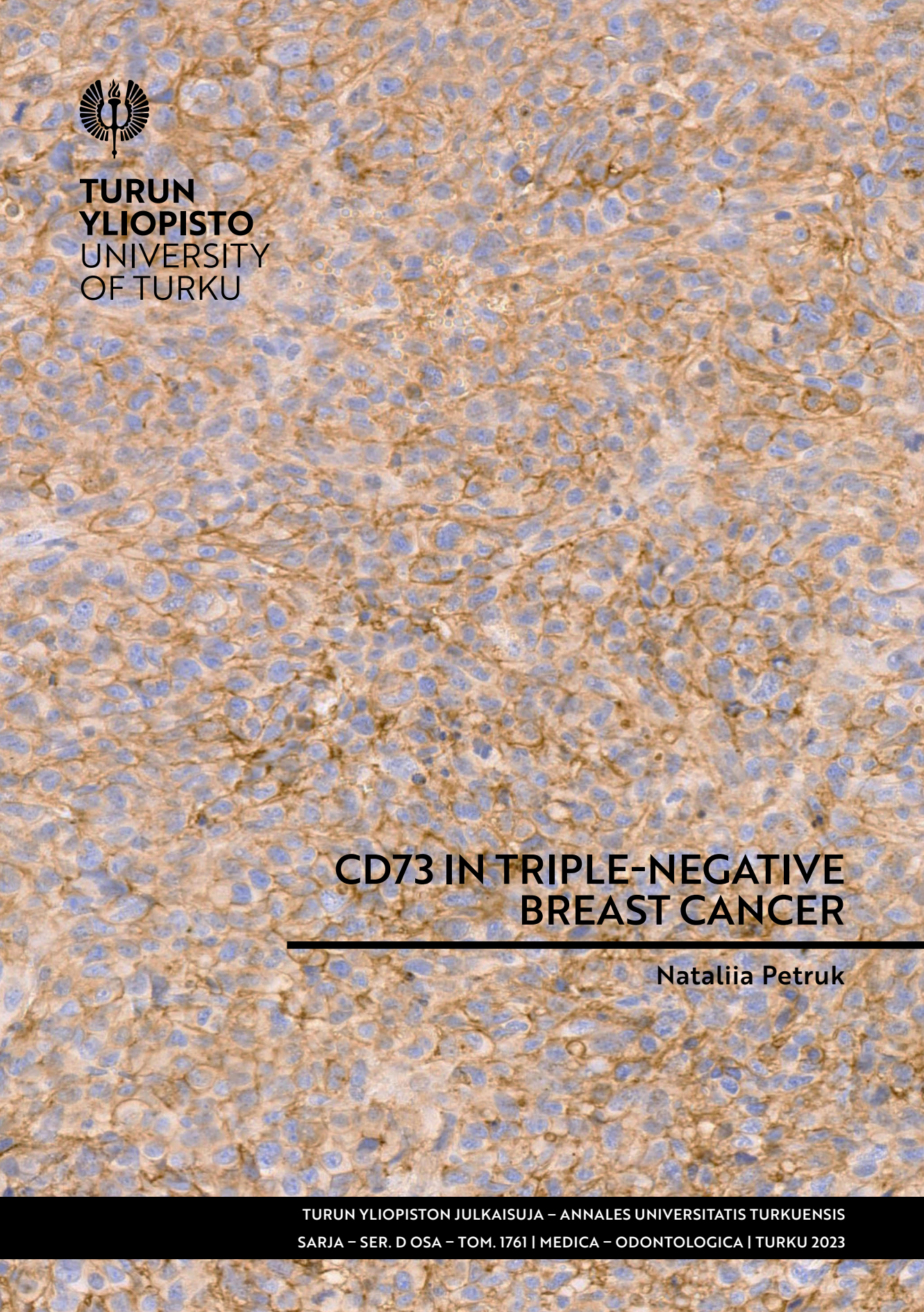

TURUN YLIOPISTO
UNIVERSITY OF TURKU

CD73 IN TRIPLE-NEGATIVE BREAST CANCER

Nataliia Petruk

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. D OSA – TOM. 1761 | MEDICA – ODONTOLOGICA | TURKU 2023

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To my family and Ukraine

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ABSTRACT

CD73 is a membrane-bound receptor that converts AMP to adenosine. Its overexpression in tumors is associated with poor outcomes in triple-negative breast cancer (TNBC) patients. TNBC patients have limited treatment options due to the lack of targeted hormonal receptors. The use of nitrogen-containing bisphosphonates (N-BPs) in the adjuvant setting has shown potential in improving overall survival, particularly among post-menopausal patients with early breast cancer (EBC). This thesis aimed to investigate the role of tumor CD73 in TNBC progression and whether tumor CD73 expression impacts treatment responses to N-BPs. Additionally, we investigated whether reformulating N-BPs enhances their immune cell targeting capabilities in the tumor microenvironment. We employed two methods to inhibit CD73: shRNA-silencing and α,β-methylene-ADP (APCP), a selective enzyme inhibitor. Our findings demonstrated that suppressing CD73 expression, rather than blocking its enzymatic activity with APCP, resulted in the inhibition of invasive properties in TNBC cells and a reduction in epithelial-mesenchymal transition. Suppressing CD73 expression attenuated the positive effects of hypoxia on cell viability and sensitized the cells to the proliferation inhibiting effects of N-BPs in vitro. In vivo experiments revealed that CD73 suppression resulted in reduced tumor growth and fewer lung metastases. In mice treated with the potent N-BP zoledronate, we observed significantly enhanced infiltration of B cells, CD8+ and CD4+ T cells into tumors with low CD73 expression. However, B cell depletion decreased CD8+ T cell infiltration into tumors with suppressed CD73 expression. These effects were not found in tumors with normal CD73 expression. Liposome-encapsulated zoledronate had reduced bone anabolic effect but significantly targeted tumor-infiltrating CD4+ T cells. This treatment also induced changes in intratumoral inflammation by shifting macrophage polarization towards the M1 phenotype. Notably, free zoledronate had only a minor impact on these events. In summary, our results provide evidence for the involvement of CD73 in the initial phases of tumor progression and the infiltration of immune cells into tumors. Furthermore, this study demonstrates that zoledronate induces immune cells infiltration into tumors. Reformulation of the drug may increase such potential.

KEYWORDS: CD73, TNBC, nitrogen-containing bisphosphonates, tumor growth, immune cells

TURUN YLIOPISTO
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TIIVISTELMÄ

CD73 on solukalvolla sijaitseva reseptori, joka muuntaa AMP:n adenosiiniksi. Kolmoisnegatiivista rintasyöpää (TNBC) sairastavilla potilailla adenosiinin yli-ilmeneminen on yhteydessä huonoihin hoitotuloksiin. Koska TNBC-potilaiden syöpäsolut eivät ilmennä sukupuolihormonireseptoreita, on hoitokeinoja rajoitetusti. Adjuvanttihoito typpeä sisältävillä aminobisfosfonaateilla (N-BP) on parantanut varhaisvaiheen rintasyöpäpotilaiden hoitotulosta. Useimmat näistä potilaista ovat postmenopausaalisia. Tämän väitöskirjan tavoitteena oli tutkia CD73:n roolia TNBC:n etenemisessä. Tavoitteena oli myös selvittää, vaikuttaako kasvaimen CD73-taso hoitovasteisiin N-BP:lle. Lisäksi tutkimme, voiko N-BP:ien lääkeformulaatioilla parantaa niiden kohdentumista kasvaimen immuunisoluihin. Käytimme kahta menetelmää CD73:n estämiseen: shRNA-hiljentämistä ja selektiivistä entsyymin estäjää α,β-metyleeni-ADP (APCP). Tuloksemme osoittivat, että CD73:n ilmentymisen tukahduttaminen, sen entsymaattisen toiminnan estämisen sijaan, vähensi TNBC-solujen invasiivisuutta ja epiteeli-mesenkymaali-muuntumista. CD73:n tukahduttaminen vähensi hypoksian vaikutusta solujen elinkykyyn ja lisäsi N-BP:ien solujakautumista estävää vaikutusta soluviljelmissä. Eläinkokeissa havaitsimme, että CD73:n inhiboiminen hidasti kasvainten kasvua ja vähensi keuhkometastaaseja. N-BP tsoledronaatti lisäsi B-solujen sekä $CD8^+$ ja $CD4^+$ T-solujen tunkeutumista niihin kasvaimiin, joissa CD73:n ilmentyminen oli vähäistä. Toisaalta kasvaimen B-solujen määrän vähentäminen hillitsi $CD8^+$ T-solujen tunkeutumista kasvaimiin, joissa CD73:n ilmentymien oli tukahdutettu. Liposomeihin kapseloidulla tsoledronaatilla oli alhaisempi anabolinen luuvaikutus kuin vapaalla tsoledronaatilla, mutta se kohdistui merkittävästi kasvaimiin tunkeutuviin $CD4^+$ T-soluihin. Tämä hoito myös lisäsi kasvaimen makrofaagien M1-tyypin polarisaatiota. Vapaan tsoledronaatin vaikutus oli heikompi. Yhteenvetona tuloksemme osoittavat, että CD73:lla on rooli kasvaimen varhaisessa etenemisvaiheessa sekä immuunisolujen tunkeutumisessa kasvaimiin. Lisäksi tämä tutkimus osoittaa, että tsoledronaatti lisää immuunisolujen tunkeutumista kasvaimiin ja tsoledronaatin pakkaaminen liposomeihin voi tehostaa tätä vaikutusta.

AVAINSANAT: CD73, TNBC, aminobisfosfonaatti, kasvaimen kasvu, immuunisolut.

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Abbreviations

ACT	Adjuvant chemotherapy
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AP	Alkaline phosphatase
APCP	5'-(α,β-methylene) diphosphate adenosine
AppCCl2p	Adenosine 5′-[β,γ-dichloromethylene] triphosphate
ApppI	1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester triphosphoric acid
ATP	Adenosine triphosphate
BC	Breast cancer
BL1	Basal-like 1
BL2	Basal-like 2
BMD	Bone mineral density
BP	Bisphosphonate
BRCA	BReast CAncer gene
CAF	Cancer-associated fibroblast
CTLA4	Cytotoxic T-lymphocyte associated antigen 4
DFS	Disease-free survival
eADO	Exracellular adenosine
e-AK	Extracellular adenylate kinase
EBC	Early breast cancer
EMP-LIP	Empty-liposome
EMT	Epithelial-mesenchymal transition
E-NPP	Ecto-nucleotide pyrophosphatase/ phosphodiesterases
E-NTPD	Ecto-nucleoside triphosphate diphosphohydrolases
ER	Estrogen receptor
FPPS	Farnesyl pyrophosphate synthase
GGPP	Geranylgeranyl diphosphate
GPI	Glycosylphosphatidylinositol
GTPases	Guanosine triphosphate-binding proteins
HER2	Human epidermal growth factor receptor 2
HIF-1	Hypoxia-induced factor-1

IHC	Immunohistichemistry
IL	Interleukin
IM	Immunomodulatory
IMP	Inosine-5-phosphate
INO	Inosine
IPP	Isopentenyl pyrophosphate
LAR	Luminal androgen receptor
LIP	Liposomes
M	Mesenchymal
M-CSF	Macrophage colony-stimulating factor
MDSC	Myeloid-derived suppressor cells
MSL	Mesenchymal stem-like
NACT	Neoadjuvant chemotherapy
N-BP	Nitrogen-containing bisphosphonates
NT-5E	ecto-5-nucleotidase
OS	Overall survival
PARP	Poly ADP ribose polymerase
pCR	Pathological complete response
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
PEG	Polyethylene glycol
PR	Progesterone receptor
RANKL	Receptor activator of nuclear factor-κB ligand
SRE	Skeletal-related events
TAM	Tumor-associated macrophage
TF	Transcription factor
TIL	Tumor-infiltrating lymphocytes
TNBC	Triple-negative breast cancer
Treg	Regulatory T cell
ZOL	Zoledronate
ZOL-LIP	Liposome-encapsulated zoledronate

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Petruk N, Tuominen S, Åkerfelt M, Mattsson J, Sandholm J, Nees M, Yegutkin GG, Jukkola A, J Tuomela J, Selander KS. CD73 facilitates EMT progression and promotes lung metastases in triple-negative breast cancer. *Sci Rep*. 2021 Mar 16;11(1):6035.

II Petruk N, Siddiqui A, Tadayon S, Määttä J, Mattila PK, Jukkola A, Sandholm J, Selander KS. CD73 regulates zoledronate-induced lymphocyte infiltration in triple-negative breast cancer tumors and lung metastases. *Front. Immunol.* Jul 18; 14:1179022.

III Nataliia Petruk, Sofia Sousa, Martine Croset, Lauri Polari, Hristo Zlatev, Katri S. Selander, Jukka Mönkkönen, Philippe Clézardin, Jorma Määttä. Liposome-encapsulated zoledronate increases inflammatory macrophage population in TNBC tumors. *European Journal of Pharmaceutical Sciences*, https://doi.org/10.1016/j.ejps.2023.106571.

1 Introduction

CD73 is a cell surface ecto-5′-nucleotidase, which main function is to convert extracellular adenosine monophosphate (AMP) into adenosine and inorganic phosphate (H. Zimmermann, 1992). Its high expression has been observed in multiple types of cancer, including triple-negative breast cancer (TNBC) (Cerbelli et al., 2020; Q. Chen et al., 2020; Y. H. Chen et al., 2021; He et al., 2021; Rocha et al., 2021; A. Tripathi et al., 2020). The widespread expression of CD73 on both malignant cells and endothelial/immune cells suggests its potential as a target for inhibiting tumor progression and improving patient survival (Buisseret et al., 2018). CD73 enhances cell migration and invasion and also chemotherapy resistance, possibly due to its immunosuppressive capability (Loi et al., 2013; Samanta et al., 2018). The overall survival rate for TNBC patients within the first five years is 82%. However, the survival is highly dependent on the cancer stage. The five-year overall survival of TNBC patients on stage I, II, III and IV is 95%, 86%, 59% and 11%, respectively (Hsu et al., 2022).

Bisphosphonates (BPs) were introduced over 50 years ago as compounds to inhibit osteoclast-mediated bone resorption. These drugs possess high affinity for bone (La-Beck et al., 2021). They effectively reduce bone fractures during osteoporosis and skeletal complications resulting from bone metastases (Yusuf et al., 2018). However, nitrogen-containing BPs (N-BPs) have shown a survival advantage in a cohort of breast cancer patients in the adjuvant setting (Wilson et al., 2018). Several preclinical studies have demonstrated direct anti-cancer effects of N-BPs (Daubiné et al., 2007; Tuomela et al., 2008). Because of BPs high affinity to bone hydroxyapatite, it is thought that these drugs do not reach inside tumor cells. Encapsulating BPs in liposomes may allow for reduced drug dosing and more precise targeting to cancer cells (La-Beck et al., 2021).

Apart from the direct anti-cancer effects, both N-BPs and CD73 modulate immune events during cancer progression. N-BPs could induce local inflammation by inhibiting the mevalonate pathway (K. Thompson & Rogers, 2004), which enhances the infiltration of γδ T cells (Galluzzo et al., 2007) and pro-inflammatory macrophages (Kaneko et al., 2018), while also reducing the amounts of immunosuppressive regulatory T cells (Hsien Liu et al., 2016). In contrast, CD73

facilitates the infiltration of immunosuppressive immune cells into tumors, contributing to tumor progression by decreasing immunoregulatory immune cells (Häusler et al., 2011; King et al., 2022).

In this thesis, we further investigated the mechanisms by which CD73 could promote TNBC progression. Considering the contrasting effects of CD73 and N-BPs on immune cells within tumors, we studied the changes in intratumoral immune cell infiltration influenced by these two factors. We also investigated whether reformulation of the potent N-BP zoledronate leads to enhanced immune cell targeting compared to unmodified zoledronate.

2 Review of the Literature

2.1 Breast cancer

Breast cancer (BC) accounted for 2.3 million cases among the total cancer incidence in 2021. Among women, BC comprised 11.7% of all cancer types in 159 countries. The mortality rate was 6,9% in 110 countries worldwide. The incidence rate of BC in postmenopausal women is highest in Australia/New Zealand and Western Europe, followed by Northern America, Northern and Southern Europe (Sung et al., 2021). In Finland, breast cancer is the most common cancer diagnosed in women. In 2021, a total of 5105 cases were reported, corresponding to an incidence rate of 167.3 per 100,000 person-years. The incidence has seen an upward trend since the 1950s. When considering age groups, women between 20 and over 70 years had the highest number of breast cancer diagnoses, followed by colon and rectum cancers. The five-year relative survival rate for patients with breast cancer was at 92%. When considering age groups, the percentage of relative survival ratio was higher in women under 55 years in comparison to older age groups. In the same year, breast cancer had the highest mortality rate (26.8 per 100,000 person-years) compared to other cancer types in women aged from 20 to over 70 years (Seppä et al., 2021).

BC is clinically classified into three main subtypes using immunohistochemical (IHC) tests based on the expression of cellular receptors: estrogen receptor (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2). The three subtypes are hormone sensitive (ER+/- or PR+/-), HER2-positive (ER+/-, PR+/- and HER2+) and triple-negative BC (TNBC; ER-/PR-/HER2-). With the addition of gene expression analysis to the subtyping, BC was further classified into luminal A (ER+ or PR+, HER2-, index of proliferative marker, Ki67 < 14%), luminal B (ER+, PR+/- or HER2+/-, index of Ki67 > 14%), HER2 (ER-/PR- and HER2-enriched) and basal (ER-/PR-/HER2-) subtypes (Uscanga-Perales et al., 2016). (Uscanga-Perales et al., 2016). However, only the receptor status is commonly clinically used and widely available.

2.2 Triple-negative breast cancer

Triple-negative breast cancer (TNBC), accounts for 15–20% of all breast cancer cases. TNBC is characterized by the absence of hormonal receptors, ER and PR, localized in the nucleus and a member of epidermal growth factor family HER2, localized on the cell membrane. Of all EBC patients, TNBC patients typically have the poorest survival outcome, due to several factors, including a high recurrence rate in distant organs, a shorter disease-free interval, and limited treatment options (Liedtke et al., 2008). The database analysis of 180,996 breast cancer incidences diagnosed in women between 2010 and 2012 demonstrated TNBC patients have demonstrated worse overall survival (OS) across all stages of breast cancer progression compared to non-TNBC patients. Typically, TNBC patients are younger than non-TNBC patients (average age 58.9 vs. 61.8 years) (X. Li et al., 2017). The study of 50,856 breast cancer patients have shown that the 5-year OS for early TNBC patients was approximately 82%. The OS for non-TNBC patients was nearly 87%. The OS of TNBC patients is also influenced by cancer stage. The 5-year OS for TNBC patients is approximately 95% for stage I, 86% – for stage II, 59% – for stage III, and 11% – for stage IV tumors (Hsu et al., 2022). A recent analysis of a Finnish TNBC cohort of 147 patients suggested that women aged between 49 and 57 years had better overall survival probabilities compared to younger or older age groups. The mortality rates associated with TNBC correlated with tumor size. Each 10 mm increase in tumor size showed a higher mortality risk in patients over 57 years old (Vihervuori et al., 2022).

