Evaluation of SAMHD1 role as a predictive and prognostic biomarker in solid tumours: deciphering its function in innate immune signalling

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I per tal de que en quedi constancia, signen aquest document a Badalona,

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A mi familia,
por su apoyo constante e incondicional

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SUMMARY

Cancer is still a major burden of disease worldwide. Due to the high variability between and within tumours and specific characteristics of each patient, it is becoming increasingly clear that the future of cancer care will be dependent on a personalized patient management. In this setting, the identification of reliable and robust predictive and/or prognostic biomarkers will be key. Over the years, growing evidence pointed towards SAMHD1 as one of these putatively valuable biomarkers. SAMHD1 is a deoxynucleotide triphosphate (dNTP) triphosphohydrolase that was first described as a viral restriction factor, although its impact on cell biology and metabolism goes far beyond. Despite the evidences suggesting an important function in hematological tumours and cancer disease progression, SAMHD1 role in solid tumours is still a matter of intense debate, mainly due to reports arguing in favour of both pro- and anti-tumourigenic effect.

Considering the need of additional studies to further delineate how SAMHD1 function might influence onset and evolution of cancer disease, in this PhD thesis we have focused on describing the role of SAMHD1 as a predictive and prognostic biomarker in solid tumours. We investigated the underlying mechanisms of SAMHD1 in the induction and regulation of tumourigenesis, bearing in mind its putative immunomodulatory function.

In the first chapter of the thesis, we performed the first in-depth study of SAMHD1's role in advanced solid tumours treated with platinum derivatives and/or antimetabolites and developed novel *in vitro knock-out* models to explore the mechanisms driving SAMHD1 function in cancer. Our results show that low (or no) expression of SAMHD1 was associated with a positive prognosis in breast, ovarian, and non-small cell lung cancer patients. In addition, our *in vitro* results show that SAMHD1 *knock-out* cells present increased DNA damage and apoptosis, and treatment with platinum-derived drugs significantly enhance γ-H2AX and apoptotic markers expression in these SAMHD1 *knock-out* cells. These results suggest that SAMHD1 depletion induces DNA damage leading to cell death and indicate a synergic effect of SAMHD1 depletion and platinum-based treatment.

Next, in the second chapter of the thesis, we focused on the characterization of the use of SAMHD1 as a prognostic biomarker in early-stage breast cancer patients. We described and validated the use of SAMHD1 expression as a prognostic biomarker in residual disease after neoadjuvant chemotherapy *in vivo.* Moreover, we developed *in vitro* 3D spheroid models to better elucidate the immunomodulatory consequences of SAMHD1 depletion. Whole-transcriptomic profiling of SAMHD1 *knock-out* tumour spheroids identified downregulation of

IL-12 signalling pathway as the molecular mechanism determining breast cancer prognosis. Interestingly, the reduced interleukin signalling in SAMHD1-KO spheroids induced changes in immune cell infiltration capacity in 3D heterotypic *in vitro* culture models.

Finally, in the last chapter of the thesis, we evaluated the role of SAMHD1 expression and function in ovarian cancer, both *in vitro* and in ovarian cancer patient cohorts. We found that SAMHD1 depletion modulates pattern recognition receptors, specifically RIG-I like receptor expression, and innate immune signalling in ovarian cancer cells. Moreover, clinical data allowed us to propose SAMHD1 as a prognostic marker in ovarian cancer, as SAMHD1-low expressing tumours showed increased progression free survival and overall survival.

Overall, our results provide strong evidence of the involvement of SAMHD1 function in cancer, indicating that SAMHD1 expression exerts a pro-tumourigenic effect in several solid tumours including breast, ovarian and non-small cell lung cancer. However, our data demonstrates that the function of SAMHD1 in cancer is context-dependent and may vary depending on the specific cancer type and cell line. In breast cancer, SAMHD1 depletion leads to a downregulation in innate immune signalling pathways, while in ovarian cancer cell lines we have demonstrated that SAMHD1 regulates the RIG-I/MDA5 signalling pathway and innate immune response is activated upon SAMHD1 depletion. In conclusion, SAMHD1 could be used a prognostic biomarker and it represents a promising therapeutic target for cancer treatment. However, further research is needed to fully understand the mechanisms of SAMHD1 to understand its role in cancer and to optimize putative treatment strategies.

RESUMEN

El cáncer sigue siendo una de las principales causas de muerte en el mundo. Debido a la gran variabilidad entre tumores y dentro de un mismo tumor y a las características específicas de cada paciente, cada vez está más claro que el futuro de la atención oncológica dependerá de una atención personalizada a cada paciente. En este contexto, la identificación de biomarcadores predictivos y/o pronósticos fiables y robustos será clave. A lo largo de los años, cada vez más evidencias apuntan a SAMHD1 como un importante biomarcador. SAMHD1 es una desoxinucleótido trifosfato (dNTP) trifosfohidrolasa que se describió por primera vez como un factor de restricción viral, aunque su impacto en biología celular y metabolismo va mucho más allá. A pesar de las evidencias que sugieren una función importante de esta proteína en los tumores hematológicos y en la progresión del cáncer, el papel de SAMHD1 en los tumores sólidos sigue siendo objeto de un intenso debate, principalmente debido a la diversidad de estudios que argumentan a favor de un efecto tanto pro-tumorigénico como anti-tumorigénico.

Teniendo en cuenta la necesidad de estudios adicionales para delinear mejor cómo la función de SAMHD1 puede influir en el inicio y la evolución del cáncer, en esta tesis doctoral nos hemos centrado en describir el papel de SAMHD1 como biomarcador predictivo y pronóstico en tumores sólidos. Hemos investigado los mecanismos subyacentes de SAMHD1 en la inducción y regulación de la tumorigénesis, teniendo en cuenta su posible función inmunomoduladora.

En el primer capítulo de la tesis, realizamos un primer estudio del papel de SAMHD1 en tumores sólidos tratados con derivados de platinos y/o antimetabolitos y desarrollamos nuevos modelos *knock-out in vitro* para explorar los mecanismos que dirigen la función de SAMHD1 en cáncer. Nuestros resultados muestran que la baja (o nula) expresión de SAMHD1 está asociada a un pronóstico positivo en pacientes con cáncer de mama, ovario y pulmón de células no pequeñas. Además, nuestros resultados *in vitro* muestran que las células *knock-out* para SAMHD1 presentan un aumento de daño en el ADN y apoptosis, que aumentan significativamente con el tratamiento con fármacos derivados del platino. Estos resultados sugieren que la ausencia de SAMHD1 induce daño en el ADN que conduce a la muerte celular, indicando un efecto sinérgico de la depleción de SAMHD1 y el tratamiento basado en platinos.

A continuación, en el segundo capítulo de la tesis, nos centramos en la caracterización del uso de SAMHD1 como biomarcador pronóstico en pacientes con cáncer de mama en estadio temprano. Describimos y validamos el uso de la expresión de SAMHD1 como biomarcador pronóstico en enfermedad residual tras quimioterapia neoadyuvante. Además, desarrollamos

modelos 3D de esferoides para dilucidar mejor las consecuencias inmunomoduladoras de la eliminación de SAMHD1. El análisis transcriptómico completo de los esferoides tumorales *knock-out* de SAMHD1 mostró una desregulación en la vía de señalización de IL-12 como el mecanismo molecular que determina el pronóstico del cáncer de mama. Curiosamente, la reducción de la señalización de la interleucina en los esferoides *SAMHD1-KO* indujo cambios en la capacidad de infiltración de células inmunitarias en modelos de cultivo 3D heterotípicos.

Finalmente, en el último capítulo de la tesis, evaluamos el papel de la expresión y la función de SAMHD1 en cáncer de ovario, tanto *in vitro* como en cohortes de pacientes con cáncer de ovario. Descubrimos que la eliminación de SAMHD1 en líneas celulares derivadas de cáncer de ovario modula tanto los receptores de reconocimiento de patrones (PRR, *Pattern recognition receptor*), específicamente RIG-I, como la señalización inmune innata. Además, los datos clínicos nos permitieron proponer SAMHD1 como un marcador pronóstico en el cáncer de ovario, ya que los tumores con baja expresión de SAMHD1 mostraron una mayor supervivencia libre de progresión y supervivencia global.

En general, nuestros resultados proporcionan pruebas sólidas de la implicación de la función de SAMHD1 en el cáncer, indicando que la expresión de SAMHD1 ejerce un efecto pro-tumorigénico en varios tumores sólidos, incluyendo el cáncer de mama, ovario y pulmón de células no pequeñas. Sin embargo, nuestros datos demuestran que la función de SAMHD1 en el cáncer depende del contexto y puede variar dependiendo del tipo específico de cáncer y de la línea celular. En el cáncer de mama, la ausencia de SAMHD1 conduce a una inactivación de las vías de señalización inmunitarias innatas, mientras que en las líneas celulares de cáncer de ovario hemos demostrado que SAMHD1 regula la vía de señalización RIG-I/MDA5 y que la respuesta inmunitaria innata se activa tras la eliminación de SAMHD1. En conclusión, SAMHD1 podría utilizarse como biomarcador pronóstico y representa una prometedora diana terapéutica para el tratamiento del cáncer. Sin embargo, es necesario seguir investigando para comprender plenamente los mecanismos de SAMHD1 a fin de entender su papel en el cáncer y optimizar las posibles estrategias de tratamiento.

RESUM

El càncer continua sent una de les principals causes de mort al món. A causa de la gran variabilitat tant intra com inter-tumoral i les característiques específiques de cada pacient, hi ha pocs dubtes que el futur de l'atenció oncològica dependrà d'una atenció personalitzada per a cada cas. En aquest context, la identificació de biomarcadors predictius i/o pronòstics fiables i robustos serà clau. Al llarg dels anys, cada vegada més evidències apunten a SAMHD1 com un d'aquests biomarcadors. SAMHD1 és una desoxinucleótid trifosfat (dNTP) trifosfohidrolasa que es va descriure per primera vegada com un factor de restricció viral, encara que el seu impacte en la biologia cel·lular i el metabolisme va molt més allà. Malgrat existeixen evidències que suggereixen una funció important d'aquesta proteïna en l'establiment i la progressió de tumors hematològics, el paper de SAMHD1 en els tumours sòlids continua sent objecte d'un intens debat, principalment a causa de la diversitat d'estudis que argumenten a favor d'un efecte tant de pro-tumorigènic com anti-tumorigènic.

Tenint en compte la necessitat d'estudis addicionals per a delinear millor com la funció de SAMHD1 pot influir en l'inici i l'evolució del càncer, en aquesta tesi doctoral ens hem centrat en descriure el paper de SAMHD1 com a biomarcador predictiu i pronòstic en tumours sòlids. Hem investigat els mecanismes subjacents de SAMHD1 a la inducció i regulació de la tumorigènesis, tenint en compte la seva possible funció inmunomoduladora.

En el primer capítol de la tesi, hem realitzat un primer estudi del paper de SAMHD1 en tumors sòlids tractats amb derivats de platins i/o antimetabòlits i hem desenvolupat nous models *knock-out in vitro* per a explorar els mecanismes que dirigeixen la funció de SAMHD1 en càncer. Els nostres resultats mostren que la baixa (o nul·la) expressió de SAMHD1 està associada a un pronòstic positiu en pacients amb càncer de mama, ovari i pulmó de cèl·lules no petites. A més, els nostres resultats *in vitro* mostren que les cèl·lules *knock-out* per a SAMHD1 presenten un augment de dany al ADN i apoptosi, el qual es veu augmentat significativament amb el tractament amb fàrmacs derivats del platí. Aquests resultats suggereixen que l'absència de SAMHD1 indueix dany en l'ADN que condueix a la mort cel·lular**,** indicant un efecte sinèrgic de la depleció de SAMHD1 i el tractament basat en platins.

A continuació, en el segon capítol de la tesi, ens hem centrat en la caracterització de l'ús de SAMHD1 com a biomarcador pronòstic en pacients amb càncer de mama en estadi primerenc. En aquest capítol, hem descrit i validat l'ús de l'expressió de SAMHD1 com biomarcador pronòstic en malaltia residual després de quimioteràpia neoadjuvant. A més, s'han desenvolupat models 3D d'esferoides per a dilucidar millor les conseqüències

inmunomoduladores de l'eliminació de SAMHD1. L'anàlisi transcriptòmic complet dels esferoides tumorals *knock-out* de SAMHD1 va mostrar una desregulació en la via de senyalització de IL-12 com el mecanisme molecular que determina el pronòstic del càncer de mama. Curiosament, la reducció de la senyalització de la interleucina en els esferoides SAMHD1-KO va induir canvis en la capacitat d'infiltració de les cèl·lules immunitàries en models de cultiu 3D heterotípics.

Finalment, en l'últim capítol de la tesi, hem avaluat el paper de l'expressió i la funció de SAMHD1 en càncer d'ovari, tant *in vitro* com en cohorts de pacients amb càncer d'ovari. Aquí, hem descobert que l'eliminació de SAMHD1 en línies cel·lulars derivades de càncer d'ovari modula tant els receptors de reconeixement de patrons (PRR, *Pattern recognition receptor*), específicament RIG-I, com la senyalització immune innata. A més, les dades clíniques ens van permetre proposar SAMHD1 com un marcador pronòstic en el càncer d'ovari, ja que els tumors amb baixa expressió de SAMHD1 van mostrar una major supervivència lliure de progressió i supervivència global.

En general, els nostres resultats proporcionen proves sòlides de la implicació de la funció de SAMHD1 en el càncer, indicant que l'expressió de SAMHD1 exerceix un efecte pro-tumorigènic en diversos tumors sòlids, incloent-hi el càncer de mama, ovari i pulmó de cèl·lules no petites. No obstant això, les nostres dades demostren que la funció de SAMHD1 en el càncer depèn del context i pot variar depenent del tipus específic de càncer i de la línia cel·lular. En el càncer de mama, l'absència de SAMHD1 condueix a una inactivació de les vies de senyalització immunitàries innates, mentre que en les línies cel·lulars de càncer d'ovari hem demostrat que SAMHD1 regula la via de senyalització RIG-I/MDA5 i que la resposta immunitària innata s'activa després de l'eliminació de SAMHD1. En conclusió, SAMHD1 podria utilitzar-se com biomarcador pronòstic i representa una prometedora diana terapèutica per al tractament del càncer. No obstant això, és necessari continuar investigant per a comprendre plenament els mecanismes de SAMHD1 a fi d'entendre el seu paper en el càncer i optimitzar les possibles estratègies de tractament.

