can help clinicians optimize dosing schedules and prevent overtreatment in patients who have achieved LDA and sufficient drug concentrations, with no predictive value for successful ADM discontinuation.

4 Discussion

In clinical setting, TDM typically involves adjusting the dosage based on blood concentrations and using pharmacometrics model to ensure that the concentration falls within the desired range to achieve optimal efficacy and avoid adverse reaction. The clinical implementation of TDM of ADM is intricate, mainly due to the need to adjust treatment plans based on different clinical scenarios and TDM results. Our study outlines the benefits of TDM in the entire

TABLE 3 Clinical studies on the benefits of TDM with ADM.

Study	Population	Primary outcome	Comparison (exposure/ intervention)	Results
Scenario A: Value of target concentration intervention				
PAILOT, Assa, 2019 (Assa et al., 2019)	Pediatric CD patients responded to ADM induction therapy	Sustained corticosteroid-free clinical remission (PCDAI<10) 18 months	Intervention (proactive TDM, N = 38): ADM was intensified in patients with DL < 5 μg/mL regardless of disease activity Comparator (reactive TDM, N = 40): ADM was intensified only in patients with LOR and DL < 5 μg/mL simultaneously	**Corticosteroid-free clinical remission at all visits** Proactive TDM: 82% (31/38) Reactive TDM: 48% (19/40) Proactive TDM is superior to reactive TDM, resulting in higher corticosteroid-free sustained remission
Papamichael, 2019 (Papamichael et al., 2019)	Adult IBD patients who received maintenance ADM therapy	Treatment failure (LOR or SAE or need for an IBD-related surgery) 3.1 years (median)	Intervention (proactive TDM, N = 53): titrating ADM to concentration typically >10 μg/mL Comparator (standard care, N = 329): empiric dose escalation and/or reactive TDM	**People had treatment failure** Proactive TDM: 17% (7/53) Standard care: 36% (119/329) Proactive TDM may be associated with a lower risk of treatment failure compared to standard care
SERENE–CD, D'Haens, 2022 (D'Haens et al., 2022)	Adult CD patients who achieved clinical response at week 12	Clinical remission (CDAI<150) 44 weeks	Intervention (TDM, N = 92): achieve DL > 5 μg/mL and not exceeding 20 μg/mL Comparator (clinically adjusted, N = 92): dose adjustment based on disease activity	**Achieved clinical remission at week 56** TDM: 66.3% (61/92) Clinically adjusted: 70.7% (65/92) Dose adjustment based primarily on DL did not provide additional clinical benefit over clinical adjustment based on symptoms and biomarkers
SERENE–UC, Panés, 2022 (Panes et al., 2022)	Adult UC patients who achieved clinical response at week 8	Clinical remission (full Mayo score ≤2 with no subscore >1) 44 weeks	Intervention (TDM, N = 92): achieve DL ≥ 10 μg/mL Comparator (40 mg ew, N = 152 or 40 mg eow, N = 145)	**Clinical remission at week 52** TDM: 36.5% (27/74) 40 mg ew: 39.5% (60/152) 40 mg eow: 29.0% (42/145) The efficacy of TDM group was comparable to that of standard dose or high dose group
Scenario B: Value of guiding treatment strategy optimization in patients experiencing treatment failure				
Roblin, 2014 (Roblin et al., 2014)	Adult IBD patients, who experienced LOR with 40 mg eow and subsequently receive dosage optimization of 40 mg ew	Clinical remission (CD: CDAI<150 and fecal calprotectin <250 μg/g stool, UC: total Mayo score<3 and endoscopic subscore≤1) 6 months	Three groups defined according to DL and ADA status at LOR Group A (N = 41): DL > 4.9 μg/mL Group B (N = 24): DL < 4.9 μg/mL and ADA negative Group C (N = 17): DL < 4.9 μg/mL and ADA positive	**Proportion of clinical remission** Group A: 29.2% (12/41) Group B: 67% (16/24) Group C: 12% (2/17) Dosage optimization should be considered in patients with low DL and ADA negative
Roblin, 2014 (Roblin et al., 2014)	Adult IBD patients who did not respond to ADM 40 mg ew and subsequently received IFX treatment	Clinical remission (CD: CDAI<150 and fecal calprotectin <250 μg/g stool, UC: total Mayo score<3 and endoscopic subscore≤1) 6 months	Three groups defined according to DL and ADA status at LOR Group A (N = 41): DL > 4.9 μg/mL Group B (N = 24): DL < 4.9 μg/mL and ADA negative Group C (N = 17): DL < 4.9 μg/mL and ADA positive	**Proportion of clinical remission** Group A: 6.9% (2/29) Group B: 25% (2/8) Group C: 80% (12/15) Switch to IFX should be considered in patients with low DL and ADA positive
Roblin, 2022 (Roblin et al., 2022)	Adult IBD patients who experienced LOR with 40 mg eow and DL > 4.9 μg/mL	Therapeutic discontinuation (CD: CDAI>220 and fecal calprotectin >250 μg/g stool, UC: total Mayo score>6 and endoscopic subscore>1, or intolerance to treatment) 24 months	Two strategies according to physician's decision Optimization group (N = 61): ADM 40 mg ew Swap group (N = 70): switching to UST or VDZ	**Proportion of therapeutic discontinuation** Optimization group: 59.6% (36/61) Swap group: 14.8% (11/70) Swapping to another class is better than dosage optimization in patients who experienced LOR and DL > 4.9 μg/mL

(Continued on following page)

TABLE 3 (*Continued*) Clinical studies on the benefits of TDM with ADM.

Study	Population	Primary outcome	Comparison (exposure/ intervention)	Results
Ulijn, 2020 (Ulijn et al., 2020)	RA patients who experienced inefficacy or toxicity with ADM 40 mg eow and subsequently received another TNFi	EULAR response (DAS28-CRP/ESR, change from baseline ≥1.2 and current DAS28-ESR<3.2 and DAS28-CRP<2.9) 3–6 months	Two groups defined according to DL or ADA status between ≥8 weeks after start ADM treatment and ≤2 weeks after ADM discontinuation DL ≥ 5 μg/mL (N = 17) DL < 5 μg/mL (N = 18) or ADA positive (N = 18) ADA negative (N = 39)	**Proportion of EULAR response** DL ≥ 5 μg/mL: 24% (4/17) DL < 5 μg/mL: 22% (4/18) or ADA positive: 44% (8/18) ADA negative: 44% (17/39) No predictive value of DL or ADA for response to second TNFi
Ulijn, 2020 (Ulijn et al., 2020)	RA patients who experienced inefficacy or toxicity with 40 mg eow and subsequently received non-TNFi treatment	EULAR response (DAS28-CRP/ESR, change from baseline ≥1.2 and current DAS28-ESR<3.2 and DAS28-CRP<2.9) 3–6 months	Two groups defined according to DL or ADA status at stopping ADM treatment DL ≥ 5 μg/mL (N = 18) DL < 5 μg/mL (N = 39) or ADA positive (N = 28) ADA negative (N = 62)	**Proportion of EULAR response** DL ≥ 5 μg/mL: 44% (8/18) DL < 5 μg/mL: 44% (17/39) or ADA positive: 43% (12/28) ADA negative: 39% (24/62) No predictive value of DL or ADA for response to non-TNFi
Scenario C: Value of guiding dose reduction or discontinuation				
Chen, 2016 (Chen et al., 2016)	Adult RA patients who had already achieved LDA (DAS28 < 3.2) or remission, switched to ADM dose-halving (40 mg monthly) and a concomitant stable dose of MTX	Persistent remission (DAS28 < 2.6) or persistent LDA (DAS28 < 3.2) 24 weeks	At baseline, 25 and 39 patients had achieved remission and LDA. After 24 weeks of dose-halving, 23 patients were persistent remission and 2 patients turned to LDA, persistent LDA in 24 and disease flare in 15	**The optimal cutoff at baseline for predicting persistent remission or LDA after 24 weeks of dose-halving:** persistent remission: 6.4 μg/mL (AUC 0.998, $p < 0.001$) persistent LDA:1.9 μg/mL (AUC 0.995, $p < 0.001$)
POET, Lamers-Karnebeek 2018 (Lamers-Karnebeek et al., 2019)	Adult RA patients been using ADM (40 mg every other week) for >1 year and had LDA (DAS28 < 3.2, or the rheumatologist's assessment of LDA with CRP <10 mg/L) for at least 6 months, stopped ADM treatment	Disease flare (>0.6 points increase of DAS28-ESR from baseline, with DAS28-ESR ≥3.2)12 months	Two groups defined according to DL at stopping point DL ≥ 5 μg/mL (N = 117) DL < 5 μg/mL (N = 93)	**Proportion of disease flare** DL ≥ 5 μg/mL: 53% (62/117) DL < 5 μg/mL: 47% (44/93) There is no predictive value of DL for flare risk after stopping ADM treatment

Inflammatory bowel disease, IBD; Crohn's disease, CD; Crohn's disease activity index, CDAI; ulcerative colitis, UC; rheumatoid arthritis, RA; therapeutic drug monitoring, TDM; loss-of-response, LOR; serious adverse event, SAE; drug level, DL; every week ew; every other week, eow; infliximab, IFX; ustekinumab, UST; vedolizumab, VDZ; tumour necrosis factor inhibitor, TNFi; methotrexate, MTX; low disease activity, LDA; european league against rheumatism, EULAR; C reactive protein, CRP; erythrocyte sedimentation rate, ESR; anti-dug antibodies, ADA.

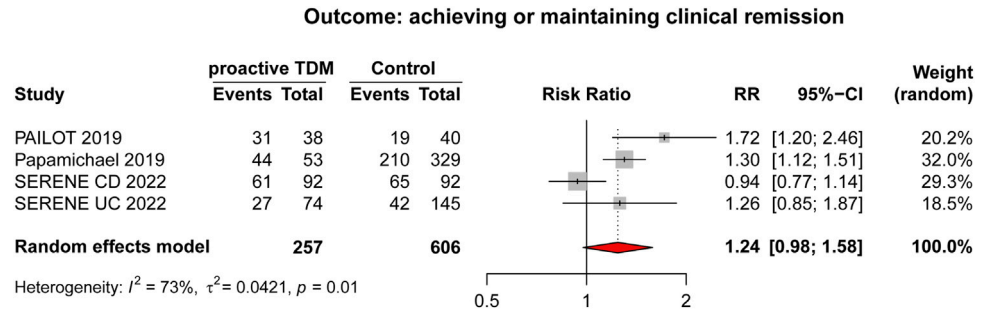

FIGURE 3
Forest plot comparing proactive TDM vs. control (reactive TDM and/or conventional management which is defined as empirical dose escalation), outcome (achieving or maintaining clinical remission).

clinical process of ADM treatment for various diseases. The comprehensive clinical scenarios and evidence are demonstrated in Figure 4.

In scenario A, people hope to obtain the drug concentration and antibody level of ADM to actively intervene and achieve better therapeutic effects. In a meta-analysis of 3 RCTs and 1 retrospective

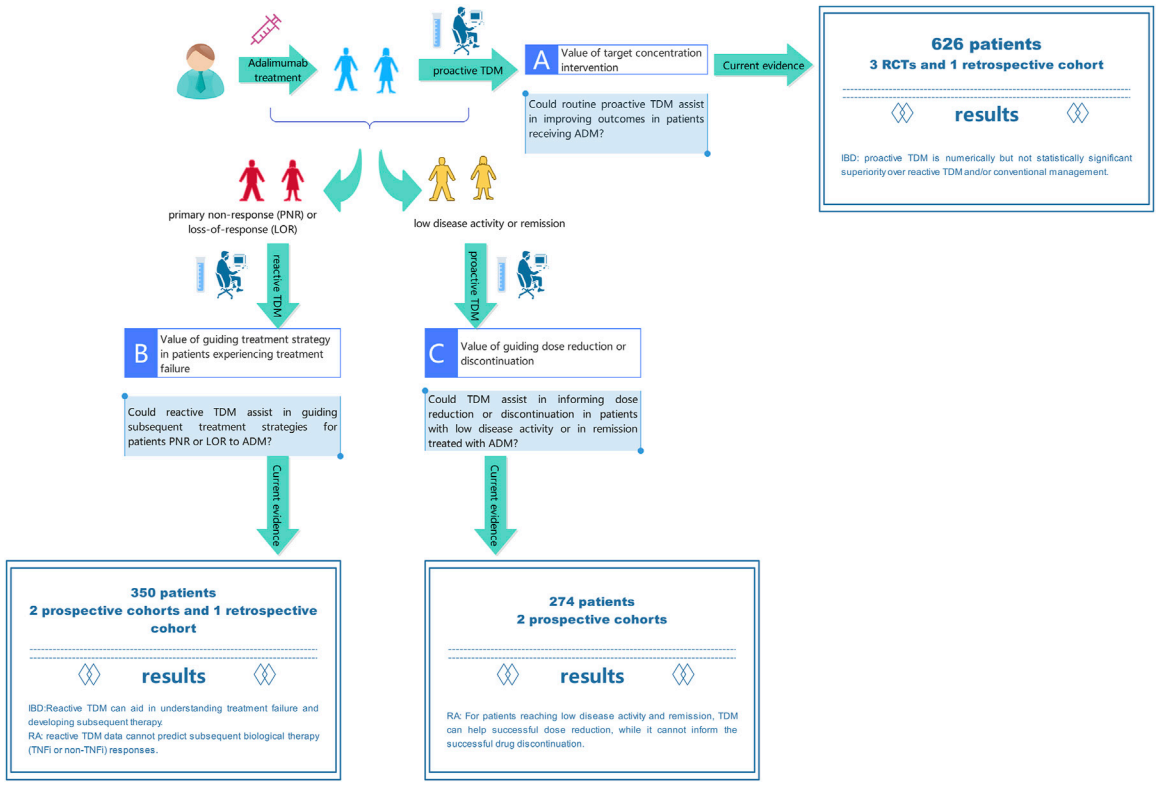

FIGURE 4
Current evidence on benefits of therapeutic drug monitoring throughout the entire process of adalimumab treatment.

cohort involving 626 IBD patients treated with ADM. Numerically but not statistically significant superiority of proactive TDM over reactive TDM and/or conventional management in achieving and/or maintaining clinical remission was observed. Our results are in line with previous studies on TNF-α inhibitors (Nguyen et al., 2022) which included 9 RCTs (6 for infliximab and 3 for ADM) in patients with IBD. There was no significant difference in the risk of failing to maintain clinical remission in patients who underwent proactive TDM vs. conventional management. Disease duration, concomitant immunomodulators, disease activity at baseline, and optimization of therapy before randomization did not modify this association. Exposure response relationship studies in IBD patients clearly demonstrate that higher anti-TNF drug concentrations are associated with clinical, biochemical, endoscopic, and histological remission (Zittan et al., 2016; Ward et al., 2017; Papamichael et al., 2018). According to reports, proactive TDM is important not only during maintenance therapy but also during induction therapy. Research has shown that ADA can develop as early as the second week in CD patients, leading to unresponsiveness. Proactive TDM can detect low concentrations at the fourth week to avoid immunogenicity and impact patient prognosis (Ungar et al., 2016). However, it seems that we have not obtained the expected evidence of benefits of ADM proactive TDM, but it is worth noting that the included literatures varied in study design, with moderate heterogeneity. The results may be influenced by factors such as patient population, sample size, study time, and detection method, etc. Therefore, more high-quality research is needed to provide additional evidence to clarify benefits of proactive TDM. Proactive TDM may be more important in more severely active patients and those with higher drug clearance rates, such as during induction therapy and in patients with acute severe UC and severe CD. These patients have a high burden of inflammation, increased drug clearance rates, and therefore a higher risk of insufficient drug exposure, immunogenicity, and treatment failure (Brandse et al., 2015; Brandse et al., 2016; Ungar et al., 2016; Battat et al., 2021). Another population with high drug clearance rates is the pediatric population (Jongsma et al., 2020; Winter et al., 2020). Assa et al. conducted relevant studies on pediatric IBD patients and demonstrated that proactive TDM can guide higher frequency treatment strategy adjustments, resulting in higher sustained response rates in the absence of corticosteroids and biological responses (Assa et al., 2019).

In scenario B, reactive TDM is performed when the patient experiencing treatment failure (Krieckaert et al., 2015; Irving and Gecse, 2022; Papamichael et al., 2022). For example, approximately one-third of IBD patients do not respond to TNF-α inhibitors treatment, and among those who initially respond, the LOR is an important clinical issue. In the first year of treatment, up to 40% of patients experience this condition (Colombel et al., 2007). For unresponsive patients, empirical dose escalation therapy may incur significant additional costs, leading to potential ineffective treatment and delaying more effective treatment. In addition, in patients with immune-mediated pharmacokinetic failure (for which ADA was established), additional drug exposure may lead to hypersensitivity reactions. Similarly, excessive drug exposure can lead to a higher risk of drug-related adverse events (such as severe

infections). Roblin's two studies confirmed that different levels of drug and ADA in the IBD population are associated with corresponding treatment adjustment strategies (Roblin et al., 2014; Roblin et al., 2022). Although there was no RCTs to demonstrate superior clinical outcomes of reactive TDM compared to empirical care, the use of TDM can elucidate the mechanism of LOR, whether the lack of response is caused by pharmacokinetic issues, insufficient drug levels, or pharmacological issues of ineffective ADA. TDM provides information for clinical decision-making in unresponsive patients and has intuitive benefits, such as preventing ineffective and potentially dangerous dose escalation in high-titer ADA patients. These results lay the foundation for the guiding the role of TDM in clinical practice and have been introduced in clinical guidelines and expert consensuses to support reactive TDM in ADM treatment (Feuerstein et al., 2017; Khan et al., 2019; Cheifetz et al., 2021; Krieckaert et al., 2023). In addition, Ulijn et al. conducted a study on RA and reported that reactive TDM data cannot predict subsequent biological therapy (TNF-α inhibitors or a non TNF-α inhibitors) responses in patients who failed treatment with ADM (Ulijn et al., 2020). On this issue, current researches have not reached a consistent convincing conclusion. In previous studies, it has been suggested that the measurement of ADM serum levels and/or ADA might be helpful for channeling the right patients to a TNF-α inhibitors or a non TNF-α inhibitors, thus increasing overall response chances (Bartelds et al., 2010; Jamnitski et al., 2011; Plasencia et al., 2013). There may be several reasons for these different results. In Ulijn's study, samples were not collected at the trough level but were randomly collected after injection of ADM. This might have reduced the association between ADA and response. Second, as this was a retrospective study, serum samples and clinical results were not always available, which may have led to selection bias. In summary, further prospective studies with larger sample sizes are needed to confirm whether drug and ADA levels indeed cannot predict disease activity.

In scenario C, due to the considerable interindividual variability in ADM concentrations and the existing exposure-response relationship, a considerable number of patients may experience overtreatment, leading to a higher risk of infection and increased costs. It is crucial in clinical practice to taper the dose to the lowest effective level, considering cost-effectiveness and potential adverse reactions. For patients who have achieved remission, sufficient ADM concentrations (≥6.4 μg/mL) can support successful ADM dose reduction (halving the dose to 40 mg monthly) (Chen et al., 2016). This approach has been validated by a RCT (l'Ami et al., 2018), RA patients with ADM concentrations >8 μg/mL can potentially prolong dosing interval to once every 3 weeks without loss of disease control, leading to reduced drug costs. While other biomarkers, involving patient, treatment, disease activity, and laboratory and imaging measurements, have not shown predictive value for successful dose reduction (Tweehuysen et al., 2017). It is hypothesized that patients who have achieved LDA and have undetectable drug concentrations may be considered for discontinuation of ADM, as the maintenance of LDA may be independent of the drug. However, data from the POET study (Chen et al., 2016) revealed that a significant proportion of patients (48%) experienced disease flare even with low or undetectable ADM concentrations, indicating that drug concentrations alone may not be sufficient to guide discontinuation decisions. Alternative strategies, such as disease activity-guided dose reduction and withdrawal or step-down approaches, may also be worth considering (van Herwaarden et al., 2015; Fautrel et al., 2016).

Our systematic review and meta-analysis summarized the benefits of TDM in the entire clinical process of ADM treatment for various diseases. However, there are some limitations to consider. In terms of data sources, limitations in data collection methods or sources may affect the reliability and universality of research results. Grey literature, as an important source of information, plays an indispensable role in literature search. Unlike traditional commercial publications, gray literature is usually published by institutions, enterprises, government agencies, professional conferences, and individuals. Its uniqueness makes it important, such as providing comprehensive information, reflecting practical experience and policy advocacy, timely grasping the latest research results, and eliminating publication bias. However, in our study, we only manually searched the trial registry and the list of references included in the trial, which made our search for grey literature incomplete and needed improvement in future research. Secondly, the included literatures varied in study design and quality. The results may be influenced by factors such as patient population, sample size, study time, and experimental environment, etc. as we excluded studies for which we were unable to extract individual ADM data; consequently, studies related to certain diseases, such as psoriasis and ankylosing spondylitis, were not included. Although evidence of benefits, including CD, UC, and RA, was ultimately included, the patient population, research perspectives, and outcome indicators of these studies were not the same, making it difficult to quantitatively summarize and perform meta-analyses for all literature results. Thirdly, it should be noted that assays used in TDM are varied and not yet standardized and may explain the deviation in results from different studies. Finally, our results are mainly based on the Western population, which means that it is difficult to generalize globally. However, within the scope of the currently published research, this article provides the latest results on the benefits of TDM in the entire process of clinical use and management of ADM.

5 Conclusion

The systematic review highlights the current evidence of TDM in ADM treatment. We addressed three clinical concerns regarding the benefits of TDM throughout the ADM treatment process. Current evidence suggests that proactive TDM is numerically but not statistically significant superiority over reactive TDM and/or conventional management in achieving and/or maintaining clinical remission. For patients experiencing treatment failure, reactive TDM can aid in understanding the reasons for treatment failure and developing subsequent treatment schedule. For patients reaching LDA or remission, monitoring drug concentrations can help identify and reduce overtreatment, while it cannot inform the successful drug discontinuation. Evidence was observed across various populations, including those with CD, UC, and RA. They encompass optimizing treatment strategies, enhancing clinical outcomes, improving drug utilization, and reducing treatment

costs. However, existing clinical trials are limited and of varying quality. More well-designed, high-quality clinical studies are needed to clarify the role of TDM in different clinical settings.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Author contributions

YL: Writing–original draft, Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration. CX: Writing–original draft, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration. XD: Writing–original draft, Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Software. ZW: Writing–review and editing, Conceptualization, Formal Analysis, Investigation, Methodology. JJZ: Writing–review and editing, Data curation, Formal Analysis, Investigation, Methodology. JGZ: Writing–review and editing, Conceptualization, Project administration, Supervision, Validation. LM: Writing–review and editing, Conceptualization, Formal Analysis, Funding acquisition, Project administration, Supervision, Validation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1376708/full#supplementary-material

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The story of clobenpropit and CXCR4: can be an effective drug in cancer and autoimmune diseases?