2.2.1 Classification of TNBC

Triple-negative breast cancer exhibits high diversity at the molecular and cellular levels, resulting in variations in cancer progression. This heterogeneity is evident in the different cell compositions found within the tumor itself and its surrounding microenvironment, influencing the behavior of the cancer (Lehmann et al., 2021). This results in varying treatment responses and conflicting clinical outcomes. The identification of TNBC subtypes is challenging due to the high heterogeneity of these tumors. The standard clinical analysis of TNBCs involves assessing the expression of ER, PR, and HER2 through IHC staining. The majority of scored TNBC tumors exhibit a basal-like phenotype, expressing genes of basal epithelial cells, such as EGFR, cytokeratins 5/6, 14 and 18, and lacking the expression of ER or/and HER2. Although not all basal-like tumors lack expression of ER, PR, or HER2 (Marra et al., 2020).

In 2011, Lehmann et al. defined six molecular subtypes and one unstable subtype of TNBC based on gene expression profiles and immune cell populations within tumors (Lehmann et al., 2011). More recently, a genomic landscape of TNBC tumors

was characterized using multi-omics analysis (Lehmann et al., 2021). These subtypes can be grouped as follows:

- Immunomodulatory (IM) – enriched for tumor infiltrating lymphocytes (TIL) and immune gene expression, involved in cytokine and antigen signaling, B cell receptor, natural killer (NK) cell, dendritic cell (DC) and T cell receptor signaling pathways.
- Mesenchymal (M) – immunosuppressed or non-permissive to TILs, with low immune gene expression, enriched for genes involved in cellular differentiation and migration. Recent RNA-seq data has demonstrated that TILs might also be margin restricted and absent from tumor cores in this subtype.
- Mesenchymal stem-like (MSL) – contained a small number of TILs, enriched for angiogenesis, migration and cellular differentiation gene expression and low for proliferation genes.
- Luminal androgen receptor (LAR) – consists of 10–15% of TNBC cases, showing chemotherapy resistance. This subtype is ER-negative and shares features with luminal tumors and enriched for genes in pathways responsible for steroid and androgen/estrogen metabolism. Luminal-like gene expression, such as cytokeratin gene KRT18 or luminal markers FOXA1 and XBP1 were increased in the LAR subtype.
- Basal-like 1 (BL1) – enriched for cell cycle and proliferation pathways gene expression. This subtype showed high mRNA expression of proliferative marker, Ki-67, and cell cycle genes which might sensitize this subtype to antimitotic treatment. BL-1 showed a high sensitivity to chemotherapy.
- Basal-like 2 (BL2) – enriched for metabolic signaling gene expression, such as epithelial growth factor, mesenchymal-epithelial transition or Wnt/β catenin pathways. BL-2 showed a low sensitivity to chemotherapy.

However, Lehmann's classification did not include IHC-confirmed ER, PR and HER2 status, but only RNA profiling datasets. In 2015, Burstein and coworkers suggested 4 stable subtypes of TNBC, considering also hormonal receptor protein status: luminal androgen receptor, mesenchymal, basal-like immune suppressed, and basal-like immune activated (Burstein et al., 2015).

2.2.2 TNBC risk factors

Risk factors for developing TNBC include age, race, genetics, breast density and age of first parity. Compared to other BC types, TNBC women are likely to be pre-

menopausal (Friebel-Klingner et al., 2021; X. Li et al., 2017). African-American women are more frequently diagnosed with TNBC. However, those women who breastfed over 6 months had lower odds to be diagnosed with TNBC (H. Ma et al., 2017). Dense breast tissue and obesity also increase the risk of developing TNBC (Friebel-Klingner et al., 2021). The age of first occurrence of menstruation is not a risk factor for TNBC (H. Ma et al., 2017). It is still unclear whether pregnancy is associated with TNBC initiation. Women who have never given birth have a lower probability of developing TNBC compared to parous women (Asztalos et al., 2015). A study of over 2500 cases showed that pregnancy was not a risk factor in TNBC, when the mean age of first parity was under 25 years (H. Ma et al., 2017). However, a low increase in risk of being diagnosed with TNBC was observed when the first completed pregnancy occurred between 25 and 30 years (Brouckaert et al., 2017). Genetic dysregulation is another factor that promotes cancer initiation. Women from breast cancer families have a higher chance of developing cancer due to mutations in certain genes. For instance, women with mutations in the BRCA (BReast CAncer) genes are at a higher risk of developing BC, and these mutations are correlated with worse patient survival (Y. Zhu et al., 2016). BRCA1 mutations occurred in 52% and BRCA2 mutations in 35% of BC families (Ford et al., 1998). A cohort of 215 TNBC patients, with a median age 54 years, exhibited a 10% prevalence of BRCA mutations (Hartman et al., 2012). A study involving 355 TNBC patients, with age 24 – 40 years showed that approximately 18.9% of them had BRCA mutations, with BRCA1 mutation occurring in 87% of those individuals (Ye et al., 2021). TNBC patients with BRCA1 mutation had a significantly worse disease-free survival (DFS) (M. Liu et al., 2021) and larger tumors (H. Chen et al., 2018) in comparison to patients with non-mutated BRCA1 gene.

2.2.3 Metastatic sites of TNBC

In 1889, Dr. Stephen Paget, an English surgeon, proposed that the development of metastases depended on interaction of cancer cells and microenvironment or pre-metastatic niche, where disseminated tumors cells migrated to (Paget, 1889). The pre-metastatic niche provides a homing site for disseminated tumors cells, leading to late recurrence. The relapse of a specific cancer type is organ-specific, with common sites for disseminated tumors cells being lymph nodes, lungs, bones, liver, and brain (Arciero et al., 2019; Kennecke et al., 2010).

The first relapse of TNBC in women typically occurs within an average of 2.6 years, while for non-TNBC patients is approximately 5 years. Recurrence in ER+ breast cancer patients can occur up to 20 years after the initial detection (Dent et al., 2009; Sopik et al., 2019). The brain is a metastatic site typically for HER2+, following by TNBC subtype. A low number of luminal A and luminal B patients

develop brain metastases (Kennecke et al., 2010). Except brain, luminal B subtype tends to metastasize to the lungs and liver (Ignatov et al., 2018). The liver is also a frequent site for metastases for $HER2^+$ subtype (Arciero et al., 2019). Bone is a frequent metastatic site among all BC subtypes (Ignatov et al., 2018). However, TNBC patients develop fewer bone metastases, which are more commonly associated with hormone receptor-positive subtypes (Dent et al., 2009; Kennecke et al., 2010; Y. Liu et al., 2014), or $ER^+/HER2^-$ (Arciero et al., 2019). Within 10 years of diagnosis, TNBC patients experience significantly more visceral metastases (lung, lymph nodes, liver) and brain metastases compared to hormone receptor-positive subtypes (Dent et al., 2009; Ignatov et al., 2018; Kennecke et al., 2010; Y. Liu et al., 2014).

2.2.4 TNBC treatment

Surgery, radiotherapy and chemotherapy are standard of care for TNBC (Figure 1). With surgery, the surgeon removes the visible disease, radiation reduces local recurrences and chemotherapy aims to eliminate microscopic disease from the body. Chemotherapies induce DNA destabilization, inhibit DNA or RNA synthesis, inhibit cell division or disruption of mitochondria biogenesis, which leads to the cytotoxic effect of these drugs. Despite the development of new therapeutic strategies, chemotherapy remains the standard-of-care for TNBC. Biomarker-based therapy or personalized medicine was included to the TNBC treatment after promising data from clinical trials that demonstrated benefits of using biomarkers to improve selection of therapies based on individual molecular or cellular characteristics (Bou Zerdan et al., 2022), however defining biomarkers for these tumors remains limited due to its heterogeneity (Lehmann et al., 2021).

2.2.4.1 Neoadjuvant treatment

It is now standard of care, that treatment of TNBC begins with chemotherapy. This is indicated when TNBC is larger than 2 cm, with or without metastatic lymph nodes (Cardoso et al., 2019). Neoadjuvant therapy is applied to reduce the primary tumor to a size, which allows surgical resection. Neoadjuvant chemotherapy (NACT) also allows targeting microscopid disease early and provides a possibility to switch between treatment regimens when patients do not response or show little response to therapy. A systemic NACT and adjuvant chemotherapy (ACT) include taxanes, anthracyclines, platinum agents, and cyclophosphamides agents. Taxanes act to inhibit mitosis by binding to microtubules. Anthracyclines intercalate into DNA and block the topoisomerases activity. While, cyclophosphamides mechanism of action

involves damage to cancer cell DNA and targeting immunosuppressive T cells (Bukowski et al., 2020).

The aim of NACT is a pathological complete response (pCR), characterized by the absence of invasive cancer cells in the breast and/or lymph nodes. Achieving pCR has prognostic value in predicting survival in patients with TNBC, luminal B, and HER2+ subtypes (Von Minckwitz et al., 2012). Since, the number of tumor-infiltrating lymphocytes (TILs) are also a predictor of the response to NACT, application of immune checkpoint inhibitors, such as programmed cell death protein 1 (PD-1) or programmed cell death ligand 1 (PD-L1) may enhance the efficacy of treatment (Denkert et al., 2018).

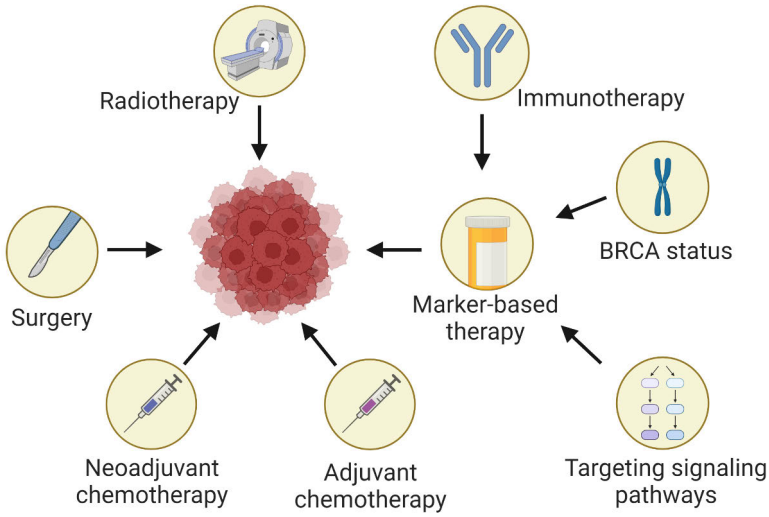

Figure 1. Treatment options for triple-negative breast cancer patients. TNBC anti-cancer treatments include surgery, radiotherapy, chemotherapy and targeted therapy. Chemotherapy consists of neoadjuvant- and adjuvant chemotherapy. Marker-based therapy includes immunotherapy, signaling pathway inhibitors, and PARP inhibitors for TNBC patients with BRCA gene mutations. Created in BioRender.com based on Von Minckwitz et al., 2012; Del Mastro et al., 2015; Robson et al., 2017; Adams et al., 2019; Lehmann et al., 2021.

High TILs enabled achieving pCR in half of the cohort, while low TILs were observed in a third of TNBC patients treated with NACT. Infiltration of TILs in tumors is a marker not exclusive to the TNBC subtype. A higher pCR rate was also observed in other BC subtypes with high TILs (Denkert et al., 2018). A meta-analysis revealed that the most effective combined therapy in TNBC was a PD-1 inhibitor plus platinum with anthracycline- and taxane-based NACT. It demonstrated the highest pCR and significant DFS or event-free survival (Lin et al., 2022).

Higher pCR rate correlated with better outcomes after NACT for TNBC patients (Von Minckwitz et al., 2012). When stratified by TNBC subtype, BL1 showed the highest pCR rate, indicating the high sensitivity of this subtype to NACT (Echavarria et al., 2018; Masuda et al., 2013). Interestingly, NACT was able to change TNBC subtype. Masuda et. al demonstrated that BL1 was the most common subtype, in a patient cohort before treatment. However, the M subtype became most frequent after NACT, suggesting that tumors undergo immunosuppression (Masuda et al., 2022). This could explain the lack of response in achieving pCR in some TNBC patients.

2.2.4.2 Adjuvant treatment

The systemic ACT was initially suggested over 40 years ago (Bonadonna et al., 1976). Adjuvant therapy is considered the main treatment for cancer that following NACT and / or surgery. It is used to target residual primary tumor cells and any latent disseminated tumor cells in distant organs (Pondé et al., 2019). ACT can also decrease the risk of residual tumor cells from dividing due to its highly cytotoxic effect. Selection criteria for ACT in BC patients include tumor size, risk of relapse or/and expression of hormonal or HER2 receptors. For instance, ACT should receive patients with tumors more than 5 mm. Endocrine therapy or trastuzumab in combination with chemotherapy is required for ER+ or/and PR+ or HER+ patients, respectively (Anampa et al., 2015).

Clinical trials have provided strong evidence for the effectiveness of the anthracyclines-taxanes combination in TNBC patients, leading to higher rates of DFS or recurrence-free survival (An et al., 2020; Del Mastro et al., 2015). Currently, numerous clinical trials are focused not only on developing novel drugs but also on exploring new drug combinations (*https://Clinicaltrials.Gov/*). This may also allow to reduce side effects of treatment. An antimetabolite drug, capecitabine, in combination with docetaxel (taxane) and epirubicin (anthracycline), allows for reduced drug doses and increased DFS or recurrence-free survival within 5 years in the adjuvant settings (J. Li et al., 2020), but not in the neoadjuvant setting (Von Minckwitz et al., 2010). Multicenter clinical trial from Finland and Sweden, FinXX, also reported increased OS for ER-, HER2- and TNBC patients treated with capecitabine in combination with chemotherapy (Joensuu et al., 2022).

2.2.4.3 Targeted therapies

Biomarkers in cancer care are used for diagnosis, prognosis of patient survival or prediction of responses to therapy. Common strategies in targeted therapy for TNBC are focused on blockage of poly ADP ribose polymerase (PARP) and immune checkpoints.

Mutated BRCA (2.2.2) is unable to repair DNA damage that sensitizes cells to PARP inhibition. In adjuvant setting, these inhibitors (olaparib, talazoparib) are a first line treatment for women with metastatic BRCA1/2 mutant BC to reduce the risk of disease progression and to prolong progression-free survival (Robson et al., 2017; Tutt et al., 2021). Combination of PARP inhibitors with chemotherapy decreased the risk of disease progression and increased the response rate in patient with advanced stage of BC (Litton et al., 2018). Defective DNA repair by homologous recombination has been considered a general characteristic of all TNBC subtypes until recently (Nik-Zainal & Morganella, 2017). In 2021, Lehmann et al. showed that a higher rate of defective DNA repair by homologous recombination has become a subtype-specific signatures for TNBC BL1 and M subtypes (Lehmann et al., 2021). However, the M-subtype was also significantly enriched for deletion of DNA repair and β-2-microglobulin genes in comparison to all other subtypes. These alterations might impair the efficacy of therapy by targeting immune checkpoints, since only M-subtype did not have immune cell pathway enrichment (Lehmann et al., 2021).

Immunotherapy is focused on inhibition of immunosuppressive receptors to fight diseases. In oncology, purpose of immunotherapy is to enhance long-lasting immune responses against cancer cells. PD-1 is a widely studied immunosuppressive receptor that inhibits immune cells from attacking cancer cells. Monoclonal antibodies targeting PD-1 (e.g., pembrolizumab, nivolumab) or its ligand, PD-L1 (e.g., durvalumab, atezolizumab), block the receptor-ligan interaction to promote effector T cell responses (Bardhan et al., 2016). TNBC tumors demonstrate higher immune cell infiltration (Z. Liu et al., 2018) and expression of PD-L1 (Mittendorf et al., 2014) which may explain the superior response of TNBC patients to this treatment compared to other BC subtypes. PD-L1 has emerged as a stratification factor for patients who exhibit a higher response rate to monotherapy with immune checkpoint inhibitors (Adams et al., 2019) and improved progression-free survival after combination of immune checkpoint inhibitors and chemotherapy (Cortes et al., 2020; L. A. Emens et al., 2021). Another immunosuppressive receptor, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) suppresses T lymphocytes activation in early stages of the immune response. Genomic analysis demonstrated that TNBC patients had a higher expression of CTLA-4 than non-TNBC patients (Z. Peng et al., 2020). CTLA-4 inhibitors (ipilimumab, tremelimumab) showed a higher overall response rate in comparison to ER^+ BC patients in the pilot study (Santa-Maria et al., 2018).

Genomic analysis has revealed that TNBC patients may benefit from other treatments. BL1 and M subtypes might respond better to the cyclin-dependent kinases CDK1/2 inhibitors (block the entry into the G2/M phase and transition into the S-phase), but BL2 and LAR subtypes could benefit from CDK4/6 inhibitor

(prevent cell transition to S-phase) treatment. Phosphorylation of AKT1 and AKT2 indicates activation of the PI3K/mTOR pathways in BL2 and LAR subtypes. However, BL2 tumors are also enriched for activating mitogen-activated protein kinase (MAPK) pathway mutations. Activation of MEK, ERK1, and ERK2 in these tumors may sensitize this subtype to EGFR/MARK signaling pathway targeted therapy (Lehmann et al., 2021).

2.2.4.4 Treatment of metastatic TNBC

Patients with advanced TNBC have in 3 times lower the five-year overall survival in comparison to non-TNBC patients, 11% and 33%, respectively. The first line treatment of advanced TNBC is the combination chemotherapy, such as platinum with taxane or cyclophosphamide with fluorouracil and doxorubicin or epirubicin. Metastatic TNBC in women, typically aged over 60, often leans toward single chemotherapy as a treatment approach in comparison to women of younger age (Hsu et al., 2022).