ABBREVIATIONS

3D	Three dimensional
5-FU	5-fluorouracilo, fluorouracil
AAM	Alternatively activated macrophages
AGS	Aicardi Goutières syndrome
AIM2	Absent in melanoma-2
AIs	Aromatase inhibitors
ALL	Acute lymphocytic leukemia
ALRs	(AIM-2)-like receptors
AML	Acute myelocytic leukemia
APC	Antigen presenting cells
APC	Adenomatous polyposis coli gene
ARA-C	Cytarabine, 1-beta-D-arabinofuranosylcytosine
ATCC	American Type Culture Collection
AZT	Azidothymidine (Zidovudine)
BC	Breast cancer
BCR	B-cell receptors
BCS	Breast-conserving surgery
BRCA1/2	Breast Cancer gene 1/2
CAFs	Cancer-associated fibroblasts
CAMs	Classically activated macrophages
CARD	Caspase activation and recruitment domain
CARDs	Caspase activation and recruitment domains
Casp	Caspase
CCC	Clear cell carcinoma
CCT	Chaperonin containing TCP1 complex
CD	Cluster of Differentiation
CDK	Cyclin-dependent kinase
CDKi	CDK inhibitors
cGAMP	cyclic GMP-AMP
cGAS	cGAMP synthase
CI	Confidence intervals
CLL	Chronic lymphocytic leukemia
CLRs	C-type lectin receptors
CML	Chronic myelocytic leukemia
CMV	Cytomegalovirus
CNN2	Calponin 2
CpG	Cytidine-phosphate-guanosine
CRD	Carbohydrate recognition domain
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRLF1	Cytokine Receptor Like Factor 2
CSF	Colony-stimulating factors
CTCF	Corrected total cell fluorescence
CTD	C-terminal domain
CtIP	CtBP-interacting protein

CTLA4	Cytotoxic T lymphocyte-associated protein 4
CXCL10	C-X-C motif chemokine ligand 10
CXCR3	C-X-C Motif Chemokine Receptor 3
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DFS	Disease free survival
dGTP	Deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
dN	deoxyribonucleoside
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dNTPase	dNTP triphosphohydrolase
DSB	Double-stranded break
dsRNA	Double-stranded RNA
EC	Endometrioid carcinoma
ECACC	European Collection of Authenticated cell cultures
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMA	European Medicines Agency
ER	Estrogen receptor
ERKs	Extracellular signal-regulated kinases
ESMO	European Society for Medical Oncology
FA	Formaldehyde
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FSC	Forward scatter
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSEA	Gene Set Enrichment Analysis
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HER2	human epidermal growth factor receptor 2
HGSC	High-grade serous carcinoma
HIV	Human immunodeficiency virus
HLA	Human leukocyte-associated
HR	Homologous recombination
HSP	Heat shock protein
ICGC	International Cancer Genome Consortium
ICO	Catalan institute of oncology
IFITM2	Interferon-induced transmembrane 2
IFN	Interferon

IFNAR	Interferon a/b receptor
IKK	IκB kinase
IL	Interleukin
IP-10	Interferon inducible protein-10
IQR	interquartile range
IRAK4	IL-1R-related kinase 4
IRF	IFN-regulatory factors
IRF	Interferon Regulatory Factors
ISG	IFN-stimulated gene
JAK	Janus kinase
JNKs	c-Jun NH2-terminal kinases
KO	*Knock-out*
LGP2	Laboratory of genetics and physiology 2
LGSC	Low-grade serous carcinoma
LOD	Limit of detection
LPS	Lipopolysaccharide
LRRs	Leucine rich repeats
LTA	Lipoteichoic acid
MAPK	Mitogen-activated protein kinase
MAPKKK	MAPK kinase kinase
MAVS	Mitochondrial antiviral signalling protein
MC	Mucinous carcinoma
MCP	Monocyte chemoattractant protein
M-CSF	Monocyte-colony stimulating factor
MDA5	Melanoma differentiation-associated protein 5
MDM	Monocyte derived macrophages
MHC	Major histocompatibility complex
MIP	Monocyte inflammatory protein
MRE11	Meiotic recombination 11
mRNA	Messenger RNA
MsigDB	Molecular Signatures Database
MTT	tetrazolium-based colorimetric method
MyD88	Myeloid differentiation factor 88
NACT	Neoadjuvant chemotherapy
NBD	Nucleotide binding domain
NF-κB	Nuclear factor kappa-B
NK cells	Natural Killer cells
NLRs	NOD-like receptors
NOD	Nucleotide oligomerization domain
NSCLC	Non-small cell lung cancer
OC	Ovarian cancer
ORR	Overall response rate
OS	Overall survival

OSCD	Overall survival since cancer diagnosis
P/S	Penicillin/Streptomycin
PAMPs	Pathogen-associated molecular patterns
PARP	Poly (ADP-ribose) polymerases
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PC	Pancreatic cancer
pCR	Pathological complete response
PCR	Polymerase chain reaction
PD1	Programmed cell death 1
PFS	Progression-free survival
PHA	Phytohemaglutinin
Poly(I:C)	Polyinosinic:polycytidylic acid
PR	Progesterone receptor
PRRs	Pattern recognition receptors
qPCR	Quantitative real-time PCR
RC	Rectal cancer
RIG-I	Retinoic acid-inducible gene-I
RLRs	(RIG-I)-like receptors
RLU	Relative light units
RNA	Ribonucleic acid
RNAi	RNA interference
RNAseq	RNA sequencing
RNR	Ribonucleotide reductase
RPMI	Roswell Park Memorial Institute
RS	Regulatory sites
RT	Reverse transcription
RT-PCR	Reverse-transcriptase PCR
SAMHD1	Sterile Alpha Motif (SAM) Histidine-Aspartic domain (HD)-containing protein 1
SD	Statistical deviation
SEM	Standard error of mean
SERDs	Selective estrogen receptor degraders
SERMs	Selective estrogen receptor modulators
siNT	non-targeting
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SSC	Side scatter
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
TAM	Tumour-associated macrophages
TBK	TANK-binding kinase 1
TCR	T-cell receptors
TGCA	The Cancer Genome Atlas

TGF	Tumour-growth factor
Th cells	T-helper cells
Thf cells	T follicular helper cells
TILs	Tumour-infiltrating lymphocytes
TKI	Tyrosine kinase inhibitors
TLRs	Toll-like receptors
TMA	tissue microarrays
TME	Tumour microenvironment
TNBC	Triple-negative breast cancer
TNF	Tumour Necrosis Factor
TNF	Tumour necrosis factor
TNM	Tumour Node Metastasis
TOR	Target Of Rapamycin
Treg	T regulatory cells
TTP	Time to progression
TYK2	Tyrosine kinase 2
WB	Western Blot
WHO	World health organization
WT	Wild type
γH2AX	Phosphorylated histone H2AX

INTRODUCTION

1. THE IMMUNE SYSTEM

The immune system is designed to execute rapid, specific, and protective responses against infections, *foreign* nucleic acids, or damaged cells, like cancer cells. In order to avoid harmful effects of potential autoreactive responses, multiple tolerance checkpoints are present in several organs (1). Any substances recognized by the immune system and that can thus stimulate an immune response is called antigen. Antigens are usually proteins presented in the surface of cells, viruses, fungi, or bacteria but they can be non-living substances such as toxins.

Human immunity is classified as innate, adaptive, and passive. The innate or non-specific immunity is present since birth and does not require pre-exposure to pathogens. It is the first to respond after identifying an alarm signal. Examples of innate immunity are cough reflex, skin, or stomach acid (2). A very important mechanism of the innate immune response is inflammation (3), a process that will be further described in the following sections. On the other hand, the adaptive or acquired immunity is only developed after the exposure to antigens. It is a specific response against a concrete pathogen; therefore, it can take some time before the responses are effective. Typically, immunological memory was an exclusive hallmark of the adaptive immune response. However, this traditional paradigm is being currently challenged as growing body of literature is showing that innate immune cells can develop immune memory and provide long-lasting protection by epigenetic modifications. This concept is termed trained immunity (4,5). Finally, passive immunity is a temporary type of immunity that derives from another person. It can occur naturally, when maternal antibodies are transferred to the foetus through the placenta or from breast milk to the gut of the infant but it can also be produced artificially, when antibody preparations derived from sera or secretions of immunized donors are transferred to non-immune individuals (6).

1.1 CELLS OF THE IMMUNE SYSTEM

The immune system is composed by different parts that work together to defend the body. The primary components of the immune system include the bone marrow and the thymus (7). All the cells of the immune system are originated from hematopoietic pluripotent stem cells (known as progenitor or precursor cells) in the bone marrow. Then, they differentiate into the common myeloid progenitor or into the common lymphoid progenitor, that circulate in the blood and through the lymphatic system to migrate to peripheral tissues.

The common lymphoid progenitor differentiates further into the four major populations of mature lymphocytes, that are mainly related with the adaptive immunity: B cells, T cells, natural killer (NK) cells and NK-T cells (8). **B lymphocytes or B cells,** which express the transmembrane

protein CD19 on their surface, differentiate in the bone marrow and express B-cell receptors (BCR) (9). Activated B cells differentiate into plasma cells that secrete antibodies participating in mounting an immune response. **T cells** differentiate in the thymus and express T-cell receptors (TCR), that bind processed antigen displayed by antigen presenting cells (APC). TCR is associated with a CD3- T cell co-receptor (10). T cells, when activated, differentiate into **cytotoxic T cells** (CD8+ T cells), responsible of eliminating infected or damaged cells, or into **helper T cells** (CD4+ T cells) that activate other cells, such as B cells and macrophages and orchestrate the adaptive arm of the immune system by producing cytokines with chemotactic, pro-inflammatory and immune-protective properties. Naïve helper T cells, depending on the antigen they encounter, have the potential to differentiate into subgroups, such as Th1, Th2 or Thf (T follicular helper), each characterized by the secretion of a specific cytokine profile. Once differentiated, Th1 effector cells are characterized by the production of pro-inflammatory cytokines. Th1 response is involved in the clearance of intracellular pathogens and tumour cells. Th2 cells are implicated in the defence against extracellular parasites (e.g. helminths) and the stimulation of the humoral response (via the B cells) (11). All activated T cells produce and signal proliferative/survival cytokines and begin to expand in number. Excessive T cell proliferation and survival could become detrimental for the body. To prevent this T cell hyperactivation, **regulatory T cells** (CD4+CD25+) dampen the immune response. Additionally, inhibitory molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death 1 (PD1) are also induced during immune responses and represent a 'checkpoint' to dampen T cell hyperactivation (12). Following the antigen-driven expansion and the death of effector cells after antigen clearance, some of the remaining T cells differentiate into **memory T cells** of two different types: central memory (located in lymphoid organs and bone marrow, present high proliferative potential) and effector memory T cells (stay in peripheral tissues in a preactivated form with rapid activation after pathogen recognition) (13). **NK cells** are distinguished by their lack of TCR or BCR but are able to recognize target cells using a complex collection of activating and inhibitory cell surface receptors (14). NK cells can be divided into functional subsets based on the relative expression of the surface markers CD56 and CD16. The two major subsets are highly cytotoxic and low cytotoxic: $CD56^{bright}$ $CD16^{dim/-}$ and $CD56^{dim}$ $CD16^{+}$, respectively (15). **NK-T cells** share characteristics from NK and T cells, expressing surface markers characteristic from both NK and conventional T cells (8).

On the other hand, cells from the myeloid lineage contribute to the innate immune system in recognizing invading pathogens and tissue damage. The myeloid progenitor is the precursor of granulocytes, macrophages, dendritic cells, and mast cells. **Granulocytes**, also known as

polymorphonuclear leukocytes, contain enzymatic granules in their cytoplasm. There are three types of granulocytes that are produced in high numbers during immune responses: (i) neutrophils, the most numerous phagocytic cells and an important cellular component of the innate immune response, due to the production of large quantities of reactive oxygen species but also substantial amounts of chemokines and cytokines, such as Tumour Necrosis Factor (TNF) and interleukin (IL)-12 (8), (ii) eosinophils, that contain toxic molecules and enzymes and play important roles against parasitic infections and (iii) basophils, whose function is similar and complementary to that of eosinophils. Basophils, together with **mast cells**, are also key regulators of the allergic responses, releasing important quantities of histamine, IL-4 and other preformed mediators that affect vascular permeability, leading to tissue inflammation and oedema. **Monocytes** are responsible of the orchestration of the immune response against infection and inflammation, by differentiating into dendritic cells and macrophages (16,17). **Dendritic cells**, the most potent types of antigen-presenting cells (APCs), specialized in taking up antigen and displaying it for the corresponding recognition by lymphocytes. Immature dendritic cells migrate from the blood to reside in the tissues and are both phagocytic and macro-pinocytic, ingesting large amounts of the surrounding extracellular fluid. Upon encountering a pathogen, they rapidly mature and migrate to lymph nodes. **Macrophages** are widely distributed in the body tissues, where they play a critical part in innate immunity, mainly due to their phagocytic activity. Monocytes and macrophages express CD14 protein on their surface. Macrophages are also closely related to inflammatory responses. Due to their heterogeneous phenotype, they can adapt to the tissues and organs in which they reside, leading to tissue-resident macrophages that have specific functions needed within the tissue (18). Based on their polarization (how macrophages have been activated at a given point in space and time), activated macrophages are usually divided into two categories: M1-like macrophages (also referred as classically activated macrophages, CAMs) usually linked to pro-inflammatory responses, and M2-like macrophages (known as alternatively activated macrophages, AAMs) mainly involved in anti-inflammatory responses (19,20). Polarization status can switch from M1 to M2 or *vice versa* in response to environmental stimuli (8). Furthermore, a key role of the cells from the monocyte/macrophage lineage is to take antigens and process them through proteolysis, to subsequently present the generated peptide fragments to T cells and activate adaptive immune response. In critical tissues subjective to pathogen invasion, specialized macrophages are responsible for this process, as Langerhans cells in the epidermis, Kupffer cells in the liver, and microglial cells in the central nervous system (8,18).