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Clobenpropit is a histamine H3 receptor antagonist and has developed as a potential therapeutic drug due to its ability to inhibit CXCR4, a chemokine receptor involved in autoimmune diseases and cancer pathogenesis. The CXCL12/CXCR4 axis involves several biological phenomena, including cell proliferation, migration, angiogenesis, inflammation, and metastasis. Accordingly, inhibiting CXCR4 can have promising clinical outcomes in patients with malignancy or autoimmune disorders. Based on available knowledge, Clobenpropit can effectively regulate the release of monocyte-derived inflammatory cytokine in autoimmune diseases such as juvenile idiopathic arthritis (JIA), presenting a potential targeted target with possible advantages over current therapeutic approaches. This review summarizes the intricate interplay between Clobenpropit and CXCR4 and the molecular mechanisms underlying their interactions, comprehensively analyzing their impact on immune regulation. Furthermore, we discuss preclinical and clinical investigations highlighting the probable efficacy of Clobenpropit for managing autoimmune diseases and cancer. Through this study, we aim to clarify the immunomodulatory role of Clobenpropit and its advantages and disadvantages as a novel therapeutic opportunity.

KEYWORDS

clobenpropit, CXCR4, autoimmune disease, CXCL12 (SDF-1α), cancer

1 Introduction

The complicated network of molecular communications orchestrating immune responses has long been pivotal in exploring pioneering therapeutic approaches against autoimmune diseases (ADs) and malignancies (Dhillon et al., 2020; Masoumi et al., 2021). Among the numerous players, the chemokine receptor CXCR4 has emerged as a critical mediator in the pathogenesis of various immune-based human disorders (Pozzobon et al., 2016; Bagheri et al., 2019; Kawaguchi et al., 2019). The role of CXCR4 in immune regulation cannot be overstated. CXCR4 involvement in immune cell trafficking (Kucia et al., 2005; Pelekanos et al., 2014), homing (Burger and Bürkle, 2007; Yellowley, 2013; Asri et al., 2016), and cell activation (Kumar et al., 2006; Hong et al., 2009) make it a pivotal player in the orchestration of immune responses (Jacobson and Weiss, 2013). Ligation of CXCL12 to CXCR4 can initiate several downstream signaling pathways, inducing cell growth and

proliferation, migration, angiogenesis, inflammation, and metastasis (Hassanshahi et al., 2010; Aminzadeh et al., 2012; Azin et al., 2012; Khorramdelazad et al., 2016; Nazari et al., 2017). Dysregulation of CXCR4 signaling has been associated with ADs, where aberrant immune responses target self-antigens, and tumorigenesis, where uncontrolled cell proliferation and evasion of immune surveillance are hallmarks (Chong and Mohan, 2009; Chatterjee et al., 2014; García-Cuesta et al., 2019; Shi et al., 2020). Therefore, inhibition of the CXCL12/CXCR4 axis can be a potential therapeutic target in cancers and ADs (Chong and Mohan, 2009; Derlin and Hueper, 2018). Numerous studies employed CXCR4 inhibitors to suppress the CXCL12/CXCR4 signals for treating cancer (Domanska et al., 2012; Biasci et al., 2020; Bockorny et al., 2020; Chaudary et al., 2021). AMD3100 is one of the most common CXCR4 inhibitors (De Clercq, 2003). Researchers showed that it could be effective in cancer therapy via the inhibition of tumor cell proliferation and reducing the infiltration of immunosuppressive cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) into the tumor microenvironment (TME) (Scala, 2015; Liu et al., 2021; Lei et al., 2022). Our recent study showed that A1, a novel fluorinated CXCR4 inhibitor, can effectively treat colorectal cancer (CRC) *in vitro* and *in vivo* (Khorramdelazad et al., 2023).

Regarding the potential association between the CXCL12/CXCR4 axis and various signaling pathways, such as the ERK pathway, it has been reported that the CXCL12/CXCR4 signaling pathway plays a proinflammatory role in experimental emporomandibular joint osteoarthritis (TMJOA) model and the bicyclam derivative AMD3100 could reduce the severity of experimental TMJOA (Wang et al., 2016). Current attention has turned towards Clobenpropit, initially recognized as a histamine H3 receptor antagonist, and its exciting proficiency in inhibiting CXCR4 (Mani et al., 2021; Bekaddour et al., 2023). The question here is, in addition to the antihistaminic properties of Clobenpropit, to what extent can this drug help treat CXCL12/CXCR4-related diseases by inhibiting CXCR4? Moreover, what interactions may occur with the simultaneous inhibition of histamine H3 receptor and CXCR4.

This review article aims to summarize the complicated molecular dance between Clobenpropit and CXCR4, clarifying the potential of Clobenpropit as a therapeutic agent in ADs and human malignancies. We will unravel the molecular mechanisms supporting the inhibitory effects of Clobenpropit on CXCR4, exploring its impact on immune cell function and immune-mediated pathologies. Additionally, we will critically analyze preclinical and clinical evidence, evaluating Clobenpropit's efficacy and safety profile in the most critical ADs and cancer. By doing so, we endeavor to contribute to the developing landscape of targeted therapy, offering new perspectives on utilizing Clobenpropit as a potential therapeutic intervention against diseases characterized by CXCR4-associated immune dysregulation.

2 Methodology

2.1 Literature search strategy

A comprehensive literature search was conducted across several databases, including PubMed, Scopus, Web of Science, and Google Scholar. The search terms included "Clobenpropit," "CXCR4," "histamine H3 receptor antagonist," "autoimmune diseases," "cancer," "CXCL12/CXCR4 axis," "cell proliferation," "migration," "angiogenesis," "inflammation," and "metastasis." The search was limited to articles published in English from 1990 to the present (May 2024).

2.2 Inclusion and exclusion criteria

Studies were included in the review if they met the following criteria: Investigated the role of Clobenpropit as a CXCR4 inhibitor; addressed the molecular mechanisms of the CXCL12/CXCR4 axis; examined the effects of Clobenpropit on autoimmune diseases or cancer; included preclinical or clinical data supporting the efficacy of Clobenpropit; peer-reviewed articles, reviews, and clinical trial reports. Moreover, studies were excluded if they did not focus on Clobenpropit or its interaction with CXCR4, were not peer-reviewed, included editorials and commentaries, lacked sufficient methodological detail or experimental rigor, or were unavailable in full-text form.

2.3 Data extraction and analysis

Two reviewers independently extracted data using a standardized form. Extracted information included study design, sample size, methods, key findings, and conclusions. Discrepancies between reviewers were resolved through discussion or consultation with a third reviewer.

3 The CXCL12/CXCR4 signaling pathway: unraveling developmental and immunological connectivity

As discussed, the CXCL12/CXCR4 signaling pathway is a vital conduit linking developmental processes with immune regulation. Through a complex interplay of chemokine ligand CXCL12 and its receptor CXCR4, this pathway influences diverse cellular behaviors crucial for tissue morphogenesis and immune surveillance (Ara et al., 2005). As we explore the intricate mechanisms and multifaceted roles of the CXCL12/CXCR4 axis in this section, we gain insight into its profound influence on developmental connectivity and immunological responses.

3.1 Importance of the CXCL12/CXCR4 axis in development

Hematopoietic stem cells (HSCs) are pivotal in hematopoiesis or generating blood cells (Nemeth and Bodine, 2007). During midgestation, HSCs originate from hemogenic endothelial cells or mesenchymal cells adjacent to the dorsal aorta, migrate to the fetal liver, and colonize the bone marrow (BM) (Nagasawa et al., 1996; Ma et al., 1998; Zou et al., 1998; Ara et al., 2003). Researchers have found that the CXCL12/CXCR4 axis facilitates the BM niche's hematopoietic stem and progenitor cell (HSPC) colonization during development by genetically modified murine models lacking CXCL12 or CXCR4. In addition, HSC maintenance

requires CXCL12/CXCR4 signaling, as evidenced by conditional deletion experiments in BM (Sugiyama et al., 2006; Tzeng et al., 2011; Ding and Morrison, 2013; Greenbaum et al., 2013). Moreover, sperm and oocytes are formed by migrating and colonizing primordial germ cells (PGCs). CXCL12/CXCR4 signaling underlies the colonization of the genital ridges by PGCs, which originate from the allantoic root (Nagasawa, 2014). In murine models, CXCL12/CXCR4 signaling is implicated in colonization (Nagasawa et al., 1996; Zou et al., 1998). Findings in zebrafish further corroborate the significance of CXCL12 in directing PGC migration toward the gonads (Knaut et al., 2003; Nagasawa, 2014).

Signaling between CXCL12 and CXCR4 is fundamental for cardiogenesis and vascular development. Its deficiency impairs membrane formation in the cardioventricular septum and compromises vascularization in multiple tissues (Tachibana et al., 1998; Ara et al., 2005; Li et al., 2013). CXCL12 plays a vital role in patterning vascular pathways during mesenteric development, specifically by facilitating interactions between arterial endothelial cells and the adjacent capillaries (Ara et al., 2005). Zebrafish studies have demonstrated that CXCR4a contributes to forming arterial networks during brain vascularization (Chong et al., 2001; Siekmann et al., 2009). CXCL12/CXCR4 signaling is intricately involved in neurogenesis, influencing migration, differentiation, and axonal guidance. Using mice deficient in CXCL12 or CXCR4, aberrant granule cell clustering in the cerebellum has been observed, hippocampal dentate gyrus morphology has been altered, and the assembly of GABAergic interneurons is disrupted, emphasizing the importance of CXCL12/CXCR4 signaling in neurodevelopmental processes (Ma et al., 1998; Zou et al., 1998; Bagri et al., 2002; Lu et al., 2002; Stumm et al., 2003; Zhu et al., 2009). Additionally, CXCL12 guides the axon trajectory of motor and sensory neurons during the development of the nervous system (Chalasani et al., 2003; Lieberam et al., 2005).

3.2 Immunological aspects of the CXCL12/CXCR4 axis

The CXCL12/CXCR4 signaling pathway is critical in immune regulation and exemplifies the nuanced interactions that govern cell migration, homing, and immune surveillance (Lu et al., 2024). This section summarizes the bio-structure, bio-function, and signaling of the CXCL12/CXCR4 axis. At the epicenter of this pathway lies CXCL12, also identified as stromal cell-derived factor-1 α (SDF-1α) (Kukreja, 2005). CXCL12 is a member of the CXC chemokine family, categorized by a conserved cysteine motif that adopts the canonical chemokine fold, comprising three anti-parallel β strands and a single α helix (N terminus post-translational, 2016). In disulfide bonds, the cysteine residues are responsible for the stability of the protein (Xu et al., 2013). The exclusive spatial arrangement of amino acid residues outlines the chemotactic specificity, supporting its ligation with the CXCR4 receptor. The CXCR4 is considered a chemokine receptor for CXCL12 and a seven-transmembrane G protein-coupled receptor (GPCR) (Teixido et al., 2018). CXCR4 possesses a multifaceted structure containing an extracellular N-terminus, seven transmembrane helices, three intracellular loops, and an intracellular C-terminus. The CXCR4 binding pocket contains the N-terminus of CXCL12, starting a cascade of conformational alterations that activate several downstream signaling occurrences (Van Hout, 2019).

Following ligation of CXCL12 to CXCR4, a series of bioevents occur, activating downstream signaling pathways that regulate immune cell performance through inducing G-protein pairing and activating heterotrimeric G proteins, principally Gαi (Liekens et al., 2010). Gαi, in turn, hinders adenylyl cyclase, reducing the production of cyclic AMP (cAMP) (Scala, 2015). Condensed cAMP levels modulate intracellular signaling, inducing cell proliferation, differentiation, migration, and survival (Castaldo et al., 2014). Moreover, activating CXCR4 induces the phosphorylation of intracellular adaptor molecules and domains, engaging and triggering kinases such as focal adhesion kinase (FAK) and mitogen-activated protein kinases (MAPK). FAK can regulate cell adhesion and migration, while MAPKs contribute to cell proliferation and survival (Rigiracciolo et al., 2021). The Ras/Raf/MEK/ERK signaling pathway is a prominent downstream branch of MAPK signaling and plays a fundamental role in cellular responses to CXCL12 (Kumari et al., 2021). The phosphoinositide 3-kinase (PI3K) pathway is also employed, activating Akt, which modulates diverse cellular processes, including cell proliferation, metabolism, and survival (West et al., 2002; Mousavi, 2020) (Figure 1). Contributing these signaling cascades in various physiological and pathological states magnificently tunes immune cell responses, pointing out their locomotion, activation, and functions (Mousavi, 2020). As discussed, the CXCL12/CXCR4 axis is crucial in immune cell trafficking, predominantly in hematopoiesis and the homing of immune cells to lymphoid organs and inflammatory milieus (Salcedo and Oppenheim, 2003; Nagasawa, 2014). Evidence revealed that this pathway is critical in embryonic development, organogenesis, and tissue repair (Cheng et al., 2014). Beyond the CXCL12/CXCR4 axis's physiological functions, its dysregulation concerns several pathological conditions, including cancer metastasis, ADs, and human immunodeficiency virus (HIV) infection (Liekens et al., 2010). The interactions of CXCR4 with other related proteins are illustrated in Figure 2.

Taken together, the CXCL12/CXCR4 axis is a testament to the complicated molecular interactions network that governs immune responses. Therefore, understanding the bio-structure of CXCL12 and CXCR4 offers essential perceptions of their functional roles. The stage-managed activation of downstream signaling pathways orchestrates a symphony of immune responses, emphasizing the pathway's significance in physiologic and pathologic conditions. As we unravel the intricacies of the immune system and its components, the CXCL12/CXCR4 axis emerges as an essential player, suggesting potential therapeutic targets for several immune-related disorders such as ADs and cancers.

4 Role of CXCL12/CXCR4 axis in pathologic states

4.1 Cancer

CAFs are believed to be the major producers of CXCL12 in the TME, as they are the most common and significant cells that secrete it (Costa et al., 2014). M2 macrophages and cancer cells also produce CXCL12. By secreting CXCL12, M2 macrophages can activate and differentiate CAF (Liu et al., 2019). Despite being widely expressed

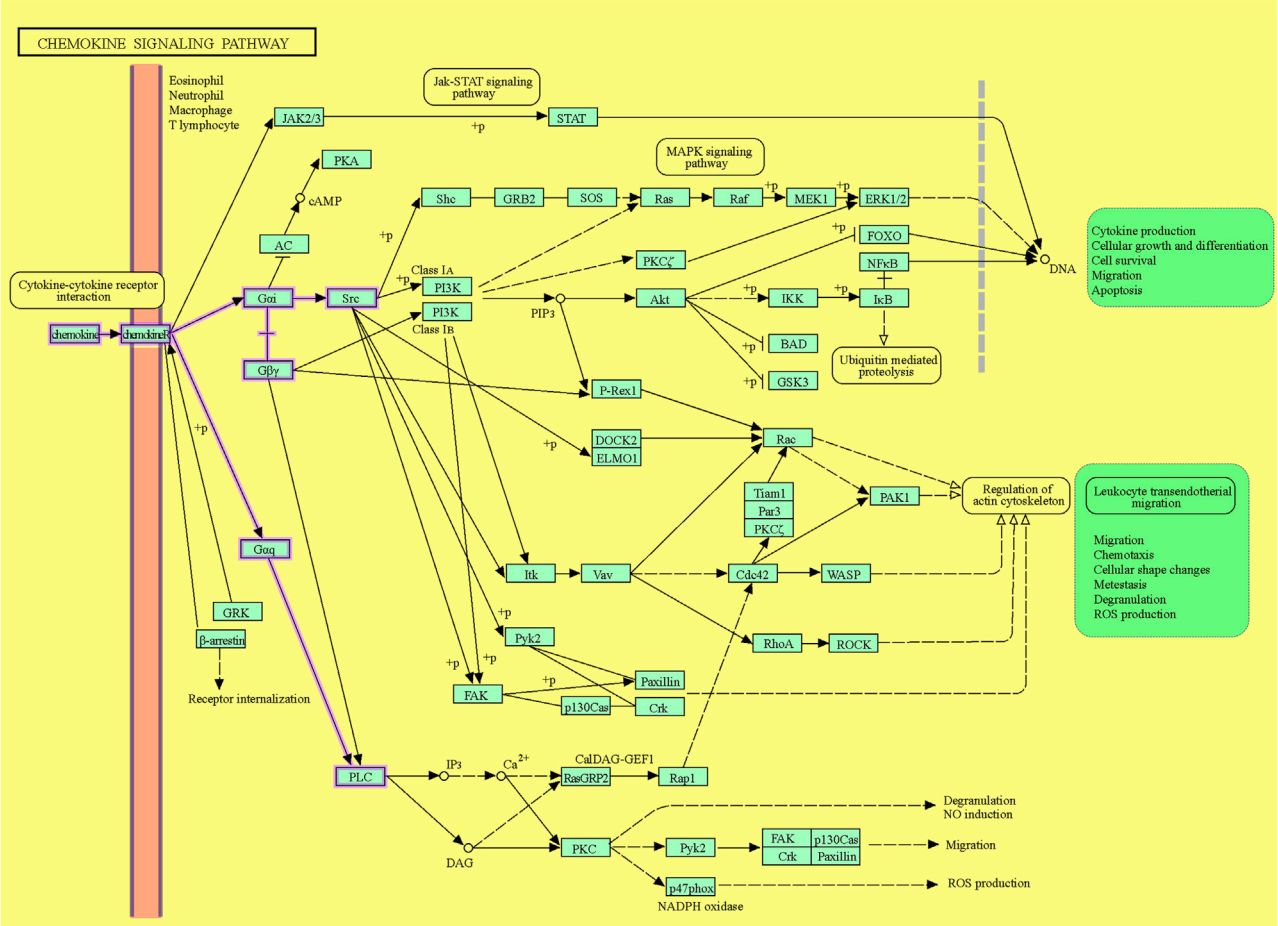

FIGURE 1
The CXCL12/CXCR4 signaling pathway. The downstream pathways are shown with violet lines (source: KEGG, https://www.kegg.jp/pathway/hsa04062+N01765).

by cancer cells from various sources, the CXCR4 receptor is typically found on cancer stem cells (CSCs) and is not present in normal mammary epithelial cells (Yi et al., 2019). This suggests that the CXCL12/CXCR4 axis can function in autocrine and paracrine manners. CXCR4, which promotes embryonic development, is required for CSC migration toward metastatic sites (Huang et al., 2010). Other cells that express CXCR4 include neutrophils, endothelial cells, lymphocytes, stromal fibroblasts, hematopoietic stem cells (HSCs), and MDSCs (Mortezaee, 2020). Accordingly, recruiting $CXCR4^{+}$ MDSCs, M2 macrophages, and Tregs can suppress anti-tumor immune responses, inducing tumor growth and progression.

Genetic mutations and epigenetic changes are at the root of cancer, one of the leading causes of premature death worldwide (Kanwal and Gupta, 2012). It has been revealed that various growth factors and signaling pathways intricately regulate primary tumorigenesis (Cross and Dexter, 1991; Guo et al., 2020). The CXCL12/CXCR4/PI3K/AKT axis is involved in the pathogenesis of several malignancies, such as adamantinomatous craniopharyngiomas, breast cancer (BCa), neuroblastoma, pancreatic intraepithelial neoplasia, medullary thyroid cancer, hepatocellular carcinoma (HCC), colorectal cancer (CRC), and glioblastoma (GB), via activation of different downstream signaling pathways, inducing tumor cell proliferation, migration, and invasion (Carmo et al., 2010; Yin et al., 2019; Yang et al., 2020; Hjazi et al., 2023; Yang et al., 2023). Additionally, CXCR7, a receptor related to CXCR4 and CXCL12, has been associated with the growth and metastasis of tumor cells in colon cancer, melanoma, and BCa (Wang et al., 2015). Metastasis is a major cause of cancer-related mortality that involves sequential invasion, circulation, infiltration, and proliferation (Ha et al., 2013). CXCL12/CXCR4 affects integrin expression, homeobox genes, tight junctions, and matrix metalloproteinases, which affect colorectal, endometrial, breast, and glioma metastasis (Yang et al., 2023).

Several studies have demonstrated that CXCL12 is overexpressed in tumor tissues of various human malignancies (Portella et al., 2021). In addition to fostering pre-metastatic niches (tumorigenic soils), it recruits tumor cells (oncogenic "seeds") to the niches, inducing tumor progression and metastasis (Yang et al., 2020). Cancer stem/progenitor cells overexpress the CXCR4 receptor, which transmits CXCL12 signals. Oncogenes are activated following the ligation of CXCL12 to CXCR4, which activates multiple downstream pathways. By activating the CXCL12/CXCR4 axis, cancer stem,

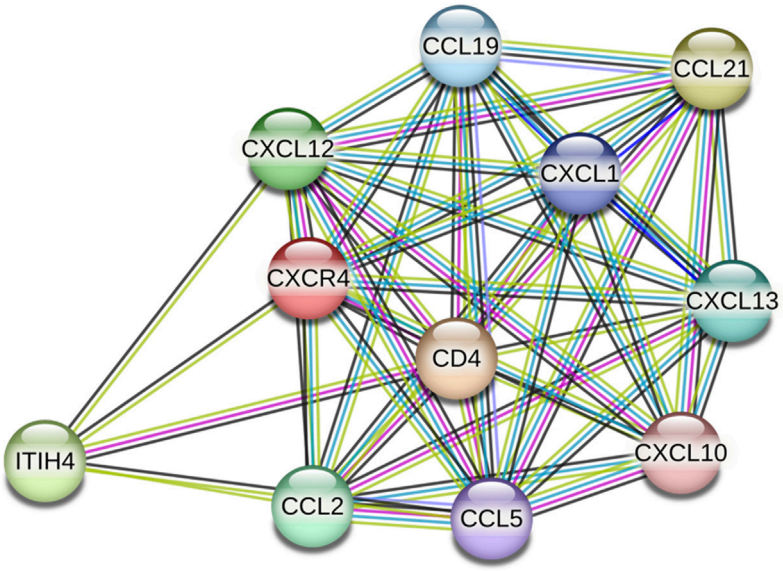

FIGURE 2
CXCR4 protein interactions (source: STRING, https://stringdb.org/cgi/network?taskId=bT78SZ6aKg1s&sessionId=b9z8Gu1eenbw&allnodes=1).

and progenitor cells are mobilized to pre-metastatic niches and undergo epithelial-mesenchymal transition (EMT) (Yang et al., 2020).

Although it has long been established that cancer-associated fibroblasts (CAFs), the primary producers of CXCL12 in the TME, can induce EMT in tumor cells, the direct involvement of CXCL12 in EMT has remained unclear (Jiang et al., 2023). This ambiguity persists because other factors secreted by CAFs, such as tumor growth factor-beta (TGF-β) and IL-6, are also known to be highly transformative. Interestingly, recent investigations have revealed that overexpression of CXCL12 in MCF7 cell lines (Breast cancer) leads to upregulation of *OCT4*, *Nanog*, and *SOX2* (DiNatale et al., 2022). These factors are well known for their roles in pluripotency and stem cell reprogramming, further confirming the close association between EMT and the stem cell program in cancer. Additionally, CXCL12-driven EMT induction in this model system was found to depend on the Wnt/β-catenin pathway.

Moreover, the significance of CXCL12 in the TME is explored by its interaction with CXCR4, which is often overexpressed in various cancers. This axis stimulates EMT and promotes tumor invasiveness and metastasis (Anastasiadou et al., 2023). The role of the Wnt/β-catenin pathway in this process highlights a critical signaling mechanism that integrates external signals from the TME with intracellular pathways governing cell differentiation and proliferation (Moon, 2005). Studies have reported that activating the Wnt/β-catenin pathway can stabilize β-catenin in the cytoplasm, translocating to the nucleus, and the subsequent activation of target genes that promote EMT and stemness properties (Jiang et al., 2007). In addition to CXCL12, the interplay between CAFs and tumor cells involves a complex network of signaling molecules. For instance, TGF-β has been extensively studied for its dual role in cancer. It acts as a tumor suppressor in the early stages and a metastasis promoter in the advanced stages (Pardali and Moustakas, 2007). Similarly, IL-6 is known to activate the JAK/STAT3 signaling pathway, contributing to EMT and cancer progression (Pardali and Moustakas, 2007). Understanding the specific contributions of these factors, including CXCL12, within the TME is crucial for developing targeted therapies to disrupt these pro-tumorigenic interactions.