TNBC patients with residual disease after NACT are likely to have a shorter overall survival than patients with other BC subtypes (Liedtke et al., 2008). Moreover, circulating tumor cells were suggested as a stratification factor for clinical trials due to their association with TNBC relapse after NACT (Radovich et al., 2020). Clinical trials investigating the response of metastatic TNBC to monotherapy or combined-treatment are ongoing. A microtubule-targeting drug, ixabepilone, alone or with anti-EGFR monoclonal antibody, cetuximab and paclitaxel alone or with anti-PD-L1 mAb, durvalumab, showed clinical activity in phase-II trial for advanced TNBC (Ghebeh et al., 2021; Trédan et al., 2015). The analysis of tumor biopsies after atezolizumab (PD-L1 inhibitor) treatment demonstrated that metastatic TNBC patients with high PD-L1 had more favorable outcomes to monotherapy in a phase I clinical trial (Leisha A. Emens et al., 2019). In another study, treatment with pembrolizumab monotherapy decreased tumor size in a third of PD-L1-positive patients with advanced TNBC and the overall response rate was increased in patients with high PD-L1 expression (Nanda et al., 2016). The antimetabolite drug capecitabine together with taxane-based chemotherapy has increased OS and sensitivity to treatment of metastatic TNBC patients with anthracycline resistance (Manjunath & Choudhary, 2021). Metastatic TNBC patients also benefit from a combination of chemotherapy and immunotherapy (Cortes et al., 2020).

Most clinical trials use treatment options focusing earlier on gene expression or abnormalities within one cancer type, but not subtypes. In ongoing clinical trials, TNBC patients with metastases are stratified into subtypes. The results of the phase I/II FUTURE trial have demonstrated a better response to PD-1 inhibitors and paclitaxel in IM-subtype or to anti-VEGFR in the BL immune-suppressed subtype

than other subtypes (Jiang et al., 2021). In LAR-subtype, androgen receptor inhibitors, such as bicalutamide and enzalutamide, showed promising results in increasing the clinical benefit rate in phase II trials (Bou Zerdan et al., 2022; Manjunath & Choudhary, 2021).

2.3 Bisphosphonates

Due to their ability to target osteoclasts, bisphosphonates (BPs) have been widely used for more than 50 years to prevent bone diseases (Russell, 2011; Widler et al., 2002). Bones undergo lifelong remodeling to ensure organ protection and body support. Bone-forming osteoblasts and bone-resorbing osteoclasts play essential roles in bone homeostasis. Functions of these cells are regulated by various factors, such as transcription factors, hormone secretion or disease progression (J. M. Kim et al., 2020). Cytokines, such as macrophage colony-stimulating factor or receptor activator of nuclear factor-κB ligand (RANKL) promote osteoclasts differentiation and survival, leading to accelerated bone loss (Shevde et al., 2000).

BPs can be classified into two groups based on the presence or absence of nitrogen in their molecular structure: non-nitrogen-containing BPs and nitrogen-containing BPs. The non-nitrogen-containing BPs, also known as first-generation BPs, include clodronate, etidronate, and tiludronate, which are clinically available options (Van Acker et al., 2016). Nitrogen-containing BPs (N-BPs), or the second and third generation BPs, include clinically available options such as zoledronate, alendronate, pamidronate, ibandronate, and risedronate (Van Acker et al., 2016).

Bisphosphonates have a low distribution in soft tissues but exhibit a high affinity for bones (Xiang et al., 2020). Only about 2% of non-nitrogen-containing BPs are absorbed in the gastrointestinal tract. Non-nitrogen-containing BPs are generally better tolerated compared to nitrogen-containing BPs in terms of side effects (Frediani & Bertoldi, 2015). When N-BPs are orally administered, their absorbance in the gastrointestinal tract is less than 1%. The presence of nitrogen in the molecular structure of N-BPs significantly enhances their anti-resorptive ability, which can be up to 10,000 times greater compared to the first-generation BPs. BP concentration within bones is not homogeneous. They tend to accumulate more in the growth plate, epiphysis, and areas of bone metastasis. This affinity of bisphosphonates for regions with higher calcium content may explain their preferential accumulation in these areas (Xiang et al., 2020).

2.3.1 Mechanisms of action

BPs are hydrophilic calcium chelator. Approximately 50% of the absorbed drug remains in the bones (Drake et al., 2008), where it effectively inhibits osteoclast-

mediated bone resorption. This mechanism helps reducing bone fractures and skeletal complications, which occur due to bone metastasis (Yusuf et al., 2018).

BPs are analogues of inorganic pyrophosphate with a high affinity to bone hydroxyapatite (Russell, 2007). The structure of BPs includes a carbon atom (P-C-P) instead of oxygen, which is found in pyrophosphate (P-O-P). The central carbon atom in the core structure of all BPs attaches to a hydroxyl group. Additionally, it binds to a phosphate group that interacts with the hydroxyapatite crystals (Sato et al., 1991; Widler et al., 2002).

Non-nitrogen-containing bisphosphonates are metabolized within cells to a nonhydrolyzable analogue of ATP called adenosine 5′-[β,γ-dichloromethylene] triphosphate (AppCCl2p) (Frith et al., 2001). This ATP analogue disrupts mitochondrial respiration by targeting ATP-ADP translocase (ANT). These events ultimately induce apoptosis of osteoclasts or peritoneal macrophages (Selander et al., 1996).

N-BPs, unlike non-nitrogen-containing bisphosphonates, do not undergo cellular metabolism. They primarily target the inhibition of farnesyl pyrophosphate synthase (FPPS) and geranylgeranyl pyrophosphate synthase (GGPPS) within the mevalonate pathway. Dimethylallyl diphosphate and geranyl pyrophosphate form a sub-pocket, which serves as the active site targeted by N-BPs. By blocking FPPS, there is an accumulation of isopentenyl pyrophosphate (IPP). As a consequence, IPP is converted to toxic analogues of ATP known as 1-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester triphosphoric acid (ApppI) within cells (Park et al., 2014). FPP and GGPP are essential for the prenylation of small guanosine triphosphate-binding (GTPases) proteins. Small GTPases such as Rab, Rac, and Rho play vital roles in osteoclast survival, membrane ruffling, and transmitting signals from cell surface receptors to intracellular signaling pathways (Russell, 2007).

2.3.2 Nitrogen-containing bisphosphonates in cancer treatment

Originally, BPs were administered to cancer patients in order to prevent bone loss and to inhibit cancer-induced skeletal complications, such as fractures and hypercalcemia. This was particularly important for postmenopausal women, who, due to the lack of estrogen, are more susceptible to fractures and osteoporosis during the progression of breast cancer (Ebert et al., 2014; Räikkönen et al., 2009). Estrogen deficiency leads to increased activity of bone resorptive regulators, and preclinical studies have shown that estrogen treatment can suppress osteoclast formation (Shevde et al., 2000). BPs have demonstrated beneficial effects when given to cancer patients, not only by preventing bone loss but also by reducing the risk of bone

metastasis, ultimately improving overall survival (Strobl et al., 2018; Winter & Coleman, 2013).

2.3.2.1 N-BPs in pre-clinical cancer research

Some cancer cells have the ability to stimulate osteoblasts in bones by secreting factors like endothelin-1 and parathyroid hormone-related protein. This leads to increased osteoblast activity. Some cancer cell, especially in breast cancer inhibit the normal activity of osteoblasts (Back et al., 2021). These cancer cells modulate the expression of RANKL, a protein that promotes the survival of osteoclasts, resulting in a decrease in bone volume (Shupp et al., 2018). The continuous bone resorption caused by the tumor growth factors, such as insulin growth factor-1 and tumor growth factor-β, released during this process further promotes the growth of tumor cells within the bone environment. N-BPs directly induce apoptosis in osteoclasts, disrupting vicious cycle of cancer cells and inhibiting the development of bone metastasis (Ottewell, 2016; Shupp et al., 2018).

N-BPs have been demonstrated to directly suppress cell viability of various cancer cells when used as a monotherapy. In the case of breast cancer cells, the uptake of zoledronate leads to the complete inhibition of FPPS, resulting in decreased cell viability. This effect is associated with the accumulation of IPP and ApppI within cells. Notably, it has been observed that achieving a comparable level of AppCCl2p as IPP/ApppI in cells requires approximately 20 times higher concentrations of clodronate when compared to N-BPs (Räikkönen et al., 2009). Recent studies have focused on investigating the anti-cancer effects of N-BPs in combination therapy or how they affect immune cells.

Impaired cell viability is closely associated with apoptosis, which involves the activation of several cascades. N-BPs can induce apoptosis by increasing expression of apoptosis-associated proteins, activation of caspases-3 and -7 cleavage or mitochondrial membrane dysfunction (Asahi et al., 2006; Ebert et al., 2014; X. Gao et al., 2015; Rachner et al., 2010). Additionally, the decreased prenylation of small GTPases, a consequence of N-BPs treatment, has been associated with apoptosis-induced events and was detected in lung cancer cells treated with zoledronate (Xie et al., 2015). Zoledronate has also demonstrated the ability to reduce the migration and invasion of breast cancer cells and exhibit an anti-tumor effect by reducing mRNA expression of extracellular matrix metalloproteinases 2 (R. Tripathi et al., 2016). Similarly, alendronate treatment has been associated with actin disruption or a decrease in actin-related cofilin in prostate cancer cells (Virtanen et al., 2018). Zoledronate has been shown to prevent tumor formation in colorectal cancer cells (X. Gao et al., 2015) and bone metastases in a murine breast cancer model (André Barrière et al., 2019; Hiraga et al., 2004). Furthermore, it has also been found to

reduce lung metastases formed by prostate (Pietrovito et al., 2019) and breast (Hiraga et al., 2004) cancer cells.

N-BPs can cause local inflammation by inhibiting FPPS in the mevalonate pathway. This inhibition leads to the accumulation of IPP, which can stimulate immune cells due to their ability to recognize accumulated IPP (K. Thompson & Rogers, 2004). Immune γδ T cells respond to N-BPs and increase the production of IFN-γ (Galluzzo et al., 2007), TNF-α, interleukin-1β (IL-1β), and IL-6 in peripheral blood mononuclear cells (Takimoto et al., 2021). Zoledronate has been shown to increase the number of peritoneal neutrophils and monocytes (Norton et al., 2011), macrophages in spleen (Shikama et al., 2010) and circulating TNF-α, IL-1β, NF-κB positive cells (de Barros Silva et al., 2016). Zoledronate also reduces the infiltration of immunosuppressive Treg cells, without affecting CD4 T cells (Hsien Liu et al., 2016).

Macrophages can adapt a wide spectrum of activation states , but conceptually the term "M1" is used often for inflammation augmenting activation states and "M2" – for inflammation downregulating/tissue regenerating activation states (Murray et al., 2014; Murray & Wynn, 2011). Macrophages can alter their activation state in response to physiological signals or pharmacological agents. Inhibition of the mevalonate pathway leads to an increased number of macrophages in the spleen and the production of pro-interleukin 1 β by these cells (Shikama et al., 2010). In addition to influencing cell infiltration, N-BPs promote macrophage polarization. For instance, zoledronate-induced polarization of M1-type macrophages occurs through NALP3 (Kaneko et al., 2018) or TLR4 inflammasome activation (W. Zhu et al., 2019), but only when cells were pre-treated with lipopolysaccharides (Kaneko et al., 2018). The addition of lipopolysaccharides to N-BPs also increases IL-1β levels, which is a factor to stimulate M1 macrophage polarization (Kaneko et al., 2018; Tamai & Kiyoura, 2018; Q. Zhang et al., 2015) and enhances the expression of the pro-inflammatory cytokine IL-6 in macrophages (Norton et al., 2011).

2.3.2.2 Clinical cancer trials with N-BPs

In line with pre-clinical results, a large meta-analysis has demonstrated that administrating N-BPs in the adjuvant setting provides a survival benefits for breast cancer patients (Wilson et al., 2018). The application of N-BPs has the potential to be a therapy with a dual effect on both bones and overall survival for postmenopausal women patients, who are more susceptible to experience slower bone turnover during the progression of breast cancer.

Clinical trials actively apply N-BPs as bone modifying agents. Agents that prevent osteoclast maturation by binding to RANKL in combination with N-BPs prevented bone turnover and skeletal-related events (SREs) in breast cancer patients

with bone metastases (Lipton et al., 2008). Postmenopausal women with hormone-receptor positive breast cancer had increased bone mineral density (BMD) and inhibited bone turnover markers within 2-year period of risedronate application (Greenspan et al., 2008). In the clinical trial BCINIS, N-BPs therapy for over 18 months resulted in increased survival rate across different breast cancer types in postmenopausal women (Rennert et al., 2017). AZURE trial results demonstrated a reduced fracture rate in both ER-positive and ER-negative BC patients in both pre- and postmenopausal patients, after adjuvant treatment with zoledronate (Wilson et al., 2018). Comprehensive meta-analysis of 26 clinical trials with over 19 000 participants reported that BP treatment (both non-N-BP and N-BP) significantly reduced mortality and distant recurrence, specifically in bones, compared to local recurrence in postmenopausal women. Likewise, in patients with bone metastases, the study showed inhibition of bone resorption and SRE. Treatment with BPs demonstrated a dual benefit by reducing distant metastases and preventing bone resorption. However, BP treatment did not have an obvious effect in premenopausal women (Coleman et al., 2015).

One downside of using N-BPs as a treatment for cancer patients with bone metastases is the risk of developing adverse effects, such as bisphosphonate-related osteonecrosis of the jaw (BRONJ). BRONJ is an uncommon side effect specific to the drug's application, occurring in less than 1% of patients, as reported in the SWOG0307 clinical trial (Kizub et al., 2021). Other adverse effects include renal dysfunction, and 10–30% of all patients treated with N-BPs may experience after drug infusion an acute-phase reaction that lasts for a few days, resembling flu-like symptoms. However, the rate of adverse effects is relatively small compared to the benefits of N-BP treatment (Drake et al., 2008; Papapetrou, 2009).

Table 1 demonstrates completed clinical trials focused on both patient survival and skeletal-related changes after N-BP treatment in other cancer types than breast cancer.

Table 1. Application of nitrogen-containing bisphosphonates in cancers.

TRIAL	TYPE OF CANCER	ENROLMENT	TREATMENT	RESULTS
NCT00083382 (Barlogie et al., 2008)	Multiple myeloma	83	Pamidronate Thalidomide Zoledronate	Combined therapy of N-BPs with antiangiogenic agents increased a survival potential.
NCT00104650 (Fizazi et al., 2009)	Advanced cancer Multiple myeloma Bone metastases	111	Pamidronate Zoledronate Denosumab	Treatment with N-BPs leaded to suppression of markers of bone turnover in patients with multiple myeloma.
NCT00321620 (Fizazi et al., 2011)	Prostate cancer Bone metastases	1904	Zoledronate Denosumab	Castration-resistant prostate cancer patients with bone metastases showed similar response to both drugs to prevent SRE.
NCT00172042 (Scagliotti et al., 2012)	Non-Small-Cell lung cancer	437	Zoledronate	No difference between zoledronate vs placebo in overall survival or prevention of bone metastases.
NCT00577642 (Patel et al., 2014)	Multiple myeloma	29	Zoledronate Myeloma therapy	Patients who responded to myeloma therapy showed also reduced SRE.
NCT00330759 (Henry et al., 2014)	Multiple myeloma Bone metastases	1779	Zoledronate Denosumab	Both drugs prevented SRE. Denosumab was more effective than zoledronate.
NCT00216060 (Hahn et al., 2014)	Metastatic prostate cancer	63	Risedronate	Risedronate altered bone turnover markers, but did not affect patient survival compared to placebo.
NCT01345019 (Raje et al., 2018)	Multiple myeloma	1718	Zoledronate Denosumab	Overall survival and SRE were similar between drugs. Adverse events were observed more after denosumab treatment.

2.3.2.3 Reformulation of N-BPs

N-BPs' half-life is several months after they bind to bones (Xiang et al., 2020), but they stay in circulation from 30 minutes to 2 hours (Van Acker et al., 2016). Several strategies to enhance their bioavailability and increase intratumoral concentrations have been suggested (Zhong & Li, 2021).

Liposomes (LIP) are lipid bilayer particles widely used in preclinical studies due to the enhanced permeability and retention effect in tumors (Maruyama, 2011). N-

BP encapsulation in liposomes allows for reduces drug dosing with better tumor targeting. Liposome-encapsulated zoledronate (ZOL-LIP) has increased circulation time and lowered affinity to bones (Shmeeda et al., 2013).These reformulated N-BPs have been shown to have better tumor targeting in several solid tumor models, with limited accumulation in normal tissues (La-Beck et al., 2021).

Calcium-bound reformulation of zoledronate increased IPP accumulation within first hours the drug was introduced to cancer cells in comparison to LIP-ZOL. However, cells retained higher concentrations of IPP upon LIP-ZOL after 24–72 hours (Zlatev et al., 2016). Another approach is to use of nanoparticles with 1 – 100 nm sizes. They have high mobility and specificity to surfaces. They are designed to release their drug load only when internalized by cells. Nanoparticle spatially controlled delivery system of N-BPs is therapy that lately applied in pre-clinical studies to target cancer cells. These drugs showed anti-cancer activity against breast and lung cancer metastases in bones (Zhong & Li, 2021). BPs can be also encapsulated also with polyethylene glycol (PEG), a polymer approved to use for drug delivery in humans. The administration of of zoledronate conjugated with PEGylated lipid bilayer-gated silica nanoparticles resulted in decreased viability and proliferation of TNBC cells in vitro (Desai et al., 2017). PEGylation increased efficacy of alendronate to reduce tumor growth (Rajan et al., 2018). Liposomal formulation combining alendronate with doxorubicin led to higher accumulation of doxorubicin in tumors and a highest probability of mice survival compared to free drug or encapsulated doxorubicin alone in a murine fibrosarcoma model (Islam et al., 2022). This PEGylated liposomal treatment of alendronate/doxorubicin also reduced tumor formation from murine lung cancer cells and TNBC cells (Shmeeda et al., 2016).

2.4 Overview of the adenosinergic pathway

In the adenosinergic pathway, ATP is hydrolyzed to extracellular nucleosides and inorganic pyrophosphate through a cascade of cell surface-located ecto-nucleotidases or a group of enzymes that convert ATP to adenosine in the cytosol (Figure 2). ATP serves as an energy source produced from glucose during aerobic and anaerobic cellular respiration. It also functions as a neurotransmitter, regulating homeostasis and preventing cell membrane disruption. Cell damage or stress factors, such as hypoxia or malignancy, can increase ATP concentration in cells (Vultaggio-Poma et al., 2020).