Innate and adaptive immune response work together to mount a functional and complete immune response (Figure 1). When a pathogen is detected, phagocytes travel through the body and digest them into smaller pieces, maturing into antigen presenting cells (APC). The maturation process involves the loss of the ability to further phagocyte, alters surface expression of major histocompatibility complex (MHC) molecules (also called the human leukocyte-associated [HLA] antigens) and other co-stimulatory molecules and develops the ability to produce increased levels of cytokines, leading to an inflammatory response. MHC proteins expressed on APCs are used to permit recognition of processed antigen by the TCR on naïve T cells. Depending on the pathogen, different T cell subsets are activated (Treg, Th1, Th2…). Finally, B cells will start the antibody production and memory T cells start to develop to respond faster in case of re-exposure to the pathogen (21).

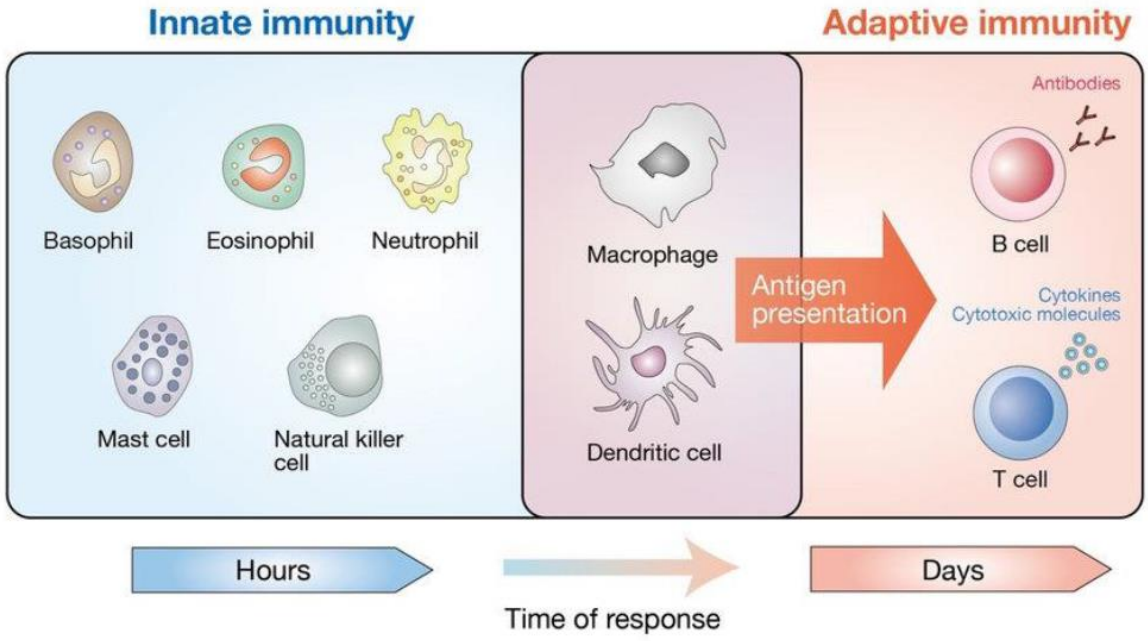


Figure 1. Coordination between innate and adaptive cells to mount an effective immune response. Innate and adaptive immune cells interact to present antigens and develop cytokines and antibodies necessary for the development of an inflammatory and antiviral response to respond to a foreign pathogen and an approximate timeline of the responses. Obtained from (22).

1.2 INFLAMMATION AND IMMUNITY

Inflammation is a key part in the immune system response to harmful stimuli, such as pathogens, damaged cells, or toxic compounds. After exposure to damaging agents, the inflammatory response is initiated, and the inflammatory transcriptional cascade subsequently ensues in sentinel innate immune cells that contribute to homeostasis restoration. Inflammation is normally beneficial for the host and can be resolved rapidly, however, uncontrolled inflammation may become chronic, leading to a variety of chronic inflammatory responses that are harmful to the organism (23,24). Although inflammatory response depends on the initial

stimulus and the specific tissue/cells involved, shared mechanisms are identified: i) recognition of signalling factors by cell surface pattern receptors, ii) activation of downstream signalling pathways or inflammatory pathways, iii) release of inflammatory effectors and other factors and iv) recruitment of inflammatory cells. At the tissue level, all these molecular processes leads to redness, swelling, heat and pain, and loss of tissue function (23).

i) Ligand-recognition mechanisms and PRRs

Pathogens and/or damaged tissue can trigger an inflammatory response through the activation of pattern recognition receptors (PRRs) that are expressed predominantly in immune cells but also in non-immune cells. PRRs are expressed on the cell membrane but they can also be found in intracellular compartments and in the cytoplasm. PRRs recognize widely conserved features in pathogens, termed pathogen-associated molecular patterns (PAMPs) but also endogenous signals known as damage-associated molecular patterns (DAMPs) released in response to cell or tissue injury (24,25). The five major pathogen sensor families include: Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs) (26) (Figure 2). Apart from extracellular elements, PRRs can also recognize endogenous factors that could indicate a loss of homeostasis if delocalized (e.g., nucleic acids). In this sense, DNA and RNA derived from external microorganisms but also from host cells play fundamental roles in the activation of the immune system (27,28). Nucleic acids are recognized by different PRRs: TLRs, RLRs and cytosolic DNA sensors that are able to distinguish between “self” and “non-self” nucleic acids (29). Sensing of non-self-nucleic acids is based on (i) localization of nucleic acids in aberrant compartments usually devoid of DNA or RNA, i.e., outside the cell membrane or at the cytosol, (ii) changes in DNA or RNA concentration and (iii) structure and conformation of nucleic acids determined by chemical modifications. Discrimination of non-self-nucleic acids is relevant in the context of autoimmune diseases or cancer, where mutations in genes, damaged cells or stress can activate nucleic acid sensors in the absence of infection (28,29). Besides, the activation of nucleic acid sensors could also lead to the induction of programmed cell death. Indeed, apoptosis, pyroptosis, and necroptosis can all be initiated by DNA and RNA sensors (28).

Toll like receptors

TLRs are type I transmembrane glycoproteins that are composed of an extracellular region (responsible for the recognition of specific ligands), a transmembrane region, and an intracellular region. The intracellular domain conducts signals by binding to different adaptor proteins, that amplify the inflammatory response. So that other cells are recruited and activated

through the transcription of genes, leading to the production and secretion of a variety of pro-inflammatory factors (30). Up to now, eleven TLRs (TLR1~TLR11) have been identified in human cells and they are expressed either in the cytoplasm or in the cell membrane. The cellular localization of TLRs determines the types of ligands and the recognition mechanism. Some TLRs (TLR1, 2, 4, 5, 6, 10) are expressed on the surface of immune cells, mainly recognizing the membrane components of pathogenic microorganisms, such as lipids, lipoproteins, and proteins, others mainly recognize the nucleic acids of microorganisms. Among them, TLR3, TLR7, TLR8, TLR9 and TLR13 recognize ssRNA and dsRNA and TLR9 preferentially detects DNA containing unmethylated cytidine-phosphate-guanosine (CpG) motif common in bacteria to activate downstream signalling pathways (29–31).

NOD-like receptors

The NOD-like receptors are intracellular PRRs present in the cytoplasm. Until now, 22 types of NLRs have been described and all of them present three domains: the central nucleotide binding domain (NBD), also known as the NACHT domain, Leucine rich repeats (LRRs) at the C-terminus, important or ligand identification, and the N-terminal effector domain, which is the protein interaction domain, such as the caspase activation and recruitment domain (CARD). Among the NLRs family, the most in-depth studies have been focused on NOD1 and NOD2 proteins. NOD1 mainly recognizes the cell wall of gram-negative bacteria and NOD2 can also recognize complete single-stranded RNA (ssRNA) of viruses (30).

RIG-I-like receptors

RLRs are intracellular PRRs specialized in RNA recognition localized in the cytosol. This protein family encompasses three members: retinoic acid-inducible gene I (RIG-I, coded by the *DDX58* gene), melanoma differentiation-associated protein 5 (MDA5, coded by *IFIH1*), and laboratory of genetics and physiology 2 (LGP2, coded by *DHX58*) (32). All three RLRs share structural features consisting of a central DExD/H box RNA helicase domain and a C-terminal domain (CTD, also known as the regulatory or repressor domain), responsible for the recognition of RNA. Additionally, RIG-I and MDA5 have two caspase activation and recruitment domains (CARDs) at the N-terminus, responsible for downstream signalling. The third RLR, LGP2, lacks CARD domains and it is known to regulate the function of RIG-I and MDA5 (32,33). Both RIG-I and MDA5 can recognize viral RNA, but despite their similarities, they recognize different dsRNA structures. RIG-I recognizes relatively short dsRNA, while MDA5 preferentially binds to long dsRNA (>1 kb).

C-type lectin receptors (CLRs)

CRLs are phagocytic PRRs. In contrast to previous receptors, phagocytic receptors recognize PAMPs to place pathogens in cytoplasmic vesicles for direct digestion. CRLs, through their carbohydrate recognition domain (CRD), recognize carbohydrates on the surface of microorganisms. They can be present either on the cell membrane as transmembrane receptors or to be secretory receptors (30).

AIM2-like receptors

ALRs are a new type of PRRs that can recognize intracellular DNA. When binding to dsDNA, ALRs form a multimeric complex called inflammasome, which trigger the release of proinflammatory cytokines. Apart from participating in the regulation of the innate immune response, they also regulate apoptosis (30).

In addition of the four major types of PRRs, there are other PRRs described as universal cytosolic DNA sensors. One of the most important is the cyclic GMP-AMP (cGAMP) synthase (**cGAS**), that recognizes and responds to cytosolic DNA in a DNA-sequence-independent but DNA length-dependent manner in various cell types (34).

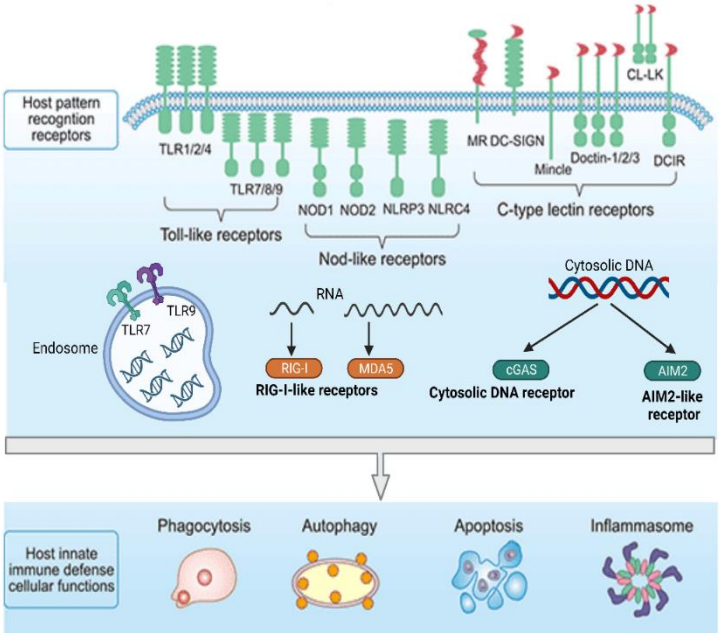


Figure 2. Major types of Pattern Recognition Receptors (PRRs). Toll-like receptors (TLRs) and C-type lectin receptors (CRLs) are transmembrane receptors composed by an extracellular region, a transmembrane region and an intracellular region that signals by binding to different adaptor proteins. Both TLRs and CRLs are present in the cytoplasm membrane but TLRs could be present also in intracellular compartments, such as endosomes, and CRLs can be secretory receptors. NOD-like receptors are intracellular PRRs present in the cytoplasm that recognize extracellular pathogens and viral ssRNA. RIG-I-like receptors (RLRs), such as RIG-I and MDA5, are intracellular PRRs specialized in RNA recognition present in the cytosol. Some other receptors specifically recognize DNA. Among them, TLR9 and TLR7, absent in melanoma 2 (AIM2) receptors and cyclic GMP–AMP synthase (cGAS) are the best described DNA-sensors in mammalian cells. Figure modified from (35). Created with BioRender.

ii) Activation of signalling pathways

Although activated by different PAMPs and DAMPs in distinct subcellular structures, PRRs trigger intracellular inflammation signalling pathways that include a variety of inflammatory mediators and regulatory proteins involved in signal transduction. The main three types of signalling molecules involved are protein kinases, adaptor proteins, and transcription factors. At the end, the signals transmitted by those signalling molecules converge into several common signalling pathways that will be briefly described in this section.

Nuclear factor kappa-B (NF-κB) signalling pathway.

The NF-κB family plays a key role in inflammation and immunity, but it is also involved in tumour development and in the formation of inflammasomes (36). The NF-κB family is composed of five related transcription factors: p50 (also named NF-κB1), p52 (also named NF-κB2), RelA (p65), RelB, and c-Rel. Under physiological conditions, NF-κB is regulated by inhibitory proteins present in the cytoplasm, IκB family, whose most important member is IκBα. NF-κB activation might occur through the canonical or non-canonical (alternative) signalling pathways. The canonical NF-κB pathway include the degradation of the inhibitory protein IκBα through its phosphorylation by IκB kinase (IKK) complex. Phosphorylation of IκB results in its degradation by the proteasome, resulting in the release and the subsequent rapid and transient nuclear translocation of NF-κB members for gene transcription activation (Figure 3). Canonical activation of NF-κB responds to diverse factors, including PRRs mentioned above (23,37) . In contrast, non-canonical NF-κB activation responds selectively to a specific group of stimuli and does not involve IκBα degradation. Here, NF-κB-inducing kinase (NIK) activates IKKα, that phosphorylates p100, the precursor of p52 (NF-κB2). Phosphorylation of p100 results in the generation of mature NF-κB2 p52, ready for nuclear translocation (37).