Collectively, the evidence suggests that CXCL12 plays a significant role in inducing EMT and stemness in tumor cells, with the Wnt/β-catenin pathway being a crucial mediator in this process. Further studies are required to fully elucidate how CXCL12 and other CAF-derived factors contribute to tumor progression and explore potential therapeutic strategies targeting these pathways (Shan et al., 2015).

According to the available knowledge, the TME plays a pivotal role in cancer progression. The hypoxic condition and high acidity, as well as expressing inhibitory immune checkpoints, such as programmed cell death protein 1/programmed cell death ligand 1 (PD-1/PD-L1), anti-cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), like lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT), and V-domain Ig suppressor of T cell activation (VISTA) are the essential features of the TME (Qin et al., 2019). Moreover, recruitment and infiltration of immunosuppressive immune cells, such as regulatory T cells (Tregs), tumor-associated macrophages (TAMs), cancer-associated fibroblasts (CAFs), and myeloid-derived suppressor cells (MDSCs) in the TME can suppress anti-tumor immune responses to tumor cells via releasing immunosuppressive mediators, such as IL-10, IL-35, and tumor growth factor beta (TGF-β) (Gao et al., 2023) (Figure 3). Chemokines recruit anti and pro-tumor immune and non-immune cells into the TME. Dysregulation of CXCL12 secretion by tumor cells and expression of CXCR4 by immunosuppressive cells induces the creation of an inhibitory TME by fostering the infiltration of the mentioned regulatory and cancer-associated cells (Mortezaee, 2020).

As discussed, the CXCL12/CXCR4 axis is involved in tumor angiogenesis and metastasis of tumor cells to distant organs. By

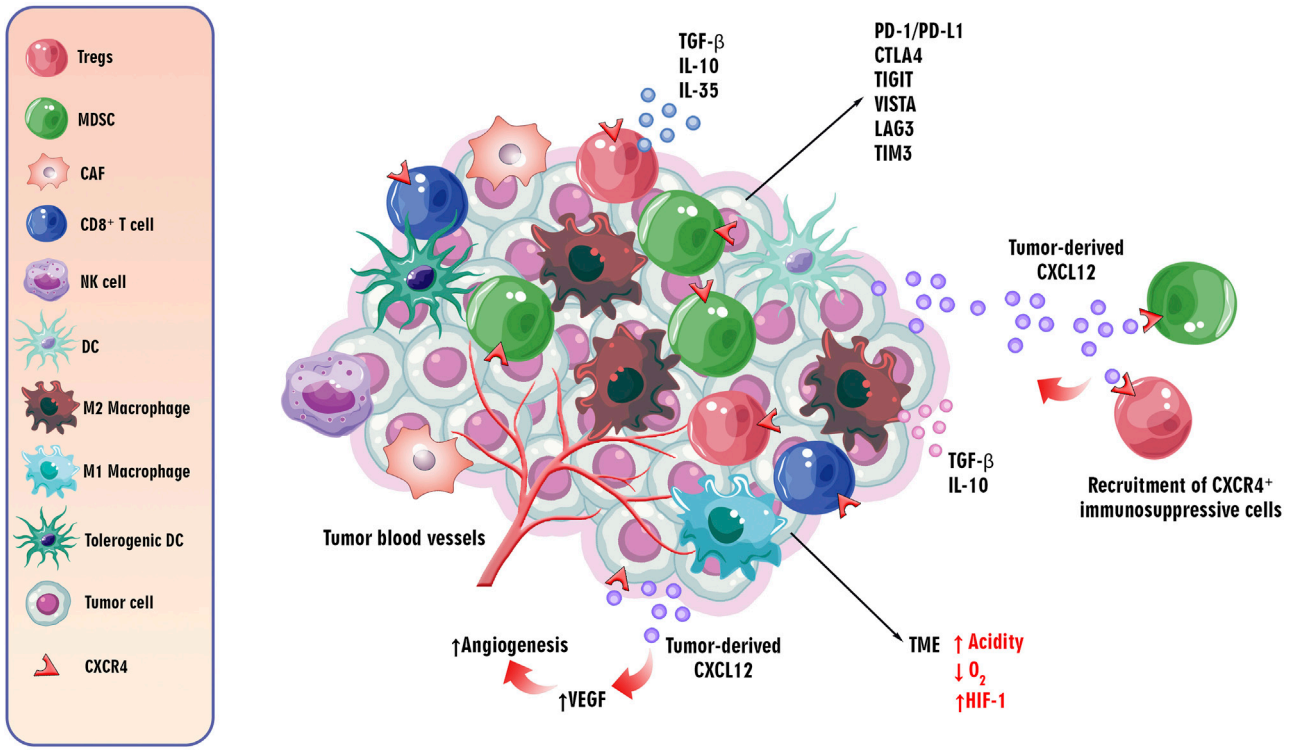

FIGURE 3
The role of/CXCL12CXCR4 axis in the TME. The critical features of the TME influence cancer progression. The TME is characterized by hypoxia, high acidity, and expression of inhibitory immune checkpoints such as PD-1/PD-L1, CTLA-4, LAG-3, TIM-3, TIGIT, and VISTA. Additionally, immunosuppressive cells like Tregs, TAMs, CAFs, and MDSCs are recruited, suppressing anti-tumor immune responses via secreting immunosuppressive cytokines, such as IL-10, IL-35, and TGF-β. Chemokines, particularly the dysregulated CXCL12/CXCR4 axis, play a role in recruiting regulatory and cancer-associated cells to the TME. The CXCR4 receptor, upregulated in hypoxic conditions, induces angiogenesis through the PI3K/AKT pathway, contributing to tumor progression by promoting VEGF expression. This axis is implicated in both tumor angiogenesis and metastasis to distant organs.

inducing vascular endothelial growth factor (VEGF) through the PI3K/AKT pathway, the CXCR4 receptor can induce tumor angiogenesis, an essential step in tumor progression (Ghalehbandi et al., 2023). Furthermore, the upregulation of CXCR4 in hypoxic conditions and its role in hypoxia-inducible factor-1 α (HIF-1 α)-induced VEGF expression can lead to angiogenesis in the TME (Kruszyna et al., 2022) (Figure 3). Accordingly, understanding the complicated mechanisms underlying tumorigenesis, progression, and the role of the CXCL12/CXCR4 axis in these processes is essential for emerging targeted therapeutic interventions in cancer therapy.

4.2 Autoimmune diseases

An AD occurs when the immune system components, such as autoantibodies and autoreactive T cells, incorrectly target specific self-antigens, damaging tissues and organs (Muñoz-Carrillo et al., 2018a). These diseases are not generalized attacks but are mediated by immune responses directed against specific body parts (Muñoz-Carrillo et al., 2018b). A particular AD targets a particular tissue or organ, resulting in diverse clinical symptoms (Pisetsky, 2023). In addition, a majority of ADs appear to have a relapsing/remitting course, where periods of active disease (flare-ups) alternate with periods of remission. Symptoms worsen during flare-ups due to increased immune activity, while symptoms decrease during remissions due to reduced immune activity (Lebel et al., 2023). As a result of genetic predisposition, environmental triggers (smoking, chemical compounds, infectious agents, radiation, and ultraviolet light), and dysregulation of immune tolerance, the underlying pathophysiological mechanisms often fail to differentiate self from non-self (Javierre et al., 2011; Capalbo et al., 2012). Therefore, diagnosing and managing these diseases requires understanding their specific target antigens and dynamic nature (Muñoz-Carrillo et al., 2018a). Activated innate immune cells, stromal cells, and tissue cells produce cytokines and chemokines, which regulate immune cell trafficking and play a significant role in ADs (Shachar and Karin, 2013; Elemam et al., 2020; Fallahi et al., 2020; Abassifard et al., 2021; Moadab et al., 2021; Abbasifard et al., 2023). Although CXCL12 was initially regarded as a homeostatic chemokine, it also plays a vital role in inflammation. Inflammatory bowel disease (IBD), multiple sclerosis (MS), rheumatoid arthritis (RA), psoriasis (PsO), type 1 diabetes (T1D), and systemic lupus erythematosus (SLE) are among the ADs that are implicated by the CXCL12/CXCR4 axis (García-Cuesta et al., 2019) (Figure 4).

CXCL12 plays a crucial role in skin homeostasis and inflammation (Abdelaal et al., 2020). In PsO as a chronic inflammatory skin disease, inflammatory leukocytes, including dendritic cells (DCs), macrophages, and T cells, accumulate, leading to pronounced inflammatory

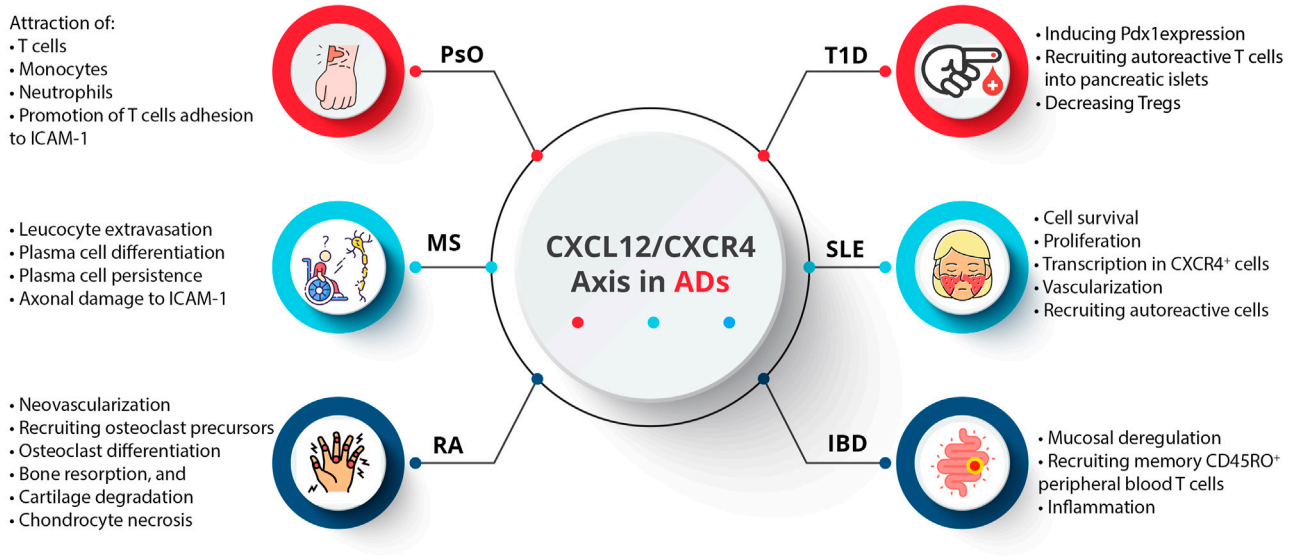

FIGURE 4
Dysregulation of the CXCL12/CXCR4 axis in ADs.

angiogenesis (Gulletta et al., 2013). VEGF-A upregulates the expression of CXCL12 in psoriatic lesions. Psoriatic skin lesions also exhibit elevated mRNA levels of *CXCR4* and *CXCL12* (Petit et al., 2007; Suárez-Fariñas et al., 2011). A study evaluated CXCL12 expression in PsO vulgaris and psoriatic arthritis (PsA) patients concerning disease activity and methotrexate (MTX) therapy, and findings showed significantly higher CXCL12 expression in PsA patients compared to PsO vulgaris patients before treatment but not after. Post-MTX therapy, PsO vulgaris patients showed a significant decrease in CXCL12 expression, while PsA patients did not show a significant change. The reduction in PASI scores correlated moderately with decreased CXCL12 expression in PsO patients. Accordingly, CXCL12 may be involved in the progression from PsO vulgaris to PsA, with MTX therapy reducing CXCL12 expression and disease severity, suggesting CXCL12 as a potential biomarker for psoriasis severity (Abdelaal et al., 2020).

MS is a demyelinating disease characterized by inflammation, progressive myelin loss within the central nervous system (CNS), and failure to remyelinate damaged axons (Carbajal et al., 2010). Leukocytes need to penetrate the brain parenchyma for tissue injury, and the unique CNS barriers challenge immune cell activation (Perry et al., 1997). CXCL12, constitutively expressed in the adult CNS and upregulated under pathological conditions, orchestrates leukocyte trafficking in the CNS (Durrant et al., 2014). Chemokines, receptors, and adhesion molecules orchestrate leukocyte trafficking (Olson and Ley, 2002). In MS patients, CXCL12 levels are elevated in serum and cerebrospinal fluid and expressed in active lesions, suggesting its involvement in disease pathology (Azin et al., 2012; Khorramdelazad et al., 2016; Bagheri et al., 2019; Marastoni et al., 2021). It has been revealed that CXCL12 localization on blood vessels specifies a potential role in leucocyte extravasation, and the CXCL12/CXCR4 axis may contribute to plasma cell differentiation and persistence. In addition, following the cleavage of CXCL12 by metalloproteases, it can convert to a neurotoxic mediator that can damage axons (Krumbholz et al., 2006).

Issues in MS suggest deficiencies in recruiting and maturing oligodendrocyte progenitor cells (OPCs), indicating the crucial role of cell replacement therapies in improving remyelination (Hughes and Stockton, 2021). In a study using a model of viral-induced demyelination, the signaling cues guiding the migration of transplanted remyelination-competent cells were investigated (Carbajal et al., 2010). While rodent-derived glial cell transplantation in MS models has been successful, the mechanisms of cell navigation within the inflammatory environment created by persistent viruses are poorly understood. The JHM strain of mouse hepatitis virus (JHMV) infection in mice induced an immune-mediated demyelinating disease similar to MS. Surgical engraftment of GFP$^+$ neural stem cells (NSCs) into the spinal cords of JHMV-infected mice resulted in migration, proliferation, and differentiation into OPCs and mature oligodendrocytes, inducing axonal remyelination. Using anti-CXCL12 blocking serum significantly reduced the migration and proliferation of engrafted stem cells. Additionally, CXCR4 antagonists, but not CXCR7, similarly inhibited migration and proliferation (Carbajal et al., 2010). These outcomes emphasize the pivotal role of the CXCL12/CXCR4 axis in recruiting engrafted stem cells to damaged CNS sites in mice with immune-mediated demyelination due to persistent viral infection.

RA is an inflammatory autoimmune disease that primarily affects the joints. This type of AD involves synovial fibroblasts, endothelial cells, and chronic inflammation (Masoumi et al., 2023). The pathogenesis of RA involves a complex interplay between immune cells and cytokines (Kondo et al., 2021). It has been revealed that activated macrophages and synovial fibroblasts are activated by T cells, causing the release of pro-inflammatory cytokines like TNF-α and IL-17 (Tu et al., 2022). Releasing pro-inflammatory cytokines and chemokines by activated macrophages contributes to inflammation and joint damage (Moadab et al., 2021). A crucial aspect of RA is the degradation of bone and cartilage, which occurs due to synovial fibroblasts and macrophages secreting

matrix metalloproteinases (MMPs) (Lefevre et al., 2015). As a result, cartilage and the bones beneath are destroyed by MMPs that destroy extracellular matrix components. T cells and macrophages collaborate on this coordinated attack, facilitated by MMPs, highlighting the importance of targeting these pathways in therapeutic interventions against RA (Siouti and Andreakos, 2019). It has been shown that chronic inflammation and bone erosion are related to the CXCL12/CXCR4 axis, contributing to bone and cartilage damage (Peng et al., 2020). It is associated with CXCL12 that neovascularization occurs in inflamed RA joints, particularly in their early stages (Yu et al., 2003). As a result, immune cells in the synovium express CXCR4. CXCL12 also induces recruiting osteoclast precursors, stimulating differentiation, bone resorption, and cartilage degradation (Grassi et al., 2004; Wright et al., 2005). Hypoxia stimulates VEGF and CXCR4 expression in inflamed joints by activating HIF-1 (Imtiyaz and Simon, 2010). In addition, *in vitro* experiments revealed that CXCL12 enhances chondrocyte necrosis, signifying the role of this CXC chemokine in cartilage damage (Xu et al., 2012).

MicroRNAs (miRs) play a significant role in the initiation and progression of RA, though the specific functions and mechanisms of miR-23 in RA are not fully understood (Evangelatos et al., 2019). An investigation demonstrated that miR-23 was downregulated, while CXCL12 was upregulated in RA samples compared to control samples (Gao et al., 2021). Overexpression of miR-23 suppressed inflammation by reducing TNF-α, IL-1β, and IL-8 expression. Mechanistically, miR-23 decreased *CXCL12* mRNA expression by binding to its 3′-untranslated region, and overexpression of CXCL12 counteracted the anti-inflammatory effects of miR-23 mimic. Additionally, CXCL12 promotes inflammation by activating NF-κB signaling (Gao et al., 2021). Therefore, miR-23 alleviates RA inflammation by regulating CXCL12 via the NF-κB pathway, suggesting that targeting miR-23 could be a potential strategy for diagnosing and treating RA.

Another investigation found that the levels of CXCR4 and CXCL12 in the serum and joint synovial fluid were significantly higher in patients with RA than in normal subjects. These levels were also higher in the RA-active group compared to both the remission and control groups. A positive correlation was also observed between the expression of CXCR4 and CXCL12 and the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), and Disease activity score in 28 joints (DAS28) scores. These outcomes suggest that CXCR4 and CXCL12 are highly expressed in RA patients, with their levels correlating positively with these clinical markers of disease activity (Peng et al., 2020).

SLE is characterized by immune complexes of autoantibodies and autoantigens circulating in the blood, leading to an inflammatory process and organ damage (Abbasifard et al., 2020). It has been revealed that chemokines, including CXCL9, CXCL10, CXCL12, and CXCL13, play crucial roles in the pathogenesis of SLE (Pan et al., 2022). The expression of CXCR4 is upregulated in various immune cell types, such as monocytes, neutrophils, T cells, B cells, and plasma cells (Badr et al., 2015). Moreover, CXCL12 expression is elevated in the kidney. CXCR4 is upregulated in SLE patients, suggesting it may be a therapeutic target for SLE patients with kidney and CNS involvement (Wang et al., 2010; Badr et al., 2015). In contrast, circulating B cells from SLE patients show altered migration and distribution of B cell compartments due to the downregulation of CXCR4 (Biajoux et al., 2012). The signaling cascades involving PI3K/AKT, MAPKs (ERK, JNK, p38), and the regulation of NF-κB nuclear translocation (IκBs) are critically involved in B cell differentiation and the production of autoantibodies during SLE disease progression (Sen et al., 2014).

However, it appears that inhibiting the CXCR4/CXCL12 axis could mitigate the autoimmune response and inflammation associated with SLE. Studies in lupus-prone murine models demonstrated that CXCR4 was upregulated in B cells, monocytes, neutrophils, and plasma cells, driven by toll-like receptors (TLRs) and pro-inflammatory cytokines. By upregulating this pathway, B cells were able to survive and migrate towards gradients of CXCL12 (Balabanian et al., 2003; von Hofsten et al., 2024).

A previous study found that NZB/W mice susceptible to lupus had elevated CXCL12 levels in the kidneys, contributing to lupus nephritis (Balabanian et al., 2003). Similar findings in other mouse models (B6.Sle1.Yaa, BXSB, MRL.lpr) were also confirmed (Wang et al., 2009). However, these findings are not replicated in human SLE patients (Wang et al., 2009). Studies report that B cells and $CD4^+$ T cells express high levels of CXCR4 when disease severity is high, while others conclude that low levels of CXCR4 in specific lymphocyte subsets are associated with disease severity (Chong and Mohan, 2009). Discrepancies may be attributed to sample size differences and patient characteristics. Rather than measuring CXCR4 and CXCL12 levels in peripheral blood, end organs could provide more insights into their role in SLE. Biopsies of lupus nephritis and cutaneous lupus skin demonstrate increased CXCL12 levels correlated with disease severity. The migration of $CXCR4^+$ cells into these organs may explain why some studies report lower levels of CXCR4 in peripheral blood (Chong and Mohan, 2009).

SLE-associated glomerulonephritis is accompanied by hyperplastic kidney lesions caused by CXCR4 dysregulation in kidney epithelial cells (Rizzo et al., 2013). This interaction may be crucial during lupus in attracting these cells to the kidney and skin, which are affected peripherally. In addition, CXCL12 binding to CXCR4 boosts cell survival, proliferation, and transcription, based on studies with mice lacking either CXCL12 or CXCR4. In mice with defective CXCL12 or CXCR4, vascularization, bone marrow myelopoiesis, and limb innervation have been observed. As a result of these findings, CXCL12/CXCR4 interactions play a crucial role in numerous physiological processes and are likely to significantly impact pathological conditions such as lupus (Wang et al., 2010).

Several chemokines are associated with T1D, including CXCL10, CCL5, CCL8, CXCL9, and CX3CL1, which are involved in insulin metabolism and pancreatic β-cell destruction (Overbergh et al., 2006). CXCL12/CXCR4 signaling is critical Field (Oliver-Krasinski et al., 2009) for the pancreatic islet to develop and differentiate. CXCL12 induces pancreatic and duodenal homeobox 1 (Pdx1) expression in the pre-pancreas region by attracting CXCR4-expressing angioblasts. All islet cell types must be formed to express neurogenin 3 (Ngn3) via Pdx1 (Oliver-Krasinski et al., 2009). CXCL12 is also vital in influencing immune processes and directing T-cell migration. Recruiting autoreactive T cells into pancreatic islets causes insulitis and

T1D. According to most studies, CXCL12 inhibition inhibits diabetes progression and insulitis, but conflicting reports suggest that adoptive cell transfer may protect against diabetes. Among the unique properties of CXCL12 are that it induces bidirectional movement of T cells and exerts a chemorepulsive effect on diabetogenic T cells while promoting normal T cell adhesion (Vidaković et al., 2015). By expressing CXCL12 in islets, autoreactive T cells are selectively repelled, and Treg cells are retained at the site. Tregs are critical in suppressing autoimmunity and are implicated in the development of T1D. It has been demonstrated that the absence of Tregs in pancreatic lymph nodes (PLNs) is correlated with T1D in non-obese diabetic mice. The restoration of euglycemia is associated with the recovery of Treg populations in PLNs, which is linked to a decrease in CXCL12 expression. In order to treat T1D effectively, we may need to enhance the CXCL12/CXCR4 axis and retain Tregs in PLNs (Vidaković et al., 2015).