2.4.1 Adenosine

Adenosine is an important molecule involved in various cellular signaling processes. Adenosine exerts its effects through activation of G (guanine nucleotide-binding)

protein-coupled adenosine receptors (A1, $A2_A$, $A2_B$, and A3) (Hunsucker et al., 2005) or regulating nucleotide balance in cells. Under normal conditions, the level of adenosine is around 20–300 nM, but it increases in situations where atmospheric oxygen levels are low, during exercise, or in response to stress conditions that could potentially damage cells (S. Li et al., 2013; Hong Liu et al., 2014). The high levels of adenosine can have both protective and detrimental effects depending on the cellular microenvironment (S. Li et al., 2013) and the specific adenosine receptor is activated. A1 and A3 receptors reduce cyclic AMP levels, while $A2_A$, $A2_B$ modulate cyclic AMP levels in cells, thereby influencing cell growth, gene or protein expression regulation. The A1 receptor is involved in proinflammatory responses, cardiovascular pathologies, and prevents lipolysis. The A3 receptor plays a role in regulating anti-inflammation. The $A2_A$ receptor is expressed on nervous system, blood vessels cells, and immune system cells, mediating the dilation of blood vessels and exhibiting anti-inflammation effects. Whereas $A2_B$ receptor is activated under hypoxic or inflammatory conditions (Borea et al., 2018). Adenosine mediates osteoclastogenesis via the $A2_B$ receptor, as demonstrated by the attenuation of mature osteoblast markers (osteocalcin and bone sialoprotein) when an $A2_B$ antagonist is added to osteoblasts after the administration of adenosine (Takedachi et al., 2012). Treatment with an $A2_B$ agonist has been shown to prevent bone loss in osteoporotic mice (Shih et al., 2019). Furthermore, an $A2_A$ antagonist has been found to inhibit osteoclast differentiation and regulate the expression of factors involved in bone homeostasis in an animal model of osteolysis (Mediero et al., 2018).

2.4.2 Ecto-nucleotidases

Ecto-nucleotidases encompass four major groups: ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-5'-nucleotidase, ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), and alkaline phosphatases (APs). The group of nucleotide-specific E-NTPDases comprises eight proteins (NTPDase 1–8) that specifically hydrolyze nucleoside triphosphates and diphosphates (e.g., ATP and ADP) while excluding monophosphates, ADP ribose, or NAD+. NTPDases play a pivotal role in modulating immune responses, controlling vascular hemostasis, and regulating inflammation. These proteins are predominantly expressed in epithelial, endothelial, or neuronal cells (Herbert Zimmermann et al., 2012). In contrast to E-NTPDases, the E-NPP family hydrolyzes ADP ribose, NAD+, ATP, and ADP, encompassing seven ectoenzymes (E-NPP 1–7). The first three enzymes, E-NPP 1–3, are transmembrane alkaline nucleotide pyrophosphatases involved in purinergic signaling pathways. Studies on E-NPP 4–5 have demonstrated that they hydrolyze ATP and GTP, and E-NPP 6–7 enzymes specifically hydrolyze phospholipids (Borza et al., 2022). These proteins play crucial roles in bone

mineralization, B cell differentiation, promoting invasion and migration in cancer cells, as well as contributing to the calcification of vascular smooth muscle cells through pyrophosphate production.

Two groups of ecto-nucleotidates (NTs) can generate adenosine. APs are homodimeric glycosylphosphatidylinositol–anchored (GPI) proteins that dephosphorylate all nucleoside triphosphates (ATP, ADP, and AMP) into their corresponding nucleosides, potentially leading to adenosine production. However, the primary source of adenosine in cells is the catalysis of ribo- and deoxyribonucleoside monophosphates into their respective nucleosides by the nucleotide-specific GPI-anchored ecto-5'-nucleotidase (5'-NT) (Sträter, 2006; Herbert Zimmermann et al., 2012).

2.4.3 Ecto-5′-nucleotidases

The activity of 5'-nucleotidases was first described in the animal brain and retina by J.L. Reis in 1934. The extracted enzymes exhibited faster dephosphorylation of adenosine-5-phosphate (AMP) and inosine-5-phosphate (IMP) compared to adenosine-3-phosphate. In the following decades, 5'-nucleotidase was identified as a membrane glycoprotein in various animal tissues, showing diverse enzymatic activities. However, in the 1970s, Stanley and Luzio demonstrated that 5'-nucleotidase can also be internalized from the cell membrane and exist in a cytosolic form. In animals, 5'-nucleotidases were found to exist in four forms: membrane-bound 5'-NT and three soluble 5'-nucleotidase forms.

The shedding of membrane-bound 5'-NT occurs under physiological conditions through the hydrolysis of its GPI-anchor by endogenous C- and D-phospholipases (Schneider et al., 2019) or proteolytic cleavage by matrix metalloproteinase 9 (Fini et al., 2003; W. Zhang et al., 2018). There are two forms of 5'-NT that exhibit higher affinity for AMP (cytoplasmic 5'-NT-I) and IMP/AMP (cytoplasmic 5'-NT-II). Cytoplasmic 5'-NT-I is a homo-oligomer found in vertebrate heart, stimulated only by ADP, with a molecular mass of 40 kDa. Cytoplasmic 5'-NT-II is also a homo-oligomer, stimulated by ATP and ADP, with an acidic pH of 6.5 and a molecular mass ranging from 52–70 kDa in vertebrates (H. Zimmermann, 1992). The most extensively studied form of ecto-5'-nucleotidases is the membrane-bound CD73.

2.5 Membrane-bound ecto-5′-nucleotidase or CD73

Over 30 years ago, the ecto-5'-nucleotidase (NT-5E) was designated as CD73 after studies, where CD73 activity was inhibited using monoclonal antibodies in human lymphocytes. These antibodies precipitated a protein with a molecular mass of 69

kDa and suppressed its enzymatic activity (L. F. Thompson et al., 1989; Thomson et al., 1990). CD73 functions as a phosphomonoesterase, primarily responsible for hydrolyzing ribonucleoside 5'-monophosphates, including AMP, uridine monophosphate, inosine monophosphate, and cytidine monophosphate. The physiological substrate of CD73, AMP, is hydrolyzed to produce extracellular adenosine and phosphate (Borea et al., 2018).

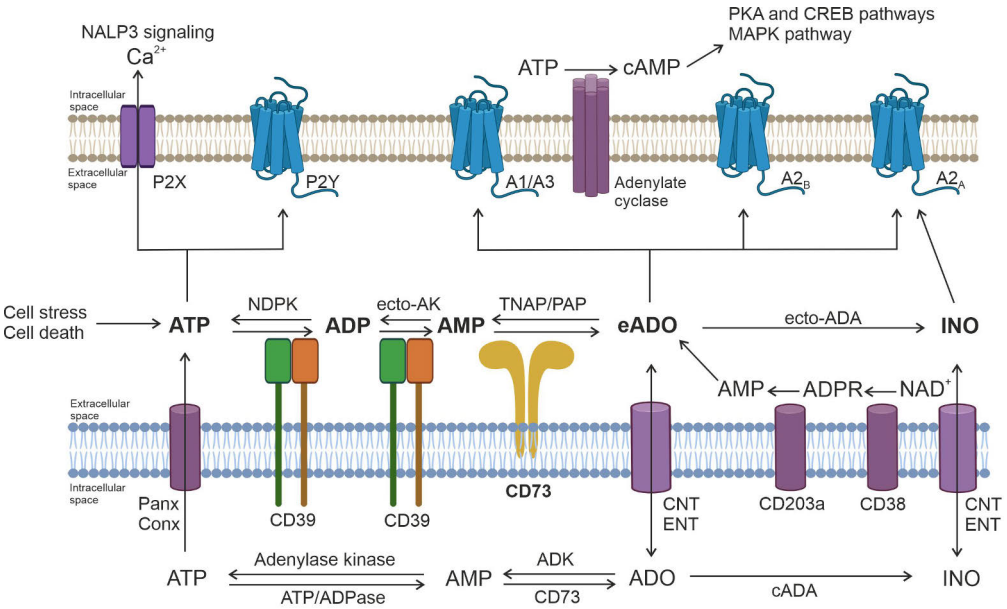

Figure 2. Adenosine signaling. ATP is released into the extracellular space through pannexin (Panx) and connexin (Conx) channels or as a result of cell stress and cell death. Extracellular ATP activates P2 purinergic receptors (P2X, P2Y), leading to the activation of NALP3 inflammasome signaling. There are two pathways for adenosine generation. In the canonical pathway, ATP is hydrolyzed to AMP by ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39), and then to extracellular adenosine (eADO) by CD73. A smaller amount of eADO is generated from AMP by tissue-non-specific alkaline phosphatase or prostatic acid phosphatase. AMP can be converted to ADP by the membrane-associated form of adenylate kinase (ecto-AK), and then back to ATP by nucleoside diphosphate kinase (NDPK). In the non-canonical pathway, NAD+ is converted to ADP-ribose (ADPR) by CD38, and then to AMP by ecto-nucleotide pyrophosphatase/phosphodiesterase family member 1 (CD203a). Additionally, eADO is catabolized to inosine (INO) by membrane-associated adenosine deaminase (ecto-ADA). Extracellular adenosine stimulates type 1 purinergic (P1) receptors: A1, A2A, A2B, A3, which activate cyclic AMP-dependent protein kinase A or mitogen-activated protein kinase cascades. Extracellular adenosine is transported to the intracellular space by nucleoside transporters (CNT/ENT). Once in the cytosol, adenosine can be catabolized to inosine by cytosolic ADA and transported back to the extracellular space by the nucleoside transporters. Cytosolic adenosine is converted to ATP by adenosine kinase (ADK) and ATP/ADPase. Modified from Allard et al., 2020. Created in BioRender.com.

2.5.1 Structure of CD73

The mature human CD73 consists of 548 amino acids and has a homodimeric structure that exists in open and closed confirmations (Figure 3). The protein has two subunits, each of which consists of two domains. The crystal structure analysis revealed that the N-terminal domain has four-layered α/β-β-β-α structure and a metal ion binding site. The C-terminal domain also consists of four-layered structure, but with a different configuration of α helix and β strands – α/β-β-α-β. The C-terminal domain has the substrate binding site and attaches to the cell membrane by glycosylphosphatidylinositol anchor (GPI-anchor). Two C-terminal domains provide dimerization of the enzyme. A single α helix connects N- and C- domains and provides large structural changes between open and closed conformations of CD73. The eukaryotic and bacterial NT5E conduct up to 114º intrachain rotation, involving both domains. However, interchain rotation involves two C-terminal domains (Knapp et al., 2012). AMP, as a substrate for CD73, binds to the C-terminal domain from the substrate binding site.

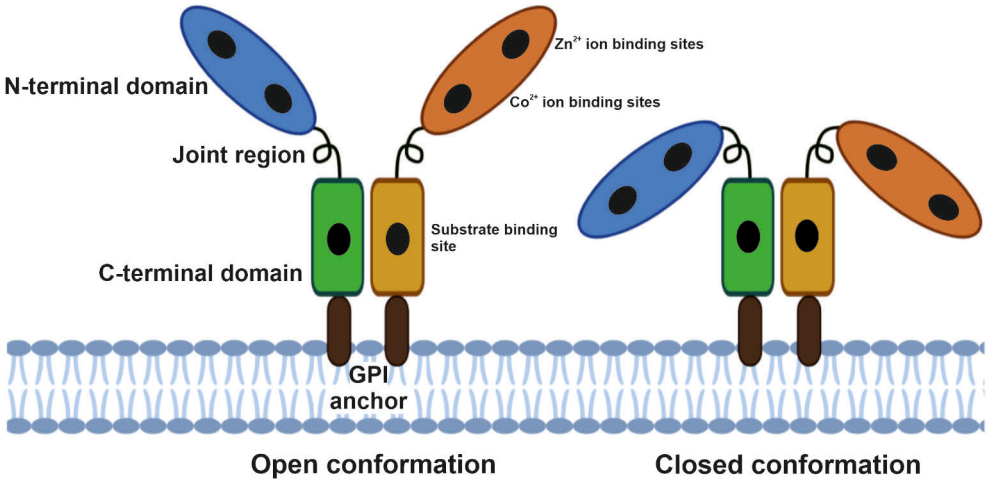

Figure 3. CD73 structure. The receptor exists in two conformations: open and closed. The homodimer is composed of N- and C-terminals. The N-terminus contains two metal ion binding sites, while the C-terminus houses the AMP binding site. The C-terminus is GPI-anchored to the cell membrane. Inhibitors induce the closure of the receptor. Inside the closed conformation, the active site with two metal ions and the substrate binding site are located. This arrangement protects the active site from AMP, thereby preventing the production of adenosine. Modified from Knapp et al., 2012. Created in BioRender.com.

2.5.2 Regulation of CD73 expression

The regulation of CD73 occurs due to the binding of transcription factors (TFs) to CD73 promotor or in the posttranslational regulation of CD73 expression by microRNAs (miRNAs) (Kordaß et al., 2018). MiRNAs are a class of small single-stranded non-coding RNA molecules. Primary transcripts of miRNAs are cleaved in the nucleus (pre-miRNA) and exported into the cytoplasm. Pre-miRNA incorporates to RNA-induced silencing complex, binds to 3' untranslated regions (3'-UTR), leading to post-transcriptional repression on mRNAs (Vidigal & Ventura, 2015). Around 16 miRNAs are involved in CD73 gene regulation, however, only 5 miRNAs demonstrate direct targeting of its expression. In cancer, miRNA-422 and miRNA-30 family negatively correlates with CD73 expression. MiRNA-340, miRNA-187 and miRNA-193b directly target CD73 in cancer cells (Kordaß et al., 2018). Other miRNAs act through indirect targeting of SP1, SMADs family, GFI-1 or HIF1α TFs (Kordaß et al., 2018).

Transcription factors recognize and bind to specific DNA response elements, which can be in the promoter, many kilobase pairs away from the promoter or in the enhancer (S. Kim & Shendure, 2019). CD73 is a direct target for SP1 and SMAD TFs, which has been shown in silico analysis of putative CD73 promotor in rat hepatocytes. First, the truncation of the luciferase constructs narrowed the region of the response element location to positions –159 to –11 bp. The analyzed minimal promotor contained two SP1 and one SMAD transcription binding sites. The mutation of each site reduced luciferase activity of the promoter. Thus, the binding of single response element of SP1 or SMAD was sufficient to increase expression of CD73 gene (Fausther et al., 2012). Another element directly binds to CD73 promoter is β-catenin in Wnt pathway. Transfection of cell to express β-catenin in a constant rate demonstrated enhanced activity of CD73 promoter in T cells. However, Hela cells have shown activation of CD73 only after additional transfection of T cell factor-1. Thus, activation of CD73 via β-catenin might require expression of T cell factor-1 and lymphoid enhancer-binding factor-1 TFs (Spychala & Kitajewski, 2004). Analysis of CD73 murine gene has revealed a presence of two putative binding site for Stat3 and GFI-1. However, GFI-1 affects ecto-nucleotidase transcription via Stat3 suppression. Stat3 TF ensures differentiation of Th17 cells. Authors showed that Th17 cells also expressed CD73 on their surface and were able to convert ADP to adenosine. The binding of Stat3 to CD73 promoter might reduce production of IFN-γ, resulting in immunosuppression (Chalmin et al., 2012).

Tumor microenvironment is typically hypoxic, which made hypoxia-induced factor-1 (HIF-1) an activator of CD73. Regulation of CD73 occurs through HIF-1 DNA motif (CCGTG) binding to the promotor of CD73 gene as was shown in epithelial cells. The transfection of cells with transient CD73 promoter showed hypoxia-inducibility after exposing cells to hypoxia. However, the graduate

truncation of the promoter sequence led to loss of hypoxia-inducibility. The truncation of the promoter sequence narrowed the region of the motif location to positions –367 to –371 in relation to the start site of major transcription. (Synnestvedt et al., 2002). In TNBC cells, HIF-1α, HIF-2α and HIF-1β bind in the first intron of CD73 in response to hypoxia. (Samanta et al., 2018). CD73 is also a direct target for the epithelial – mesenchymal transition (EMT) transcriptional factor SNAI1 in TNBC cells. SNAI1 binding motifs (CAGGTG and CACCTG) are present in the proximal promotor of CD73 gene. Such binding increases expression of CD73 and vimentin (mesenchymal marker) and decreases expression of E-cadherin (epithelial marker). SNAI1 binding to CD73 in epithelial-like TNBC cells affects also CD73 function that leads to increased amount of extracellular adenosine level (Hasmim et al., 2022). EMT transcription factors also regulate CD73 expression through affecting immune cells. For example, infiltrated MDSCs in tumors produce cytokines and TGF-β, which induce Snail1 expression (Lambies et al., 2019), thereby enhancing CD73 expression in cells (Hasmim et al., 2022). Another study investigated the effect of M2-type macrophages on EMT in TNBC cancer cells and demonstrated that M2-type macrophages induce Snail protein expression in those cancer cells (Xiangzhou Chen et al., 2022).

2.5.3 Expression of CD73 in cells

CD73 is expressed on various type of cells. On epithelial cells it maintains epithelial homeostasis (Hara et al., 2022), vascular smooth muscle cells (Sutton et al., 2020), intestinal mesenchymal cells that reduce inflammation during colitis (Hidalgo-Garcia et al., 2021). The high expression of CD73 was detected on endothelial cells in blood (Sutton et al., 2020) and lymph (Eichin et al., 2021) vessels. In bones, CD73 is expressed on both osteoblasts and osteoclasts (Mediero et al., 2018; Shih et al., 2019). Osteoprogenitor cells or osteoclasts with the lack of estrogen receptors demonstrated decreased CD39/CD73 level, resulting in decreased level of adenosine in murine model of postmenopausal osteoporosis. Estrogen also decreased the expression of CD39 and CD73 on hematopoietic or nonhematopoietic cells in bone marrow (Shih et al., 2019). CD73 modulated bone mineralization and osteoclastogenesis (Takedachi et al., 2012), possibly through adenosine receptors (Mediero et al., 2018; Shih et al., 2019). Analysis of immune cells demonstrated that most of human B cells, CD8 T cells, innate-like T cells and small subset of CD4 T cells expressing CD73, whereas human Tregs rarely show its expression (Schneider et al., 2019). In mouse, its expression is on CD8, CD4 T cells and Treg cells (Shevchenko et al., 2020), natural killer cells, peritoneal macrophages and only mature and germinal center B cells (Schneider et al., 2019). Many studies showed a high CD73 expression in different cancer cells as will be discussed further (Cerbelli

et al., 2020; Y. H. Chen et al., 2021; He et al., 2021; Rocha et al., 2021; Tahkola et al., 2021; A. Tripathi et al., 2020).