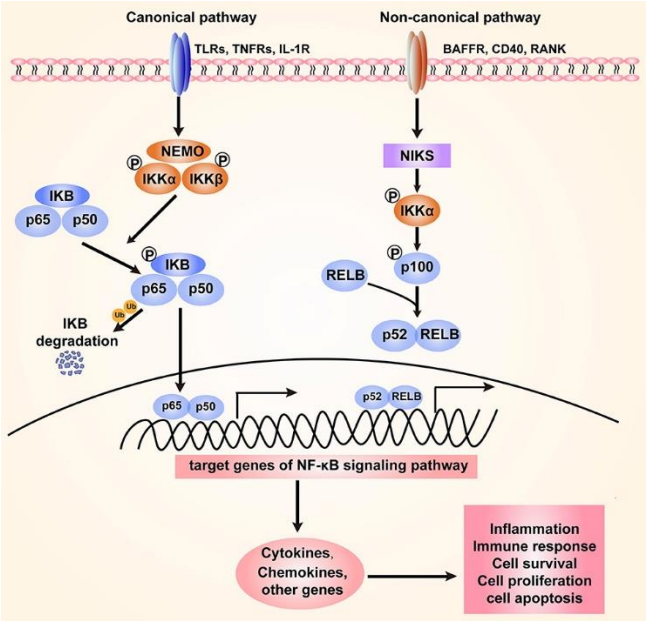


Figure 3. The canonical and non-canonical NF-κB signalling pathway. Canonical NF-κB signalling pathway is mediated by the phosphorylation and degradation of inhibitory protein IκB by IκB kinase (IKK) protein complex. NF-κB is then released with the subsequent rapid and transient nuclear translocation of NF-κB members for gene transcription activation. The non-canonical pathway is dependent on the processing of NF-κB2 (p100), precursor of p52, which is further activated and translocated to the nucleus. Nuclear translocation of NF-κB members to the nucleus induces the expression of cytokines, chemokines and other genes that regulate several cellular processes. Obtained from (38).

NF-κB signalling pathway can be activated by typical PPRs-mediated signalling. TLRs signalling, depending on the different adaptor proteins, can be divided into myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent pathways. In the MyD88-dependent pathway, the C-terminus of MyD88 binds to the intracellular domain of TLRs, and the N-terminus of MyD88 recruits IL-1R-related kinase 4 (IRAK4). The different proteins are sequentially activated and recruited, leading to the activation of the IκB kinase (IKK) complex through phosphorylation, with the subsequent release of NF-κB that is translocated into the nucleus (30). In the case of the MyD88-independent pathway (present in TLR3 and TLR4 sensing), TLRs directly interact with 2 TIR adaptor proteins, TIRAP and TRIF, inducing the transduction of downstream factors without passing through MyD88 (39).

The signal pathway mediated by NLRs recruits downstream receptors that at the end activates the IκB kinase (IKK) complex, leading to the release and the subsequent rapid and transient nuclear translocation of NF-κB (30).

After recognition of viral RNA, activated RIG-I and MDA5 induce downstream signal transduction by binding with mitochondrial antiviral signalling protein (MAVS), an important adaptor protein. MAVS interact with different downstream signalling molecules and activate the protein kinase IKK, activating the NF-κB pathway (30).

Mitogen-activated protein kinase (MAPK) pathway.

Mitogen-activated protein kinase (MAPKs) are a family of serine/threonine protein kinases that direct cellular responses to a variety of stimuli. The typical MAPK pathway consist of a series of three phosphorylation events in which a MAPK kinase kinase (MAPKKK or MAP3K) phosphorylates a MAPK kinase (MAPKK or MAP2K, also called MEKs or MKKs), which in turn phosphorylates a MAPK (23). In mammals, MAPK family is comprised by the extracellular signal-regulated kinases (ERKs-1 and -2), the c-Jun NH2-terminal kinases (JNKs) and the p38 isoforms (p38s). ERK1/2 is generally activated by any agonist that engage the Ras pro-oncoprotein, such as some growth factors and mitogens. Ras, in turn, recruits the Raf family of MAP3Ks, that activate two ERK-specific MAP2Ks: MEK1 and MEK2. p38 MAPK and JNKs are also activated by cellular stress and inflammatory cytokines (40).

Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway is a highly conserved pathway that plays critical roles in immune system and inflammation. It is composed of ligand-receptor complexes, JAKs (JAK1, JAK2, JAK3 and TYK2) and STATs (STAT1-4, STAT5a, STAT5b and STAT6). The classical JAK/STAT signalling starts when receptor-associated JAKs are activated by ligands and phosphorylate one other, forming a docking site for STATs. At this docking site, cytoplasmic STATs are recruited and phosphorylated, suffering a conformational change that leads to their dimerization. These dimers translocate then into the nucleus and bind to specific DNA-binding sites regulating gene expression (Figure 4) (23,41,42).

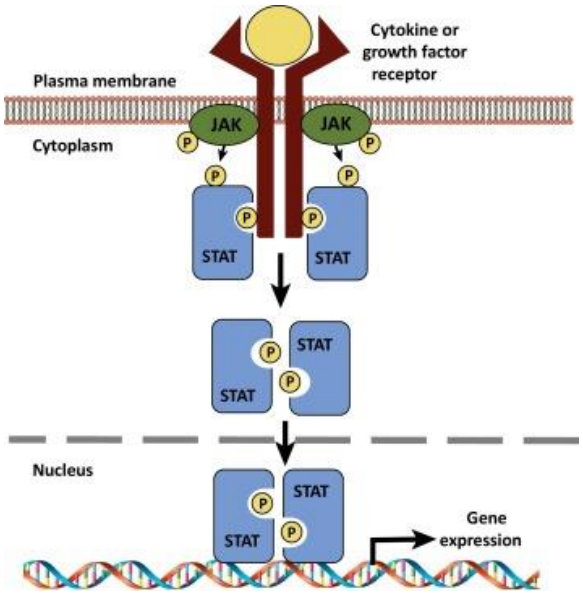


Figure 4. Janus kinase-signal transduction and activation of transcription (JAK–STAT) pathway. Binding of ligands, i.e., cytokine or growth factors to JAK-associated receptors leads to the activation and phosphorylation of JAK. This induces the phosphorylation and dimerization of STAT, that migrates to the nucleus and binds to target gene promoters to regulate gene transcription and expression. Obtained from (43).

The TBK1–IRF3/7 signalling.

Interferon Regulatory Factors 3 and 7 (IRF3 and IRF7) are key transcription factors that promote the synthesis of type I IFN. Upon activation, TBK1 phosphorylates the transcription factors IRF3 and IRF7, which leads to their dimerization and translocation to the nucleus. Both IRFs can be activated through two signal pathways: the MyD88 independent pathway (downstream of TLR3/TLR4) through the adaptor protein TRIF and in the cytosolic RNA and DNA sensing pathway through RIG-I-MAVS. Once activated, IRFs dimerize and merge into the nucleus to work (30).

IRF3 and IRF7 are critical to production of type I interferons downstream of pathogen recognition receptors. But there are several other members of the IRF family that have been implicated in the regulation of the immune response. For example, IRF5 is also important in the production of type I IFN, IRF9 regulates interferon-driven gene expression and IRF4, IRF8, and IRF5 regulate myeloid cell development and phenotype. Thus, all of them play important roles in regulating inflammatory responses (44).

iii) Inflammatory factors

Cytokines are the key modulators of inflammation. They are small soluble proteins released by immune cells that have a specific effect on the interactions and communications between cells, either to facilitate or to inhibit inflammation (pro- or anti-inflammatory cytokines, respectively) (45). Cytokine is the general term to name those proteins, but cytokines include:

- chemokine: cytokines with chemotactic activities.
- interleukins (ILs): type of cytokine first thought to be expressed by leukocytes alone but have later been found to be produced by many other body cells.
- colony-stimulating factors (CSF): control the production and differentiation of blood cells. CSFs include granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF).
- interferons (IFNs): broad class of cytokines essential for mobilizing immune responses.
- tumour-growth factors (TGFs): cytokines that stimulate tumour growth.

Cytokines modulate the immune response and inflammation by a complex network of interactions that can also lead to cellular damage, ultimately leading to death (23). In a steady state, cells produce very low amounts of proinflammatory cytokines. Upon innate immune activation, large amounts of IFN and other proinflammatory cytokines are produced and the signalling pathway is rapidly amplified to drive an strong immune response (46). In general, cytokines are produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Secreted cytokines bind to their respective receptors resulting in the

production of other proinflammatory cytokines (47,48). Cytokines can act on the same cell type or to act on several different cell types (pleiotropy).

Pro-inflammatory cytokines

Proinflammatory cytokines are produced predominantly by activated macrophages, dendritic cells and CD4+ T cells. They are involved in the up regulation of inflammatory reactions. Major pro-inflammatory cytokines include IL-1, IL-6, IL-18, and TNF-α (45). IL-1 is subdivided in IL-1α and IL-1β. IL-1β is potent pro-inflammatory cytokine, induced mainly by lymphocytes, macrophages, and monocytes in response to microbial molecules. IL-6 is a pleiotropic cytokine that not only affects the immune system, but also acts in other physiological events, such as regulating cell growth, as well as gene activation, proliferation, survival, and differentiation. IL-6 is produced by a variety of cell types including monocytes, fibroblast, and endothelial cells. Binding of IL-6 to its receptor initiates cellular events including activation of JAK (Janus Kinase) kinases. TNF-α has an important role comprising the inflammatory response both locally and in the circulation. TNF-α triggers the expression of vascular endothelial cells as well as enhances the leukocyte adhesion molecules that stimulate immune cell infiltration.

Chemokines

Chemokines are low molecular weight secreted proteins that primarily function in the activation and migration of leukocytes although some of them also possess a variety of other functions (45). Chemokines include CCL5 (RANTES), monocyte chemoattractant protein or MCP-1, monocyte inflammatory protein or MIP-1α, and MIP-1β, IL-8 also called growth related oncogene or GRO/KC). CXCL10 (also referred to as interferon inducible protein-10, IP-10) is one of the most common proinflammatory chemokines. It is induced by interferon (IFN)-γ and it is responsible for the recruitment of a variety of immune cells to sites of infection through interaction with its cellular receptor CXCR3 (49).

Anti-inflammatory cytokines

The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory cytokine response (45). Major anti-inflammatory cytokines include interleukin (IL)-1 receptor antagonist, IL-4, IL-10, IL-11, and IL-13. IL-4 and IL-13 belong to the T helper 2 (Th2) cytokine family and present important immunomodulatory activities. These cytokines display the capacity to antagonize Th1-driven proinflammatory immune response and downregulate synthesis of many proinflammatory cytokines (50). Interleukin 10 (IL-10), which is produced in almost all leukocytes, is a potent anti-inflammatory cytokine that plays a crucial, and often essential, role in preventing inflammatory and autoimmune pathologies (51).

Although some cytokines present specific roles in inflammation, occasionally, cytokine function may vary depending on various circumstances and they can present dual-function (anti-inflammatory or pro-inflammatory) depending on the site of expression and concentration. For example, leukemia inhibitory factor, interferon-alpha, IL-6, and transforming growth factor (TGF)-β can exhibit both pro-inflammatory and anti-inflammatory properties depending on the context and the signalling pathways that are activated (45).

In addition, a dysregulated cytokine production can lead to severe inflammation. Dysregulation of the immune response may lead to a massive increase of cytokine and chemokine levels which is referred to as cytokine release syndrome or cytokine storm. This phenomenon is characterized by an aggressive pro-inflammatory response in combination with an insufficient anti-inflammatory response, which results in the loss of homeostasis of the immune response (52)

2. CANCER

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. In 2020, there were 18.1 million estimated cancer cases around the world. Of these, 9.3 million cases were in men and 8.8 million in women (53). Breast and lung cancers were the most common cancers worldwide (11.7% and 11.4% of the total number of new cases diagnosed in 2020). The third most common cancer was colorectal cancer with 1.9 million new cases in 2020 (10% of new cases). Among women, the most common cancer is breast cancer while in men, lung and prostate cancer account for the major number of cases (Figure 5) (54). Despite recent advances in cancer biology and treatments, cancer is still a leading cause of death worldwide, accounting for nearly 10 million of deaths in 2020. The most common cancers leading to death were lung, colorectal and liver (55).