Recently, an investigation reported that $CD57^+$ $CD8^+$ T cells, also known as effector memory cells, contribute to tumor and virus immunity and are associated with autoimmunity (Zhong et al., 2024). However, there needs to be more knowledge of how they contribute to T1D. Upon examining a T1D patient with a STAT3 mutation, these cells were observed to be increased. The $CD57^+$ $CD8^+$ T cells of T1D patients undergo significant changes during disease progression. In longitudinal studies, their prevalence was associated with declining function of the $CD4^+$ cells. There is evidence that these cells are critical to the pathophysiology of T1D, as they produce cytotoxic cytokines, increase glucose uptake, and produce pro-inflammatory cytokines. Erk1/2 signaling enhances $CD57^+$ $CD8^+$ T cell expansion and function *in vitro* via the CXCL12/CXCR4 axis. Changes in serum CXCL12 levels were noted during the peri-remission phase of T1D. T1D mice treated with LY2510924, a CXCR4 antagonist, showed reduced infiltration of T cells and improved insulin sensitivity (Zhong et al., 2024). Based on these findings, $CD57^+CD8^+$ T cells play a crucial role in driving T1D responses, which may be a potential therapeutic option to delay the progression of the disease.

An essential function of intestinal epithelial cells (IECs) in the normal intestinal mucosa is migration, barrier maturation, and restitution, which are all mediated by cAMP. In recent studies, CXCR4 and CXCL12 are present in lamina propria T cells (LPTs), which have been implicated in IBD pathogenesis. IECs of IBD patients express CXCR4 more, and CXCL12 is upregulated in inflamed mucosa. In all sources of peripheral blood T cells (PBTs) and LPTs, CXCL12 functions as a potent chemoattractant, whether they are normal or IBD-related. As evidenced by the accumulation of $CXCR4^+$ cells near CXCR12-expressing IECs, interactions between CXCL12 and CXCR4 contribute to mucosal deregulation, specifically impacting memory $CD45RO^+$ LPTs (Werner et al., 2013). CXCL12 directs the proliferation of epithelial endocrine precursor cells in the human development of islet cells and phosphatidyl inositol-3 and AKT kinase (Oliver-Krasinski et al., 2009; Weir et al., 2011). It has been found that some IBDs, such as ulcerative colitis and Crohn's disease, are caused by dysregulated immune responses in a genetically susceptible individual in response to environmental triggers (Scharl and Rogler, 2012; Wallace et al., 2014). It has been shown that intestinal epithelial cells and lamina propria cells express CXCL12 and CXCR4 upregulation in IBD patients. The CXCL12/CXCR4 axis is associated with IBD progression and severity, as it recruits memory Th1 cells, particularly T cells (Agace et al., 2000; Katsuta et al., 2000). It has been reported that polymorphisms in this axis contribute to the recruitment of memory Th1 cells (Mrowicki et al., 2014).

Collectively, it should be noted that the dual role of the CXCL12/CXCR4 axis in ADs like SLE and T1D indicates that targeting this axis may not always be practical. Increased CXCL12 levels in lupus-prone mice have been associated with improved SLE symptoms when treated with specific peptides, suggesting its role in the disease's pathogenesis (Chong and Mohan, 2009). In T1D, the CXCL12/CXCR4 axis plays a significant role in promoting pancreatic β-cell survival. Studies show that CXCL12 helps protect β-cells from apoptosis and streptozotocin (STZ)-induced diabetes by activating the AKT pathway, which promotes cell survival. Blocking CXCR4 induces apoptosis and reduces cell survival markers in β-cells, while overexpressing CXCL12 in β-cells enhances resistance to apoptosis and diabetes (Yano et al., 2007). However, the phenotype of recruited $CXCR4^+$ cells can be crucial in the ADs pathogenesis. These findings highlight the complexity of the CXCL12/CXCR4 axis, which can contribute to disease pathogenesis in SLE and offer therapeutic benefits in T1D, suggesting that blanket targeting of this axis may not be universally beneficial.

5 Targeting CXCR4 in pathologic conditions

This section summarized the importance of receptor and ligand inhibition in all types of diseases and autoimmune diseases.

5.1 Cancer

The CXCL12/CXCR4 axis plays a pivotal role in tumor progression and metastasis, making it a promising therapeutic target in cancer. By interacting with its receptor CXCR4, CXCL12 promotes migration, invasion, and angiogenesis in cancer cells, a chemokine abundantly expressed in the TME. This ligation is disrupted by inhibitors, which inhibit metastatic spread and stimulate antitumor immune responses. Various cancers have shown promising outcomes when CXCR4 inhibitors are used, such as AMD3100 (Zhou et al., 2020). Thus, the CXCL12/CXCR4 axis has become a compelling target for novel cancer therapies to enhance treatment efficacy and prevent metastasis.

Considering the role CXCR4 plays in tumor progression and metastasis, its inhibition offers significant potential for cancer treatment (Chatterjee et al., 2014). The expression of CXCR4 is associated with increased invasiveness and distant metastases in several cancers (Yang et al., 2020). It has been shown in preclinical and early clinical studies that blocking CXCR4 can disrupt the interaction between cancer cells and the microenvironment, preventing cancer cells from migrating to secondary sites (Zlotnik, 2008). A strategy to impede cancer progression and improve treatment outcomes is effective in early clinical studies targeting CXCR4. Due to these findings, CXCR4 inhibitors are being

explored as potential cancer therapies that could impede metastasis and boost cancer therapy efficacy.

An investigation of patients with microsatellite stable pancreatic (PDA) or CRC who failed to respond to immune therapy using T cell checkpoint inhibitors in the context of cancer biology (Biasci et al., 2020). Based on their observations that cancer cells in these tumors are coated with CXCL12, the researchers proposed a possible explanation for this lack of response. Further, they note that stimulation of CXCR4 inhibited the migration of these immune cells mediated by other chemokines. For 7 days, the researchers continuously infused AMD3100 to patients to assess the relevance of these findings. Using transcriptomic analysis, they compared biopsies taken before and after treatment of metastatic lesions. This immunological response, which appears to be predictive of a positive clinical response to T-cell checkpoint inhibition, was found to be induced by the CXCR4 inhibitor. The non-response to immunotherapy may be linked to CXCL12 in cancer cells in some pancreatic and CRCs with specific characteristics (microsatellite stable). Researchers observed that AMD3100 inhibited the tumor's immune response, making it more susceptible to T cell checkpoint inhibitors by disrupting its immune response (Biasci et al., 2020). These findings suggest that targeting the CXCR4 pathway could enhance immunotherapy effectiveness in these cancer types. As a result of intrinsic/acquired resistance, antiangiogenic therapies provided limited survival benefits to cancer patients (Haibe et al., 2020). It was crucial for improving treatment outcomes to understand and target resistance mechanisms, especially in cancers that required antiangiogenic therapy, such as colon cancer. Anti-VEGFR2 treatment increased CXCL12/CXCR4 expression in orthotopic CRC models and conditional *Apc* mutant spontaneous rectal tumors (Jung et al., 2017). In response to CXCR4 signaling, anti-VEGFR2 innate immune cells were recruited to the CRCs, including $Ly6C^{low}$ monocytes and $Ly6G^{+}$ neutrophils (Jung et al., 2017). These pathways can also be successfully targeted genetically and pharmacologically, including AMD3100, which significantly enhanced response to treatment. These strategies can be readily translated into the clinic. The effectiveness of PD-1 inhibitors in pancreatic ductal adenocarcinoma (PDAC) is limited, suggesting alternative pathways should be explored (Kabacaoglu et al., 2018).

In metastatic PDAC, BL-8040 was combined with pembrolizumab and chemotherapy in a study (Bockorny et al., 2020). There was a 34.5% disease control rate (DCR) in cohort 1 (chemotherapy-resistant patients), as well as a median overall survival (mOS) of 3.3 months in second-line therapy. As a result of BL-8040, $CD8^{+}$ T cells were infiltrated in cohort 2 more efficiently, and immunosuppressive cells were reduced. Combined with pembrolizumab and chemotherapy, the tumor objective response rate (ORR) was 32%, DCR was 77%, and the median response duration was 7.8 months. A randomized trial is needed to confirm the effectiveness of dual CXCR4 and PD-1 blockade in PDAC (Bockorny et al., 2020). An investigation was conducted to assess the efficacy of a combined approach incorporating radiation therapy (RT) with cisplatin (RTCT) and the CXCR4 inhibitor X4-136, which was considered suitable for clinical use (Chaudary et al., 2021). This study found that RTCT alone increased CXCL12/CXCR4 signaling, intratumoral accumulation of myeloid cells, and PD-L1 expression.

In contrast, X4-136 was introduced along with RTCT to counteract these effects, enhancing the primary tumor response and reducing metastases. Furthermore, X4-136 alleviated late histologic changes caused by delayed RT toxicity by reducing acute toxicity in intestinal crypt cells. Study findings indicate that this combination therapy can minimize adverse effects on normal tissues, including the intestines, while improving cervical cancer treatment outcomes. It is suggested that clinical trials should be conducted to explore these benefits further, as well as the possibility of applying this approach to other cancer types for which RTCT is a curative (Chaudary et al., 2021).

It has been shown that immune checkpoint blockade (ICB) therapies are less effective in triple-negative breast cancer (TNBC) due to insufficient T cell infiltration. Immunostimulatory approaches have been developed in the field (Kruszyna et al., 2022) to enhance ICB response. As part of a novel strategy aimed at improving AMD3100's therapeutic efficacy (Lu et al., 2021), a liposomal formulation targeting CXCR4 was developed. A dual blocker and targeting moiety, AMD3100 acted both extracellularly and intracellularly to inhibit CXCR4 activation. AMD3100 was encapsulated within the liposome and coated on its surface. Based on the results of the Liposomal-AMD3100 study, AMD3100 was more effective in remodeling the immune and stromal microenvironment than AMD3100 free, suggesting that the liposomal formulation had an improved pharmacodynamic profile. A murine TNBC model (4T1) demonstrated increased antitumor effects and longer survival times when anti-PD-L1 was combined with Liposomal-AMD3100 (Lu et al., 2021). Accordingly, ICB therapy can be applied to previously ICB-insensitive cancer types by delivering CXCR4 inhibitors liposomal to activate the immune system. It has been revealed that CXCR4 is overexpressed and functional in CRC, which has prompted researchers to examine whether it can enhance standard CRC therapy (Xu et al., 2018). In a CRC HCT116 xenograft model, a study assessed the efficacy of a novel peptide antagonist of CXCR4, Peptide R (Pep R) (D'Alterio et al., 2020). Pep R was administered to mice bearing xenografts of HCT116 with chemotherapeutic agents 5-Fluorouracil (5FU) and oxaliplatin, or 5FU combined with radiotherapy (RT-CT). Compared to chemotherapy alone or Pep R alone, which resulted in 2- and 1.6-fold reductions of the relative tumor volume (RTV) after 2 weeks, the combination of chemotherapy and Pep R significantly reduced the RTV fourfold. According to *in vitro* experiments, Pep R inhibited HCT116 cell growth and further reduced the ability of those cells to clone. It was also explored whether Pep R could target epithelial-mesenchymal transition (EMT). A decrease in ECAD expression and an increase in ZEB-1 and CD90 expression were observed with chemotherapy treatment. Pep R restored the pre-treatment expression levels. Pep R also reduced a population of $CD133^{+}CXCR4^{+}$ cells in HCT116 and HT29 cells, considered stem-resistant cancer cells (D'Alterio et al., 2020). In general, the findings suggest that targeting CXCR4 with Pep R enhances the effectiveness of colon cancer treatment by reducing stem-resistant cancer cell proliferation, reversing EMT-induced markers, and inhibiting cell growth. Clinical studies are needed to explore this further.

In our recent study, we showed the potential significance of N, N″-thiocarbonylbis(N′-(3,4-dimethylphenyl)-2,2,2-trifluoroacetimidamide) (A1) as a potent inhibitor of the CXCR4 chemokine receptor in the

context of CRC therapy (Khorramdelazad et al., 2023). Compared with established CXCR4 inhibitors, A1 exhibited notable inhibitory activity, as demonstrated *in silico*. The investigation further revealed that A1 induced a cytotoxic effect on CT26 mouse CRC cells, leading to apoptosis and G2/M cell cycle arrest, in contrast to the limited impact of the control molecule AMD3100. A1's effectiveness extended to reducing cell proliferation, particularly when combined with CXCL12, and downregulating the expression of CXCR4 receptors in treated cells. The dual-functionality of A1, acting as both a CXCR4 inhibitor and a cytotoxic agent, suggests its potential as a promising candidate for enhancing CRC treatment strategies (Khorramdelazad et al., 2023).

In another investigation, glioblastoma multiforme (GBM), a highly invasive and resistance-to-treatment brain tumor, was addressed. GBM's resistance and invasiveness are contributed to by aberrant p53 function influenced by overexpressed MDM2 and MDM4 proteins, as well as an increase in CXCR4 expression (Daniele et al., 2021). This study examined whether inhibiting the p53-MDM axis could enhance the sensitivity of GBM cells. A dual MDM2/4 inhibitor, RS3594, and a CXCR4 antagonist, AMD3100, were used to treat human GBM cells and GBM stem-like cells and in addition to inhibiting neurosphere growth and inducing differentiation of GBM cells, AMD3100 and RS3594 demonstrated synergistic effects on cancer stem components (Daniele et al., 2021). It appears that simultaneous blockade of CXCR4 and MDM2/4 may offer potential therapeutic benefits in reducing GBM proliferation and invasiveness.

Among the therapeutic challenges associated with triple-negative breast cancer (TNBC), which lacks molecular targets, this study addressed. TNBC tumor growth and metastasis are implicated in the CXCR4/SDF-1 axis, which may be targeted as a therapeutic target. TNBC cells were investigated for their response to Saikosaponin A (SSA), a compound derived from Radix bupleuri (Wang et al., 2020). In mouse models, SSA significantly reduced TNBC cell proliferation, colonization, migration, and invasion, inhibiting primary tumor growth and reducing lung metastasis. Notably, SSA decreased CXCR4 expression without affecting CXCR7. Consequently, MMP-9 and MMP-2 expression were inhibited, and the Akt/mTOR pathway was inactivated. Accordingly, SSA tends to exert its effects by inhibiting CXCR4 expression, which makes it an attractive candidate therapeutic agent for TNBC patients (Wang et al., 2020).

5.2 Autoimmune diseases

Several pathological processes, such as cancer and inflammatory diseases (Bekaddour et al., 2023), are implicated in aberrant CXCR4/CXCL12 signaling (Mousavi, 2020). EPI-X4 was discovered to be an endogenous peptide antagonist and inverse agonist of CXCR4, suggesting that it could be developed as a therapeutic (Harms et al., 2021). A modified EPI-X4 derivative with increased anti-CXCR4 activity, referred to as JM#21, was engineered by researchers using molecular docking analysis and rational drug design. Among other things, JM#21 suppressed human immunodeficiency virus (HIV)-1 infection more effectively than AMD3100, a small molecule CXCR4 antagonist approved for clinical use. JM#21 did not cause toxic effects in zebrafish embryos, demonstrating its safety. A mouse model of atopic dermatitis revealed that it attenuated allergen-induced immune cell infiltration and prevented skin inflammation. As a novel and potent CXCR4 antagonist, EPI-X4 JM#21 is positioned in the text as a first-in-class inhibitor with therapeutic efficacy in treating atopic dermatitis, highlighting its importance (Harms et al., 2021). This study supports the clinical development of CXCR4 antagonists to address various diseases associated with CXCR4, including asthma and atopic dermatitis.

A study aimed at developing a CXCR4 inhibitor suitable for topical use in treating psoriasis to minimize systemic toxicity (Boonsith, 2017). As a topical drug for psoriasis, the researchers developed PAMD, a polycation derived from AMD3100. In addition to its adaptability for chemical modification, PAMD can be combined with other drugs and form nanocarriers. Due to the localized nature of psoriasis as a skin disease, topical delivery of PAMD improved safety and compliance by targeting the disease locally. A challenge was overcoming the skin's main barrier, the stratum corneum (SC), which necessitated modifying the technique. It was found that the modified, negatively charged PAMD demonstrated low toxicity in HaCaT cells and good retention and penetration in both healthy and psoriatic skin models after adding citraconic anhydride for a negative charge and oleic acid for lipophilicity. As a result of the modified polymer's characteristics, several factors influenced the penetrant's success, including its size, charge, and partition coefficients. The topical administration of AMD3100 and subcutaneous injection of PAMD.COO- significantly reduced psoriasis symptoms in an IMQ-induced psoriasis mouse model. It was found that blocking CXCR4/SDF-1 reduced skin inflammation, as demonstrated by lower mRNA levels of pro-inflammatory cytokines *IL-1β*, *IL-6*, and *TNF-α*. CXCR4 antagonistic polymers were shown to have similar therapeutic effects to AMD3100 administration by topical application. The polymer's anti-psoriatic activity in mice did not appear to be affected by its penetration, indicating its efficacy in localized treatment without compromising therapeutic outcomes (Boonsith, 2017).

Researchers investigated the role of the CXCL12/CXCR4 axis in chronic PsO-like skin inflammation, where elevated levels of the angiogenic chemokine CXCL12 and its receptor CXCR4 had been previously observed. Using two experimental models, they found that the CXCL12/CXCR4 axis upregulates blood vessels and macrophages in inflamed skin (Boonsith, 2017). With AMD3100, skin inflammation, inflammation angiogenesis, and accumulation of inflammatory cells were effectively reduced in both models. Anti-CXCL12 antibodies had similar anti-inflammatory effects. These findings were confirmed *in vitro*, suggesting that the CXCL12/CXCR4 axis plays a crucial role in inflammation and inflammatory angiogenesis. Taking advantage of these molecular mechanisms may provide insights into the mechanisms underlying vascular activation in psoriasis, a chronic inflammatory skin disease (Boonsith, 2017). Another study explores the role of CXCR4, expressed by basal keratinocytes (KCs), in inflamed skin using a mouse model with specific loss of CXCR4 in K14-expressing cells (Takekoshi et al., 2013). Despite no apparent skin defects in these mice, they showed increased ear swelling, greater epidermal thickness, and enhanced parakeratosis in an IL-23-mediated psoriasiform dermatitis model. This suggests that CXCR4 plays a regulatory role in keratinocyte proliferation.

Further experiments *in vitro* demonstrated that CXCL12, a chemokine, blocked IL-22-induced keratinocyte proliferation and worked synergistically with IL-22 to upregulate suppressor of cytokine signaling 3 (SOCS3), a key regulator of signal transducer and activator of transcription 3 (STAT3), indicating that SOCS3 is required for CXCR4-mediated growth inhibition. In human psoriatic skin, both CXCR4 and SOCS3 were upregulated in the junctional region at the border of psoriatic plaques (Takekoshi et al., 2013). These findings indicate that CXCR4 surprisingly hinders keratinocyte proliferation and mitigates the effects of proliferative cytokines, providing insights into its role in skin inflammation, particularly in PsO.

They play a crucial role in migrating leukocytes across the blood-brain barrier (BBB) during the inflammatory response in the CNS (Pachter et al., 2003). CXCL12 is highly expressed by microendothelial cells throughout the CNS, suggesting it may help maintain the BBB. With AMD3100, a specific antagonist of the CXCL12 receptor CXCR4, this hypothesis was tested in experimental autoimmune encephalomyelitis (EAE) (McCandless et al., 2006). A study demonstrates that infiltrating leukocytes migrate more rapidly into the CNS parenchyma when CXCR4 activation is lost. CXCL12 is expressed on the basolateral surface of spinal cord endothelial cells under normal conditions (McCandless et al., 2006; Liu and Dorovini-Zis, 2009). As EAE progresses, this polarity is lost in vessels where mononuclear cells have extensively invaded the parenchyma. EAE worsened by inhibiting CXCR4 activation during disease induction since mononuclear cells infiltrated the white matter, resulting in decreased perivascular cuffs and more inflammation. It appears that CXCL12 serves as an anti-inflammatory factor in EAE, limiting the infiltration of autoreactive effector cells into the parenchyma by localizing CXCR4-expressing mononuclear cells to the perivascular space (McCandless et al., 2006).

A novel mutant chemokine designed to antagonize CXCR3 and CXCR4 was used to investigate the role of these receptors in T lymphocyte activation and migration to the central nervous system (Kohler et al., 2008). *CXCL11(4–79)* antagonist was developed from truncation mutants with the highest affinity for CXCR3. CXCR3 ligands (CXCL9, CXCL10, and CXCL11) strongly inhibited mouse T-cell migration with this antagonist. *CXCL12(P2G2)*, another synthetic receptor antagonist, minimally activated these receptors but inhibited activating T cells' migration in response to the drug. These synthetic receptor antagonists inhibited EAE by interfering with the action of CXCR3 and CXCR4 in a mouse model of multiple sclerosis called experimental autoimmune encephalomyelitis (EAE). They also reduced $CD4^+$ T cell accumulation in the CNS. The results of further investigation indicate that *CXCL12(P2G2)* inhibits the sensitization phase of the immune response, but *CXCL11(4–79)* inhibits the effector phase. In treating CNS autoimmune diseases, the findings suggest that targeting both CXCR4 and CXCR3 simultaneously may be beneficial (Kohler et al., 2008).

CXCL12 is implicated in the pathological development of RA, particularly concerning the abnormal migration of peripheral immune cells in joints (Ding et al., 2023). There is controversy surrounding the impact of low-dose methotrexate (MTX) on CXCL12 signaling responses in RA despite its widespread use. According to clinical data, low-dose MTX treatment was associated with clinically relevant downregulation of the CXCR4 on peripheral T cells. By suppressing CXCR4 expression, low-dose MTX significantly decreased cell migration in *in vitro* experiments with $CD3^+$ T cells. A significant increase in genomic hypermethylation was observed across the promoter region of the *CXCR4* gene in $CD3^+$ T cells treated with low-dose MTX. A significant improvement in arthritis pathology was demonstrated by low-dose MTX-mediated downregulation of CXCR4. It was also found that conditional disruption of the *cxcr4* gene in peripheral immune cells reduced inflammation in arthritis mice's joints and lungs. It is noteworthy, however, that genetic modification in these mice did not affect their clinical scores for arthritis. By downregulating CXCR4 expression, low-dose MTX may impair immune cell migration and exert anti-inflammatory effects on RA patients (Ding et al., 2023). These findings indicate the MTX's potential therapeutic effects for RA by revealing how it influences CXCL12 signaling and immune cell behavior.

The chemokine *CXCL12* gene polymorphism has been linked to T1D in humans. CXCL12 levels are elevated in the bone marrow of non-obese diabetic mice, a model predisposed to T1D (Leng et al., 2008). NOD mice accumulate naive T cells, Tregs, and hematopoietic stem cells (HSCs) in their bone marrow (BM). AMD3100, an antagonist of CXCR4, mobilizes T cells and HSCs from their BM. By simultaneously inhibiting insulitis and preventing diabetes, this treatment is simultaneously effective. AMD3100 can treat or prevent T1D in humans by altering T cell and HSC trafficking, which supports the hypothesis that elevated CXCL12 expression promotes T1D in NOD mice (Leng et al., 2008).

An emphasis was placed on the role of CXCR4 in diabetic neuropathy, a common cause of painful diabetic neuropathy (PDN). Observations of elevated CXCR4 levels in peripheral nerve samples from diabetic patients prompted an investigation of the effects of three agents in a streptozotocin (STZ)-induced PDN model in rodents and a naive rat model activating CXCR4/CXCL12 signals (da Silva Junior et al., 2020). A diabetic neuropathy model was induced in Wistar rats through intraperitoneal injection of STZ, providing a platform for measuring rat hypersensitivity, levels of IL-6, and the concentration of calcium $[Ca^{2+}]i$ inside diabetic synaptosomes. The outcomes designated a significant decrease in hypersensitivity in diabetic rats following intrathecal administration of Phα1β or intraperitoneal administration of AMD3100, while ω-conotoxin MVIIA did not show an equivalent effect. In naïve rats with activated CXCR4/CXCL12 axis, CXCL12 administration induced hypersensitivity, which was alleviated by Phα1β or AMD3100 after 2 h of treatment, contrasting with the lack of effect detected with ω-conotoxin MVIIA.