2.6 The role of CD73 in cancer progression

CD73 supports tumor progression via creating an immunosuppressive microenvironment or as a signal molecule that supports cancer cell division and migration. CD73 maintains the balance of immunomodulating ATP and immunosuppressive adenosine ratio in cells, affecting recruiting of immune cells into tumors (Figure 4). This possible due to the different distribution of CD73 from cells to tissues, adenosine short half-life (< 1s) and sensibility of cells to adenosine. Adenosine acts as an anti-inflammatory molecule that promotes tumor progression in synergy with immunosuppressive cells (Minor et al., 2019; Schneider et al., 2019; Silva-Vilches et al., 2018).

2.6.1 Effects of cancer cell-expressed CD73 on immune cells in tumors

2.6.1.1 Immunosuppressive cells

Some types of immune cells support survival of malignant cells, subsequently contribute to their dissemination and activation in distant sites. To these immunosuppressive cells belong tumor-associated macrophages (TAMs), regulatory T cells (Treg), myeloid-derived suppressor cells (MDSCs), cancer-associated neutrophiles or fibroblasts (Cassetta et al., 2019; M. L. Chen et al., 2005; Lesokhin et al., 2012; H. Peng et al., 2020).

Myeloid-derived suppressor cells are immature neutrophils and monocytes lineage cells with immunosuppressive features to support cancer cell dissemination (Lesokhin et al., 2012). Cancer patients with high MDSC infiltration have worse OS (Tomiyama et al., 2022). Low CD73 expression reduces monocytic MDSCs infiltration in tumors (King et al., 2022; Montalbán del Barrio et al., 2016) and their low number did not show suppressive effect on $CD4^+$ or $CD8^+$ T cells (King et al., 2022). MDSCs are expanded under granulocyte-macrophage colony-stimulating factor stimulation and promote immunosuppressive function by secreting transforming growth factor β (R. Li et al., 2022). A low amount of adenosine decreased secretion of these two factors (King et al., 2022). MDSCs are precursor cells of TAM in tumors (Kumar et al., 2016).

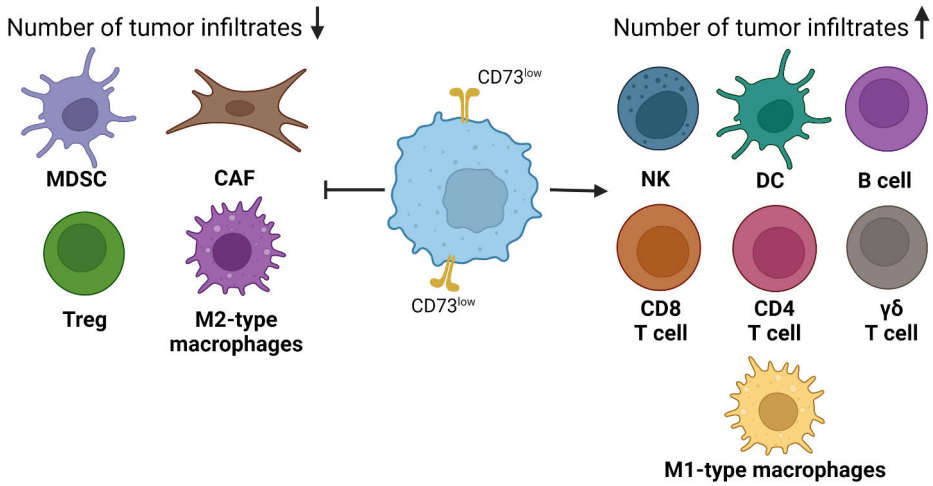

Figure 4. Effect of tumor cell-expressed CD73 on immune cells in tumor microenvironment. Low CD73 expression on cancer cells reduces the number of immunosuppressive cells in tumors, including myeloid-derived suppressor cells (MDSCs), cancer-associated fibroblasts (CAFs), regulatory T cells (Tregs), and M2-type macrophages. Conversely, low CD73 expression on cancer cells enhances the number of immunomodulating cancer cells, such as natural killer (NK) cells, dendritic cells (DCs), and effector B and T cells. Created in BioRender.com.

High number of TAMs indicates tumor aggressiveness and poor survival across breast cancer subtypes (Cassetta et al., 2019). Macrophages under high adenosine exhibited lower expression of DNA repair-related genes and enrichments of immunosuppression-related genes, particularly PD-L1. CD73 inhibition could attenuate this effect, reducing IL-10 expression and increasing TNF-α expression in macrophages (Noh et al., 2022). Co-culture of ovarian cancer cells, highly expressing CD73, stimulated monocyte-derived cell polarization to M2-type macrophages (Montalbán del Barrio et al., 2016). These macrophages decreased CD4 T cell numbers in the co-culture experiments. This effect again was attenuated by CD73 inhibition (Montalbán del Barrio et al., 2016). Whereas, in pancreatic tumor-bearing mice, low CD73 expression increased a number of M1-type macrophages but did not alter numbers of M2-type macrophages or Treg cells (King et al., 2022).

Cancer-associated fibroblasts (CAFs) – tumor stromal cells, exhibiting immunosuppressive phenotype. Cancer cells can stimulate differentiation of fibroblasts into CAFs by release of growth factors. CD73 is expressed on CAFs and cancer cells (H. Peng et al., 2020; Yu et al., 2020). The total CD73 expression in tumors correlated with CAF infiltration. These cells could generate 20 times higher amount of adenosine, than T cells or myeloid cells and they required CD73 expression for immunosuppression of T cells (Yu et al., 2020). Tumor cell-expressed

CD73 expression positively correlated with CAFs infiltration. These cells could increase CD73 expression and diminish sensitivity of $CD73^+$ cancer cells to chemotherapy in pre-clinical study (H. Peng et al., 2020).

Regulatory T cells (Treg) are an immunosuppressive subset (5–10%) of $CD4^+$ cells that support cancer progression. Treg cells inhibit cytotoxic activity of effector T cells to target cancer cells (M. L. Chen et al., 2005). High CD73 expression in tumors correlated with high expression of Treg signature genes in patients with renal cell carcinoma (A. Tripathi et al., 2020). Adenosine, released by cancer cells under stress conditions, such as radiation increased a number of Treg cells in tumors. CD73 blockage attenuated this effect (Wennerberg et al., 2020). Treg cells express CTLA4, which is a target in immunotherapy and high expression of each has been shown in TNBC (Z. Peng et al., 2020). Blockage of CD73 sensitized cancer cells to CTLA4 and PD-L1 inhibitors. However, the synergy of these inhibitors required the presence of cytotoxic $CD8^+$ T cells (B. Allard et al., 2013).

2.6.1.2 Immunomodulating cells

Types of immune cells which display immunomodulating function in malignancy are natural killer cells, dendritic cells, T cells and B cells (M. L. Chen et al., 2005; Häusler et al., 2011; King et al., 2022; Okuno et al., 2020; Wennerberg et al., 2020).

Natural killer cells are innate immune cells with ability to lyse transformed cells. Low CD39 or CD73 expression or blockage of enzymatic activity of receptors increased lysis ability of NK cells against cancer cells (Häusler et al., 2011). In TNBC, a high natural killer cells infiltration correlates with better DFS and OS. Moreover, a greater number of patients had a tumor size over 2 cm and more lymph metastases when a number of stromal natural killer cells was low (Tian et al., 2016).

Dendritic cells are antigen-presenting cells, which prime T cells in cancer progression. Adenosine increased expression of genes with Treg signature on dendritic cells (Pang et al., 2021). CD73 inhibition in tumors enhanced a number of conventional dendritic cells, but only synergizing with tumor radiation. Infiltrations of this subset of dendritic cells induced by radiation primed CD8 T cells (Wennerberg et al., 2020).

T cells are adaptive immune system cells, which are cytotoxic for cancer cells, except immunosuppressive activity of Treg cells (M. L. Chen et al., 2005). A whole-tumor gene analysis has demonstrated that TNBC tumors which have a large infiltration of CD8 T cells ensure higher survival rate. Tumors with higher number of CD8 T cells exhibit also greater infiltration of memory CD4 T cells, M1-type macrophages and B cells (Oshi et al., 2020). Cancer cells demonstrate direct and indirect interaction with immune cells. Cancer cells secrete exosomes, which can directly interact with immune cells. It has been shown that exosomes have CD73

ectonucleotides activity, thus being able to hydrolyze AMP to adenosine in bladder cancer cells. These exosomes were able to inhibit anti-cancer effects of T cells *in vitro* (Clayton et al., 2011). Cancer cells stimulate immune cells to secret cytokines, thereby supporting cancer cell survival by evading immune surveillance. The blockage of CD73 enzymatic activity could enhance activation of cytotoxic T cell response via production of IFN-γ and TNF-α (Tu et al., 2022). Increased infiltration of CD8 T cells to tumors could be regulated via adenosine receptors. Genome-wide knockout of adenosine receptor $A2_B$ in mice promoted infiltration of CD8 T cells to tumors. In this model CD8 T cells showed ability to target cancer cells, resulting in smaller tumor formation in comparison to wild-type mice. The activation of CD8 T cells is dependent on dendritic cells, which infiltrated tumors in higher amounts in mice with knockdown of $A2_B$ (S. Chen et al., 2020). The level of cytokine secretion in CD73 knockdown tumors was reduced resulting in ´´ enhanced infiltration of $CD4^+$ and $CD8^+$ T cells. Since tumor growth was decreased more in immunocompetent mice than in athymic mice, presence of both of these cell types are required to reduce tumor size in low CD73 tumor conditions (King et al., 2022). However, low CD39 or CD73 expression on cancer cells promoted greater proliferation of $CD4^+$ T cells (Häusler et al., 2011), showing anti-cancer activity (King et al., 2022).

γδ T cells are a small subset of T cells that belong to innate immune system and contribute to anti-tumor response. Infiltration of these cells was observed in tumors following chemotherapy, which correlated with improved OS of cancer patients (J. Wang et al., 2017). The suppression of Treg cells by γδ T cells requires the expression of adenosine receptor $A2_A$ on γδ T cells, while an adenosine analogue increased γδ T cell number (Liang et al., 2018). Cancer samples with high CD73 expression exhibited a low infiltration of CD8 and γδ T cells (Q. Chen et al., 2020).

B cells are adaptive immune system cells that function to produce antibodies. Infiltration of B cells has demonstrated anti-tumor activity, leading to better overall survival of cancer patients, but in the presence of T cells (Shi et al., 2013). On the contrary, a small subset of B cells known as regulatory B cells has demonstrated pro-tumor activity. Tumor growth progression may also be supported by B cell-induced immunosuppressive cytokines (Horikawa et al., 2011). Also, some studies have shown that the lack of B cells in tumor microenvironment reduced tumor growth (Bodogai et al., 2013; Maglioco et al., 2017), by increasing the number of effector immune cells in lymph nodes (Maglioco et al., 2017). B-cell infiltration has been correlated with a high number of CD8 T cells in tumors (Shi et al., 2013). Moreover, a high number of T cells and B cells but not B cells alone have been associated with better outcomes in TNBC, (Wortman et al., 2021).

2.6.2 Effects of CD73 on cancer cells

Manipulation with CD73 expression has allowed to investigate the mechanism in which CD73 is involved in cancer progression (C. Liu et al., 2022; X. L. Ma et al., 2019; Xu et al., 2020; H. Zhang et al., 2022). CD73 promotes the survival on cancer cells (C. Liu et al., 2022; Xu et al., 2020; H. Zhang et al., 2022). Cells with lower CD73 expression exhibited decreased cell viability and proliferation (Xu et al., 2020; H. Zhang et al., 2022). CD73 promotes cells motility through RhoA/LIMK/cofilin signaling, and high expression of CD73 leads to increased phosphorylation of cofilin (Xu et al., 2020). The cofilin pathway regulates protrusion formation by activating actin polarization (Hotulainen et al., 2005; Virtanen et al., 2018). Protrusion formation and cell motility are associated with EMT in cancer cells. Breast cancer cells with EMT features are more invasive and have a higher ability to form metastases. While cancer cells with mesenchymal-epithelial transition exhibit slower migration (Lüönd et al., 2021). The influence of CD73 on cancer cells migration has emphasized the involvement of invasion-related factors. Studies have demonstrated a correlation between CD73 and EMT genes (Reinhardt et al., 2017). The regulation of EMT by CD73 is also associated with AKT and EGFR signaling (Z. Gao et al., 2017; C. Liu et al., 2022; H. Zhang et al., 2022). AKT signaling is involved in cancer cell survival and proliferation (Datta et al., 1997), and can be activated by EGFR (Okano et al., 2000). Genomic analysis has shown a positive association between CD73 expression and EGFR and AKT1 (Z. Gao et al., 2017). CD73 suppression decreased phosphorylation of EGFR or AKT, leading to G2/M phase cell cycle arrest and a switch to an epithelial-like phenotype in cancer cells (X. L. Ma et al., 2019; H. Zhang et al., 2022). Suppression CD73 expression reduced tumor growth in pre-clinical studies of cancer cells (King et al., 2022; Xu et al., 2020).

There are currently no existing methods to reduce CD73 expression in humans that would result in a decrease in adenosine levels. Therefore, CD73 is blocked pharmaceutically using CD73 inhibitors, which maintain a membrane-bound and soluble form in the closed conformation (Figure 3). In this conformation, the active site is protected from solvents, preventing the production of adenosine (Bhattarai et al., 2015; Junker et al., 2019). The production of adenosine by CD73 plays a key role in modulating immune suppressive responses in cells (D. Allard et al., 2019). Among the CD73 inhibitors, 5'-(α,β-methylene) diphosphate adenosine (APCP) is considered one of the most potent inhibitors. APCP induces a closed conformation of CD73, forming the active site with two metal ions and the substrate binding site inside the conformation (Knapp et al., 2012). While APCP significantly decreases the enzymatic activity of CD73, it does not affect breast cancer tumor growth (Stagg et al., 2010). To enhance the activity of APCP, its structure has been used as a base to combine with commercially available purine nucleoside analogues such as clofarabine. This recombinant inhibitor efficiently reduced CD73 enzymatic activity

in comparison to APCP (Dumontet et al., 2018). APCP is a competitive inhibitor, and its efficacy and metabolic stability are lower when the substrate concentration is high. Allosteric inhibitors are non-competitive because they have the ability to bind to the target outside the binding site, which can increase the stability of CD73 inhibition (Rahimova et al., 2018). The monoclonal antibody MEDI9447, which is an allosteric inhibitor, was capable of blocking soluble and membrane-bound CD73 in a non-competitive manner by binding to the N-terminal site. This monoclonal antibody was also able to bind to CD73 in both open and closed conformations, resulting in reduced AMP hydrolysis, although it did not prevent AMP binding (Geoghegan et al., 2016). Allosteric inhibitors reduced CD73 enzymatic activity, leading to decreased viability of human and murine TNBC cancer cells and reduced tumor growth (Hay et al., 2016; Tu et al., 2022), making CD73 a valuable target in clinical investigation.

2.6.3 CD73 as a prognostic biomarker in cancer

CD73 is expressed in various cancer types, such as TNBC (Cerbelli et al., 2020), pancreatic cancer (Tahkola et al., 2021), gastric cancer (He et al., 2021), renal cell carcinoma (A. Tripathi et al., 2020), esophageal squamous cell carcinoma (Y. H. Chen et al., 2021) or lung adenocarcinoma (Rocha et al., 2021). High expression of CD73 in cancer has been associated with cancer aggressiveness, patient survival or response to treatment. Analysis of online databases revealed a high CD73 expression in pancreatic cancer, which correlated with worse OS or shorter DFS, as well as with a high PD-L1 expression on cancer cells (Q. Chen et al., 2020). These findings were confirmed on patient samples, where positive expression of CD73 was negatively associated with the OS of patients with pancreatic ductal adenocarcinoma (Tahkola et al., 2021; L. Zhou et al., 2019). In renal cell carcinoma, the 5-year disease-free survival (DFS) or 10-year overall survival (OS) rates were the lowest in cohorts of patients with high CD73 expression compared to cohorts with negative CD73 expression. The percentage of tumors with high CD73 expression increased during cancer progression, with the highest percentage observed in grade IV tumors, indicating high cell division and increased angiogenesis (A. Tripathi et al., 2020). CD73 was suggested as an independent prognostic marker for worse DFS or OS in esophageal squamous cell carcinoma. Patients with low CD73 expression and high PD-L1 expression showed a higher partial response to immunotherapy, while patients with low CD73/PD-L1 expression had a higher disease progression (Y. H. Chen et al., 2021). Among TNBC patients, low tumor CD73 expression was associated with prolonged disease-free survival compared to high CD73 expression. The poor outcome associated with CD73 in TNBC may be due to immune evasion, as adenosine produced by CD73 may protect cancer cells from adaptive anti-tumor

immune responses (Buisseret et al., 2018). Furthermore, TNBC patients with high CD73 expression showed a low response to anthracycline treatment, in the neoadjuvant setting (Cerbelli et al., 2020; Loi et al., 2013). The association of CD73 with poorer survival in cancer patients emphasizes its potential as a target for improving patient survival or response to anti-cancer treatment.

2.6.4 Clinical application of CD73 targeting

Oleclumab is one of the CD73 monoclonal antibodies that has been used in clinical trials. Several phase I/II clinical trials are currently recruiting breast cancer patients to investigate the use of anti-CD73 antibodies or inhibitors in addition to standard therapy (*https://Clinicaltrials.Gov/*). In one of the ongoing trials, breast cancer patients are being treated with paclitaxel followed by dose-dense doxorubicin-cyclophosphamide. Patients in Arm 2 receive an anti-PD-L1 antibody, durvalumab, while patients in Arm 3 receive oleclumab (NCT03875573, 147 participants). Oleclumab is also being administered in combination with chemotherapy and durvalumab for TNBC patients (NCT03616886, 129 participants). Another clinical trial is investigating the use of an anti-CD73 antibody in combination with an adenosine $A2_A$ receptor antagonist or pembrolizumab in advanced cancers, including TNBC (NCT03454451, 378 participants). A novel monoclonal antibody called PT199 has been developed to block soluble and surface CD73. In a phase I trial, patients with solid tumors, including breast cancer, will receive the anti-CD73 monoclonal antibody either alone or in combination with an anti-PD-1 monoclonal antibody (NCT05431270, 41 participants). The inhibition of CD73 by oleclumab will also be studied in the phase II of the Neo-CheckRay clinical trial. Breast cancer patients will be stratified based on ER-positive, HER2-negative, or Luminal B types. In Arm 1, patients will receive radiotherapy and chemotherapy. Arm 2 will include Arm 1 and a PD-L1 inhibitor, and Arm 3 will involve Arm 1 with the addition of both PD-L1 and CD73 inhibitors. The results of this trial will focus on the safety of combining radiotherapy with immunotherapy and CD73 inhibitors, as well as investigating the pCR rate achieved with these treatments (De Caluwé et al., 2021).