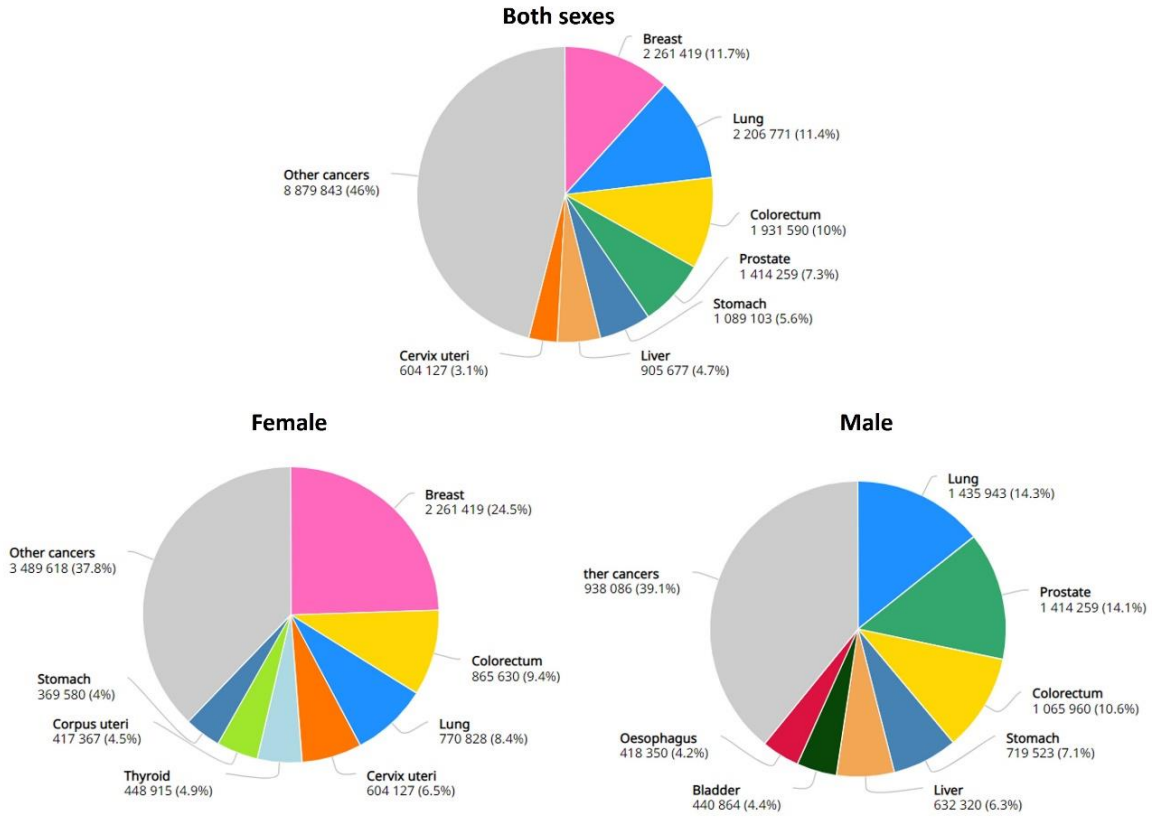


Figure 5. Estimated number of new cancer cases in 2020 in the world. Upper graph: both sexes, bottom graphs: stratified by sex (female on the left and male on the right). Obtained from International Agency for Research on Cancer, World Health Organization (WHO) (54).

Most cancers have a multifactorial aetiology and are attributable to a varying blend of genetic and environmental factors (56). Although environmental factors are gaining attention, over recent decades several genes causing predisposition to cancer have also been identified. Inherited cancer predisposition usually occurs because of germline alterations in either tumour suppressor genes or in oncogenes. Tumour suppressor genes are involved in limiting neoplastic processes, for example, by controlling cell cycle and proliferation or participating in DNA repair. Oncogenes are typically derived from proto-oncogenes that encode proteins involved in the regulation of cell proliferation, growth, and differentiation, such as growth factors, signal transducers or transcription factors. Mutations either in tumour suppressor genes or in oncogenes may lead to uncontrolled cell replication and cancer development, since tumour suppressor genes loss their protective functions and oncogenes become activated (57). Examples of oncogenes include *HER2* (*human epidermal growth factor receptor 2*), important in breast cancer, and *N-myc*, relevant in neuroblastoma. Among tumour suppressor genes, the most relevant are *BRCA1/2 (Breast cancer 1/2 genes)*, *p53* (tumour protein p53) or *APC (Adenomatous polyposis coli gene)*.

2.1 IMMUNE SYSTEM, INFLAMMATION AND CANCER: THE TUMOUR MICROENVIRONMENT

It is well known that immunity and inflammatory responses have key roles in different stages of tumour development, from initial phases to metastasis. The first indication of a possible link between immune system and cancer was in the 19th century, when Rudolf Virchow described for the first time the presence of leukocytes within tumours (58). However, the protective function of the immune system in cancer was proposed in 1960 by Frank M. Burnet and Lewis Thomas (59,60). They speculated that lymphocytes could act as sentinels in recognizing and eliminating transformed cells before they manifested the disease (61). It was not until late 1990s, with the support of *in vivo* experimental data, when the link between cancer and immune system was fully demonstrated and accepted (62–64). Since then, the relation between cancer and immune system has been extensively studied and characterised and molecular understanding of cancer has been considerably improved. Cancer research has switched from a cancer-centric model towards the concept of tumour microenvironment (TME) (65). Tumour microenvironment is defined as the specialized ecosystem that surrounds a tumour, including tumour-infiltrating immune cells, stromal cells, blood vessels and non-cellular components such as the extracellular matrix (ECM) (Figure 6) (66). The complex interactions between these components are critical in tumour development and may change the progression of the tumour: they can either supress or promote tumour growth. Therefore, it is important to understand the intercellular interactions and functions (67).

Among all components in the TME, cancer-associated fibroblasts (CAFs) are the most abundant stromal cells. They are highly heterogenous and show both tumour-suppressive and tumour-promoting activities. They secrete many kinds of cytokines that are described to influence tumour growth and progression (68,69). Tumour-infiltrating immune cells present in the TME are also a critical player in tumour progression and immunotherapeutic response, acting as a double-edge sword that can either promote or supress tumour development. The most abundant immune cells present in the TME are T lymphocytes, especially CD8+ cytotoxic T lymphocytes, but NK cells, macrophages, CD4+ T helper cells, and antigen presenting cells (APCs) are also important components that initiate an antitumoural response, especially at early stages of cancer development. Moreover, immune cells that stimulate tumour growth by promoting immune suppressive environment and tumour cell survival, are also present, for example regulatory T cells (Tregs) or M2 macrophages (70). In terms of physico-chemical characteristics of the TME, hypoxia is the most important hallmark during tumour development. Hypoxia induces cancer progression and resistance to anti-tumoural treatments (71).

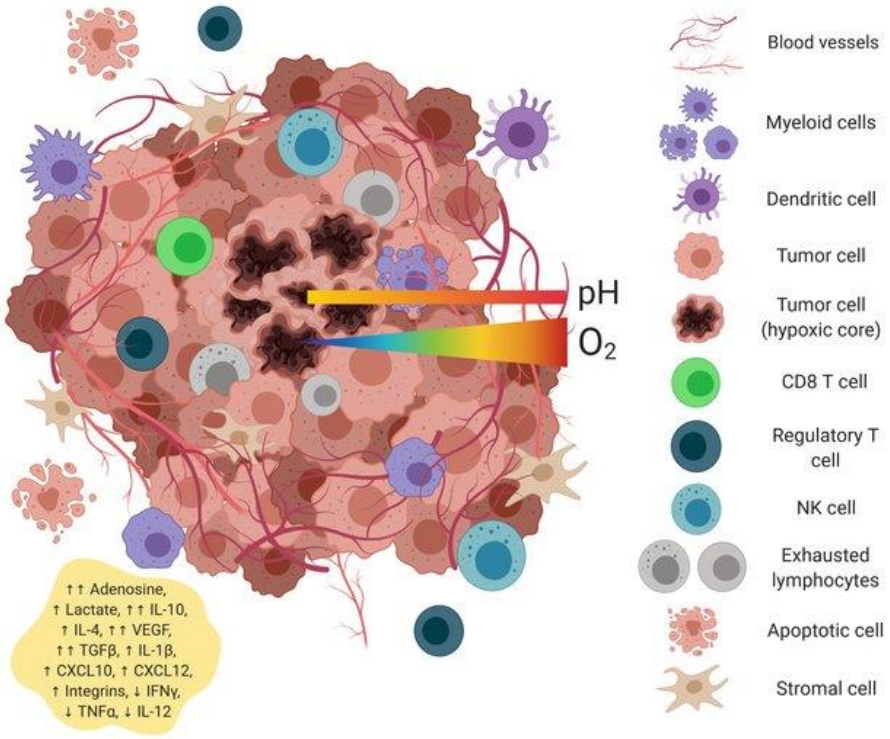


Figure 6. Components of the tumour microenvironment. The tumour microenvironment is a complex ecosystem of heterogeneous tumour cells, stromal cells, and a variety of immune cells residing in a network of dysregulated vasculature and collagen. Tumour microenvironment is often hypoxic, especially the central part of the tumour. It is also characterised by an acidic pH and poor nutrient loads. Tumour-infiltrating immune cells of both the myeloid and lymphoid lineages are found within the TME. Obtained from (72).

According to a recent classification based on the activation and infiltration of the immune cells into the tumour site, three immunoprofiles are distinguished: i) hot tumours that present high T lymphocyte infiltration, ii) cold tumours or non-inflammatory tumours that are scant of any immune cell infiltration nor inflammatory signals and iii) tumours with immune exclusion where immune cells are at the periphery or in the stromal tissue (67).

2.2 GENERAL CLASSIFICATION OF CANCER

Although cancer is a very complex and diverse group of diseases, an initial classification is almost mandatory as starting point for cancer management (73). Typically, internationally accepted classifications for cancers are based on primary site of origin (tissue or organ). Within each specific major type, finer subgroups can be defined by specific type and histological grades, and finally by spread according to the Tumour Node Metastasis (TNM) system (74,75). However, depending on tumour type, other classifications can be applied, i.e., molecular markers. Moreover, the recent onset of new data generated by high-throughput technologies has provided new insights into the diversity of human cancers and the opportunity to discover new cancer subtypes (75).

➢ Based on classification by **primary site of origin** cancers can be solid tumours or hematological tumours (Table 1) (74,75).

Table 1. Broad classification of cancers based on primary site of origin.

Type	Subtypes		Origin	Examples
Solid tumors	Carcinomas (epithelial cells)	Adenocarcinoma	Organ or gland	Breast, stomach, lung cancers
		Squamous cell carcinoma	Squamous epithelium	Esophageal cancer, NSCLC*
	Sarcomas		Connective and supportive tissues	Osteosarcoma
	Lymphomas		Lymphatic system	Hodgkin's lymphoma
Hematological tumors	Leukemia		Bone marrow (excessive immature white blood cells)	AML, CML, ALL, CLL*
	Myeloma		Plasma cells of bone marrow	-

NSCLC, non-small cell lung cancer, AML, Acute myelocytic leukemia, CML, Chronic myelocytic leukemia, ALL, Acute lymphocytic leukemia, CLL, Chronic lymphocytic leukemia.

➢ Classifications by **histological grade** combine protein expression features and morphological–structural observations. It mainly refers to the abnormality of the tumour cells with respect to surrounding normal tissues. The grade is expressed numerically from 1 to 4. Cells that are well differentiated closely resemble normal specialized cells and belong to low grade tumours. Cells that are undifferentiated are highly abnormal with respect to surrounding tissues. These are high grade tumours (74,75).

Grade 1 – well differentiated cells with slight abnormality.
Grade 2 – cells are moderately differentiated and slightly more abnormal.
Grade 3 – cells are poorly differentiated and very abnormal.
Grade 4 – cells are immature and primitive and undifferentiated.

➢ Cancers can also be classified according to their stage. The most widely used system for scoring tumour spread is the TNM Classification (76). It rates the size or extent of the primary tumour (T), the degree of regional spread or lymph nodes involvement (N), and the presence of distant metastasis (M). Each of these three categories has several numbered classes:
Stage 0 – *in situ* cancer or limited to surface cells.

Stage I – cancer limited to the tissue of origin.
Stage II – limited local spread.
Stage III – extensive local and regional spread.
Stage IV – advanced cancer with distant spread and metastasis.

2.3 CANCER TREATMENT OPTIONS

Cancer treatment has experienced important changes in the last decades from only including typically surgery, radiation therapy, and chemotherapy towards a group of targeted and more specific therapies resulting from the growing knowledge of all the different cancer pathways, which also have improved significantly treatment outcome and patient quality of life. The type of treatment depends on each particular situation, based on the cancer type or on its stage.

Cancer treatments may be used as (Figure 7):

- **Primary treatment:** the aim is to completely remove cancer and kill all the cancer cells. Any cancer treatment can be used as a primary treatment but the most common is surgery.
- **Adjuvant treatment:** the goal is to kill any cancer cells that may remain after primary treatment to reduce the chance that the cancer will recur. Any cancer treatment can be used as an adjuvant therapy. Common adjuvant therapies include chemotherapy, radiation therapy and hormone therapy.
- **Neoadjuvant treatment:** treatments used before the primary treatment to make this primary treatment easier or more effective.
- **Palliative treatment:** the objective is to relieve side effects of treatment or signs and symptoms caused by cancer itself.

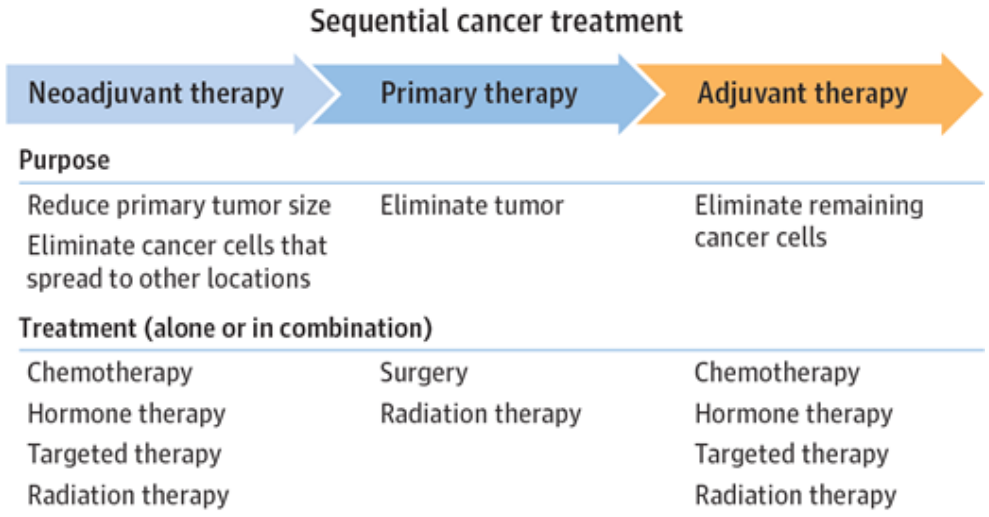


Figure 7. Sequential cancer treatment. Neoadjuvant therapy is used before primary treatment to improve the results. Then, primary treatment consists of the complete removal of the tumours or tumoural cells. Adjuvant therapy is used after primary treatment to kill any cancer cells that may remain after primary treatment. Obtained from (77).

In this thesis, we have evaluated several types of solid tumours, including rectal, lung, and pancreatic cancer, but more in depth, ovarian and breast cancers. Although these tumours arise

in different organs and have unique genetic and molecular characteristics, they share some common features. Both cancers are more prevalent in women and are associated with hormonal factors, such as estrogen exposure. In addition, both breast and ovarian cancers can be caused by inherited genetic mutations, such as *BRCA1* and *BRCA2*.