Moreover, the study investigated the modulation of IL-6 levels and calcium influx in spinal cord synaptosomes, revealing a decline in both parameters following treatment with the examined agents. Conclusively, Phα1β, ω-conotoxin MVIIA, and AMD3100 revealed effectiveness in decreasing hypersensitivity in STZ-induced PDN in diabetic rats and naïve rats with activated CXCR4/CXCL12 axis. The results propose potential therapeutic avenues, with Phα1β implicating voltage-dependent calcium channels in its repressing effects on PDN (da Silva Junior et al., 2020).

It is expected to find vascular, glomerular, and tubulointerstitial lesions in a renal biopsy taken from a patient with SLE (Weening et al., 2004). The Bowman's capsule parietal epithelial cells of proliferative glomerulonephritis become activated, and $CD133^+CD24^+$ progenitor cells invade the glomerular tuft (Rizzo et al., 2013). It has been shown that an injury to podocytes results in

dysregulated progenitor cells expressing CXCR4, along with high expression of CXCL12 in podocytes, which uses renal biopsies and rat models. In rat models, similar changes are observed in the expression of angiotensin II (Ang II) type-1 (AT1) receptors, which appear to be associated with parietal epithelial cell proliferation. Angiotensin-converting enzyme inhibitors have been shown to normalize the Ang II/AT1 receptor/CXCR4 pathways and to result in regression of lesions in patients with severe forms of glomerular proliferative disorders (Rizzo et al., 2013).

The migration, proliferation, and survival of B1a lymphocytes in the peritoneal cavity are influenced by CXCL12 in normal mice. In NZB/W mice, where these cells are self-reactive and expand, they exhibit increased sensitivity to CXCL12 (Ding et al., 2023). CXCL12 is produced constitutively in the peritoneal cavity, spleen, and glomeruli of mice with nephritis. CXCL12 is specific to the NZB genetic background and modulated by IL-10. In NZB/W mice, antagonists of CXCL12 or IL-10 are used early in life to prevent autoantibodies, nephritis, and mortality. Beginning anti-CXCL12 monoclonal antibody treatment later in life, autoantibodies are inhibited, kidney-related symptoms are eliminated, and B1a lymphocytes and T lymphocytes are suppressed. In this lupus mouse model, abnormally sensitive PerB1a lymphocytes to CXCL12 and IL-10 contribute to the development of autoimmunity (Ding et al., 2023). These data suggest the potential for preventing or mitigating autoimmune manifestations by targeting the CXCL12/CXCR4 axis.

Ulcerative colitis (UC) and IBD are examined to explore the immunological significance of the CXCL12/CXCR4 chemokine axis (Mikami et al., 2008). A multifaceted approach to the study is taken, starting with assessing CXCR4 expression on peripheral T cells in patients with active UC, which revealed significant increases compared with normal controls. According to this study, CXCL12/CXCR4 interaction is associated with UC pathophysiology, where increased expression correlates positively with disease activity. Using a murine model of dextran sulfate sodium (DSS)-induced colitis, the study further demonstrates that CXCR4 expression is elevated on leukocytes and that CXCL12 expression increases in colonic tissue when colitis is induced. A CXCR4 antagonist effectively reduces colonic inflammation in the DSS colitis model and the IL-10 knockout mouse model, suggesting it may be a promising therapeutic intervention. In mesenteric lymph node cells, the antagonist decreases pro-inflammatory cytokines, tumor necrosis factor, and interferon (IFN) production while preserving IL-10 production (Mikami et al., 2008). These findings illustrate potential therapeutic avenues for treating IBD, particularly UC, by targeting the CXCL12/CXCR4 chemokine axis, offering new avenues for intervention in this complex inflammatory condition.

6 What is clobenpropit?

Clobenpropit is a potent imidothiocarbamic ester characterized by isothiourea with S-3-(imidazole-4-yl) propyl and N-4-chlorobenzyl substituents. Functioning as a highly effective histamine H3 antagonist and inverse agonist (pA2 = 9.93), it demonstrates notable activity as a partial agonist at H4 receptors. This compound induces eosinophil shape change with an EC_{50} of 3 nM. Clobenpropit serves as both an H3-receptor antagonist and an H4-receptor agonist. Classified as an imidazole, an imidothiocarbamic ester, and an organochlorine compound, it is a conjugate base of Clobenpropit (2+) (Pubchem, 2024). It is essential to emphasize that Clobenpropit exhibits a distinctly low affinity for histamine H1R and H2R, registering pKis of 5.2 and 5.6, respectively (Esbenshade et al., 2003). Regarding its pharmacological impact, Clobenpropit is a concentration-dependent inhibitor of [3H]-dopamine transport in SH-SY5Y cells. The inhibitory effect is pronounced, with a maximum inhibition of 82.7% ± 2.8% and an IC_{50} value of 490 nM (pIC_{50} 6.31 ± 0.11) (Mena-Avila et al., 2018). Furthermore, Clobenpropit distinguishes itself as a subunit-selective noncompetitive antagonist when interacting with recombinant N-methyl-D-aspartate (NMDA) receptors. Its inhibitory activity is particularly potent against the NR1/NR2B receptor, with an IC_{50} of 1 μM (Mena-Avila et al., 2018). In therapeutic applications, a combination regimen involving.

6.1 Histamine, histamine receptors, and clobenpropit

The biogenic amine histamine, synthesized from histidine, has been studied in pharmacology since its discovery in the early 20th century by Sir Henry H. Dale (Dy and Schneider, 2004). Histamine is widely distributed throughout the body and primarily mediates inflammatory processes. The fact that it binds to four GPCR subtypes–H1, H2, H3, and H4 – accounts for its pleiotropic regulatory role in cellular events (Chazot and Tiligada, 2008). In addition to exhibiting differential expressions in different types of cells, histamine shows a broad spectrum of activities. Upon activation, the H3 receptor inhibits cAMP formation, accumulates Ca^{2+}, and activates the MAPK pathway. As a target for ligands in treating such conditions, it is implicated in central nervous system disorders (Zampeli and Tiligada, 2009) (Figure 5).

Meanwhile, leukocyte chemotaxis to inflammation sites is mediated by the H4 receptor, mainly expressed in immune cells, including mast cells, monocytes, eosinophils, DCs, T-cells, and NK cells. The H4 receptor more readily absorbs Histamine than the H1 receptor, and activation of the receptor increases intracellular Ca^{2+} concentration (Hofstra et al., 2003) (Figure 5). As an endogenous agonist for the liver-expressed chemokine LEC/CCL16, it contributes to the trafficking of eosinophils (Nakayama et al., 2004).

6.1.1 Histamine receptors in cancer

Various cancer types exhibit heterogeneous outcomes due to the intricate interplay between disparate pathways related to histamine metabolism, the unique landscape of the TME, and the H4 histamine receptor's central role in signaling cascades (Massari et al., 2020). Histamine has been shown to play a significant role in multiple stages of tumorigenesis, primarily through the H4 receptor, impacting diverse cell types, including cancer cells (Nguyen and Cho, 2021). There is a consistent pattern emerging across a broad spectrum of cancer types, including BCa, CRC, oral tongue squamous cell carcinoma, gastric cancer, melanoma, laryngeal squamous cell carcinoma, bladder urothelial carcinoma, and uterine corpus endometrial carcinoma (Nguyen and Cho, 2021).

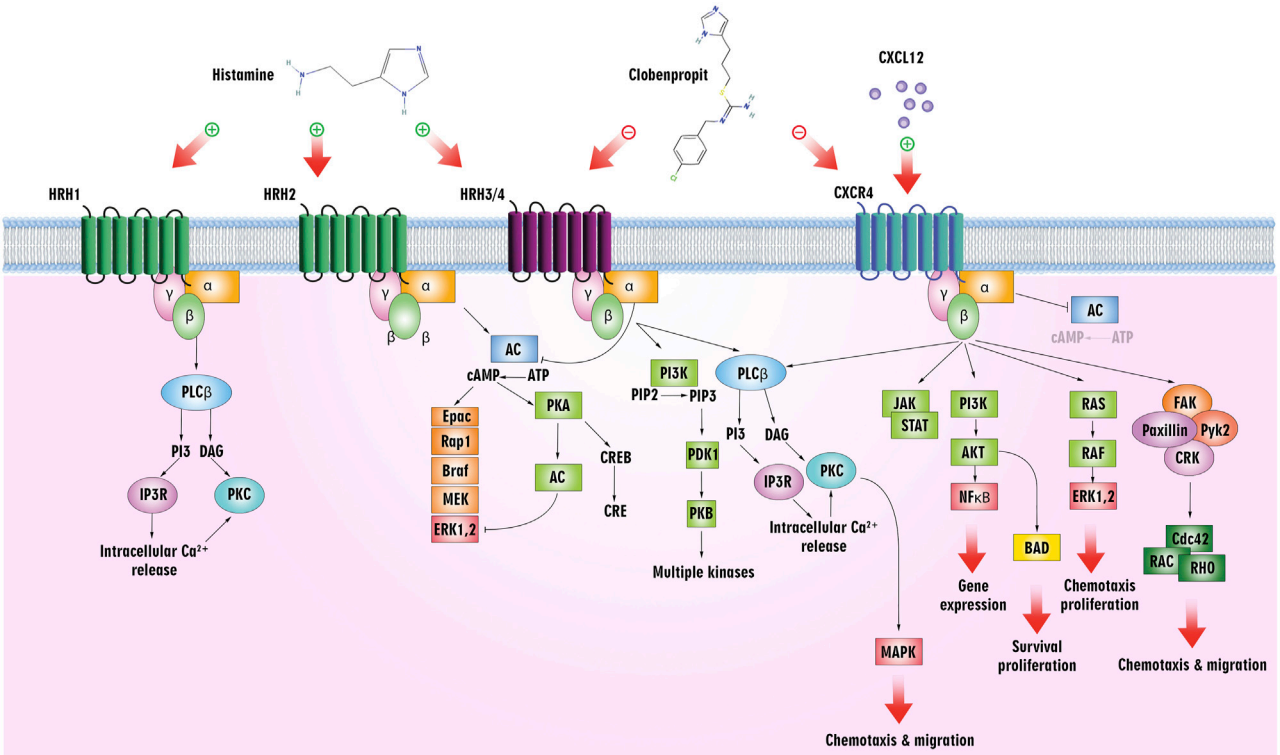

FIGURE 5
Histamine/histamine receptors and the CXCL12/CXCR4 axis. Clobenpropit binds to H3/H4 and CXCR4 receptors and can inhibit the downstream pathways. Inhibition of these receptors can lead to a decrease in cell proliferation, migration, and production of cytokines, which are used in the treatment of cancer and ADs.

Compared with normal tissues, tumors exhibit a significant reduction in the expression of the *H4 receptor* gene and/or protein (Nicoud et al., 2019). Additionally, H4 receptor expression correlates with clinicopathological characteristics, suggesting that cancer cell differentiation and tumor progression may be influenced by H4 receptor expression (Fang et al., 2011). The correlation indicates that H4 receptors can be used as a novel prognostic biomarker, providing valuable insights into disease prognosis. It has been reported that partial differentiation in pancreatic cancer is associated with inhibition of cell proliferation through the H1 and H2 receptors (Cricco et al., 2000). Histamine inhibits cell proliferation through the H2 receptor and modulates mitogen-activated protein kinase and Bcl-2 family proteins through the G_0/G_1 phase (Martín et al., 2002; Cricco et al., 2004; Cricco et al., 2006). Moreover, a previous study suggests that the H3 and H4 receptors play a role in pancreatic cancer cell proliferation, with the H3 receptor increasing proliferation and the H4 receptor decreasing cell proliferation (Cricco et al., 2008).

Malignancies of the bile ducts, such as cholangiocarcinoma (CCA), are associated with EMT, increasing invasion potential (Vaquero et al., 2017). It has been demonstrated that Clobenpropit, a potent H4HR agonist, inhibits the growth of mammary adenocarcinoma by acting on four receptors (H1-H4) (Patnaik et al., 2018). A study found that cholangiocytes and CCA cells express H1-H4 HRs, and the H3HR inhibits cell proliferation. CCA proliferation, invasion, and EMT phenotypes were significantly reduced by Clobenpropit *in vitro*, affecting the extracellular matrix (ECM). Moreover, Clobenpropit inhibited xenograft tumor growth by disrupting focal contact proteins and altering epithelial and mesenchymal markers *in vivo*. Genetic manipulation confirmed that H4HR was explicitly involved in these effects. Using Clobenpropit to modulate H4HR, CCA cells disrupt their EMT processes, ECM breakdown, and invasion potential (Patnaik et al., 2018).

In another preclinical investigation, for 15 weeks, mice were fed diets containing different test chemicals (terfenadine, cimetidine, or Clobenpropit) to induce colorectal carcinogenesis (Tanaka et al., 2016). Azoxymethane and dextran sodium sulfate (DSS) induced colorectal carcinogenesis in male ICR mice. During week 18, diets containing cimetidine (Hrh2) and Clobenpropit (Hrh3 antagonist/inverse agonist) significantly reduced colonic adenocarcinoma diversity. Colorectal carcinogenesis induced by AOM-DSS was not affected by terfenadine (Hrh1 antagonist). Immunohistochemical analysis revealed varying intensities of adenocarcinoma cells expressing Hrh1, Hrh2, Hrh3, and Hrh4. Inflammation-related colorectal cancer may be accelerated by Hrh2, Hrh3, and Hrh4, according to Clobenpropit, an Hrh3 antagonist and Hrh4 receptor agonist. Additionally, the study provided insight into the molecular aspects of colorectal carcinogenesis that are influenced by histamine receptors by identifying the mRNA expression of pro-inflammatory cytokines

TABLE 1 The most important therapeutic impacts of Clobenpropit in various disorders.

Disease	Mechanism and therapeutic outcomes	Ref
JIA	• Inhibits the production of pro-inflammatory cytokines and chemokines in inflammatory monocytes • Anti-inflammatory effects • Alleviating the hypersecretion of cytokines and chemokines associated with flare-ups	García-Cuesta et al. (2019)
RA	• Reduces cartilage destruction • Reduces bone remodeling • Reduces immune cell tissue infiltration • Reduces pannus formation in animal models of RA • Modulates cytokine production • Anti-inflammatory effect, comparable to the positive response observed with prednisone	García-Cuesta et al. (2019)
AD	• Reduces AβP deposits • Mitigates neuronal and glial reactions • Protective effects against BBB breakdown and edema formation	Gao et al. (2021)
SLE	• Inhibits pDCs and IFN-α production • Downregulating the expression of TRAIL • Inhibits TLR7-activated pDCs by CXCR4	Wang et al. (2010b)
CCA	• Modulates H4 receptors • Disrupts EMT • Reduces invasion potential • Decreases tumor growth	Weir et al. (2011)
Pancreatic cancer	• Combination of Clobenpropit and gemcitabine induces significant apoptosis in tumor cells • Inhibits tumor cell migration • Upregulation of *E-cadherin* • Downregulation of *vimentin* and *MMP-9* • Downregulation of *Zeb1* • Inhibits tumor cell invasion • Inhibits tumor growth	Scharl and Rogler (2012)

and inducible inflammatory enzymes in colonic mucosa (Tanaka et al., 2016) (Table 1).

6.1.2 Histamine receptors in autoimmune diseases

Various human immune cells express the H4 receptor, which indicates its role in immunomodulation (Nguyen and Cho, 2021). The similar tissue distribution indicates similar physiological roles for this receptor across species despite interspecies differences in amino acid sequence and receptor characteristics. ADs, particularly RA, are associated with histamine. Histamine is considered a pro-inflammatory mediator in arthritic diseases despite its anti-inflammatory properties (Adlesic et al., 2007; Ohki et al., 2007; Grzybowska-Kowalczyk et al., 2008). It indicates that H4 receptors may be related to RA by their expression varying with its severity and duration in synovial cells (Grzybowska-Kowalczyk et al., 2007). Patients with osteoarthritis and RA are found to have H4 receptors within synovial and vascular wall cells, as well as within fibroblasts and macrophages (Grzybowska-Kowalczyk et al., 2008). It has been suggested that the H4 receptor plays a functional role in normal cartilage in rats and that histamine contributes systemically to the arthritic phenotype (Zampeli et al., 2008). This raises fascinating questions about the mechanisms mediated by the H4 receptor in cartilage. According to a comprehensive investigation, Clobenpropit significantly inhibits the production of pro-inflammatory cytokines and chemokines in inflammatory monocytes derived from blood and synovial fluid from individuals with Juvenile Idiopathic Arthritis (JIA) (Bekaddour et al., 2023). A remarkable aspect of Clobenpropit's anti-inflammatory effects is that it modulates the inflammatory signature observed in patients with JIA rather than targeting specific cytokines. Clobenpropit potentially alleviates the hypersecretion of cytokines and chemokines associated with flare-ups in JIA patients, according to these *ex vivo* findings (Bekaddour et al., 2023). A significant anti-inflammatory effect of Clobenpropit has been demonstrated in animal models of RA, in which IL-6 promotes osteoclast activation, synoviocyte proliferation, and recruitment to inflammatory sites, resulting in synovial pannus development (Lipsky, 2006; Bekaddour et al., 2023). By consistently reducing cartilage destruction, bone remodeling, immune cell tissue infiltration, and pannus formation in collagen-induced arthritis (CIA) mice, the medication clobenpropit significantly reduced cartilage damage, bone remodeling, and immune cell tissue infiltration.

In addition, Clobenpropit diminishes disease progression in arthritic mice and reduces paw thickness, similar to a positive response observed with prednisone as a reference corticosteroid. In a rat model of Alzheimer's disease (AD) induced by amyloid beta peptide (AβP) infusion, the therapeutic potential of BF 2649 (an H3 receptor inverse agonist) and Clobenpropit was explored. The animals were treated daily for 1 week after 3 weeks of AβP administration. Interestingly, the findings showed that both drugs significantly reduced AβP deposits and mitigated neuronal and glial reactions in the brain. Additionally, a remarkable reduction in BBB breakdown was detected following the treatments and exhibited protective effects against edema formation. Clobenpropit demonstrated superior effects compared to BF 2649. These findings indicate that blocking H3 receptors and stimulating H4 receptors may offer therapeutic benefits in treating AD pathology, providing novel insights into potential treatment strategies (Patnaik et al., 2018).

In Parkinson's disease (PD), dopaminergic pathways are abnormal, α-synuclein levels are elevated, and tau is

phosphorylated in cerebrospinal fluid (CSF). There is a heightened number of histaminergic nerve fibers in specific brain regions of PD, such as the substantia nigra pars Compacta (SNpc), the striatum (STr), and the caudate putamen (CP), as well as an upregulation of H3 receptors and a downregulation of H4 receptors in postmortem cases. By treating SNpC and STr with chronic BF 2649 or Clobenpropit and administering monoclonal antihistamine antibodies (AHmAb), PD-induced brain pathology was reduced significantly. As a result of these findings, revealing the involvement of histamine receptors in PD, novel insights can be gained into developing potential drug strategies for treating the disease, opening up a previously untapped area of research in this field (Sharma et al., 2021).

The immune system's type I IFNs have vital antiviral and immunomodulatory effects, but prolonged exposure can lead to autoimmune reactions. In SLE, patients exhibit ongoing IFN-α production due to endogenous IFN-α inducers, specifically small immune complexes containing DNA or RNA. These inducers act on naturally IFN-α producing cells, or plasmacytoid dendritic cells (pDC). pDCs are central to innate and adaptive immunity but, through IFN-α production, may contribute to autoimmunity (Rönnblom et al., 2003). It was detected that the histamine receptor 4 (H4R) played a crucial role in mediating the inhibitory impact of histamine on human pDC (Gschwandtner et al., 2011). In this regard, a study explored the effects of Clobenpropit on this process, and the findings showed a more potent inhibitory effect compared to histamine, resulting in a significant reduction of approximately 90% in the levels of IFN-α secreted and membrane TNF-related apoptosis-inducing ligand (TRAIL) expression following HIV-1 stimulation (Smith et al., 2017). Notably, Clobenpropit exhibited no cytotoxic effects at a concentration of 10 μM. This inhibitory effect of Clobenpropit was comparable to that observed with A151, a TLR7 antagonist. Accordingly, it is possible to use this inhibitory impact of Clobenpropit on pDCs and IFN-α production in treating patients with SLE.

An investigation showed that $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocyte subsets are activated in systemic juvenile idiopathic arthritis (Macaubas et al., 2012). Their in-depth analysis of protein expression at the single-cell level also revealed the presence of a mixed M1/M2 phenotype at the individual cell level during the flare phase. Consistent with an M2 phenotype, following exposure to lipopolysaccharides (LPS), monocytes express IL-1β; however, they do not release it in systemic juvenile idiopathic arthritis. Despite the inflammatory nature of active monocytes in systemic juvenile idiopathic arthritis, circulating monocytes exibit remarkable anti-inflammatory features. In addition, the perseverance of some of these phenotypes throughout the clinically inactive disease phase claims that this condition mirrors a compensatory response against hyperinflammation (Macaubas et al., 2012).

It has been found that monocytes express the H4R protein, which IFN-γ upregulates (Damaj et al., 2007). Clobenpropit and 4-methylhistamine are H4R agonists that cause monocytes to mobilize $Ca2^{+}$ (Roßbach et al., 2009). In addition, H4R agonists inhibited CCL2 protein production in transmigration assays because they reduced the recruitment of monocytes in supernatants. CCL2 production was downregulated at mRNA and protein levels. Consequently, Clobenpropit can induce a $Ca2^{+}$ influx in monocytes, inhibiting CCL2 production and reducing monocyte recruitment by activating the H4R on monocytes (Dijkstra et al., 2007). It has also been reported that small molecules can inhibit CXCR4 and reduce the polarization of the M1 to M2 phenotype (Song et al., 2021). Therefore, Clobenpropit can also inhibit this phenotype by switching through CXCR blocking. This dual ligation of Clobenpropit to CXCR4 and H_4R receptors may be beneficial in regulating mixed M1/M2 monocyte-mediated anti-inflammatory responses by decreasing their recruitment and inhibiting prolonged M2-type polarization in some disorders, such as systemic juvenile idiopathic arthritis (Table 1).

Collectively, these data designate the importance of understanding the intricacies of histamine signaling pathways and receptor-specific functions for immune-related disorders and could provide valuable insights into potential therapeutic interventions targeting these pathways.