3 Aims

Due to the high heterogeneity of TNBC, patients respond differently to standard therapy. Therefore, there is a need for biomarkers that can identify patients who would benefit from bisphosphonate treatment. In recent years, there has been growing interest in CD73 as a potential biomarker in cancers, which has prompted this thesis project to further investigate how CD73 contributes to TNBC progression. Additionally, this project aims to study the effects of nitrogen-containing bisphosphonates on TNBC cells and whether the reformulation of zoledronate can improve tumor targeting.

Aim 1. To compare the effects of suppressing CD73 expression or blocking its enzymatic activity on TNBC cell viability and motility. Furthermore, to investigate the effect of suppression of CD73 expression on TNBC tumor growth and formation of metastases *in vivo*.

Aim 2. To investigate the effect of CD73 suppression on tumor progression and the infiltration of lymphocytes into tumors and lung metastases in the presence of zoledronate.

Aim 3. To compare the effects of zoledronate and liposome-encapsulated zoledronate on tumor growth and tumor-associated macrophages in TNBC tumors.

4 Materials and Methods

4.1 In vitro assays

4.1.1 Cell culture (I, II, III)

Triple negative breast cancer cells lacking of ER, PR, and HER2 expression (ATCC, Manassas, VA, USA) were cultured in complete Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% MEM NEAA, 1% L-glutamine and 1% penicillin-streptomycin (all from Gibco, Life Technologies, Paisley, UK). Human T47D cells with estrogen receptor alpha positive (ER^+) expression (ATCC, Manassas, VA, USA) were cultured in complete RPMI medium supplemented with 20% heat-inactivated fetal bovine serum, 10µg/ml insulin, 1% L-glutamine and 1% penicillin-streptomycin (all from Gibco, Life Technologies, Paisley, UK). Cells were cultured in an incubator at 37°C with 5% CO_2. For hypoxia experiments, cells were placed in 1% or 5% O_2 hypoxia for 24 h (InvivO$_{2,}$ Ruskin Technology Ltd.). 4T1 cells were labeled using the pmiRVec retroviral vector, which contains the luciferase2 open reading sequence.

CELL LINE	CELL TYPE	CULTURE MEDIA	MANUSCRIPT
4T1	mouse TNBC	DMEM	I, II, III
MDA-MB-231	human TNBC	DMEM	I, II, III
T47D	human ER^+	RPMI	II
4T1.Luc2	mouse TNBC	DMEM	III

4.1.2 CD73 suppression (I, II)

Approach 1. CD73 expression was downregulated in the 4T1 cells through stable small hairpin RNA (shRNA) transduction, using pSUPER-puro control shRNA (sh-NT) and pSUPER-puro CD73 shRNA (sh-CD73) mouse-specific lentiviral particles, according to the manufacturer's recommendations (Mission lentiviral transduction particles, Sigma-Aldrich). Stably transfected cells were selected using 4 µg/ml puromycin (Gibco, Life Technologies, UK) in complete culture medium.

Approach 2. For enzymatic CD73 inhibition, parental MDA-MB-231, 4T1, and T47D cells were treated for 24 h with 100 μM of Adenosine 5'-(α,β-methylene) diphosphate (APCP, Merck Life Science OY, Finland) prior to further use.

4.1.3 Quantitative and TaqMan RT-PCR (I, II and III)

Total RNA was isolated with RNeasy RNA isolation kit according to the manufacturer's instructions (Qiagen). Quantitative real-time PCR (qPCR) was performed using the SYBR Green qPCR kit (Bio-Rad Finland Oy). CD73 primers were purchased from Bio-Rad Finland Oy. TBP was used as the housekeeping gene in qPCR. For TaqMan RT-PCR, the reaction was performed using TaqMan Universal Master Mix II (Thermo Fisher Scientific, 4427788). Ribosomal 18S RNA was used as the housekeeping gene in TaqMan RT-PCR. Primer sequences and TaqMan probes are presented in Table 2. The results were analyzed using the delta-delta Ct-method by adjusting the Ct-values to those of the housekeeping gene.

Table 2. Primers for quantitative polymerase chain reaction.

GENE	SEQUENCE	MANUSCRIPT
mSP1	Frw 5'– 3' GCA AGA CCT CAC ATC TCC GA Rev 5'– 3' TTT CCC TTG GGT CTT ACT CAC C	I
hSP1	Frw 5'– 3' AGG CGA GAG GCC ATT TAT GT Rev 5'– 3' TTC TCT CCC ATA GGC TCT TGC	I
hTBP	Frw 5'– 3' ACT TCA CAT CAC AGC TCC CC Rev 5'– 3' GAA TAT AAT CCC AAG CGG TTT G	I
mTBP	Frw 5'– 3' GCA GCC TCA GTA CAG CAA TC Rev 5'– 3' CTG CGG TAC AAT TCC AGA GC	I, II
IL-1B	Mm00434228_m1	III
IL-6	Mm00446190_m1	III
IL-10	Mm00439615_g1	III
Ribosomal 18s	MM03928990_g1	III

4.1.4 RNA sequencing (II)

RNA-Seq (RNA sequencing) analysis of 4T1 sh-NT and 4T1 sh-CD73 cells was carried out by LC Sciences (Houston, Texas). To prepare the poly(A) RNA sequencing library, Illumina's TruSeq-stranded-mRNA sample preparation protocol was followed. The integrity of the RNA was assessed using Agilent Technologies 2100 Bioanalyzer. Poly(A) tail-containing mRNAs were purified using oligo-(dT) magnetic beads through two rounds of purification. Reads containing adaptor contamination, low quality bases, and undetermined bases were removed using

Cutadapt (Martin, 2011) and in-house perl scripts. The sequence quality was verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). HISAT2 (D. Kim et al., 2015) was utilized to map the reads to the mouse genome available at ftp://ftp.ensembl.org/pub/release-101/fasta/mus_musculus/dna/. Expression levels of mRNAs were determined using StringTie (Pertea et al., 2015) by calculating FPKM. Differential expression analysis of mRNAs was performed using the R package DESeq2 (Love et al., 2014) for comparisons between different groups, and the R package edgeR (Robinson et al., 2009) for comparisons between two samples. Differentially expressed mRNAs were identified based on the parameters of false discovery rate (FDR) below 0.05 and an absolute fold change of ≥ 2.

4.1.5 Thin layer chromatographic (TLC) analysis of CD73 activity (I, II)

MDA-MB-231 and 4T1 cells were seeded and allowed to attach overnight. RPMI-1640 medium containing 5 mM β-glycerophosphate and 400 μM AMP with tracer [2, 8-^{3}H] AMP (American Radiolabeled Chemicals Inc., Campro Scientific, The Netherlands) was added to 4T1 cells with CD73 suppression. MDA-MB-231 and 4T1 cells for CD73 enzymatic inhibition were pre-treated for 30 min with 100 μM of APCP prior to addition of [^{3}H]AMP substrate. After incubation for 30 min at 37˚C, aliquots of the mixture (8 μl, ~5 x 10^4 dpm/spot) were applied onto Alugram SIL G/UV254 sheets (Macherey-Nagel, Germany) for TLC. [^{3}H]AMP and its dephosphorylated metabolite [^{3}H]adenosine were separated by TLC with isobutanol/isoamyl alcohol/2-ethoxyethanol/ammonia/H_20 (9:6:18:9:15). Ultraviolet light was used to visualize bands with separated nucleotides. The areas containing radioactive substances that migrated together with their corresponding standards were scraped from the TLC sheet into scintillation vials. They were then extracted from silica using 0.1N HCl, and Wallac-1409 β-spectrometer was used to quantify [^{3}H]adenosine level.

4.1.6 Western blotting (I, II and III)

Cells or tissue lysates were prepared in RIPA buffer (Thermo Fisher Scientific) and run on 8–12% gels as indicated in the manuscripts. Membranes were incubated with primary antibodies at 4ºC overnight. After incubation with secondary antibodies, the emitted fluorescence was detected using the Li-Cor Odyssey® CLx imaging system. The list of antibodies is presented in Table 2.

4.1.7 Immunofluorescence stainings (I, II)

Cells were seeded at the density of 1×10^4 on coverslips and stained as indicated in the manuscripts. DAPI was used as a nuclear counterstain. All images were captured with a Nikon Ti2-E fluorescence microscope. CD73 staining intensity and cell protrusions were measured manually using ImageJ/Fiji 1.52 software (Schindelin et al., 2012).

4.1.8 Proliferation assay (I, II)

Cells were seeded onto 96-well plates and allowed to attach overnight. The cells were then treated as indicated in the manuscripts. Cell growth was assessed after the cells reached full confluency. Images for cell growth were acquired using the IncuCyte S3 imaging system (Essen Bioscience). The confluency was analyzed using IncuCyte 2018B software.

4.1.9 Cell migration assay (I, III)

Cells were seeded onto 96-well plates and allowed to attach overnight. The cells were then treated as indicated in the manuscript. Scratch wounds were made using the WoundMaker (Essen Bioscience). The confluency of the wound was monitored for 24 h using the IncuCyte S3 imaging system (Essen Bioscience). The confluences were analyzed using IncuCyte 2018B software.

4.1.10 Cell viability (I, II and III)

Cells were seeded onto 96-well plates and allowed to attach for 24 h. Treatment options for cell viability assay were zoledronate (ZOL) (II, III), alendronate (II), pamidronate (II), clodronate (II) and ApppI (III). Cell viability was measured using the Cell Proliferation Assay Kit (Dojindo, Biotop Oy, Denmark) and quantified using a Tecan microplate reader (Tecan AG, Austria).

4.1.11 Organotypic 3D cultures (I)

Cells were seeded to form organotypic 3D cultures in a Matrigel to collagen I media in ratio 8:2. To assess the number of dead and living cells, the organoids were stained using the Calcein AM fl (Invitrogen). Organoid projections were segmented using the AMIDA software.

4.1.12 Apoptosis and cell cycle assays (II)

Cells were seeded in 6-well plates and allowed to attach overnight. The treatment was done as indicated in the manuscript. The apoptosis assay was performed using Apoptosis Staining / Detection Kit. The cell cycle assay was performed using the Click-iT™ EdU Pacific Blue™ (ThermoFisher Scientific). Flow cytometry samples were analyzed using Flow Cytometry Analyzer BD LSRFortessa™ (BD Bioscience). The data were analyzed using Flowing Software 2.5.1 (Turku, Finland).

4.1.13 Liposome preparation (III)

Empty liposomes (EMP-LIP) and negatively charged zoledronate encapsulated in liposomes (ZOL-LIP) were prepared using the reverse-phase evaporation method (Sousa, Auriola, et al., 2015). The liposome solution was prepared immediately before use in experiments.

4.1.14 Measurement of IPP/ApppI accumulation in breast cancer cells (III)

Cells were plated in 6-well plates and allowed to attach overnight prior to treatment. The amounts of IPP and ApppI were determined in dried acetonitrile/water cell extracts by HPLC-ESI-MS, following the previously described method(Mönkkönen et al., 2000, 2006). The quantification of the molecules was performed using LCquan 2.0 software (Thermo Scientific, Carlsbad, CA, USA).

4.1.15 Colony formation assay (III)

Cell suspensions from bones and lungs were isolated as indicated in the manuscripts. Single cells were seeded in 6-well plates and allowed to attach overnight. Afterward, a 6-thioguanine (Sigma) antibiotic selection was applied for two weeks, allowing the selective growth of antibiotic-resistant tumor cells. Colonies of tumor cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet. The number of colonies was counted using Fiji-ImageJ (1.52p) software (Schindelin et al., 2012).

4.2 In vivo assays

4.2.1 Animal models (I, II and III)

Animals were cared for in accordance with the Project Authorization Board of Finland (license No ESAVI/7015/2020) and in accordance with the 2010/EU/63 EU Directive on the protection of animals used for scientific purposes. In the orthotopic tumor model, cells were inoculated into the 4th mammary fat pads. In the experimental metastases model, cells were inoculated intravenously via the tail vain. Animals were treated as indicated in the Figure 5. Body weight and tumor dimension were measured once a week. Animals were euthanized by CO_2 inhalation.

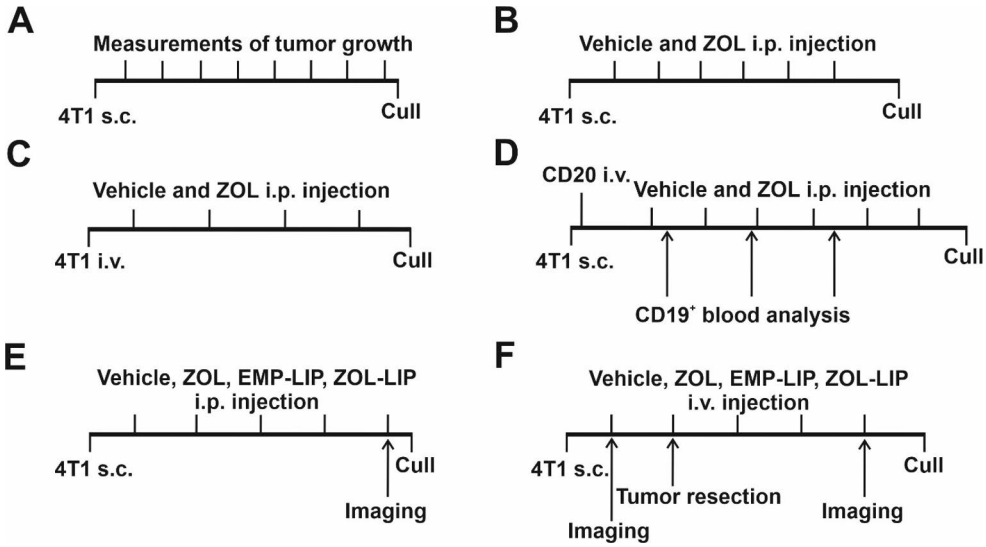

Figure 5. Schematic representation of animal experiments in manuscripts. **A)** Orthotopic tumor model (I). **B)** Orthotopic tumor model with zoledronate treatment (II). **C)** Experimental metastases model with zoledronate treatment. **D)** Orthotopic tumor model with zoledronate and CD20 antibody treatment (II). **E)** Orthotopic tumor model with zoledronate and ZOL-LIP treatment (III). **F)** Orthotopic tumor model with zoledronate and ZOL-LIP treatment with tumor resection (III). s.c. subcutaneously; i.v. intravenously.

4.2.2 B cell depletion (II)

In the B cell depletion model, animals were treated with Ultra-leaf Purified mouse anti-CD20 antibody (BioLegend, 152104) and representative control antibodies. To confirm B cell depletion, splenocytes were isolated and the presence of B cells in spleen samples was detected at different time points. The data were analyzed with Flowing Software 2.5.1 (Turku, Finland).

4.2.3 In vivo bioluminescence imaging (III)

Mice were anesthetized with 2.5% isoflurane before receiving 150 µg/kg of D-luciferin in 1x PBS (Gold Biotechnology, St. Louis, MO) intraperitoneally. Bioluminescence imaging of the mice was performed using a charge-coupled device camera–based bioluminescence imaging system, the IVIS Spectrum (Perkin Elmer, Waltham, MA, USA). The corresponding images were analysed with Living Image software (PerkinElmer, Waltham, MA, USA).

4.2.4 Flow cytometry (II)

Blood from the heart was collected in K2E tubes (BD Microtainer, 1307939) to prevent coagulation. The whole blood was stained with conjugated antibodies against CD19, CD8 and CD3. Cells isolated from the lungs were stained with conjugated CD3 and CD19 antibodies. The analysis of samples was performed Flowing Software 2.5.1 (Turku, Finland). The list of antibodies is presented in Table 2.

4.2.5 Histology (I, II and III)

Dissected tumor and lung tissues were fixed with 10% formalin overnight, followed by changing the buffer to 70% ethanol for an additional 24 h. The tissues were then embedded in paraffin and cut into 5 µM sections. The slides with tissue sections were deparaffinized and hydrated through a xylene and alcohol series for immunohistochemistry and immunofluorescence staining. Lung and liver samples were staining with hematoxylin and eosin, and scanned with Pannoramic 250 slide scanner (3DHISTECH Ltd, Hungary) to count the number of metastases.

4.2.6 Immunohistochemical and immunofluorescence stainings (I, II and III)

The tissue slides were stained as indicated in the manuscripts. The slides with immunohistochemical staining and immunofluorescence staining were scanned using Pannoramic 250 slide scanner (3DHISTECH Ltd, Hungary) and Pannoramic Midi fluorescence slide scanner (3DHISTECH Ltd, Hungary), respectively. For staining analysis, scripts were created in QuPath-0.2.0 software (Bankhead et al., 2017). The staining results were analyzed blindly using QuPath-0.2.0 software. The list of primary antibodies is presented in Table 2.

4.2.7 Bone histomorphometry (II, III) and micro-CT analysis (II)

The decalcified bones were embedded in paraffin. Osteoclasts were stained with tartrate-resistant acid phosphatase method (Merck, Germany). Bone histomorphometry was analyzed using the TrapHisto software (van 't Hof et al., 2017). The number of osteoclasts was manually counted using Fiji-ImageJ (1.52p) software (Schindelin et al., 2012). Quantitative analysis of femurs was performed using a Skyscan 1272 X-ray computer tomography scanner (Bruker, Kontich, Belgium).

4.2.8 Statistical analyses (I, II and III)

The results are presented as mean ± SD of three independent experiments. All analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc, San Diego, CA, USA). Data were analyzed for statistical significance using two-tailed Student's t - test and one-way ANOVA. Differences with a p-value <0.05 were reported as statistically significant. Comparisons between CD73 and E-cadherin or vimentin mRNA expressions in human breast cancer specimens were based on cBioPortal Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) database (1904 samples).

4.2.9 Ethical approval (I, II and III)

All procedures involving animal studies were performed in accordance with the Project Authorization Board of Finland ESAVI/7015/2020 (I, II) and ESAVI/3257/04.10.07/2014 (III) in accordance with the 2010/EU/63 EU Directive on the protection of animals, which are used for scientific purposes. The animal experiments were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, as well as statutes 1076/85 and 1360/90 of The Animal Protection Law in Finland and EU Directive 86/609 and national permissions from the French Government.

Table 3. List of antibodies.