2.4 OVARIAN CANCER

Ovarian cancer (OC) is a group of diseases that originates in the ovary or in the related areas of the fallopian tubes and the peritoneum. It is the fifth cause of cancer death among women and the most lethal gynecologic neoplasia (78). A significant proportion of ovarian cancer cases are the consequence of genetic mutations in genes associated to DNA repair machinery. In this sense, mutations in the tumour suppressor genes *BRCA1* and *BRCA2* are the most important known predisposition for ovarian cancer (79,80). Different investigations suggest that these genes are involved in two fundamental cellular processes: transcriptional regulation and repair of DNA double-strand breaks (DSBs) via the homologous recombination (HR) pathway (81).

The major histologic subtypes of ovarian carcinoma are high-grade serous carcinoma (HGSC), endometrioid carcinoma (EC), clear cell carcinoma (CCC), low-grade serous carcinoma (LGSC) and mucinous carcinoma (MC) (82,83).

The main treatment for newly diagnosed cancer consists of surgery, whose extent is determined by the cancer stage and other patient characteristics. After surgery, adjuvant chemotherapy, is used in those cancers with higher grades and/or specific histologies, such as HGSC and CCC typically platinum-based chemotherapy (carboplatin and cisplatin) (84). In addition to platinum-based treatments, paclitaxel and bevacizumab are approved by the European Medicines Agency (EMA) for the treatment of newly diagnosed, advanced-stage ovarian cancer. Other treatment combinations with promising results are platinum-based chemotherapy agents and anti-angiogenic agents (bevacizumab, nintedanib, trebananib and pazopanib) or other drugs such as doxorubicin and gemcitabine. In the case of neoadjuvant chemotherapy (NACT), carboplatin and paclitaxel are also the most effective treatments. However, long term outcomes are still unsatisfactory mainly due to the high incidence of recurrence after the initial treatment. In recurrent cancer, chemotherapy with anti-angiogenic agents such as Bevacizumab and trabectedin and the three PARP inhibitors (iPARPs) -olaparib, niraparib and rucaparib- represent an important therapeutic alternative (83,85). PARP inhibitors are drugs that target poly (ADP-ribose) polymerase (PARP), an enzyme that is involved in the repair of single-strand DNA breaks. A substantial benefit of iPARPs among patients with *BRCA* mutations has been demonstrated. In BRCA1/2 mutated patients, the homologous recombination pathway is already impaired, and

the additional inhibition of the PARP pathway leads to the accumulation of DNA damage, resulting in cell death. PARP inhibitors take advantage of the DNA repair deficiencies that are already present in BRCA1/2 mutated tumours, leading to selective killing of tumour cells (86,87).

Recently, several studies associated OC growth and metastasis with its intrinsic tumour microenvironment. Ovarian cancer is characterised by early metastases in the disease process. Malignant cells shed from the primary tumour and spread throughout the peritoneal cavity to proliferate preferentially in “milky spots”. Milky spots are aggregates of immune cells, including macrophages, lymphocytes, and plasma cells supplied by blood and lymphatic vessels, which function as secondary lymphoid organs. Therefore, all these elements together contribute to the formation of the ovarian cancer TME (88). As ovarian cancer is a highly heterogeneous tumours, its associated TME also is very varied, not also among histology types but also between patients that received different treatments. Moreover, increasing number of studies are pointing out the critical role of the innate immune system in ovarian cancer development and progression. Although MDA5 and RIG-I expression have been found to be upregulated in ovarian cancer, their role is complex and their functions are not fully understood (89). According to some authors, activation of MDA5 and RIG-I triggers immunogenic cancer cell death (90). But recent studies have shown that MDA5 and RIG-I signalling can also promote the survival and growth of tumour cells (91). Thus, the relationship between the TME, innate immunity, and MDA5/RIG-I signalling in ovarian cancer is an active area of research, and further studies are needed to fully understand the mechanisms underlying these interactions.

2.5 BREAST CANCER

Breast cancer (BC) is a disease in which cells in the breast grow out of control. With more than 2 million new cases in 2020, breast cancer is the most common cancer diagnosed in women and the second common cause of death from cancer among women worldwide (73,92). Breast tumours tend to spread lymphatically and haematologically, leading to distant metastasis and poor prognosis. The risk factors of breast cancer include both lifestyle factors (i. e. smoking or physical activity) and intrinsic individual characteristics (i.e., female sex, genetic mutations, and older age). However, the survival rate improves significantly with early diagnosis, emphasizing the importance of screening programs to reduce incidence rate, especially in women of older ages or with a family history of breast cancer (93).

The basis for the diagnosis and subsequent classification of breast cancer remains standard pathomorphological diagnostics, including histological type of tumour and grade, degree of advancement according to TNM classification, infiltration by cancer cells and the expression of

receptors: steroid receptors -estrogen and progesterone-, HER-2 receptor and cellular proliferation index Ki-67 (94).

Independently from histological subtypes, breast cancer can be molecularly classified based on gene expression. The consensus clustering includes four main breast cancer subtypes: Luminal A, Luminal B, HER2-enriched and triple-negative breast cancers. All luminal breast cancers are estrogen receptor (ER)-positive and represent the most common breast cancer subtype, representing almost 70% of all breast cancer cases in Western populations (73).

- Luminal A tumours are characterized by the presence of estrogen-receptor (ER) and/or progesterone-receptor (PR) and absence of HER2. As they present low expression of cell proliferation genes, they tend to grow more slowly than other cancers. Clinically, they are low grade and thus, they have a better prognosis (73).
- Luminal B tumours present higher expression of proliferation-related genes, therefore, they tend to grow faster. They have higher grade with slightly worse prognosis. They are ER-positive and may be PR negative and/or HER2 positive (73).
- HER2-enriched group is characterized by the high expression of the HER2 and the absence of ER and PR. It represents 10-15% of breast cancers. In general, HER2-enriched subtype presents higher expression of proliferation-related genes, therefore, they grow faster than luminal cancers and can have worse prognosis. However, the introduction of HER2-targeted therapies considerably improved the prognosis of this cancer subtype (73).
- Triple-negative breast cancer (TNBC) or basal-like cancer is a very heterogeneous group of ER-negative, PR-negative, and HER2-negative cancers. TNBC is considered more aggressive than other cancers and is often associated with the worst prognosis. It accounts for about 20% of all breast cancers but it is more common in African-American women (95).

Different subgroups are determined based on pathological morphology using available immunohistochemical (IHC) markers. So, in practice, to estimate prognosis of patients with breast cancer or the best initial therapy, a combination of routine markers such as ER, PR and HER2, together with evaluation of the proliferation markers Ki-67 is used. Ki-67 is a nuclear protein present in proliferating cells that is used as a cellular proliferation index (94). Moreover, evaluation of other biological markers is used in specific breast cancer subtypes, such as cytokeratin 5/6 or epidermal growth factor receptor (EGF) to identify basal-like cancers among the TNBC (73).

According to the Guidelines of the European Society for Medical Oncology (ESMO), the choice of therapy for early breast cancer is dependent to medical decision based on tumour size, feasibility of surgery and clinical phenotype but also on patient decision to preserve the breast. Moreover, surgery could be mastectomy (complete breast removal) or breast-conserving surgery (BCS), also known as partial mastectomy. Chemotherapy is often used as a systemic treatment of either primary or secondary breast cancer and could be either adjuvant or neoadjuvant. Adjuvant chemotherapy is delivered after the primary treatment, to destroy remaining cancer cells. Neoadjuvant chemotherapy is delivered before the main treatment, for example, to help to reduce the size of a tumour. It is used for inflammatory or locally advanced breast cancers or in small tumours with worse prognostics molecular subtypes (HER2 or TNBC). Currently, treatment includes carboplatin, cyclophosphamide, 5-fluorouracil/capecitabine, taxanes (paclitaxel, docetaxel) and anthracyclines (doxorubicin, epirubicin). The choice of the proper drug is of major importance since different molecular breast cancer subtypes respond differently. A local treatment option for breast cancer is radiotherapy, which is typically provided after surgery and/or chemotherapy to minimize the possibility of breast cancer recurrence. In patients with Luminal breast cancer (ER-positive), other treatment option is endocrinal or hormonal therapy that can be used either as neoadjuvant or adjuvant. Endocrinal therapy aims to lower the estrogen levels or prevents breast cancer cells to be stimulated by estrogen. Drugs that block ERs include selective estrogen receptor modulators (SERMs) (tamoxifen, toremifene) and selective estrogen receptor degraders (SERDs) (fulvestrant) while treatments that aim to lower the estrogen levels include aromatase inhibitors (AIs) (letrozole, anastrazole, exemestane). Finally, other treatment option for breast cancer patients is targeted therapy, for example, everolimus (TOR inhibitor) or palbociclib, ribociclib and abemaciclib (cyclin 4/6-dependent kinase inhibitors, CDKi). Although chemotherapy is considered to be effective and wide variety of treatment options seem to be available, the use of most of breast cancer treatments are accompanied by several side effects including hair loss, nausea/vomiting, or diarrhoea.

The immune landscape of breast cancer is characterized by cellular and molecular heterogeneity and a highly inflammatory microenvironment, with significant variation observed across patients, subtypes, and disease setting. Different attempts tried to classify breast cancers based on immune subtypes, without a clear agreement yet, but all authors agree in the lack of an immunologically quiet breast cancer profile (96). This highlights the importance of improving the understanding of the complexity of host-tumour interactions in the TME, that may lead to the possibility of targeting elements within the microenvironment to expand clinical responses

to immune therapies (96). Tumoural immune infiltration has been shown to be related to clinical outcome through the modulation of treatment response, but the importance of the composition of the infiltrating immune cells is still being determined. Although breast cancer is not regarded as an immune hot tumour, a high number of tumour-infiltrating lymphocytes (TILs) can be found in high grade, hormone receptors negative or HER2 positive cancers. TILs in breast cancer are predominately T-cells with much fewer B-cells (97). Natural killer cells (NK) and neutrophils have also been found in a strong proportion in ER-positive breast tumours, while cytotoxic T cells and naïve and memory T cells are found in smaller proportions. Eosinophils and monocytes are important cells as it has been suggested that they may function as indicators of cancer prognosis. However, the presence of these cell populations is still controversial. They may exert both pro-tumour and anti-tumour effects depending on the composition of the TME and on their phenotypes (98,99). With respect to the HER2-positive breast cancer type, there are not many reports about the infiltrating immune mass, and it is mainly represented by DCs, mast cells and T lymphocytes. In reference to cytokine profile, it vary among different breast cancer stages and many cytokines are pleiotropic with both tumour-promoting and antitumour effects (96). It is also well-known that the immune system shows remarkable sex-differential responses, thus, this fact potentially suggests that sex hormones such as estrogens address these events. ERs participate in many immune system functions (100).

2.5 CANCER BIOMARKERS

A cancer biomarker is a characteristic that is measured as an indicator of risk of cancer, occurrence of cancer, or patient outcome (101). However, biomarkers can also potentially indicate the best treatment option for each patient, or at least, assist in the decision-making. Testing patient's biomarker status before the administration of corresponding treatment has been an emerging procedure in clinical practice during last decades, opening the gate towards a more personalized medicine. Biomarkers can be categorized as prognostic and predictive. A prognostic biomarker provides information about the patient's overall cancer outcome, regardless of therapy, whilst a predictive biomarker gives information about the effect of a therapeutic intervention (102). Biomarkers can be either molecular, cellular, physiologic, or imaging-based (101). Larger molecules such as nucleic acids, genetic alterations, and protein molecules, as well as intact cells, are utilized as biomarkers in the diagnosis of cancer. They can be observed in blood in the form of circulating tumour cells, DNA, and RNA enabling liquid biopsies a useful clinical technique (103). Along this thesis, we will evaluate the potential use of SAMHD1 protein as prognostic and predictive biomarker in different solid tumours.

3. SAMHD1

Sterile Alpha Motif Histidine Aspartate domain-containing protein 1 (SAMHD1) is a protein that regulates the level of intracellular dNTPs. By its deoxynucleotide triphosphohydrolase function, it degrades dNTPs into its constituent deoxyribonucleoside (dN) and inorganic triphosphate (104,105). Although SAMHD1 was initially investigated as a viral restriction factor, its functions go far beyond and will be described along next sections.

3.1 STRUCTURE AND REGULATION OF SAMHD1

SAMHD1 is a 62 amino acid protein comprise of an N-terminal sterile alpha motif (SAM), a Histidine-aspartic domain acid containing domain (HD), and a C-terminus domain. Although the specific role of the SAM domain remains unclear, this kind of domains are commonly involved in protein-protein and protein-DNA/RNA interactions (106). The HD domain of SAMHD1 contains the dNTPase active site, regulatory sites, and the necessary interfaces for enzyme oligomerization. The C-terminus of SAMHD1 is important for stabilizing the oligomeric state of the enzyme and nucleic acid interaction (107,108).

SAMHD1 exists in a monomer-dimer equilibrium and only tetramerizes when nucleotides bind to its regulatory sites and activate the catalytically competent holoenzyme (108,109). Each SAMHD1 monomer contains two allosteric regulatory sites (RS1 and RS2) and activating nucleotide triphosphates must sequentially bind at each site to induce a conformational change that promote tetramerization and subsequent catalytic activation (107–109).

Additionally, SAMHD1 function is regulated by post-translational modifications such as phosphorylation (110). When cells are in a non-dividing/quiescent state (G0 phase of the cell cycle), they present low dNTP levels and SAMHD1 predominates in an active dephosphorylated form. When cells enter G1 phase, cyclin-dependent kinases 1 and 2 (CDK1/2) phosphorylate SAMHD1 at the C-terminal Tyrosine 592 residue (P-T592 phosphorylation), leading to an increase in dNTPs available for the subsequent DNA replication. Phosphorylation negatively regulates SAMHD1 tetramerization and dNTPase activity, leading to inactivation of SAMHD1 (Figure 8).