6.2 Molecular docking

In this study, we explored Clobenpropit interactions in CXCR4 binding pocket for CXCL12 with the aid of molecular docking technique, following our previously performed simulations (Khorramdelazad et al., 2023), and the obtained results were then compared to those of known potent CXCR4 inhibitors, including BPRCX807 (Song et al., 2021), BPRCX714 (Song et al., 2021), AMD3100 (Zhou et al., 2020), WZ811 (Li et al., 2016), MSX-122 (Zhang et al., 2008), as well as ITD, the crystallographic ligand (3ODU) (Table 2). A comparison between the studied ligands' mode of interactions with that of Clobenpropit shows that the ligand sits very close to those of WZ811 and MSX-122. All three ligands establish π-π stacked and π-π T-shaped electrostatic interactions with residues W94, H113, and Y116. While the hydrophobic pattern is similar in the three ligands, the presence of methanimidamide and imidazole moieties in Clobenpropit structure provides π-cation interactions and salt-bridge formations with E32, W94, D97, and R183 that are not observed in WZ811 and MSX-122 structures and subsequently results in the better binding energy of Clobenpropit for CXCR4 binding pocket for CXCL12. A comparison between AMD3100 and Clobenpropit demonstrates that the presence of positively charged amine groups in AMD3100 structure mediates the formation of salt bridges and charge-charge interactions that enhance AMD3100 binding energy for CXCL12 binding groove significantly (Figure 6). It has already been observed that the creation of salt bridges between ligand and protein acts as clips that stabilize protein structure and can be used as a valuable tool for drug design (Spassov et al., 2023).

6.3 Therapeutic effectiveness of clobenpropit via CXCR4 inhibition

It has been revealed that Clobenpropit inhibits mammary adenocarcinoma spread by acting as a specific H3 antagonist and H4 agonist by decreasing invasion potential (Medina et al., 2008). Several studies have shown that Clobenpropit modulates

TABLE 2 Binding energy calculation and the type of proposed compound interactions over CXCR4 binding site for CXCL12. The molecules binding energy values (Except for that of Clobenpropit) are obtained from Nazari et al. (2017).

Compound	MMGBSA (Kcal/mol)	Glide score	Interaction over CXCR4 binding site			
			H-bond	Hydrophobic	Electrostatic	Salt bridge
BPRCX807	−69.67 ± 3.03	−10.32	C186, H203, E288	E32, L41, Y45, D97, W94, A98, V112, H113, Y116, I185, C186, D187, A188, Y190, F199, Q200, H203	W94, H113, Y116	E32, D97, D187, R188
BPRCX714	−64.12 ± 3.03	−8.85	N33, D97, E288	E32, L41, Y45, D97, W94, A98, V112, H113, Y116, I185, C186, D187, A188, Y190, F199, Q200, H203	W94, H113, Y116	E32, D97, D187, E288
AMD3100	−59 ± 3.32	−8.61	—	L41, Y45, D97, W94, A98, V112, H113, Y116, I185, C186, D187, A188, E288	W94, A98	E32, D97, D187, E288
ITD	−47.50 ± 2.00	−7.75	—	L41, Y45, D97, W94, A98, V112, H113, Y116, I185, C186, D187, A188, E288	W94, D97, E288	D97, E288
Clobenpropit	−53.47 ± 0.820	−6.21	—	E32, L41, Y45, W94, D97, A98, H113, Y116, R183, C186	W94, V112, H113, Y116, R183	E32, A98
WZ811	−41.80 ± 0.801	−5.51	Y116, E288	D97, W94, A98, V112, H113, Y116, I185, C186, D187, A188, E288	W94, H113, Y116, C186	E32, D97
MSX-122	−36.71 ± 2.666	−4.76	Y116	D97, W94, A98, V112, H113, Y116, I185, C186, D187	E32, W94, D97, H113, Y116, R183, C186	—

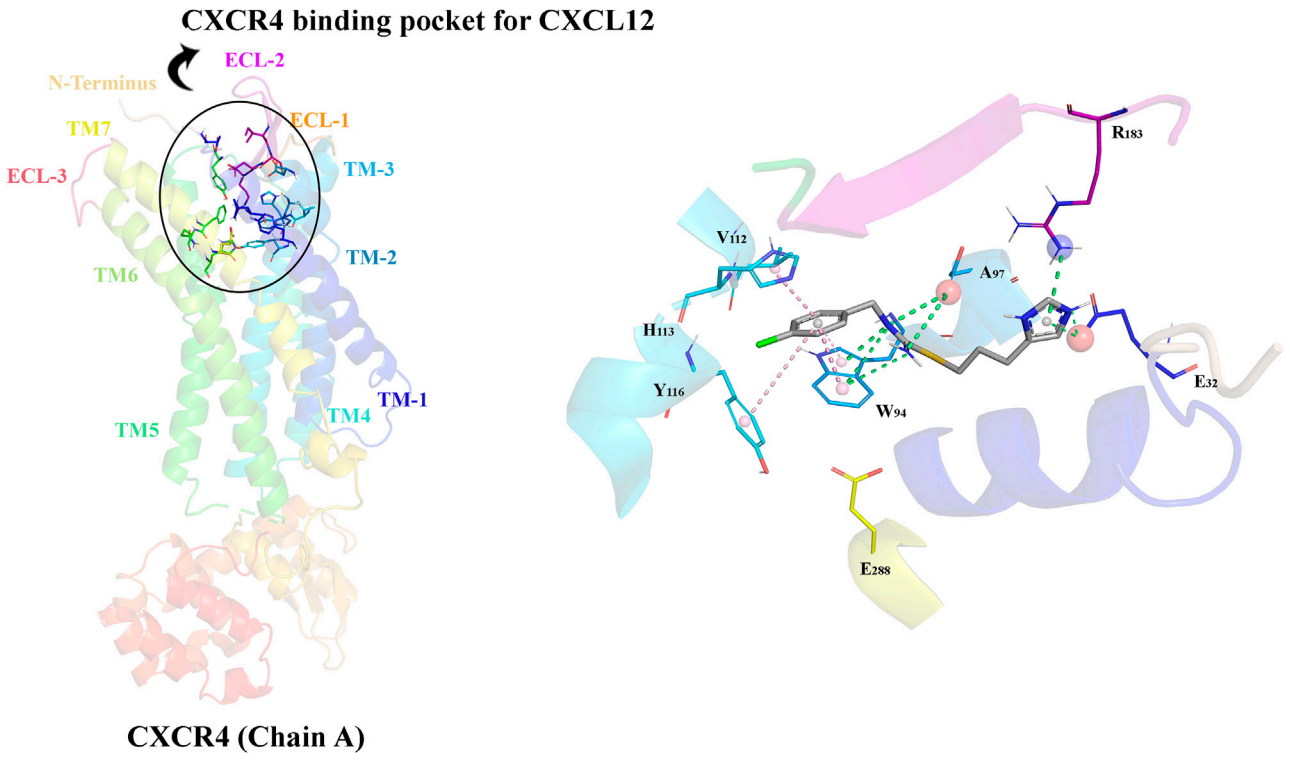

FIGURE 6
The overall structure of CXCR4 (monomer) and the CXCR4 binding site (groove) for CXCL12 along with a close view of Clobenpropit interactions with CXCR4. Green dashes represent salt bridges and velvet dashes represent π- π stacked and π- π T-shaped electrostatic interactions.

H4 receptors, disrupts EMT, reduces invasion potential, and decreases tumor growth in CCA (Meng et al., 2011). EMT also plays a vital role in pancreatic cancer metastasis and progression. In addition, Zeb1 is known to make human pancreatic cancer cells resistant to chemotherapy (Arumugam et al., 2009). Therefore, therapeutic agents targeting the EMT process could restore pancreatic cancer's resistance to chemotherapy.

Combining Clobenpropit (50 μg) and Gemcitabine (5 μg) was reported to be effective (Paik et al., 2014). A study using

clobenpropit in combination with Gemcitabine demonstrated that H4 receptors were present and manifested as two subunits, while H3 receptors were not expressed. Clobenpropit inhibited cell migration and increased apoptosis. It has been shown that Clobenpropit induces changes in gene expression associated with cell adhesion and migration, including the upregulation of *E-cadherin*, while the downregulation of *vimentin* and matrix metalloproteinase 9 (*MMP-9*). Compared with Gemcitabine alone, Clobenpropit significantly inhibited tumor growth in a Panc-1 xenograft mouse model, decreasing tumor weight. Correspondingly, Clobenpropit and Gemcitabine synergized to increase apoptosis in the mouse model, owing to the upregulation of *E-cadherin* and the downregulation of *Zeb1*. According to these findings, Clobenpropit, particularly when combined with Gemcitabine, shows efficacy at impeding tumor progression and inducing programmed cell death, suggesting that it may be a promising treatment for pancreatic cancer (Paik et al., 2014).

The CXCR4 receptor is a potential therapeutic target in chronic and acute inflammatory disorders (Smith et al., 2017).

IFNs-I derived from pDCs serve as a cornerstone of antiviral defense and are vital to preventing viral propagation (Khorramdelazad et al., 2022). Dysregulated IFNs-I production, however, can have adverse effects in inflammatory and autoimmune disorders, which is why pDCs must be tightly regulated (Herbeuval and Shearer, 2007). The impact of monoamines and polyamines on pDC function has been uncovered, suggesting that a common receptor mechanism is involved. Researchers have discovered that polyamine derivatives such as spermine phenylguanide and spermidine phenylguanide inhibit HIV-1 CXCR4-tropic strains, suggesting an intricate interaction with this receptor (Wilkinson et al., 2011). In addition, muroid and human pDCs cannot be developed in the BM without CXCL12/CXCR4 signaling (Kohara et al., 2007; Tassone et al., 2010). In particular, the internalization of CXCR4 plays a pivotal role in attenuating pDC activation, delineating an important immunomodulating mechanism. According to findings in a study, Clobenpropit induces CXCR4 internalization in pDCs, whereas FFN-511 (a fluorescent amine mimicking serotonin) colocalizes strongly with CXCR4 (Smith et al., 2017). Therefore, inhibiting CXCR4 by Clobenpropit could reduce the pathologic effects of dysregulated pDC-derived IFNs-I production in inflammatory and autoimmune diseases.

In TLR7-activated human pDCs, histamine and its analog Clobenpropit inhibited the production of all subtypes of IFN by engaging CXCR4. In the broncho-alveolar wash, Clobenpropit administration through intranasal spray significantly reduced type I and III IFN secretion in mice infected with IAV (Smith et al., 2017). CXCR4, not histamine receptors, exclusively mediated the anti-IFN activity of Clobenpropit. Clobenpropit may inhibit monocyte-driven inflammation, particularly in RA, due to its prominent expression in various immune cells, including monocytes and macrophages.

The anti-inflammatory activity of Clobenpropit on monocytes has been demonstrated by competitive experiments using AMD3100 as a CXCR4 antagonist and siRNA-based approaches for reducing CXCR4 expression (Smith et al., 2017). In addition to emphasizing the role of CXCR4 signaling in IFN pathway regulation, this study indicates CXCR4's role in regulating inflammation in a broad range of cell types, including monocytes and pDCs. Considering the prevalence of type I IFNs in RA and their functional activity, the concurrent suppression of IFNs and anti-inflammatory effects of CXCR4 present significant clinical advantages (Rönnblom and Eloranta, 2013). Researchers have found that individuals with active RA have higher levels of CXCR4 and its natural ligand, CXCL12, in serum and joint synovial fluids. CXCR4 and CXCL12 expression levels were also higher in the group with active RA compared to the group in remission (Peng et al., 2020). It has been suggested that targeting CXCR4 as a therapeutic strategy for RA patients could hold considerable promise because of the increased accessibility of CXCR4.

Compared to JAK inhibitors, Clobenpropit demonstrates its impact at an earlier stage of the inflammatory process (Bekaddour et al., 2023). Instead of directly targeting cytokine-mediated signaling, Clobenpropit intervenes one step upstream by inhibiting the production of inflammatory cytokines. Compared to JAK inhibitors, this unique approach may offer distinct benefits regarding therapeutic effectiveness. In mouse models of RA with Clobenpropit treatment, the marked reduction in disease progression suggests using Clobenpropit-like molecules to target CXCR4 as a potential therapeutic strategy for arthritic conditions (Bekaddour et al., 2023). Clobenpropit is a small molecule with no side effects in *in vivo* preclinical models, indicating that it could be a possible breakthrough drug for treating RA. Clobenpropit modulates cytokine production to exert a comprehensive anti-inflammatory effect by targeting the widely expressed immune cell receptor CXCR4. Clobenpropit combines these attributes to highlight it as a compelling RA treatment candidate (Bekaddour et al., 2023). Regarding the role of CXCR4 in TLR7-mediated inflammation and promising results following treatment of TLR7-dependent lupus-like model with IT1t (a CXCR4 inhibitor), it is possible that administrating Clobenpropit reduces systemic inflammation by suppressing type I IFNs production by pDCs and anti-dsDNA autoantibodies, preventing glomerulonephritis in SLE (Smith et al., 2019) (Table 1).

Clobenpropit and AMD3100 have different mechanisms of action (Bhatt et al., 2010). AMD3100's main mechanism is its ability to block CXCR4 and activate CXCR7, whereas Clobenpropit modulates histamine H3 and CXCR4 receptors (Kalatskaya et al., 2009; Liao et al., 2013). Compared with AMD3100, clobenpropit exerts unique effects on target pathways, potentially resulting in synergistic or complementary therapeutic outcomes (Figure 5). However, it is noteworthy that AMD3100 may be limited by side effects and resistance development in specific clinical contexts, even though it has demonstrated efficacy in specific clinical contexts (Sideeffects, 2024). In contrast, preclinical studies suggest that clobenpropit may be more tolerated and sustainable as a therapeutic option based on its promising efficacy and safety profiles (Yang et al., 2002). Consideration should also be given to the specific disease context. According to the severity and stage of the condition, some drugs may be more effective than others. Clobenpropit is expected to provide crucial insights for clinical decision-making through comprehensive preclinical investigations and comparative studies.

On the other hand, while several novel small molecules targeting CXCR4 are in clinical and preclinical development phases,

Clobenpropit may stand out for several reasons (Debnath et al., 2013; Lu et al., 2024). Clobenpropit demonstrates superior efficacy compared to early-stage drugs. According to our *in silico* studies, Clobenpropit might exhibit the same or even stronger binding affinity or more potent inhibitory effects on CXCR4-mediated pathways crucial for disease progression compared with novel CXCR4 inhibitors, such as WZ811 and MSX-122. This efficacy could be demonstrated through preclinical studies or even early clinical trials. Moreover, Clobenpropit may possess a more favorable safety profile. This aspect is critical in drug development, especially in autoimmune and cancer diseases where patients may already be compromised in terms of their health status. A better safety profile can translate into reduced adverse effects and improved patient compliance and outcomes (Bekaddour et al., 2021). Clobenpropit might exhibit favorable pharmacokinetic and pharmacodynamic properties, such as better bioavailability, longer half-life, or more predictable metabolism (Ishizuka et al., 2008). These factors contribute to the drug's effectiveness and suitability for clinical use. As discussed, Clobenpropit may act through novel mechanisms beyond mere CXCR4 antagonism (Bekaddour et al., 2021). This could involve additional pathways or synergistic effects that enhance its therapeutic potential, offering a unique advantage over other CXCR4-targeting agents (McHugh, 2019). Clobenpropit may have advanced further along the clinical development pipeline compared to the early-stage drugs if applicable. This could imply a more comprehensive understanding of its efficacy, safety, and dosing regimens through advanced clinical trials or real-world data.

7 Concluding Remarks

Since the CXCL12/CXCR4 axis plays a crucial role in cancer and AD pathogenesis, inhibiting it seems like a promising therapeutic approach. Notably, several inhibitors for CXCR4 have been conceptualized and developed, with AMD3100 standing out as one of the most significant achievements, having been approved by the FDA in 2008 and showing efficacy in the treatment of cancer and AIDS, as well as transplantation (De Clercq, 2019). Our molecular docking analyses have unveiled promising binding scores for Clobenpropit compared to other potent CXCR4 inhibitors. Furthermore, the compound's dual inhibitory action on H3 and H4 histamine receptors warrants attention, given the pivotal roles of these receptors in fostering cancer and ADs, such as RA. Clobenpropit's concurrent and synergistic impact on these receptors may yield favorable outcomes in the context of cancer and AD treatment.

Even with the intriguing dual mechanism of action, the current shortage of comprehensive studies hampers a conclusive determination of Clobenpropit's efficacy in inhibiting CXCR4. Further investigations across preclinical and clinical phases are imperative to elucidate the precise role of Clobenpropit in the treatment of malignancies and ADs through CXCR4 inhibition. These studies would not only contribute to substantiating the therapeutic potential of Clobenpropit but also shed light on its intricate interplay with histamine and CXCR4 receptors, thus advancing our understanding of its therapeutic utility.

Author contributions

MA: Investigation, Writing–original draft, Writing–review and editing. KB: Investigation, Visualization, Writing–original draft, Writing–review and editing, Software. HK: Investigation, Visualization, Writing–original draft, Writing–review and editing, Conceptualization, Methodology, Supervision, Validation.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Association of hemoglobin, albumin, lymphocyte, and platelet score with risk of all-cause and cause-specific mortality among cancer survivors: NHANES 1999-2018

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Background: The HALP score, comprising hemoglobin, albumin, lymphocyte, and platelet levels, serves as an indicator of both nutritional and inflammatory status. However, its correlation with all-cause and cause-specific mortality among cancer survivors remains unclear. Therefore, this study aims to investigate the relationship between HALP scores and mortality outcomes in this population.

Method: We extracted cohort data spanning ten cycles (1999-2018) from the U.S. National Health and Nutrition Examination Survey (NHANES). Mortality rates, determined using the National Death Index (NDI) as of December 31, 2019, were assessed. Weighted multivariate logistic regression analyzed the association between HALP scores and cancer prevalence. Kaplan-Meier analyses and weighted multivariate-adjusted Cox analyses investigated the link between HALP scores and all-cause and cause-specific mortality in cancer survivors. Restricted cubic spline (RCS) analysis was employed to assess nonlinear relationships. Furthermore, multi-parametric subgroup analyses were conducted to ensure the robustness of the results.

Results: Our study included 41,231 participants, of whom 3,786 were cancer survivors (prevalence: 9.5%). Over a median follow-up of 91 months (range: 51-136), we observed 1,339 deaths, including 397 from cancer, 368 from cardio-cerebrovascular disease, and 105 from respiratory disease. Elevated HALP scores showed a consistent association with reduced cancer incidence (P for trend <0.001). In multivariable-adjusted Cox regression analyses, HALP scores were significantly inversely associated with all-cause mortality, cancer mortality, cardio-cerebrovascular disease mortality, and respiratory disease mortality in cancer survivors (P for trend < 0.05). Nonlinear relationships between HALP scores and all-cause and cause-specific mortality in cancer survivors were evident through RCS regression modeling (P for nonlinearity < 0.01). Kaplan-

Meier analyses demonstrated that higher HALP scores were indicative of a poorer prognosis.

Conclusion: Our findings indicate a notable inverse correlation between HALP scores and both all-cause and cause-specific mortality among cancer survivors.

KEYWORDS

cancer survivors, HALP score, all-cause mortality, cause-specific mortality, national health and nutrition examination survey

1 Introduction

Cancer remains the primary cause of mortality worldwide, particularly prevalent in low- and middle-income nations (1). According to the World Health Organization's (WHO) 2019 statistics, cancer ranks as the primary or secondary cause of death before age 70 in 112 out of 183 countries, and as the third or fourth leading cause in 23 countries (2). Despite a decline in cancer mortality rates from 1991 to 2021, credited to reduced tobacco use, enhanced cancer detection, and advancements in therapies, the global cancer burden is projected to escalate due to demographic shifts and changes in risk factors such as obesity, sedentary lifestyles, and altered fertility rates (3–8). The increasing number of cancer survivors underscores the pressing need for a robust predictive indicator to monitor and enhance their long-term health outcomes.

The Hemoglobin, Albumin, Lymphocyte, and Platelet (HALP) score, introduced by Chen et al. in 2015, initially predicted prognosis in gastric cancer patients (9). Its potential as a biomarker for various illnesses has since garnered attention. Mounting evidence links nutrition, inflammation, and cancer outcomes (10–13). Matsushita et al. suggested a potential link between diet, nutrition, and prostate cancer, possibly mediated by the gut microbiota (14). Dietary-induced inflammation has been implicated in various cancers, such as CRC, liver cancer, prostate cancer, and kidney cancer (15–17). Recent research underscores the significant impact of diet on both mucosal and systemic immune systems, influencing inflammation in tumor cells and their response to cancer therapy (18).

Given HALP score's ability to assess immune and nutritional status, it can reflect tumor tolerance by integrating immune markers, nutrition, and inflammation. While studies have explored its predictive significance in different cancers, findings vary (19–21). In a multicenter study, lower HALP values correlated with increased risks of mortality and cancer-related deaths in patients with locally advanced colorectal cancer (19). However, a separate study found no significant association between HALP scores and long-term survival in patients with retroperitoneal soft tissue sarcoma (21). Therefore, a comprehensive investigation into HALP score's association with all-cause and cause-specific mortality in cancer survivors is crucial.

Drawing on NHANES data spanning 1999 to 2018, our cohort study delves into the correlation between the HALP score and all-cause and cause-specific mortality in cancer survivors and to assess the impact of the HALP score on cancer survivors. Our study will provide valuable reference metrics for optimizing treatment and clinical management of cancer survivors.

2 Methods

2.1 Study population

The NHANES, conducted by the National Center for Health Statistics (NCHS), is a nationally representative cross-sectional study employing a stratified multistage random sample design to assess the health and nutritional status of the US population (22). Before implementation, NHANES surveys undergo thorough review and approval by the Disclosure Review Board of the NCHS. Comprehensive details regarding ethical approval and informed consent procedures are available through the NCHS (23). This study employed a nationwide cross-sectional design, utilizing secondary analyses of publicly accessible and deidentified NHANES data. As such, additional institutional review board approval or informed consent was not required. Additional details can be found at http://www.cdc.gov/nchs/nhanes.

For our cohort study, we included 101,316 participants from ten NHANES cycles spanning 1999 to 2018. Exclusion criteria encompassed individuals under 20 years old, pregnant individuals, those with missing cancer or HALP score data, and participants with incomplete follow-up or covariate information. Ultimately, 41,231 participants were included in the study (Figure 1).