PRIMARY ANTIBODY	IHC	IF	WB	FACS	MANUFACTURER
CD73 (I, II)	1:200		1:1000		NBP2-158015, Novus
E-cadherin (I)	1:100		1:1000		24E10, CST
Vimentin (I)	1:200		1:1000		D21H3, CST
HIF-1α (I)			1:500		610958, BD Bioscience
p27 (II)			1:500		sc-528, Santa Cruz
α- tubulin (I, II)			1:20000		ab4074, Abcam
β-actin (I, III)			1:10000		A1978, Sigma-Aldrich
pHH3 (I, II)	1:500				9701, CST
Cleaved caspase 3 (II)	1:500				9664, CST
Ki-67 (II)		1:500			ab15580, Abcam
CD45R/B220 (II)	1:200				550286, BD Bioscience
CD8 (II, III)		1:100		1:100	557668, BD Bioscience
CD4 (II, III)	1:500				NBP1-19371, Novus
CYR61 (II)	1:2000				NB100-356, Novus
CD3 (II)				1:100	APC-65077, Proteintech
CD4 (II)				1:100	100411, BioLegend
CD4 (II)				1:100	100408, BioLegend
CD21 (II)				1:100	123412, BioLegend
CD23 (II)				1:100	101628, BioLegend,
CD19 (II)				1:100	115508, BioLegend
CD34 (II, III)	1:200				sc-18917, Santa Cruz
CD3 (III)		1:100			ab33429, Abcam
CD206 (III)	1:300				ab64693, Abcam
CD68 (III)	1:100				ab125212, Abcam
INOS (III)	1:300				ab15323, Abcam
NF-kB (III)			1:1000		75338, Full Moon Biosystems
IkBα (III)			1:1000		ab76429, Abcam
p- IkBα (III)			1:500		Full Moon Biosystems
Rab4 (III)			1:500		610888, BD Biosciences
CD80 (III)	1:500				BS-2211R, ThermoFisher Scientific

5 Results

1.1 CD73 suppression prevents cell motility and EMT progression in vitro (I)

Hypoxia is increasing in the tumor microenvironment as tumor progression occurs and the hypoxia levels are higher in the center than in the tumor periphery. TNBC cells were studied under normoxic and hypoxic conditions (1% and 5% O2). Two approaches were employed to suppress CD73 in murine 4T1 and human MDA-MB-231 TNBC cells. In the first approach, the enzymatic activity of CD73 was blocked using the selective inhibitor APCP in 4T1 and MDA-MB-231 cells. In the second approach, CD73 expression was suppressed through stable lentiviral transfection in 4T1 cells. Treatment with APCP or CD73 shRNA resulted in decreased cell viability, proliferation, migration, and protrusion length in normoxic conditions for 4T1 cells (I, Figure 1, 2, and 4). The organoid formation assay demonstrated that CD73 suppression prevented the invasion of 4T1 cells in normoxia. Hypoxia had a similar effect (I, Figure 3). MDA-MB-231 cells were less sensitive to enzymatic blockage, which led to decreased cell viability and protrusion length, but did not significantly alter proliferation, migration, or protrusion length in normoxia (I, Figure 1, 2, and 4). Both hypoxic conditions increased cell viability in all cell lines, although cell viability remained lower in cells treated with APCP or subjected to CD73 shRNA suppression (I, Figure 2). Hypoxia did not significantly affect cell migration. Protrusion length was not changed by hypoxia in 4T1 cells but was increased in MDA-MB-231 cells. APCP treatment decreased protrusion length in 4T1 and MDA-MB-231 cells (I, Figure 4). Both approaches to decrease CD73 increased E-cadherin expression and decreased vimentin protein expression in normoxia. Hypoxia increased vimentin expression, but CD73 suppression kept it lower than in the control groups in vitro.

5.1 CD73 suppression decreases the progression of established tumors and metastases (I)

Suppression of CD73 expression prevented tumor formation, with only half of the mice forming tumors 30 days after cell inoculation (I, Figure 5). CD73 suppression

decreased tumor growth compared to the control group (I, Figure 5). The proliferative marker pHH3 was decreased in CD73-suppressed tumors (I, Figure 5). CD73 suppression reduced the number of lung metastases and prevented their formation compared to the control (I, Figure 5). Similarly, in line with the in vitro effects of CD73 suppression on EMT, tumors showed a significant increase in E-cadherin and decrease in vimentin expression *in vivo*, indicating reduced EMT progression when CD73 was suppressed (I, Figure 6). CD73 might specifically mediate lung metastasis formation, as the number of liver metastases was not significantly changed in the CD73-suppressed group compared to the control group (I, Additional Files).

5.2 CD73 suppression sensitizes TNBC cells to N-BP treatment in vitro (II)

IC50 doses of N-BPs were significantly lower in CD73-suppressed cells compared to cells with normal CD73 expression after 48 h and 72 h of treatment (II, Additional Files). Cell viability was notably decreased after 72 h with all indicated N-BPs; therefore, those doses were chosen for further experiments. N-BPs prevented cell proliferation and caused G1 phase cell cycle arrest in CD73-suppressed cells compared to control cells (II, Figure 2). Furthermore, N-BPs induced more apoptosis in CD73-suppressed cells, as shown by the increased number of apoptotic cells and the positive number of caspase 3/7 cells *in vitro* (II, Figure 2).

5.3 Zoledronate reduces tumor growth and lung metastases independently from CD73 expression (II)

Zoledronate decreased tumor growth in both the control and CD73-suppressed groups when tumors were allowed to grow for 31 or 34 days, respectively (II, Figure 3 and 5). However, the volume of ZOL-treated CD73-suppressed tumors at sacrifice was notably lower than that of ZOL-treated control tumors when tumors were allowed to grow for 34 days (II, Figure 5). ZOL reduced the number of osteoclasts in bones and increased bone volume, which was demonstrated as an internal control in the manuscript (II, Additional files). The proliferative marker pHH3 was decreased in CD73-suppressed tumors in comparison to control tumors. Cleaved caspase-3 was elevated in the CD73-suppressed group upon ZOL treatment (II, Additional Files). There was a trend towards decreased expression of an angiogenesis marker, CD34 in the CD73-suppressed group compared to the control group, but had no effect on expression of another marker, CYR-61 (II, Additional Files). CD73 suppression reduced the number of lung metastases and prevented their

formation compared to the control group, regardless of the influence of ZOL treatment (II, Additional Files and II, Figure 5). Although these results were not included in the original publication, we demonstrated that lung metastases formed from CD73-suppressed cells had a significantly lower number of Ki-67 positive cells. ZOL did not influence the number of Ki-67 positive cells in lung metastases (5.4 session in Results, Figure 6).

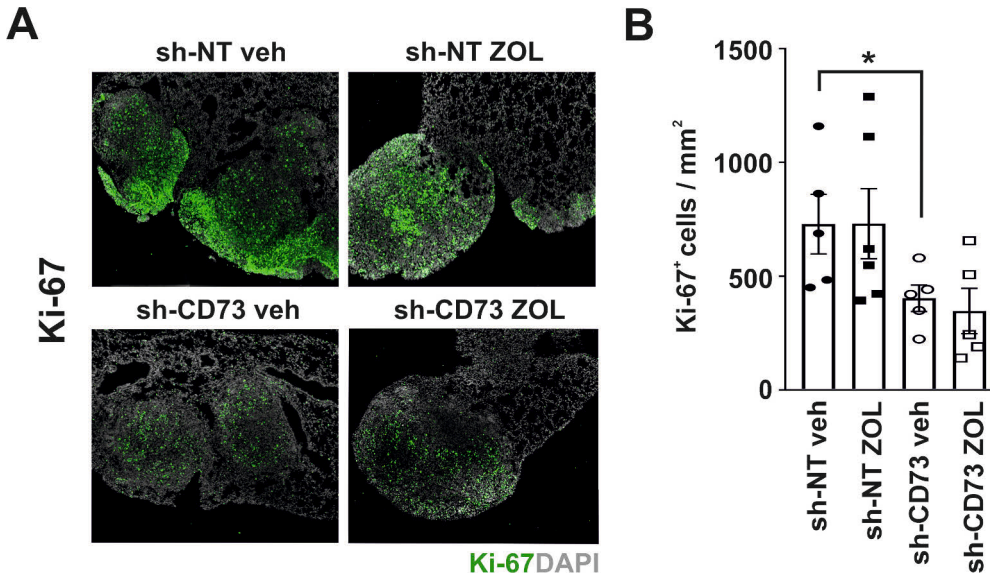

Figure 6. CD73-suppressed cells express less Ki-67. A) Representative images of Ki-67 staining in lung metastases formed from sh-NT and sh-CD73 cells. Scale bar – 200 µm. **B)** Number of Ki-67 positive cells in lung metastases formed from sh-NT and sh-CD73 cells. Data is expressed as mean ± SEM, by one-way ANOVA with a Sidak post-test. * $P < 0.05$; sh-CD73 vs. sh-NT groups.

5.4 CD73 suppression enhances immune cell infiltration upon zoledronate treatment (II)

Clustering the sequenced data revealed that cells with suppressed CD73 expression played a role in signaling related to immune responses (II, Figure 1 and Additional Files). The suppression of CD73 expression did not modify the presence of B220 B cells and CD4 T cells within tumors. However, there was a tendency for an increased infiltration of CD8 T cells in tumors with suppressed CD73 expression, when compared to control tumors. The administration of ZOL led to an increased infiltration of B220, CD4, and CD8 immune cells in tumors for both experimental groups. Nonetheless, statistically significant differences were only observed in the group with CD73 suppression (II, Figure 3). To examine the effects of ZOL on TILs in lung metastases, we employed an experimental lung metastasis model. This model

typically yields larger lung metastases without the influence of primary tumors. Similar to immune cell infiltration in primary tumors, there was a tendency for an increase towards the infiltration of B220 B cells, CD4, and CD8 T cells in response to ZOL treatment in lung metastases. This trend seemed slightly more noticeable in the CD73-suppressed group compared to the control group. However, none of these observed changes reached statistical significance (II, Figure 4).

5.5 B cell depletion augments zoledronate effects on growth of CD73-expressed tumors (II)

In mice, B cell depletion was achieved by administering a single intravenous injection of anti-CD20 IgG through the tail vein (II, Figures 5 and 6). One dose of anti-CD20 IgG (100μM per animal) decreased the absolute number of CD19-positive lymphocytes in the spleen when compared to the group treated with control IgG (II, Additional Files). In mice bearing CD73-expressing tumors, both anti-CD20 treatment and combination of anti-CD20 + ZOL treatment notably reduced tumor growth in both groups, when compared to the groups treated with vehicle alone. In mice bearing CD73-suppressed tumors, the most pronounced reduction in tumor growth was observed with anti-CD20 treatment on its own, compared to the vehicle-treated group. While, the combination of anti-CD20 + ZOL treatment was as effective as ZOL alone in comparison to the groups treated with vehicle alone (II, Figure 5). These trends were also consistently reflected in the analysis of post-mortem tumor sizes (II, Figure 5). A similar trend with reduced sizes of lung metastases was also observed with these treatments (II, Figure 5).

5.6 CD73 suppression makes tumors less permissive to CD8 T cells infiltration upon zoledronate treatment and B cell depletion (II)

In primary tumors, zoledronate + control IgG increased the infiltration of B220 B cells, CD8 T cells, and CD4 T cells in control and CD73-suppressed groups compared to the representative groups with vehicle + IgG (II, Figure 6). This effect was inhibited by anti-CD20 treatment in both groups. However, the addition of ZOL to anti-CD20 treatment increased the number of B220 B cells and CD4 T cells only in CD73-suppressed tumors. The number of CD4 T cells was increased in the anti-CD20 with ZOL treatment compared to anti-CD20 alone in CD73-expressed tumors. The number of CD8 T cells was significantly decreased in the anti-CD20 alone and anti-CD20 with ZOL treatment in CD73-suppressed tumors (II, Figure 6). Anti-CD20 treatment reduced the number CD19-positive cells in circulation and in the lungs in the CD73-suppressed group compared to the vehicle anti-CD20 treated

group. However, contrary to tumor infiltration, the number of CD8 T cells in circulation was not altered in the CD73-suppressed group upon anti-CD20 treatment alone. and even increased when the anti-CD20 group was treated with ZOL (II, Figure 6).

5.7 ZOL-LIP accumulates in tumors more but shows lower bone anabolic effect than zoledronate (III)

The formulation of nitrogen-containing bisphosphonates through liposome encapsulation may enhance tumor targeting (La-Beck et al., 2021). ZOL-LIP significantly inhibited the migration of 4T1.luc2 cells compared to the vehicle or EMP-LIP groups, whereas ZOL had only a slight effect (III, Figure 1). Five doses of ZOL or ZOL-LIP over 24 days markedly reduced tumor growth compared to the vehicle group (III, Figure 2). Five doses of ZOL-LIP also significantly increased the expression of Rab4 (a small GTPase) in tumors (III, Additional Files). However, a single dose of treatment did not affect tumor growth on the day of tumor resection (III, Figure 3). There was a trend towards lower bioluminescence in ZOL- LIP group compared to the vehicle or EMP-LIP groups. None of the treatments resulted in changes in animal weights (III, Figure 2, 3).

ZOL-LIP notably reduced the total number of osteoclasts in the area compared to the vehicle group (III, Figure 6). Both ZOL and ZOL-LIP decreased the number of osteoclasts per bone volume compared to the respective controls. Although ZOL and ZOL-LIP increased bone volume, the bone volume remained significantly lower in the ZOL-LIP group than in the ZOL group (III, Figure 6).

5.8 ZOL-LIP treatment reduces M2-type macrophages in tumors at the accelerated growth phase (III)

The intratumoral changes upon vehicle, ZOL, EMP-LIP and ZOL-LIP treatment were studied in two tumor growth phases: a slow growth phase until day 12 post-inoculation and a later accelerated growth phase after day 12 post-inoculation. In the slow phase, there was a trend towards reduced number of CD206+ macrophages and increased number of iNOS+ and CD80+ (M1-type) upon ZOL-LIP treatment. None of treatment affected CD68+ cell number in tumors (III, Figure 3). In the later accelerated growth phase, ZOL-LIP significantly decreased the number of CD206+ macrophages and increased CD68+ and CD80+ macrophages in tumors compared to the vehicle group. The CD206/CD68 ratio was notably decreased with ZOL-LIP compared to the vehicle group. Although none of the treatments increased the

number of iNOS+ macrophages, ZOL-LIP group had the highest number of iNOS+ cells. The expression of CD34 (angiogenesis marker) was decreased in the ZOL and ZOL-LIP groups compared to the respective controls (III, Figure 4). ZOL and ZOL-LIP treatment increased infiltration of CD4+ T cells into tumors, but it reached significance only following ZOL-LIP treatment compared to EMP-LIP or vehicle groups (III, Figure 5). None of treatment have significant effect on CD3+ or CD8+ T cell infiltrations into tumors (III, Figure 5). ZOL-LIP notably increased NF-κB expression and decreased IκBα expression in tumors compared to EMP-LIP tumors (III, Figure 5). There was a trend towards increased mRNA levels of IL-1β and IL-6 upon ZOL or ZOL-LIP, while the treatment did not have effects on IL-6 in tumors (III, Figure 5).

6 Discussion

6.1 CD73 in cellular processes

Low tumor CD73 expression in cancer cell is associated with prolonged disease-free survival compared to high CD73 expression among TNBC patients (Buisseret et al., 2017). CD73 is involved in influencing cellular processes that support cancer progression, such as cell invasiveness or motility (P. Zhou et al., 2007). To characterize the cellular changes induced by the suppression of CD73 expression, we performed RNA-seq analysis on 4T1 cells. Through the Gene Ontology Biological Process database, we identified three clusters associated with inflammation and immune responses, as well as one cluster related to cell division and replication. Moreover, KEGG enrichment analysis of the most significantly altered genes revealed their involvement in apoptosis, cell cycle regulation, and cytokine activity (Petruk et al., 2023). Suppression of CD73 expression or the use of a selective inhibitor resulted in decreased cell viability in both mouse and human TNBC cells. Specifically, we observed reduced cell proliferation and migration in mouse TNBC (4T1) cells, but not in human TNBC (MDA-MB-231) cells upon treatment with the CD73 inhibitor, APCP. However, APCP did not affect proliferation and migration when CD73 was overexpressed in lung cancer cells (Z. W. Gao et al., 2021). This can be attributed to the competitive nature of APCP as an inhibitor, as its efficiency and metabolic stability may be compromised in the presence of high concentrations of the substrate. Alternatively, the use of allosteric inhibitors targeting CD73 (Rahimova et al., 2018) have shown promise in suppressing CD73-induced events in cancer cells (Hay et al., 2016; Tu et al., 2022).

Extension of cell protrusions, such as lamellipodia and filopodia, help cells to migrate (Lauffenburger & Horwitz, 1996). In the context of CD73 expression, we found that decreased CD73 expression or enzymatic activity led to a reduction in the length of cell protrusions in 2D-cultured 4T1 cells, but not in MDA-MB-231 cells. This effect was also observed in 3D-culture, where cells with suppressed CD73 expression exhibited smaller cell protrusions. Notably, in organoids derived from cells with unmodified CD73 expression, spontaneous invasion was observed around day 6, and clear signs of invasion were observed by day 8. In contrast, organoids derived from cells with suppressed CD73 expression maintained a well-differentiated phenotype and did not exhibit invasive properties even on day 13 (Petruk et al., 2021).

Cell invasiveness is associated with EMT, making especially breast cancer cells with EMT feature, more prone to form metastases (Lüönd et al., 2021). Notably, CD73, an ecto-enzyme involved in adenosine production, is directly regulated by EMT transcription factors in breast cancer cells. Previous studies have revealed significant correlations between EMT transcription factors SNAI1, 2, TWIST1, 2 and ZEB1, 2 with CD73 expression, in HER2+ breast cancer (Turcotte et al., 2017) or with SNAI1 in TNBC patient cohort (Hasmim et al., 2022) in METABRIC breast cancer patient database (Curtis et al., 2012). The binding of SNAIL1 to CD73 promotes EMT transition, leading to an increased accumulation of adenosine in the extracellular environment (Hasmim et al., 2022). We and others have shown a mutual regulation between CD73 and EMT (Lupia et al., 2018; Petruk et al., 2021). CD73 is involved in promoting EMT toward a mesenchymal phenotype by increasing vimentin expression and decreasing E-cadherin expression *in vitro* (Xu et al., 2020) and *in vivo* (X. L. Ma et al., 2019). CD73 downregulation reverses these effects, resulting in smaller tumors with epithelial-like features and fewer metastases (X. L. Ma et al., 2019). In ovarian cancer cells, suppression of CD73 expression and inhibition of its enzymatic activity have been shown to decrease the expression of key regulators of EMT, SNAIL1, Twist1, and ZEB1 (Lupia et al., 2018). In our study, we investigated the effects of blocking CD73 enzymatic activity in 4T1 and MDA-MB-231 cells and suppressing CD73 expression in 4T1 cells. We observed an increase in E-cadherin expression, while vimentin expression remained unchanged in 4T1 and MDA-MB-231 cells with blocked CD73 enzymatic activity. However, suppression of CD73 expression in 4T1 cells resulted in decreased vimentin expression *in vitro*.