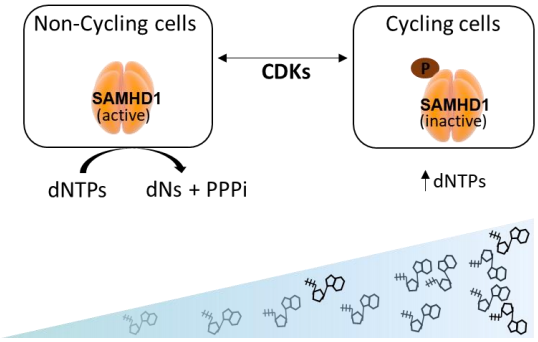


Figure 8. Model for SAMHD1 dNTPase activity. In non-cycling cells, SAMH1 is active and maintains cellular dNTP homeostasis by degrading dNTPs into their corresponding dNs and inorganic phosphate. When cells enter G1 phase, SAMHD1 is inactivated by phosphorylation by cyclin-dependent kinases (CDKs), leading to an increase in dNTPs available for the subsequent DNA replication. Modified from (111).

3.2 CELLULAR FUNCTIONS OF SAMHD1

The central cellular function of SAMHD1 is to maintain the intracellular dNTP pool at a proper level for DNA replication but below a potentially mutagenic threshold (112). dNTPase activity of SAMHD1 is crucial to maintain regulated cellular dNTP pools that are essential for genome stability and proper DNA replication. Therefore, SAMHD1 is not only implicated in maintaining fidelity of DNA synthesis but also in cell cycle regulation and proliferation control (113) (Figure 9). Interestingly, SAMHD1 has also a role in DNA repair by promoting the degradation of nascent DNA at stalled replication forks (114,115). Recently, it has been described that SAMHD1 is also implicated in other important cellular processes. Due to its dNTPase activity, SAMHD1 is essential for preserving genome integrity by maintaining homeostasis of cellular dTNPs levels. Imbalanced dTNP pools could lead to gene mutations, genomic instability, and DNA damage, all accompanied by activation of IFN signalling (116–119).

Moreover, besides its canonical dNTP hydrolase activity, SAMHD1 is involved in other activities. Several studies have demonstrated that SAMHD1 participates in the DNA damage response (DDR) process by recruiting CtBP-interacting protein (CtIP) endonuclease to DNA damage sites (114). In addition, SAMHD1 also plays a non-catalytic role during DNA replication by recruiting and stimulating the exonuclease activity of Double Strand Break Repair Nuclease (MRE11) at the stalled replication forks (115).

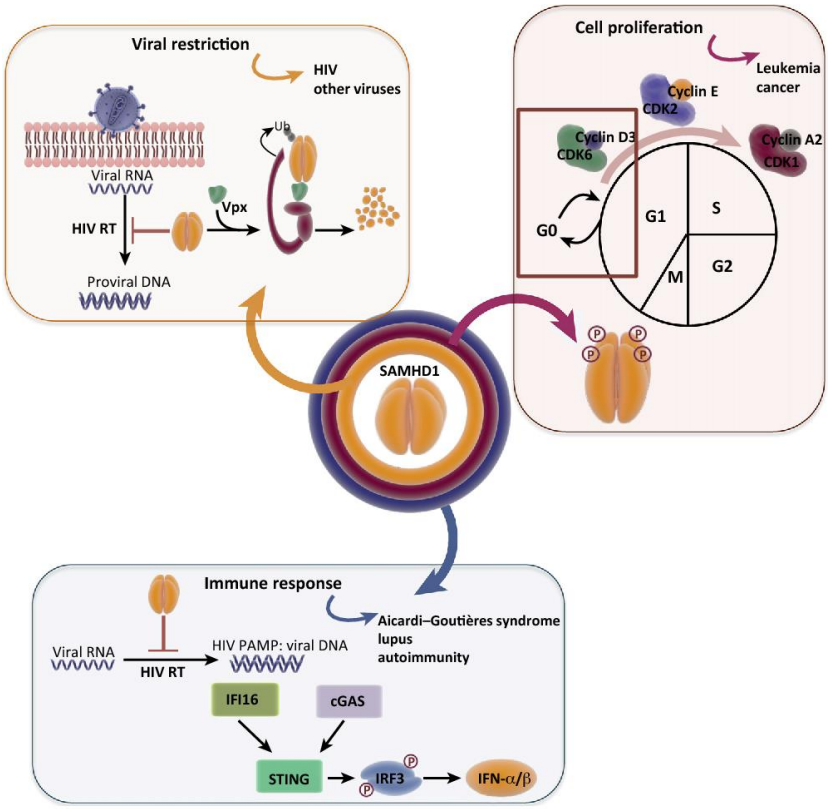


Figure 9. Functions of SAMHD1. SAMHD1 sits at the crossroads of several cellular processes. As SAMHD1 controls intracellular dNTP levels, it plays an essential role in cell-cycle progression and proliferation. By its dNTPase activity, SAMHD1 also restricts viral replication of several viruses including HIV-1, by limiting the pool of dNTPs available for viral reverse transcription. Mutations in SAMHD1 has been associated with interferonopathies such as Aicardi-Goutières syndrome, characterised by high expression of type I IFN. Therefore, SAMHD1 is crucial in multiple processes and play an important role in health and disease. Figure obtained from (111).

3.3 ROLE OF SAMHD1 IN HEALTH AND DISEASE

Although it is at the crossroads of several cellular processes, SAMHD1 is well known for its role as a viral restriction factor that limits the permissiveness of cells to diverse viruses such as human immunodeficiency virus 1 (HIV-1), hepatitis B virus (HBV) or cytomegalovirus (CMV) (reviewed in (111)). Through its dNTPase activity, SAMHD1 inhibits retroviral replication at the reverse transcription (RT) step by maintaining the intracellular pool of dNTPs below the threshold required for reverse transcription of the viral RNA genome into DNA (111).

Moreover, SAMHD1 also plays an important role in other pathological processes, including cancer development, autoimmune diseases, or modulation of immune response. Germ-line mutations in SAMHD1 cause Aicardi Goutières syndrome (AGS), a rare congenital neurodegenerative autoimmune disorder, characterized by a dysregulated interferon (IFN) signalling (120). In addition, due to its role in dTNP metabolism, its involvement in cancer development has been extensively investigated, albeit its specific role is controversial. On the one hand, SAMHD1 has been reported to be mutated or dysregulated in several cancer types

and it is considered a tumour suppressor. For example, it has been found to be frequently mutated in chronic lymphocytic leukemia (CLL) and colorectal cancer (121,122), but SAMHD1 mutations have also been found in other cancers, including myeloma (123), breast cancer (124), lung carcinoma (125), pancreatic cancer (126) and glioblastoma (127). On the other hand, it has been proposed SAMHD1 as a tumour gene driver or promoter. Increased SAMHD1 expression has been associated with cancer progression and metastasis in some other cancer types such as colon cancer or NSCLC (128,129).

Role of SAMHD1 as a modulator of nucleotide analogue efficacy.

The dNTPase hydrolase activity of SAMHD1 is important for the activity of nucleoside analogues, an important class of antivirals and anticancer drugs. Nucleoside analogues, after phosphorylation by intracellular kinases, are structurally similar to endogenous dTNPs. It has been shown that SAMHD1 can modify *in vitro* efficacy of several of these antinucleoside analogues, either used in the treatment of viral infections (130–133) or as chemotherapeutic drugs in the treatment of cancer (134–136). Specifically, in the field of HIV SAMHD1 modifies the efficacy of several analogues with varying potency and efficacy depending on the specific cell type assessed (130–132). Modulation of the efficacy of antinucleoside drugs by SAMHD1 could be directed either to improve their action by depleting the intracellular pool of dNTP competitors (130,133) or to limit their action by directly using the triphosphate compounds as substrates, as in the case of Cytarabine (Cytosar-U®, Ara-C), which is the first line therapeutic agent treatment for acute myeloid leukaemia (AML) (134,136). Several attempts to predict the effect of SAMHD1 on drug efficacy did not show any clear correlation. However, it is hypothesized that compounds whose activity is enhanced in the absence of SAMHD1 are enzyme substrates, and compounds that lose activity in the absence of SAMHD1 would be competing with the intracellular dNTP pool, which is lower when SAMHD1 is active (137). Accordingly, anti-folate drugs such as pemetrexed showed higher activity when SAMHD1 effectively limits the dNTP pool. When SAMHD1 is active, some nucleoside analogues (cladribine, clofarabine, and nelarabine) show limited activity while others such as capecitabine, floxuridine and fluorouracil show enhanced potency (137). Based on this, SAMHD1 inhibition is considered a promising strategy to overcome tumour resistance to chemotherapeutic agents and SAMHD1 expression has been proposed as a potential biomarker for the stratification of patients with cancer diagnosis that have to be treated with antimetabolites (137).

Overall, the intricate relationship between cancer and SAMHD1 opens a realm of possibilities for novel therapeutics interventions. However, further research is needed to completely dilucidate the role of SAMHD1 in each cancer type.

HYPOTHESIS AND OBJECTIVES

At present, it is becoming increasingly clear that the future of cancer care will be dependent on a personalized patient management. In this setting, the identification of reliable and robust predictive and/or prognostic biomarkers will be key to maximize the possibilities of success of personalized medicine in clinical practice.

SAMHD1 is a deoxynucleotide triphosphate (dNTP) triphosphohydrolase that catalyzes the conversion of dNTP to deoxyribonucleoside (dN) and triphosphate, being key for the control of intracellular dNTP pool balance. Albeit SAMHD1 was first described as a viral restriction factor, its impact on cell biology and metabolism goes far beyond. SAMHD1 has been recognized to be essential for preserving genome integrity and the fidelity of DNA synthesis. dNTP pool imbalances caused by SAMHD1 deficiency may lead to DNA damage, accompanied by excessive interferon (IFN) production, with the subsequent immune activation and systemic inflammatory injury. Moreover, due to SAMHD1 central role in cellular dNTP metabolism, it has also been linked to cancer development and disease progression. On one hand, SAMHD1 has been shown to modulate *in vitro* efficacy of several antinucleotide analogues used in the treatment of viral infections and hematological cancers. On the other hand, although the importance of SAMHD1 in hematological cancer treatment is clear, its specific role in solid tumours is somehow controversial and more research is needed. In this regard, SAMHD1 may represent a valuable predictive biomarker of treatment response but also it may serve as a putative prognostic value of cancer progression in different tumour types.

The main objective of this PhD thesis is **the evaluation and characterization of SAMHD1 as a potential biomarker** of solid tumour onset, evolution, and treatment response, as well as the elucidation of the immunomodulatory function of SAMHD1 in cancer patients.

The specific objectives are the following:

1. Evaluate the prognostic and predictive value of SAMHD1 expression in different advanced solid tumours treated with antimetabolites.
2. In depth evaluation of SAMHD1 role in breast and ovarian cancer and its impact in antitumour immune response.
    2.1. To develop novel *in vitro* models to explore the mechanisms driving SAMHD1 function in cancer development and treatment response.
    2.2. To delineate the immunomodulatory consequences of SAMHD1 depletion in breast and ovarian cancer.
    2.3. To clinically evaluate the prognostic and/or predictive value of SAMHD1 expression in different breast and ovarian cohorts of patients.

MATERIALS AND METHODS

PATIENT COHORTS AND SAMPLES

Along this work, to support our *in vitro* results, we use three different cohorts, each of them corresponds to one chapter of this thesis. The first cohort here described is the pan-cancer cohort that enfolds 128 patients with solid tumours treated with antimetabolites. Then, the neoadjuvant breast cancer cohort includes 182 patients received neoadjuvant chemotherapy (NACT) with anthracycline-based regimens with or without taxanes. Finally, the ovarian cancer cohort include patients from the pan-cancer cohort but is presented in more detailed way towards ovarian cancer parameters.

All studies were conducted in accordance with the ethics principles of the Declaration of Helsinki and approved by the Research and Ethics Committee of Hospital Germans Trias i Pujol. Tumour samples were obtained from the Biobank of the *Institut d'Investigació Germans Trias i Pujol*. All patients provided written informed consent.

1. Pan-cancer cohort

The pan-cancer cohort used in the present work was composed by a total of 128 patients with solid tumours treated with different chemotherapy agents at the Medical Oncology Service, ICO-Badalona, from 2012 to 2018. The cohort included different tumour types, selected based on the use of antimetabolites as part of their anticancer treatment: 46 samples of patients with advanced breast cancer (BC) treated with capecitabine, 22 samples of advanced ovarian cancer (OC) patients treated with cisplatin or carboplatin in combination with gemcitabine or gemcitabine in monotherapy, 16 samples from non-small cell lung cancer (NSCLC) patients treated with cisplatin or carboplatin in combination with gemcitabine or pemetrexed or with gemcitabine in monotherapy, 14 samples of advanced pancreatic cancer (PC) patients treated with nab-paclitaxel in combination with gemcitabine and 30 samples of locally advanced rectal cancer (RC) treated with radiotherapy in combination with capecitabine. Clinical characteristics are described on table 2. Extended clinical data can be found on (138).

Cohort variables: the following demographic, clinical and biological data and treatment algorithms were collected for all study participants: sex, date of birth, date of cancer diagnosis, date of metastasis, number of lines of therapy for advanced disease previous to cohort therapy, date of starting cohort therapy, date of end cohort therapy, cause of need cohort therapy, evaluation of overall response rate (ORR), date of last follow-up, status at last follow-up, and toxicity parameters according to National Cancer Institute Common Toxicity Criteria (139). Tumour stage was classified according to TNM classification of the Union International Cancer Control (140). For ovarian cancer patients, additional variables were studied: histologic subtype

(high grade serous *versus* others), BRCA genes status (pathologically mutated *versus* wild type, variants of unknown significance or unknown). All treatments were obtained from review of medical records.

Table 2. Clinical characteristics of patients and tumours from the pan-cancer cohort.