2.2 Definition of hemoglobin, albumin, lymphocyte, platelet score

Blood samples were collected during examinations at Mobile Examination Centers (MECs) and subsequently analyzed in the laboratory. The HALP score comprises serum levels of hemoglobin, albumin, lymphocytes, and platelets. Hemoglobin, lymphocyte, and

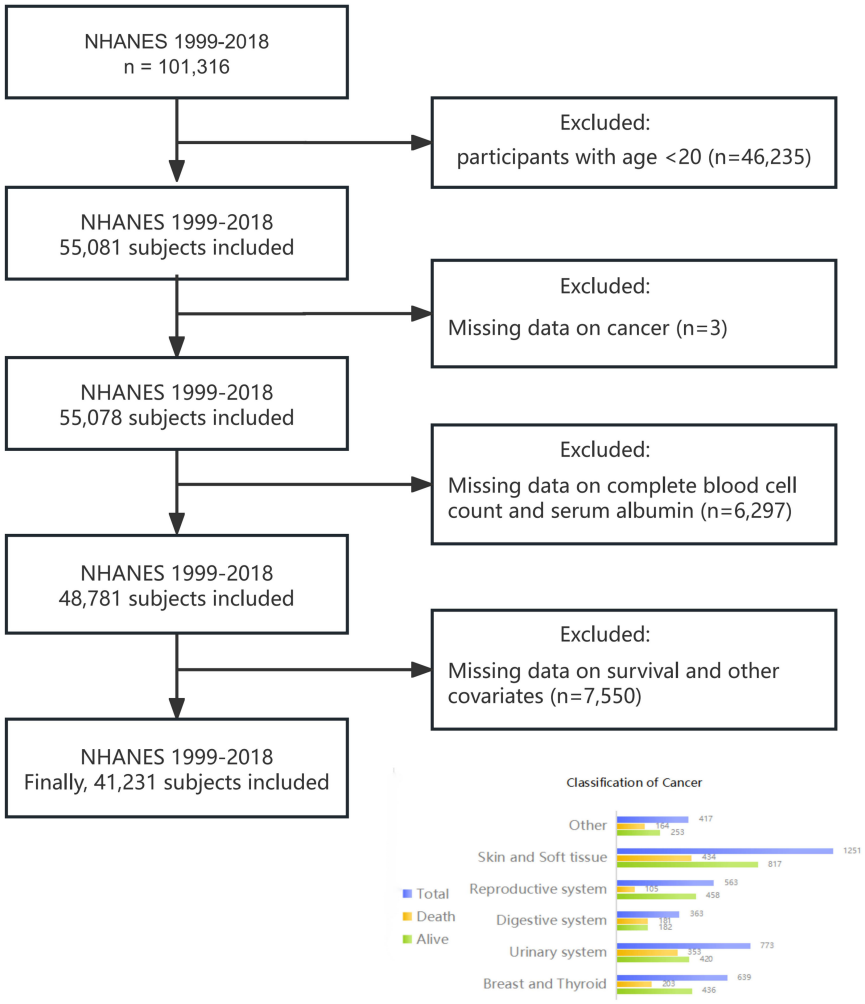

FIGURE 1
Flowchart of study design.

platelet levels are measured using a hematology-analyzing device (UniCel DxH 800 analyzer), while serum albumin levels are determined using Roche Modular P and Roche Cobas 6000 chemistry analyzers. The HALP score is calculated using the formula: hemoglobin (g/L) × albumin (g/L) × lymphocytes (10^9/L)/platelets (10^9/L) (24, 25).

2.3 Definition of cancer survivor

Self-reported cancer history data were sourced from the "Medical Conditions" section of NHANES, gathered via professionally self-administered questionnaires (26). Cancer survivors were identified by their response to the question: "Have you ever been told by a doctor or other health professional that you had cancer or malignancy of any kind?" Positive responses categorized individuals as cancer survivors, while negative responses classified them as non-cancer individuals. Cancer survivors were categorized based on their responses to the question "What kind of cancer?" The classification comprised six categories, as illustrated in Figure 1.

2.4 Mortality outcomes

Mortality data were obtained from NHANES Public-Use Linked Mortality Files, available until December 31, 2019. Causes of death were documented using ICD-10 (International Statistical Classification of Diseases, 10th version) codes (27). Our analysis focused on all-cause and cause-specific deaths, including malignant neoplasms (ICD-10: C00-C97), cardio –cerebrovascular disease (ICD-10: I00-I09, I11, I13, I20-I51, I60-I69), and respiratory diseases (ICD-10: J40-J47, J09-J18). The follow-up period for the study extended from the date of initial diagnosis until the date of death or December 31, 2019, whichever came first.

2.5 Definitions of covariates

Covariates in this cohort study included age (years), gender (male or female), ethnicity (Mexican American, other Hispanic, non-Hispanic white, non-Hispanic black, or other race), educational attainment (below high school, high school, or above high school), marital status (married/cohabiting, widowed/divorced/separated, or never married), and body mass index (BMI) categories (<18.5, 18.5-25.0, 25.0-29.9, or >29.9 kg/m^2) (28). Income was assessed using the Poverty Income Ratio (PIR), classified as ≤1.0, 1.1-3.0, and >3.0 based on US Department of Health and Human Services guidelines. Never smokers were individuals who had smoked fewer than 100 cigarettes in their lifetime. Those who had smoked more than 100 cigarettes and were currently smoking were classified as current smokers, while those who had smoked more than 100 cigarettes but had quit were classified as former smokers (29). Alcohol consumption was dichotomized into non-drinker or drinker (≥12 drinks in a year). Physical activity was quantified as metabolic equivalent (MET) minutes of moderate to vigorous exercise per week according to World Health Organization guidelines (30). Diabetes mellitus was determined by self-report, glycated hemoglobin ≥6.5%, or fasting blood glucose ≥126 mg/dL (7.0 mmol/L). Hypertension was defined by medication use or self-reported diagnosis. Complete blood count parameters, serum albumin levels, high-density lipoprotein cholesterol (HDL-C), and total cholesterol (TG) levels were also collected from the database.

2.6 Statistical analysis

We utilized NHANES-recommended weights, specifically the 2-year cycle of Mobile Examination Center (MEC) exam weights (wtmec2yr), for statistical analyses, given that HALP scores were derived from four laboratory measurements. Continuous data were presented as medians [first quantile (P25) and third quantile (P75)] and compared using the nonparametric Wilcoxon rank sum test or independent samples t-test, as applicable. Categorical variables were reported as percentages (%) and assessed using the chi-squared test or Fisher's exact test, if appropriate.

In this cohort analysis, three logistic regression models were employed to estimate adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association between HALP scores and cancer prevalence. Similarly, three Cox regression models were utilized to calculate adjusted hazard ratios (HRs) and 95% CIs for all-cause mortality, cancer mortality, cardio-cerebrovascular disease mortality, and respiratory disease mortality among cancer survivors. Model 1 was unadjusted, while Model 2 was adjusted for age, sex, and race/ethnicity. Model 3 included additional adjustments for education level, family poverty income ratio, MET minutes per week, drinking status, smoking status, BMI, self-reported diabetes, and self-reported hypertension.

Restricted cubic spline regression analyses were conducted to explore dose-response relationships between HALP scores and all-cause and cause-specific mortality among cancer survivors, with knots placed at the 5th, 35th, 65th, and 95th percentiles of each exposure variable. Kaplan-Meier analyses were utilized to evaluate the association between HALP scores and long-term mortality in cancer survivors. Additionally, subgroup analyses were performed to investigate the relationship between HALP scores and mortality outcomes based on age, sex, BMI, smoking status, self-reported hypertension, self-reported diabetes, and different types of cancer.

3 Results

3.1 Baseline characteristics

Table 1 presents baseline characteristics and weighted estimates of the study population. Our analysis included 41,231 individuals, representing 17.04 million noninstitutionalized US residents. Among them, 3,786 were cancer survivors, aged 20 to 85 years, with a mean age of 49.47 ± 18.14 years. Race distribution was as follows: Mexican Americans: 2.2%, Other Hispanics: 2.3%, Non-Hispanic Whites people: 87%, Non-Hispanic Blacks people: 5.0%, and Others: 3.4%. Cancer survivors were more likely to be older women, highly educated, higher-income earners, divorced, smokers, and drinkers. Additionally, they exhibited higher waist circumferences, lower physical activity levels, and a higher prevalence of comorbid hypertension or diabetes. Median (P25, P75) values of hemoglobin, serum albumin, lymphocyte count, platelet count, and HALP scores in cancer survivors were 14.10 (13.20, 14.90) g/dL, 4.20 (4.00, 4.40) g/dL, 1.80 (1.50, 2.40)×10^9/L, 235 (198, 280)×10^9/L, and 46 (35, 62), respectively, significantly lower than non-cancer participants. Significant differences in HALP scores and HALP-related parameters were observed between individuals with and without cancer ($P< 0.05$).

3.2 HALP score and cancer prevalence

Table 2 illustrates the relationship between HALP scores and cancer prevalence using weighted multivariate regression models. All three logistic regression models revealed a negative association between HALP score and cancer incidence. The ORs and 95% CIs for the highest tertile compared to the lowest tertile were as follows: OR=0.61 (0.57-0.67), p for trend<0.001; OR=0.83 (0.75-0.89), p for trend<0.001; and OR=0.61 (0.75-0.89), p for trend<0.001, respectively.

3.3 HALP score and mortality

Over a median follow-up of 91 (51, 136) months, 1,339 (35.37%) out of 3,786 cancer survivors succumbed to all-cause mortality, with 397 (10.49%) attributed to cancer, 367 (9.70%) to cardio-cerebrovascular disease, and 105 (2.77%) to respiratory disease. As shown in Table 3, higher HALP scores were significantly associated with a reduced risk of all-cause mortality and cause-specific mortality among survivors, evident in both crude and multivariable-adjusted Models 1 and 2 (all p for trend <0.05). The multivariable-adjusted HRs and 95% CIs for the highest tertile

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TABLE 1 Baseline characteristics of adults in NHANES 1999–2018.

Characteristics	Overall, N = 41231 (100%)	Cancer survivors		P Value
		No, N = 37445 (91%)	Yes, N = 3786 (9.5%)	
Sex, %				<0.001
Female	21,245 (52%)	19,251 (51%)	1,994 (58%)	
Male	19,986 (48%)	18,194 (49%)	1,792 (42%)	
Age, %				<0.001
20-35 years	10,733 (28%)	10,562 (30%)	171 (5.0%)	
35-60 years	16,754 (48%)	15,858 (49%)	896 (34%)	
60+ years	13,744 (25%)	11,025 (21%)	2,719 (61%)	
Race/ethnicity, %				<0.001
Non-Hispanic White	18,748 (69%)	16,036 (67%)	2,712 (87%)	
Non-Hispanic Black	8,269 (11%)	7,782 (11%)	487 (5.0%)	
Mexican American	7,082 (8.3%)	6,834 (8.9%)	248 (2.2%)	
Other Race - Including Multi-Racial	3,686 (6.9%)	3,535 (7.2%)	151 (3.4%)	
Other Hispanic	3,446 (5.3%)	3,258 (5.6%)	188 (2.3%)	
Education level, %				<0.001
Below high school	10,876 (17%)	10,019 (17%)	857 (14%)	
High school	9,580 (24%)	8,711 (24%)	869 (22%)	
Above high school	20,775 (59%)	18,715 (59%)	2,060 (64%)	
Marital status, %				<0.001
Married/cohabiting	25,000 (64%)	22,680 (64%)	2,320 (66%)	
Widowed/divorced/separated	9,066 (18%)	7,823 (17%)	1,243 (28%)	
Never married	7,165 (17%)	6,942 (19%)	223 (5.6%)	
Family PIR, %				<0.001
≤1.0	7,790 (13%)	7,307 (14%)	483 (8.6%)	
1.1–3.0	19,301 (40%)	17,493 (40%)	1,808 (39%)	
>3.0	14,140 (47%)	12,645 (46%)	1,495 (52%)	
Smoking status, %				<0.001
Never smoker	22,345 (54%)	20,669 (55%)	1,676 (45%)	
Former smoker	10,200 (25%)	8,682 (23%)	1,518 (38%)	
Current smoker	8,685 (21%)	8,093 (22%)	592 (17%)	
Drinking status, %				<0.001
Drinker	5,332 (11%)	4,743 (11%)	589 (14%)	
Nondrinker	35,899 (89%)	32,702 (89%)	3,197 (86%)	
Body mass index, %				0.3
Underweight, kg/m^2	647 (1.6%)	585 (1.6%)	62 (1.7%)	
Normal, kg/m^2	11,339 (29%)	10,335 (30%)	1,004 (28%)	
Obese, kg/m^2	13,750 (34%)	12,440 (33%)	1,310 (35%)	
Overweight, kg/m^2	14,761 (35%)	13,454 (35%)	1,307 (35%)	

(Continued)

TABLE 1 Continued

Characteristics	Overall, N = 41231 (100%)	Cancer survivors		P Value
		No, N = 37445 (91%)	Yes, N = 3786 (9.5%)	
Age, years	46.0 (33.0, 59.0)	44.0 (32.0, 57.0)	64.0 (53.0, 75.0)	<0.001
Family PIR	2.98 (1.49, 5.00)	2.93 (1.46, 5.00)	3.40 (1.81, 5.00)	<0.001
BMI , kg/m2	28 (24, 32)	28 (24, 32)	28 (24, 32)	0.3
Waist Circumference (cm)	97 (87, 108)	97 (86, 108)	100 (90, 111)	<0.001
Self-reported hypertension, %	14,286 (31%)	12,162 (29%)	2,124 (51%)	<0.001
Self-reported diabetes, %	6,124 (11%)	5,305 (10%)	819 (17%)	<0.001
MET minute/week	1,890 (0, 5,460)	2,100 (0, 5,460)	1,680 (0, 5,040)	<0.001
Hemoglobin (g/dL)	14.30 (13.30, 15.40)	14.40 (13.40, 15.40)	14.10 (13.20, 14.90)	<0.001
Albumin (g/dL)	4.30 (4.10, 4.50)	4.30 (4.10, 4.50)	4.20 (4.00, 4.40)	<0.001
Platelet count (10^9/L)	246 (209, 290)	247 (210, 291)	235 (198, 280)	<0.001
Lymphocyte count (10^9/L)	2.00 (1.60, 2.50)	2.00 (1.70, 2.50)	1.80 (1.50, 2.40)	<0.001
HDL-C, mg/dl	51 (41, 63)	51 (41, 62)	52 (42, 65)	0.003
Total cholesterol, mg/dl	194 (168, 222)	194 (168, 222)	196 (170, 225)	0.2
HALP score	50 (39, 65)	51 (39, 66)	46 (35, 62)	<0.001
HALP score classifcation				<0.001
Tertile 1	13,606 (31%)	12,012 (30%)	1,594 (40%)	
Tertile 2	13,606 (34%)	12,470 (35%)	1,136 (31%)	
Tertile 3	14,019 (35%)	12,963 (35%)	1,056 (29%)	

PIR, poverty income ratio; HDL-C, High-density lipoprotein cholesterol; HALP score, Hemoglobin, albumin, lymphocyte, and platelet score.
Continuous variables are described as medians [interquartile ranges]. Categorical variables are presented as numbers (percentages). N reflect the study sample while percentages reflect the survey-weighted.

compared with the lowest tertile for all-cause mortality, cancer mortality, cardio-cerebrovascular disease mortality, and respiratory disease mortality among cancer survivors were 0.61 (0.49-0.76), 0.91 (0.70-1.17), 0.67 (0.49-0.91), and 0.60 (0.35-0.97), respectively.

Furthermore, Kaplan-Meier analysis revealed a comprehensive association between HALP scores and both cause-specific and all-cause mortality in cancer survivors. Higher HALP scores were correlated with reduced all-cause mortality (p=0.0033), decreased cancer mortality (p<0.0001), lowered cardio-cerebrovascular disease mortality (p<0.0001), and lessened respiratory disease mortality (p=0.00054) among cancer survivors (Figure 2).

TABLE 2 Logistic regression analysis between HALP score and prevalence of cancer among adults in NHANES 1999–2018.

	HALP score			P for trend
	Tertile 1	Tertile 2	Tertile 3	
Range	<41.41	41.41-58.47	>58.47	
Crude	1.00 [Reference]	0.69 (0.63, 0.74)	0.61 (0.57, 0.67)	<0.001
Model 1	1.00 [Reference]	0.81 (0.74, 0.88)	0.81 (0.75, 0.89)	<0.001
Model 2	1.00 [Reference]	0.81 (0.74, 0.88)	0.83 (0.75, 0.89)	<0.001

Data are presented as OR (95% CI); Model 1 was adjusted as age (continuous), MET (continuous), sex (male or female), and race/ethnicity (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black or Other); Model 2 was adjusted as model 1 plus education level (below high school, high school, or above high school), family poverty income ratio (≤1.0,1.1–3.0, or >3.0), drinking status (nondrinker, drinker), smoking status (never smoker, former smoker, or current smoker), BMI (<18.5, 18.5- 25.0, 25.0-29.9, or >29.9), self-reported diabetes (yes or no), and self-reported hypertension (yes or no).

3.4 Restricted cubic spline analysis

We employed weighted restricted cubic spline curves to explore the nonlinear relationship between HALP scores and all-cause, as well as cause-specific mortality, while controlling for potential confounders. Illustrated in Figure 3, the restricted cubic spline analysis unveiled a nonlinear association between HALP scores and cardio-cerebrovascular disease mortality in cancer survivors (nonlinear P=0.0015). Notably, lower HALP scores were linked to an elevated risk of cardio-cerebrovascular disease mortality in this population.

Furthermore, nonlinear associations were also observed between HALP scores and all-cause mortality (nonlinear P<0.0001), cancer mortality (nonlinear P<0.0001), and respiratory disease mortality (nonlinear P<0.0001) in cancer survivors. Importantly, the results of the two linear regressions indicated that the probability of all-cause mortality, cancer mortality, and respiratory disease mortality progressively decreased to the lowest point at HALP scores of 47.24, 42.47, and 45.04, respectively, before increasing with rising HALP scores.

TABLE 3 Cox regression analysis between HALP score and long-term mortality among cancer survivor in NHANES 1999–2018.

	HALP score			P for trend
	Tertile 1	Tertile 2	Tertile 3	
All-cause mortality				
No. deaths/total	670/1594	357/1136	312/1056	
Crude	1.00 [Reference]	0.63 (0.54, 0.74)	0.58 (0.49, 0.68)	<0.001
Model 1	1.00 [Reference]	0.66 (0.54, 0.80)	0.68 (0.55, 0.83)	<0.001
Model 2	1.00 [Reference]	0.64 (0.51, 0.79)	0.61 (0.49, 0.76)	<0.001
Cancer mortality				
No. deaths/total	190/1594	97/1136	110/1056	
Crude	1.00 [Reference]	0.69 (0.53, 0.89)	0.86 (0.67, 1.10)	0.016
Model 1	1.00 [Reference]	0.72 (0.56, 0.94)	0.95 (0.73, 1.22)	0.040
Model 2	1.00 [Reference]	0.72 (0.55, 0.93)	0.91 (0.70,1.17)	0.038
Cardio–cerebrovascular disease mortality				
No. deaths/total	196/1368	94/1367	77/1367	
Crude	1.00 [Reference]	0.64 (0.50, 0.83)	0.56 (0.42, 0.74)	<0.001
Model 1	1.00 [Reference]	0.72 (0.54, 0.95)	0.70 (0.52, 0.95)	0.019
Model 2	1.00 [Reference]	0.70 (0.52, 0.92)	0.67 (0.49, 0.91)	0.008
Respiratory disease mortality				
No. deaths/total	62/1368	20/1367	23/1367	
Crude	1.00 [Reference]	0.44 (0.26, 0.72)	0.55 (0.33, 0.88)	0.002
Model 1	1.00 [Reference]	0.50 (0.30, 0.80)	0.67 (0.41, 1.07)	0.011
Model 2	1.00 [Reference]	0.47 (0.27, 0.78)	0.60 (0.35, 0.97)	0.006

Data are presented as HR (95% CI); Model 1 was adjusted as age (continuous), MET (continuous), sex (male or female), and race/ethnicity (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black or Other); Model 2 was adjusted as model 1 plus education level (below high school, high school, or above high school), family poverty income ratio (≤1.0,1.1–3.0, or >3.0), drinking status (nondrinker, drinker), smoking status (never smoker, former smoker, or current smoker), BMI (<18.5, 18.5- 25.0, 25.0-29.9, or >29.9), self-reported diabetes (yes or no), and self-reported hypertension (yes or no).

3.5 Subgroup analyses

To further evaluate the robustness of the relationship between HALP scores and all-cause, as well as cause-specific mortality in cancer survivors, subgroup analyses were conducted based on sex, age, BMI, smoking, hypertension status, and diabetes status. The findings indicated a largely consistent dose-response relationship between HALP scores and all-cause and cause-specific mortality across subgroups, particularly in all-cause mortality. However, the association between HALP scores and cancer mortality in the subgroup analyses was less pronounced (Table 4). Moreover, no statistically significant interaction p-values were detected (Table 4).

To delve deeper into the influence of HALP scores across different tumor types, subgroup analyses were conducted according to tumor classification. Results presented in Table 5 indicate a notable impact of HALP scores on all-cause mortality among patients with thyroid and breast cancers, as well as those with tumors of the digestive system, and skin and soft tissue tumors. This observation suggests a tumor-specific effect of HALP scores on cancer survivors.

4 Discussion

In our cross-sectional study spanning 1999 to 2018, we investigated the association between HALP scores and cancer prevalence, as well as long-term mortality in the US population. Following adjustment for multiple variables, a significant negative relationship emerged between HALP score and cancer incidence. Among cancer survivors, we observed a notable nonlinear association between HALP score and all-cause, as well as cause-specific mortality (including cancer, cardio-cerebrovascular disease, and respiratory disease mortality), with higher HALP scores correlating with reduced mortality rates. Notably, HALP scores of 47.24, 42.47, and 45.04 corresponded to the lowest all-cause, cancer, and respiratory disease mortality, respectively. Kaplan-Meier analysis further revealed that lower HALP scores were associated with shorter survival times among cancer survivors. Importantly, these findings remained robust across multiple subgroup analyses. In addition, subgroup analyses based on different types of cancer revealed that the effect of HALP scores on all-cause mortality in cancer survivors may be tumor-specific. Overall, our results suggest

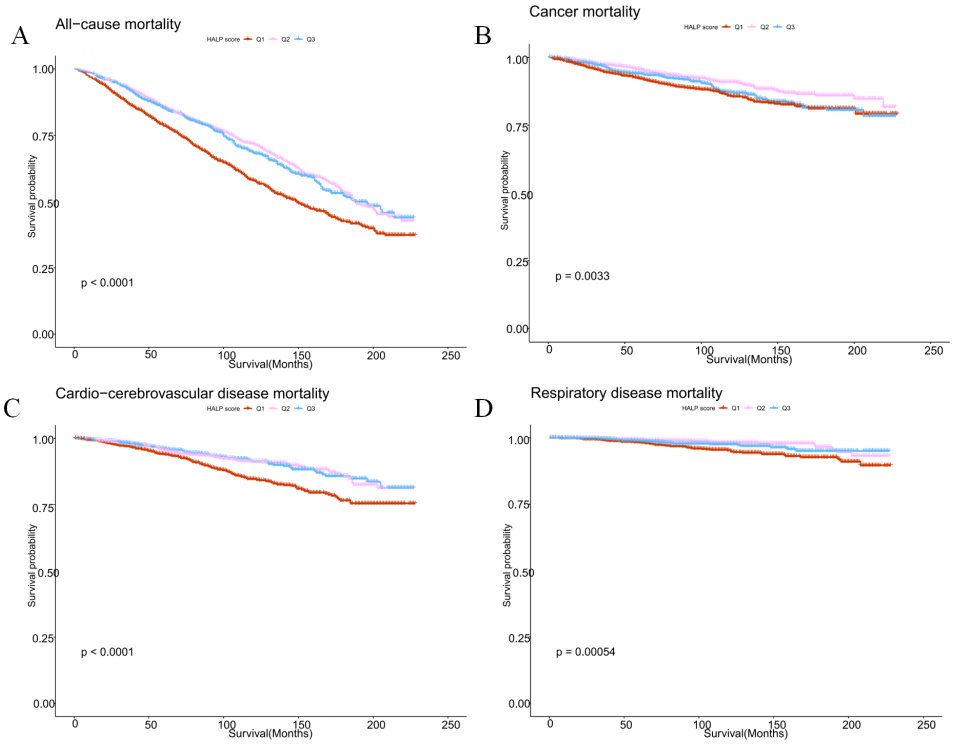

FIGURE 2
Kaplan-Meier survival survival estimates between HALP scores and all-cause mortality **(A)**, cancer mortality **(B)**, cardio-cerebrovascular disease mortality **(C)**, and respiratory disease mortality **(D)** in cancer survivors.

that HALP score holds significant potential as a valuable predictor of outcomes for cancer survivors.