Adenosine plays a significant role in the tumor microenvironment, particularly under conditions of increased hypoxia. Tumor oxygen levels in solid tumors are highly dynamic, typically characterized by a hypoxic environment. Hypoxia has been found to suppress the infiltration of anti-inflammatory immune cells, promote the transcription of angiogenesis factors, and enhance the survival of breast cancer cells (Xiguang Chen et al., 2020). In solid tumors, hypoxia accelerates the production of ATP (Vijayan et al., 2017) or induces CD73 expression through HIF-1 activation (Synnestvedt et al., 2002). This also has been observed in TNBC cells. Inhibition of HIF using acriflavine attenuates CD73 mRNA expression and its expression is significantly correlated with HIF-1α and HIF target-gene mRNAs (Samanta et al., 2018). In our study, we confirmed that hypoxia increases both CD73 mRNA expression and protein levels in TNBC cells. Hypoxia also enhances the viability of parental 4T1 and MDA-MB-231 cells, while treatment with APCP (CD73 inhibitor) or suppression of CD73 expression attenuates the effects of hypoxia on cell viability. We did not observe any significant influence of hypoxia on cell migration or invasion, but hypoxia prolonged protrusions in MDA-MB-231 cells. We and others have shown that hypoxia regulates EMT (Azab et al., 2012; Petruk et al., 2021). In our study, we found that hypoxia increases E-cadherin expression in 4T1 cells when treated with

APCP or when CD73 expression is suppressed. Vimentin expression, on the other hand, is increased in response to hypoxia in cells treated with APCP or in cells with suppressed CD73 expression. However, only the suppression of CD73 expression or APCP treatment in MDA-MB-231 cells slightly reduced hypoxia-induced vimentin expression.

It has been shown that N-BPs cause a direct suppressive effects on cancer cells (Sandholm et al., 2016; Virtanen et al., 2018). Treatment with N-BPs significantly reduced the viability of 4T1 cells with suppressed CD73 expression compared to cells with unmodified CD73 expression. Among the N-BPs, zoledronate exhibited the most pronounced growth inhibition of cells with suppressed CD73 expression. Previous studies have shown that cancer cells may exhibit varying sensitivity to N-BPs. Zoledronate suppressed proliferation and induced apoptosis in MDA-MB-231 cells (representative of triple-negative breast cancer), while MCF-7 cells (representative of luminal A subtype) demonstrated greater tolerance to treatment. This resistance in MCF-7 cells has been attributed to reduced expression of caspase-3 (Rachner et al., 2010). In our study, the inhibition of CD73 enzymatic activity did not affect the cell viability of parental triple-negative breast cancer or luminal A cancer cells upon zoledronate treatment. However, the suppression of CD73 expression sensitized 4T1 cells to the inhibitory effects of N-BPs. This was evidenced by delayed proliferation, increased apoptotic rate, and G1-phase cell cycle arrest in CD73-suppressed cells treated with N-BPs compared to treated cells with unmodified CD73 expression. Moreover, zoledronate increased the expression of p27 (G1 -phase cell cycle regulator) in CD73-suppressed cells. A previous study associated CD73 expression and G-phase cell cycle arrest with the suppression of the AKT/ERK pathway. The regulation of this process was mediated by the lack of CD73 expression on cancer cells (L. Zhou et al., 2019). Zoledronate also caused G1-phase cell cycle arrest through the AKT/ERK pathway, reducing the expression of the cell cycle regulator cyclin D1 and CDK4 (L. Wang et al., 2019). Consistent with cell cycle arrest, we observed a higher number of apoptotic cells and caspase 3/7 positive cells in 4T1 cells with CD73 suppression upon zoledronate treatment.

6.2 CD73 is a target to prevent tumor growth

High CD73 expression was associated with worse outcome for patients with different types of cancer, including TNBC (Buisseret et al., 2018; Q. Chen et al., 2020; He et al., 2021). Our research supports these findings by revealing that cells with suppressed CD73 expression exhibited reduced *in vitro* growth and migration, leading to significantly smaller tumors in immunocompetent mice. Moreover, *in vivo* experiments demonstrated that tumors with suppressed CD73 expression showed increased levels of E-cadherin and decreased levels of vimentin, compared to tumors with unmodified CD73 expression. Consistently, analysis of mRNA CD73 expression

in a breast cancer patient cohort from the METABRIC database revealed a significant correlation between CD73 expression and E-cadherin as well as vimentin mRNA expression.

The evaluation of potential tumor biomarkers before and after anti-cancer treatment holds promise for avoiding adverse effects of therapy. In our study, we found that N-BPs had no effect on CD73 expression or enzymatic activity in TNBC cells both *in vitro* and *in vivo*. Similarly, neoadjuvant therapy did not alter CD73 expression in breast cancer patients (Cerbelli et al., 2020). However, in the context on treatment, bisphosphonates inhibited CD73 expression in endothelial cells (Sharma et al., 2016), but increased CD73-derived adenosine level in bone cells (J. Z. Zhou et al., 2014). Our research demonstrated that tumors with suppressed CD73 expression were significantly smaller compared to control tumors. Zoledronate treatment resulted in a similar reduction in tumor formation in both groups after 31 days of inoculation. However, when tumors were allowed to grow for 34 days, zoledronate significantly decreased tumor formation specifically in the CD73-suppressed tumors compared to the control tumors. The anti-cancer effects of zoledronate were associated with an enhanced expression of pro-apoptotic markers, such as cleaved caspase-3, and down-regulation of cyclin D1 and CDK4, which promote cell progression into the S-phase of the cell cycle (L. Wang et al., 2019). In the case of TNBC cells, N-BPs have been shown to suppress proliferation and increase apoptosis by facilitating the cleavage of caspases-3 and -7 (Ebert et al., 2014; Rachner et al., 2010). Mitochondrial membrane dysfunction caused apoptosis of colorectal cancer cells after N-BP treatment (X. Gao et al., 2015). In prostate cancer cells, the shift in the ratio of Bcl2/Bax apoptosis-associated protein expression has been demonstrated upon N-BP treatment (Asahi et al., 2006). We observed that zoledronate increased the number of cleaved caspase-3 positive cells in tumors with CD73 suppression, but had no significant effect on these cells in control tumors.

TNBC patients develop fewer bone metastases, but significantly more visceral metastases in comparison to those with hormone receptor-positive subtypes (Dent et al., 2009; Ignatov et al., 2018; Kennecke et al., 2010; Y. Liu et al., 2014). In our studies, we observed that mice bearing tumors with suppressed CD73 expression had significantly lower numbers and smaller sizes of lung metastases compared to mice with unmodified CD73 expression tumors. However, we did not observe any bone metastases in tumor-bearing mice and the number of liver metastases showed no significant change between the two groups. The observed reduction in lung metastases in mice with CD73-suppressed tumors could be attributed to a slower dissemination of cancer cells. Apart from migration, invasion, and EMT, angiogenesis is another factor that supports cancer cell dissemination (Lee et al., 2009). Tumor cells with higher oxygen demands may trigger the activation of pro-angiogenic signaling pathways to promote the formation of new blood vessels. This phenomenon, known as the "angiogenic switch," can also contribute to decreased treatment efficacy

(Lugano et al., 2020). We observed a reduced number of CD34-positive cells (an angiogenic marker) in tumors with ZOL or ZOL-LIP treatment in comparison to tumors with vehicle or EMP-LIP treatment.

The development of metastases also depends on the timing of dormant cell activation in distant organs. Ki-67 is commonly used as a marker of cell proliferation. Ki-67 status is considered high when more than 20% of tumor cells show nuclear immunoreactivity. Furthermore, high Ki-67 predicted worse DFS (Pan et al., 2017), which led to the use of Ki-67 status as a marker to stratify TNBC to high and low proliferative tumors (Choi et al., 2022; Schneeweiss et al., 2019). To investigate whether metastases arising from CD73-suppressed cancer cells exhibited altered proliferation rates, we utilized an experimental metastasis model where cancer cells were intravenously injected. Lung metastases derived from cancer cells with suppressed CD73 expression had significantly fewer Ki-67 positive cells compared to those with unmodified CD73 expression. High expression of proliferative marker Ki-67 demonstrated a significant association with high pCR in TNBC (Darb-Esfahani et al., 2009). A study from University of Turku has shown the correlation of Ki-67 index with TNBC patient's age. The increase of Ki-67 index on each 10% increased the risk of mortality in those patients, particularly in patients over the age of 57 (Vihervuori et al., 2022).

6.3 Effects of tumor cell-expressed CD73 on immune cells

Adjuvant bisphosphonates have been shown to increase the survival of postmenopausal women with breast cancer (Rennert et al., 2017). In both in pre-clinical and clinical studies, N-BPs have been found to possess pro-inflammatory properties and activate immune cells (Dieli et al., 2003). CD73 expression on tumor cells has a prognostic significance in TNBC (F. Jin et al., 2021; Xing et al., 2022). The worse outcome linked to CD73 in TNBC patients could result from immune evasion, as adenosine protects cancer cells from adaptive anti-tumor immune response (Minor et al., 2019; Schneider et al., 2019; Silva-Vilches et al., 2018; Stagg et al., 2010).

The discrepancy between our *in vitro* and *in vivo* results, particularly when tumor-bearing mice were treated with zoledronate, could be attributed to the immune response. We demonstrated that zoledronate induces the accumulation of $CD8^+$ and $CD4^+$ T cells, and B cells in primary tumors as well as lung metastases. However, this effect was partially regulated by tumor CD73 expression, as immune cell infiltration was significant only in tumors with CD73 suppression. In tumors, high $CD8^+$ and $CD4^+$ T cells amounts associate with a longer DFS and OS, but $CD4^+$ cells did not show such correlation when $CD8^+$ T cell infiltration was low (Oshi et al., 2020). In our study, depletion of B cells significantly inhibited tumor growth regardless of

tumor CD73 expression. Tumors with suppressed CD73 expression were less permissive for $CD8^+$ T cells upon zoledronate treatment and B cell depletion. However, a previous study showed that the blockage of CD73 enzymatic activity did not alter $CD8^+$ T cell infiltration into tumors from mice with B cell depletion. The presence of T cells was still required to achieve anti-tumor effects of B cell depletion and blockage of CD73 enzymatic activity, the CD73 inhibitor did not show any tumor growth suppression in athymic mice (Forte et al., 2012). Furthermore, anti-CD20 antibodies can deplete CD20-positive B cells and CD20-positive $CD8^+$ or $CD4^+$ T cells. CD20-positive T cells showed activity similar to CD20-negative T cells (Schuh et al., 2016) and can potentially contribute to the inhibition of cancer growth, similar to $CD8^+$ T cells. Throughout animal experiments, our stably transfected 4T1 cells maintained low CD73 expression. Thus, tumor cells could produce less CD73-derived adenosine, but CD73 expression was not modified in tumor stromal cells. It is worth noting that CD73 inhibitors target all CD73-expressing cells. Therefore, the differential outcomes between low CD73 expression in tumors and CD73 blockade might be influenced by other CD73-expressing cells. Studies have shown that anti-CD73 antibodies alone were not effective in decreasing the growth of TNBC cells (Loi et al., 2013; Qiao et al., 2019) or colon adenocarcinoma cells (Wurm et al., 2021) *in vivo*. Moreover, the efficiency of anti-CD73 monotherapy required the activation of immune responses (R. Jin et al., 2020; Stagg et al., 2010). CD73 is a promising agent for combined therapy to suppress the growth of different tumor cells (Tsukui et al., 2020; Tu et al., 2022; Wurm et al., 2021), including TNBC (F. Jin et al., 2021; Xing et al., 2022). It is plausible that the inflammatory effects of adjuvant N-BPs, which promote TIL accumulation, play a role in impeding the transition of microscopic disease to macroscopic metastasis in post-menopausal women. CD73 holds potential for novel approaches to cancer treatment and therapeutic interventions. Especially in the realm of immunotherapy and the modulation of tumor immune microenvironment, given that breast cancers are often characterized as "cold tumors," with limited inflammatory infiltration (Y. T. Liu & Sun, 2021).

6.4 Reformulation of zoledronate to target tumors

Nitrogen-containing bisphosphonates were primarily used to prevent osteoporosis and regulate bone turnover (Roelofs et al., 2006). However, clinical studies have demonstrated that N-BPs are a beneficial treatment option for postmenopausal women, reducing the risk of breast cancer recurrence and improving patient survival (Coleman et al., 2015; Kroep et al., 2016; Rennert et al., 2017; Strobl et al., 2018). N-BPs exert their effects on tumor growth through both indirect and direct mechanisms. These mechanisms involve influencing signaling pathways in immune cells and inducing apoptosis in cancer cells, achieved by inhibiting the mevalonate pathway (Ebert et al., 2014; Rachner et al., 2010; K. Thompson & Rogers, 2004). However,

the therapeutic application of N-BPs in cancer treatment has been limited due to the occurrence of side effects (Gralow et al., 2020; Seider et al., 2018).

By reformulating bisphosphonates, it may be possible to achieve the desired therapeutic effects using lower doses of the drug, without compromising its anti-cancer properties (La-Beck et al., 2021). One approach to enhance the delivery of zoledronate is through its encapsulation in liposomes, which has been shown to increase drug accumulation in the spleen, bone, and tumors (Shmeeda et al., 2013). Liposome reformulation of N-BPs limited the accumulation of the drug in normal tissues (La-Beck et al., 2021). In our study, we used treatment doses that were clinically achievable, equivalent to a standard clinical dose of 4 mg (Ottewell et al., 2008). We observed a reduction in the overall anabolic effect on bone in the ZOL-LIP group compared to the free zoledronate group. Furthermore, the number of osteoclasts was still decreased not only in the ZOL group but also in the liposome-encapsulated zoledronate (ZOL-LIP) group. This observation suggests that the reduction in osteoclasts may be attributed to the phagocytosis (W. Wang et al., 1997) of ZOL-LIP by osteoclasts, rather than the direct accumulation of zoledronate in osteoclasts through bone resorption.

Tumor-associated macrophages (TAMs) have been associated with poor prognosis in cancer patients (Cassetta et al., 2019; Mehta et al., 2021). In solid tumors, pro-inflammatory macrophages have been shown to secrete pro-inflammatory cytokines that associate with tumor growth suppression (Salmaninejad et al., 2019). In TNBC, an M2-type TAM gene signature was found to have also inflammatory and angiogenic properties (Bao et al., 2021). In our study, both zoledronate and ZOL-LIP reduced the number of CD34-positive cells, indicating suppression of angiogenesis. The infiltration of M2-type macrophages was higher in TNBC tumors compared to Luminal or HER2 types. Conditioned media from M2-type macrophages increased migration, invasion, EMT, and cancer stem cell markers in cancer cells, and these effects were attenuated by inhibitors of β-catenin and AKT (Xiangzhou Chen et al., 2022). Also, conditioned media from TNBC cancer cells differentiated macrophages to M2-type (Sousa, Brion, et al., 2015). Previous studies demonstrated that ZOL-LIP was taken up by breast cancer cells and promoted the expression of M1 markers in monocyte/macrophage cultures, even in the presence of factors secreted by breast cancer cells (Sousa, Auriola, et al., 2015; Zlatev et al., 2016). In our study, we observed a significant increase in the number of CD68 macrophages and the expression of NF-κB protein in tumors following longer ZOL-LIP treatment. In contrast, free zoledronate did not have a notable effect on these parameters. Activation of M1-type pro-inflammatory macrophages relies on the expression of iNOS (Paul et al., 2019) or CD80 (Raggi et al., 2017), ZOL-LIP treatment further augmented iNOS expression and significantly increased the number of $CD80^+$ cells compared to zoledronate treatment alone. Additionally, ZOL-LIP treatment resulted in a reduction of $CD206^+$ M2-type macrophages within tumors, although this effect was dependent

on drug accumulation within the tumor microenvironment. However, a single dose of treatment did not significantly affect macrophage infiltration.

The uptake of N-BPs by tumor cells leads to the accumulation of IPP/ApppI (Jauhiainen et al., 2009). ApppI level was detectable in tumors 48 h after the last drug injection, but not after 24 h (Benzaïd et al., 2011, 2012). Notably, N-BPs can be taken up by tumor cells and TAMs (Junankar et al., 2015). *In vitro* experiments demonstrated that both ZOL-LIP and free zoledronate similarly inhibited the prenylation of the small GTPase Rap1A in macrophages (Sousa, Auriola, et al., 2015). However, in our *in vivo* study, ZOL-LIP treatment significantly increased the accumulation of another small GTPase, Rab4, within tumors, indicating a distinct mechanism of action. Taken together, our findings suggest that the liposome encapsulation of zoledronate may enhance intratumor inflammation compared to free zoledronate treatment by greater targeting of tumors and enhanced M1-type macrophage infiltration to tumors. In the future, it would be interesting to investigate effects ZOL-LIP on disseminated cancer cells activation and whether it could modulate immune cells infiltration in metastatic niche.

7 Summary/Conclusions

Based on the results, the following conclusion can be made:

1. Suppression of CD73 expression, but not its enzymatic activity, reduced cell viability under hypoxic conditions. The migration, invasiveness, and progression of epithelial-mesenchymal transition (EMT) were also reduced when CD73 expression was suppressed in TNBC cells. Addition of N-BPs enhanced the inhibitory effects of CD73 suppression, leading to cell cycle arrest and increased apoptosis *in vitro*.

2. Lack of CD73 expression in TNBC cells reduced tumor growth and decreased the development of lung metastases. Treatment with zoledronate increased immune cell infiltration in both tumors and lung metastases when CD73 expression was suppressed.

3. Depletion of B cells demonstrated a similar tumor-suppressing effect as zoledronate treatment. CD73 suppression made tumors less permissive for infiltration of $CD8^+$ T cells in response to zoledronate and B cell depletion.

4. Liposome encapsulation of zoledronate exhibited greater accumulation in tumors compared to free zoledronate, leading to proinflammatory effects within tumors and reduced targeting of bones.

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