Immunity and nutrition play pivotal roles in cancer development and progression. Chronic inflammation is known to be carcinogenic, contributing to increased stem cell proliferation and local mutagenic effects through long-term cell turnover stimulation (10, 31, 32). The relationship between neoplasia and the immune system is encapsulated in the '3E hypothesis,' which delineates the stages of initial elimination of transformed cells by immunological effector cells, followed by equilibrium between malignant cells and the immune response within a smoldering neoplastic lesion, and ultimately, the escape of cancer cells from immunological control (33). Systemic inflammation, a hallmark of the tumor microenvironment, significantly influences disease progression and prognosis in cancer survivors (34, 35). Numerous studies have investigated various systemic inflammatory biomarkers and have demonstrated their high predictive value for the prognosis of different cancer types (36–40).

Malnutrition is often linked to tumor progression, stemming from inadequate nutritional intake, increased tumor consumption, or the effects of anticancer therapy. International point prevalence studies report malnutrition rates ranging from 31% to 39% among lower gastrointestinal cancer patients (41, 42). Malnutrition can compromise immunity, trigger metabolic disturbances, and diminish treatment tolerance in cancer survivors, all of which can influence the efficacy of oncological treatment and patient prognosis (43, 44). A study investigating penile cancer patients undergoing inguinal lymph node dissection (ILND) revealed that the preoperative albumin alkaline phosphatase ratio (AAPR) reliably predicts pathologic lymph node-positive (pN+) status (45). Similarly, in bladder cancer patients, an elevated preoperative fibrinogen-to-albumin ratio (FAR) has been identified as a potential predictor of malignancy and advanced grade (46). These findings underscore the significant predictive role of nutritional status in cancer patients.

The significance of inflammatory response and nutritional status in cancer prognosis is increasingly recognized. Nøst et al. (47) analyzed the UK Biobank data and found that the systemic immune-inflammatory index (SII), neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR) correlated positively with the risk of 7 out of 17 cancers, while the lymphocyte-to-monocyte ratio (LMR) correlated negatively. Ouyang et al. (48) identified preoperative SII as an independent prognostic marker in pediatric osteosarcoma. Regarding nutrition, Kheirouri et al. (49) linked high preoperative Controlling Nutritional Status (CONUT) scores to lower overall survival (OS) and cancer-specific survival (CSS) across various cancers. Another study validated the Cholesterol-modified Prognostic Nutritional Index (CPNI) for predicting breast cancer prognosis (50). These findings highlight the combined impact of inflammation and nutrition on cancer outcomes, suggesting that effective prognostic models should integrate both factors.

HALP scores, derived from hemoglobin, albumin, lymphocyte, and platelet levels, serve as indicators of the host's inflammatory and nutritional status. Hemoglobin, a pivotal factor in tumor progression, is frequently depleted in cancer survivors,

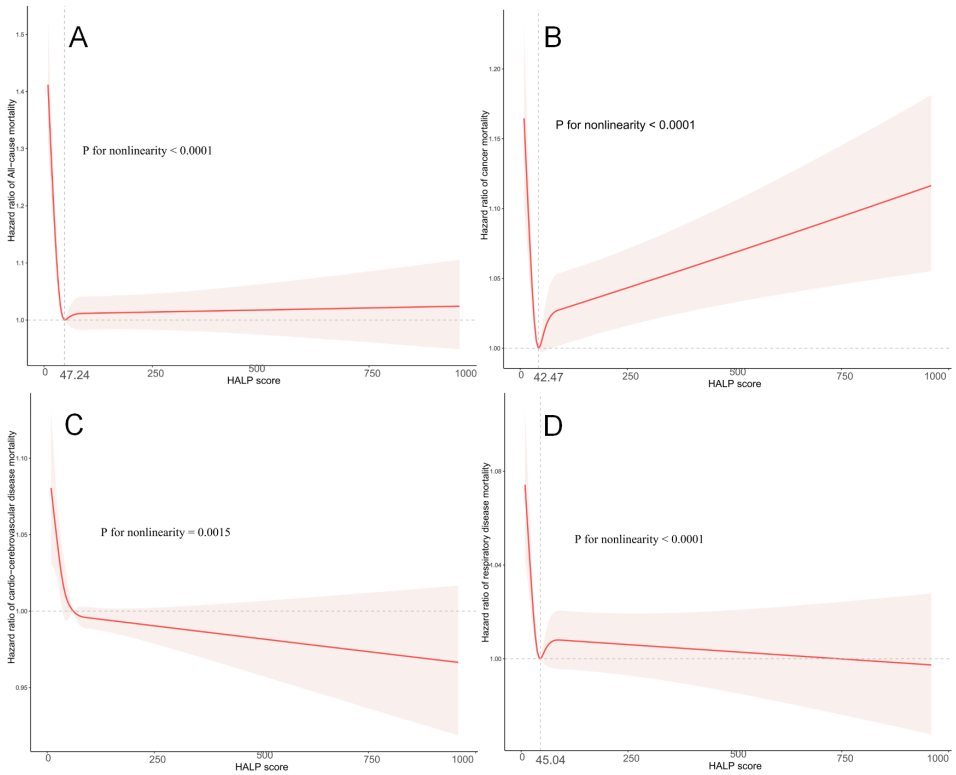

FIGURE 3
Restricted cubic spline analysis to assess the association between HALP score and all-cause mortality **(A)**, cancer mortality **(B)**, cardio-cerebrovascular disease mortality **(C)**, and respiratory disease mortality **(D)** in cancer survivors. Adjusted for age (continuous), MET (continuous), sex (male or female), ethnicity (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black or Other), education level (below high school, high school, or above high school), family poverty income ratio (≤1.0, 1.1–3.0, or >3.0), drinking status (nondrinker, drinker), smoking status (never smoker, former smoker, or current smoker), BMI (<18.5, 18.5- 25.0, 25.0-29.9, or >29.9), self-reported diabetes (yes or no), and self-reported hypertension (yes or no).

contributing to hypoxia (51), a driver of tumor advancement and treatment resistance (52). Numerous studies have established a correlation between hemoglobin levels in cancer patients and both survival outcomes and disease progression (53–55).

Serum albumin, synthesized by the liver, constitutes a crucial component of total serum protein and reflects the host's inflammatory and nutritional profile. Hypoalbuminemia, stemming from malnutrition, hypermetabolism, systemic inflammation, or heightened cytokine release, compromises the immune response to cancer cells (56). Extensive research has underscored the association between hypoalbuminemia and poor survival across various cancer types (57, 58).

Moreover, lymphocytes play a pivotal role in the host's anti-cancer defense mechanisms. They secrete cytokines such as interferon-γ and tumor necrosis factor-α (TNF-α), which enhance prognosis by inducing apoptosis and impeding cancer cell proliferation, invasion, and migration (59, 60). Consequently, a decline in lymphocyte levels correlates with a poorer prognosis for cancer survivors.

Additionally, recent research suggests that platelets participate in various signaling pathways implicated in tumor immunity and progression (61). Several studies have demonstrated that elevated pretreatment platelet counts are associated with reduced survival rates in cancer patients (62–64). Taken together, these findings underscore the potential of the HALP score as a valuable prognostic tool for assessing cancer survivors.

The HALP score has demonstrated promising prognostic value across various cancer types, including gastric cancer (9), esophageal squamous cell carcinoma (65), colorectal cancer (19), renal cell carcinoma (66), bladder cancer (20), and small cell lung cancer (67). However, the association between HALP scores and the risk of all-cause and cause-specific mortality across all cancer types remains unexplored. Using NHANES 1999-2018 data, our study unveiled a significant nonlinear relationship between HALP scores and all-cause and cause-specific mortality. Higher HALP scores were consistently associated with reduced all-cause and cause-specific mortality. Notably, HALP scores of 47.24, 42.47, and 45.04 were linked to the lowest levels of all-cause mortality, cancer mortality, and respiratory disease mortality, respectively. Our study bridges this gap in research and provides an invaluable tool for the treatment and management of cancer survivors.

Although survival rates for cancer survivors remain poor, improvements in treatment strategies and care have positively impacted the prognosis of cancer survivors. With increased survival, non-cancer causes of death have become more and more important. In Japan, the reported number of deaths from cancer in 2021 was 381,505 (26.5%) among cancer survivals, and cancer was still the leading cause of death, however, heart disease was the

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TABLE 4 Subgroup analysis of HALP score and long-term mortality among cancer survivor in NHANES 1999–2018.

	HALP score			P for trend	P for interaction
	Tertile 1	Tertile 2	Tertile 3		
All-cause mortality					
Age					0.835
<60 years	1.00 [Reference]	0.55 (0.31, 0.95)	0.52 (0.30, 0.87)	0.025	
>60 years	1.00 [Reference]	0.62 (0.51, 0.75)	0.58 (0.47, 0.71)	<0.001	
Sex					0.719
Male	1.00 [Reference]	0.59 (0.45, 0.76)	0.56 (0.43, 0.74)	<0.001	
Female	1.00 [Reference]	0.69 (0.46, 1.03)	0.64 (0.41, 0.99)	0.037	
BMI					0.364
<25.0	1.00 [Reference]	0.56 (0.37, 0.85)	0.85 (0.55, 1.31)	0.024	
25.0-29.9	1.00 [Reference]	0.71 (0.51, 0.98)	0.75 (0.53, 1.06)	0.079	
>29.9	1.00 [Reference]	0.74 (0.50, 1.09)	0.55 (0.36, 0.82)	0.015	
Smoke					0.586
Yes	1.00 [Reference]	0.68 (0.53, 0.87)	0.61 (0.47, 0.78)	<0.001	
No	1.00 [Reference]	0.58 (0.38, 0.87)	0.67 (0.43, 1.03)	0.021	
Diabetes					0.389
Yes	1.00 [Reference]	0.75 (0.51, 1.09)	0.64 (0.43, 0.95)	0.037	
No	1.00 [Reference]	0.61 (0.47, 0.79)	0.64 (0.49, 0.84)	<0.001	
Hypertension					0.158
Yes	1.00 [Reference]	0.73 (0.56, 0.96)	0.62 (0.46, 0.82)	0.002	
No	1.00 [Reference]	0.55 (0.39, 0.76)	0.70 (0.49, 0.98)	0.001	
Cancer mortality					
Age					0.711
<60 years	1.00 [Reference]	0.80 (0.36, 1.69)	0.76 (0.36, 1.60)	0.700	
>60 years	1.00 [Reference]	0.68 (0.51, 0.91)	0.90 (0.68, 1.18)	0.028	
Sex					0.640
Male	1.00 [Reference]	0.69 (0.49, 0.96)	0.81 (0.59, 1.13)	0.041	
Female	1.00 [Reference]	0.76 (0.50, 1.16)	1.03 (0.67, 1.57)	0.400	
BMI					0.306
<25.0	1.00 [Reference]	0.78 (0.47, 1.28)	1.14 (0.69, 1.84)	0.400	
25.0-29.9	1.00 [Reference]	0.87 (0.55, 1.36)	1.19 (.76, 1.86)	0.500	
>29.9	1.00 [Reference]	0.63 (0.39, 0.98)	0.73 (0.46, 1.13)	0.100	
Smoke					0.662
Yes	1.00 [Reference]	0.72 (0.52, 0.98)	0.86 (0.63, 1.17)	0.120	
No	1.00 [Reference]	0.71 (0.44, 1.11)	1.07 (0.68, 1.67)	0.200	
Diabetes					0.263
Yes	1.00 [Reference]	0.86 (0.51, 1.44)	0.82 (0.47, 1.40)	0.700	
No	1.00 [Reference]	0.69 (0.51, 0.93)	1.00 (0.75, 1.34)	0.032	

(Continued)

TABLE 4 Continued

	HALP score			P for trend	P for interaction
	Tertile 1	Tertile 2	Tertile 3		
Cancer mortality					
Hypertension					0.921
Yes	1.00 [Reference]	0.76 (0.54, 1.05)	0.95 (0.68, 1.31)	0.200	
No	1.00 [Reference]	0.71 (0.46, 1.08)	0.96 (0.63, 1.45)	0.200	
Cardio–cerebrovascular disease mortality					
Age					0.711
<60 years	1.00 [Reference]	0.46 (0.17, 1.14)	0.17 (0.04, 0.50)	0.004	
>60 years	1.00 [Reference]	0.67 (0.51, 0.88)	0.65 (0.49, 0.87)	0.002	
Sex					0.640
Male	1.00 [Reference]	0.65 (0.43, 0.97)	0.68 (0.44, 1.02)	0.045	
Female	1.00 [Reference]	0.77 (0.52, 1.11)	0.64 (0.41, 0.98)	0.044	
BMI					0.306
<25.0	1.00 [Reference]	0.66 (0.39, 1.08)	0.85 (0.51, 1.38)	0.300	
25.0-29.9	1.00 [Reference]	0.60 (0.36, 0.99)	0.67 (0.38, 1.15)	0.100	
>29.9	1.00 [Reference]	0.85 (0.53, 1.35)	0.60 (0.36, 1.00)	0.150	
Smoke					0.662
Yes	1.00 [Reference]	0.75 (0.52, 1.07)	0.78 (0.53, 1.12)	0.200	
No	1.00 [Reference]	0.66 (0.43, 0.98)	0.55 (0.34, 0.88)	0.016	
Diabetes					0.263
Yes	1.00 [Reference]	0.61 (0.36, 1.01)	0.64 (0.37, 1.07)	0.094	
No	1.00 [Reference]	0.77 (0.54, 1.09)	0.70 (0.47, 1.02)	0.120	
Hypertension					0.921
Yes	1.00 [Reference]	0.76 (0.55, 1.05)	0.71 (0.50, 1.01)	0.010	
No	1.00 [Reference]	0.60 (0.33, 1.08)	0.63 (0.33, 1.17)	0.200	
Respiratory disease mortality					
Age					0.798
<60 years	1.00 [Reference]	0.22 (0.04, 0.90)	0.29 (0.08, 0.99)	0.035	
>60 years	1.00 [Reference]	0.47 (0.27, 0.79)	0.61 (0.35, 1.02)	0.010	
Sex					0.395
Male	1.00 [Reference]	0.28 (0.12, 0.58)	0.39 (0.19, 0.77)	<0.001	
Female	1.00 [Reference]	0.67 (0.37, 1.17)	0.78 (0.43, 1.39)	0.300	
BMI					0.313
<25.0	1.00 [Reference]	0.36 (0.14, 0.80)	0.40 (0.16, 0.88)	0.011	
25.0-29.9	1.00 [Reference]	0.62 (0.22, 1.61)	0.71 (0.25, 1.86)	0.600	
>29.9	1.00 [Reference]	0.38 (0.11, 1.04)	0.76 (0.30, 1.80)	0.200	
Smoke					0.981
Yes	1.00 [Reference]	0.49 (0.27, 0.87)	0.60 (0.33, 1.05)	0.030	
No	1.00 [Reference]	0.43 (0.08, 1.55)	0.64 (0.15, 2.14)	0.400	

(Continued)

TABLE 4 Continued

	HALP score			P for trend	P for interaction
	Tertile 1	Tertile 2	Tertile 3		
Respiratory disease mortality					
Diabetes					0.077
Yes	1.00 [Reference]	0.90 (0.28, 2.66)	1.75 (0.65, 4,79)	0.400	
No	1.00 [Reference]	0.39 (0.19, 0.72)	0.40 (0.19, 0.75)	0.001	
Hypertension					0.499
Yes	1.00 [Reference]	0.36 (0.16, 0.75)	0.57 (0.28, 1.10)	0.016	
No	1.00 [Reference]	0.62 (0.31, 1.17)	0.59 (0.28, 1.17)	0.200	

Data are presented as HR (95% CI), which was adjusted as age (continuous), MET (continuous), sex (male or female), race/ethnicity (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black or Other), education level (below high school, high school, or above high school), family poverty income ratio (≤1.0,1.1-3.0, or >3.0), drinking status (nondrinker, drinker), smoking status (never smoker, former smoker, or current smoker), BMI (<18.5, 18.5- 25.0, 25.0-29.9, or >29.9), self-reported diabetes (yes or no), and self-reported hypertension (yes or no). In addition, the corresponding subgroup analyses require the exclusion of the corresponding variables (e.g., age subgroup analyses require the exclusion of age).

TABLE 5 Subgroup analysis by cancer of HALP score and all-cause mortality among cancer survivor in NHANES 1999–2018.

	HALP score			P for trend
	Tertile 1	Tertile 2	Tertile 3	
Breast and Thyroid				
No. deaths/total	118/639	48/639	37/639	
Crude	1.00 [Reference]	0.65 (0.41, 1.01)	0.54 (0.36, 0.80)	0.005
Model 1	1.00 [Reference]	0.76 (0.44, 1.31)	0.53 (0.32, 0.85)	0.031
Model 2	1.00 [Reference]	0.67 (0.37, 1.19)	0.49 (0.29, 0.82)	0.021
Urinary system				
No. deaths/total	174/773	98/773	81/773	
Crude	1.00 [Reference]	0.77 (0.55, 1.08)	0.68 (0.48, 0.97)	0.075
Model 1	1.00 [Reference]	0.76 (0.52, 1.10)	0.93 (0.63, 1.38)	0.3
Model 2	1.00 [Reference]	0.71 (0.48, 1.05)	0.84 (0.56, 1.27)	0.2
Digestive system				
No. deaths/total	103/363	46/363	32/363	
Crude	1.00 [Reference]	0.70 (0.42, 1.14)	0.51 (0.30, 0.87)	0.038
Model 1	1.00 [Reference]	0.59 (0.34, 1.03)	0.47 (0.26, 0.84)	0.023
Model 2	1.00 [Reference]	0.54 (0.29, 1.00)	0.39 (0.20, 0.74)	0.009
Reproductive system				
No. deaths/total	51/563	31/563	23/563	
Crude	1.00 [Reference]	0.73 (0.44, 1.20)	0.52 (0.30, 0.89)	0.052
Model 1	1.00 [Reference]	0.88 (0.48, 1.58)	0.75 (0.39, 1.40)	0.7
Model 2	1.00 [Reference]	0.75 (0.34, 1.60)	0.48 (0.20, 1.13)	0.2
Skin and Soft tissue				
No. deaths/total	190/1251	130/1251	114/1251	
Crude	1.00 [Reference]	0.67 (0.51, 0.89)	0.57 (0.43, 0.76)	<0.001
Model 1	1.00 [Reference]	0.75 (0.61, 1.07)	0.68 (0.56, 0.89)	0.03

(Continued)

TABLE 5 Continued

	HALP score			P for trend
	Tertile 1	Tertile 2	Tertile 3	
Skin and Soft tissue				
Model 2	1.00 [Reference]	0.78 (0.56, 1.08)	0.66 (0.47, 0.93)	0.047
Other				
No. deaths/total	86/417	34/417	44/417	
Crude	1.00 [Reference]	0.53 (0.32, 0.87)	0.56 (0.35, 0.89)	0.011
Model 1	1.00 [Reference]	0.67 (0.37, 1.20)	0.72 (0.41, 1.24)	0.3
Model 2	1.00 [Reference]	0.68 (0.36, 1.26)	0.60 (0.33, 1.07)	0.2

Data are presented as HR (95% CI); Model 1 was adjusted as age (continuous), MET (continuous), sex (male or female), and race/ethnicity (Mexican American, Other Hispanic, Non-Hispanic White, Non-Hispanic Black or Other); Model 2 was adjusted as model 1 plus education level (below high school, high school, or above high school), family poverty income ratio (≤1.0,1.1–3.0, or >3.0), drinking status (nondrinker, drinker), smoking status (never smoker, former smoker, or current smoker), BMI (<18.5, 18.5- 25.0, 25.0-29.9, or >29.9), self-reported diabetes (yes or no), and self-reported hypertension (yes or no).

second highest cause of death, accounting for as high as 214,710 (14.91%) recorded deaths, followed by cerebrovascular disease (104,595 deaths, 7.26%) (68). As a result, the proportion of non-cancer mortality among cancer survivors will increase in the future. Thus, a valuable predictive parameter must properly predict not only the risk of all-cause and cancer mortality in cancer survivors but also the risk of non-cancer mortality. To our knowledge, our study represents the first comprehensive investigation into the relationship between HALP scores and all-cause and cause-specific mortality among cancer survivors. Our findings demonstrate significant associations between HALP scores and all-cause mortality, cancer mortality, cardio-cerebrovascular disease mortality, and respiratory disease mortality in this population. Our study introduces a reliable monitoring metric for the management of cancer survivors, facilitating accurate prognosis prediction and enabling timely interventions that can markedly enhance their outcomes.

An interesting observation in our results is that we found a significant effect of the HALP score on all-cause mortality in patients with breast cancer, thyroid cancer, digestive system tumors, and skin and soft-tissue tumors, suggesting that its effect varies across tumor types. Consistent with prior research, Zhao et al. demonstrated the HALP score's independent prognostic value in early-stage breast cancer, correlating with poorer recurrence-free survival (69). Similarly, Duzkopru et al. identified the HALP score as a prognostic indicator in patients with metastatic gastric cancer (70). These findings underscore the tumor-specific impact of the HALP score on cancer survivors, warranting further investigation into its underlying mechanisms. In addition, in our subgroup analysis, we found a consistently robust correlation between HALP score and all-cause mortality in cancer survivors. However, certain specific mortality results did not reach significance, likely due to the limited data available. Therefore, additional validation through large-scale studies is necessary.

Our study demonstrates several strengths. Firstly, our cohort analysis draws from ten NHANES cycles spanning 1999 to 2018, ensuring robustness with ample data and a lengthy investigative period. Secondly, our choice of the HALP score as a parameter offers a comprehensive assessment of both inflammation and nutritional status, surpassing single inflammation metrics. This approach enriches the evaluation of cancer survivors' physical well-being and enhances outcome prediction reliability. Thirdly, our study pioneers the comprehensive examination of HALP scores' association with all-cause and specific mortalities in cancer survivors, including cancer, cardio-cerebrovascular disease, and respiratory disease mortalities. These findings provide invaluable insights for prognostic management in this population. Lastly, employing diverse analytical methodologies such as RCS nonlinear analysis, subgroup scrutiny, and Kaplan-Meier analysis further consolidates the HALP score's validity as a reliable mortality risk indicator for cancer survivors.

Several limitations must be acknowledged in our study. Firstly, NHANES data relies on self-reports from patients, which could introduce recall bias. Secondly, despite our efforts to adjust for known confounding factors such as age, gender, and smoking status, there may still be unidentified confounders affecting our results. Thirdly, the HALP score utilized in our study was derived from a single complete blood count parameter and serum albumin measurement, which may not fully capture individuals' overall health status, potentially leading to bias.

5 Conclusions

By conducting a comprehensive survey of cancer survivors across the United States, our study unveiled a significant nonlinear relationship between HALP scores and both all-cause and cause-specific mortality. Elevated HALP scores were consistently associated with lower long-term mortality rates among cancer survivors. Therefore, the HALP score emerges as a practical and cost-effective tool for identifying high-risk groups within this population. Our findings underscore the promising potential of HALP scores in prognosticating outcomes for cancer survivors, offering valuable insights for clinical decision-making in this demographic.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by the NCHS Ethics Review Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

JF: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. XY: Formal analysis, Methodology, Validation, Writing – original draft. YZ: Methodology, Validation, Writing – original draft. JZ: Supervision, Validation, Writing – review & editing. XW: Conceptualization, Methodology, Supervision, Writing – review & editing. DZ: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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