Variable	Type of Tumour				
	Rectal (*n*=30)	**Ovarian** (*n*=22)	**Lung** (*n*=16)	**Breast** (*n*=46)	**Pancreas** (*n*=14)
Age (y), Mean	62.77	62.82	61.88	54.02	63.57
Interquartile range (IQR)	(37–80)	(51–82)	(47–83)	(29–84)	(45–80)
Gender					
Male, *n* (%)	19(63.3%)	-	13 (81.3%)	-	9 (64.3%)
Female, *n* (%)	11(36.7%)	22 (100%)	3 (18.7%)	46 (100%)	5 (35.7%)
Line of Therapy					
Neoadjuvant, *n* (%)	30(100%)	-	-	-	-
First, *n* (%)	-	1(4.5%)	-	15 (32.6%)	13 (92.9%)
Second, *n* (%)	-	7 (31.8%)	16 (100%)	15 (32.6%)	1 (7.1%)
Third, *n* (%)	-	6 (27.3%)	-	8 (17.4%)	-
≥Fourth, *n* (%)	-	8 (36.4%)	-	8 (17.4%)	-
Overall Response Rate (ORR) *					
Complete response (CR), *n* (%)	-	3(13.6%)	0 (0%)	3 (18.7%)	-
Partial response (PR), *n* (%)	6 (20%)	8 (36.4%)	5 (31.3%)	1 (2.2%)	-
Stable disease (SD), *n* (%)	21(70%)	3 (13.6%)	6 (37.5%)	17 (37%)	2 (14.3%)
Progressive disease (PD), *n* (%)	2 (6.7%)	5 (22.8%)	2 (12.5%)	15 (32.6%)	4 (28.6%)
Non-evaluable (NE), *n* (%)	1 (3.3%)	3 (13.6%)	-	13 (28.2%)	8 (57.1%)
TTP #, months, median (IQR)	26.00 (0.00–56.79)	10.00 (4.68–15.31)	6.00 (2.75–9.23)	12.97 (9.06–16.89)	6 (4.3–7.46)
OS £, months, median (IQR)	77.85 (68.11–87.59)	18.00 (9.68–26.31)	13.00 (1.25–24.74)	28.81 (18.95–38.67)	11.00 (7.33–14.66)
DFS &, months, median (IQR)					
All	nd	15.00 (10.40–19.59)	8 (2.1–13.88)	29.83 (17.83–41.82)	6 (4.3–7.46)
Excluding “de novo” ¥	nd	24.0 (7.86–40.13)	17 (4.05–29.94)	40.41 (22.77–58.04)	15 (11.90–18.09)
OSCD α, months, median (IQR)	nd	66 (33.03–98.96)	24.00 (10.28–37.72)	93.83 (72.33–115.32)	17.00 (0–51.83)

IQR, interquartile range, * ORR: the proportion of patients who have a partial (PR) or complete response (CR) to therapy, # TTP: time to treatment progression, the time from date of treatment initiation to date of progression or death resulting from any cause (whichever occurred first), £ OS: the time from date of cohort treatment initiation to date of death resulting from any cause, & DFS: disease-free interval, the time from curative therapy (surgery) to distance relapse, ¥ Excluding “de novo”: excluding patients with initial advanced disease without further resection (DFS = 0), α OSCD: overall survival since cancer diagnosis, the time from cancer diagnosis to date of death resulting from any cause, nd. not determined.

2. Neoadjuvant Breast Cancer cohort

The neoadjuvant breast cancer cohort includes 182 treated patients from ICO Badalona diagnosed with stage II and III breast cancer between 2002 and 2012. Included patients were not eligible for conservative breast treatment and represented candidates for primary therapy according to the decision of the Breast Cancer Committee of our institution (Figure 10A).

All included patients received neoadjuvant chemotherapy (NACT) with anthracycline-based regimens with or without taxanes. In patients with HER2 amplification, trastuzumab was added to taxanes-based chemotherapy. After surgery, patients were evaluated and received additional anticancer therapy according to the anatomopathological result as follows, trastuzumab in HER2+ cases, adjuvant hormone therapy in estrogen receptor (ER) and/or progesterone receptor (PR) positive cases. Patients undergoing lumpectomy also received breast radiotherapy and regional nodal radiation was delivered at the discretion of the breast committee. Patients without residual infiltrating cancer disease in the breast and axilla were considered to have had a pathological complete response (pCR). Thus, the analysis of post NACT tumour biopsies was finally performed in 151 patients, excluding those patients with pCR and in which there was post chemotherapy tumour tissue available (Figure 10B). Table 3 summarizes patients characteristics of the entire cohort and patients' characteristics of the cohort in which tumour biopsies were available.

Cohort variables: the following demographic, clinical and biological data and treatment algorithms were collected for all study participants: sex, date of birth, menopausal status, clinical stage, post chemotherapy nodal status, estrogen receptor status, progesterone receptor status, HER2 status Ki67 in surgical specimen, histological grade in surgical specimen, subtypes (according to the ER, PR, HER2 status and Ki67), type of neoadjuvant chemotherapy, clinical response, pathological response, type of surgery, complementary hormonal therapy, complementary radiotherapy, date of relapse, type of relapse, date of last follow-up, status at last follow-up. tumour stage was classified according to the TNM classification of the Union International Cancer Control (140). Treatments were obtained from review of medical records.

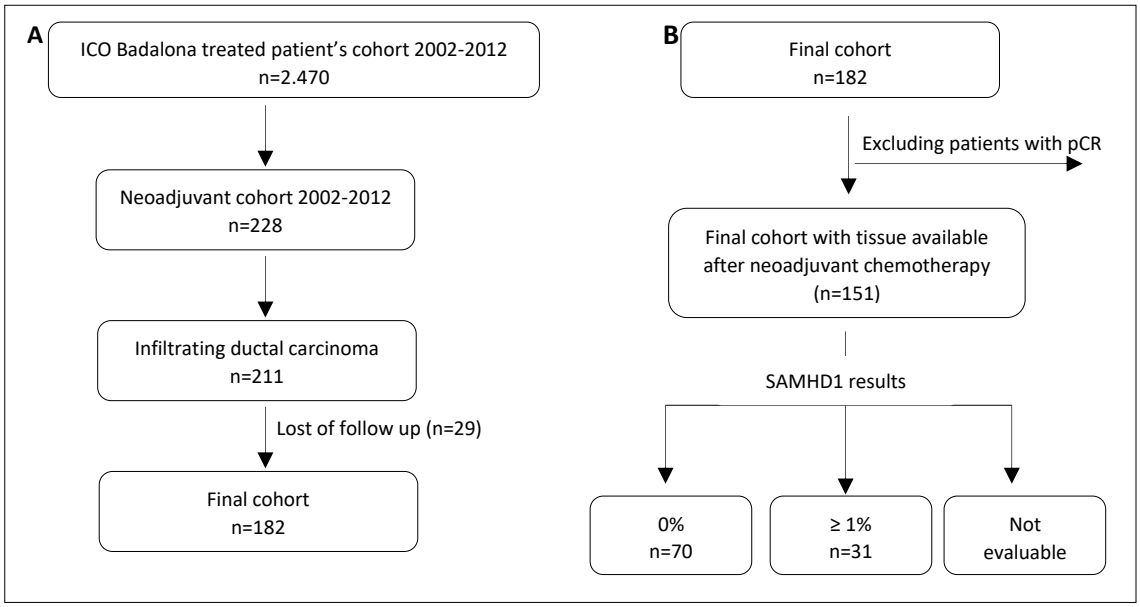


Figure 10. Flow chart of breast cancer patients included in the neoadjuvant cohort. (A) Flow chart of patients of the whole cohort. (B) Flow chart of SAMHD1 analyzed patients and SAMHD1 results. Of 182 patients originally included, only 151 had sufficient tumour tissue to perform SAMHD1 assessment.

Table 3. Clinical characteristics of patients and tumours from the neoadjuvant breast cancer cohort.

Variable	Entire cohort (n=182)	Patients with SAMHD1 post NACT (n=151)	*p*
Age (y), Mean (IQR), DT	50.07 (25-79)	50.28 (24-79)	.319
Menopausal status, n (%)			.240
Yes	78 (42.9%)	67 (55.6%)	
Not	104 (57.1%)	84 (44.4%)	
Clinical Stage			.222
II	74 (40.7%)	59 (39.1%)	
III	108 (59.3%)	92 (60.9%)	
Estrogen Receptor (ER)			.198
<1	67 (36.8%)	59 (39.1%)	
1-10	12 (6.6%)	11 (7.3%)	
>10	103 (56.6%)	81 (53.6%)	
Progesterone Receptor (PR)			.169
<1	99 (54.4%)	85 (56.3%)	
1-10	7 (3.8%)	7 (4.6%)	
>10	76 (41.8%)	59 (39.1%)	
HER2 (IHQ&)			.482
Negative	144 (79.1%)	120 (79.5%)	
Positive	38 (20.9%)	31 (20.5%)	
Ki67 Post surgery (mean)	12.43(0-80)	12.67 (0-80)	.571
Histological grade post-surgery			
I	13 (7.1%)	13 (8.6%)	
II	67 (36.8%)	62 (41.1%)	
III	46 (25.3%)	39 (25.8%)	
Unknown	56 (30.8%)	37 (24.5%)	
***Subtypes**			.663
Luminal A	44 (24.2%)	41 (27.2%)	
Luminal B	40 (21.9%)	38 (25.2%)	
Luminal B HER2 positive	18 (9.8%)	14 (9.2%)	
HER2 positive	22 (12.2%)	17 (11.2%)	
Triple Negative	58 (31.9%)	41 (27.2%)	
Pre-surgery Chemotherapy			
Chemotherapy (Anthracyclines and taxanes) plus Trastuzumab	16 (8.8%)	12 (7.9%)	.206
Anthracycline based regimens	52 (28.6%)	47 (31.1%)	
Anthracycline and taxane based regimens	114 (62.6%)	92 (60.9%)	
Clinical response			.621
CR	57 (31.3%)	46 (30.5%)	
PR	101 (55.5%)	83 (55.0%)	
ED	20 (11%)	18 (11.9%)	
PD	4 (2.2%)	4 (2.6%)	
Recurrence			.159
Yes	65 (35.7%)	51 (33.8%)	
Not	117 (64.3%)	100 (66.2%)	

*Luminal A: ER positive, PR>20%, HER2 negative ki67<14%, Luminal B: ER positive, PR<20%, HER2 negative, and/or ki67≥14%, Luminal B HER2 positive: ER and/or PR positive, HER2 positive, HER2: HER2 positive, Triple Negative: ER negative, PR negative, HER2 negative.

Construction of tissue microarrays (TMA) and immunohistochemical methods

Three different areas/tumour were selected and included into the TMA (cylinders of 0.6mm in diameter of each block of paraffin-embedded tissue) using a TMA workstation MTA-1 (Beecher instruments). Then, TMAs were cut in 5-micron sections for analysis by immunohistochemistry of SAMHD1 expression (1:200, polyclonal rabbit anti-SAMHD1 antibody, cat. nº 12586-1-AP, Proteintech), using an automated detection system (ultraView, Ventana 9 after antigen retrieval). The specificity of the polyclonal antibody was validated by western blot analysis in cell lines (Figure 11A) and by immunohistochemistry using paraffin-embedded tissue (Figure 11B). Evaluation of the immunostained slides was performed blinded to any clinical data by experienced pathologists, reporting the level of SAMHD1 expression as the percentage of positive tumour cells. Independent triplicate evaluations were performed for each tumour. TMA sections were also stained for ER, PR, basal cytokeratins, HER2, Ki67 following standardized methods used for routine clinical testing. All immunohistochemical analyses were performed in the histopathological unit of Hospital Germans Trias i Pujol. Rectal tumours were evaluated in paraffin-embedded tissue slides following the same procedure described above but from whole tissue slides.

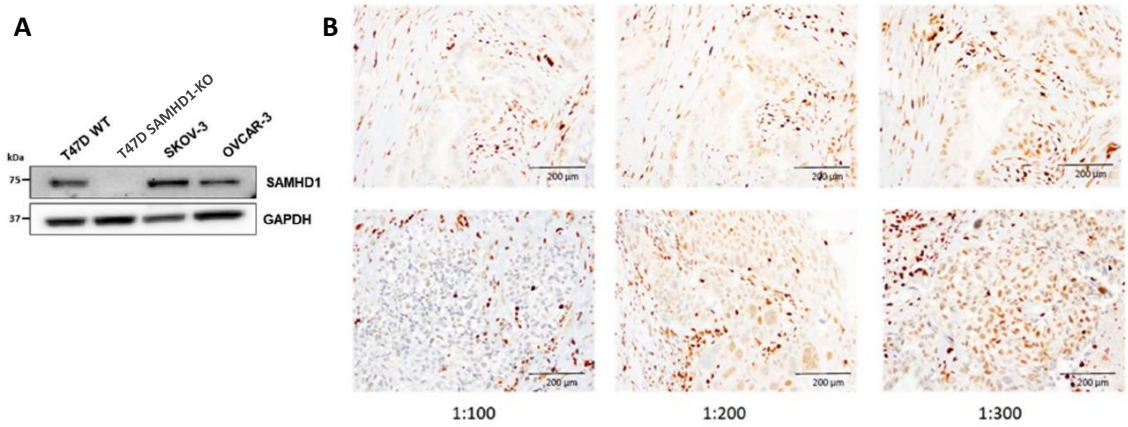


Figure 11. Validation of SAMHD1 antibody specificity. (A) Detection of SAMHD1 protein in whole cell lysates. Western blot analysis of parental T47D and two ovarian cancer cell lines (SKOV-3 and OVCAR-3) resulted in a single band at the expected protein size. No SAMHD1 expression was detected in the SAMHD1-KO clone. (B) Formalin-fixed, paraffin-embedded blocks of pancreas (upper panels) and lung (lower panels) cancer samples were prepared, and the sections were immunostained with the same SAMHD1 antibody at different antibody concentrations (1:100, 1:200, 1:300). In both cases, a clear strong nuclear staining for SAMHD1 was detected in both tissues, showing a clear dose-response depending on the antibody concentration. All images were obtained at 200x magnification.

STATISTICAL ANALYSIS OF CLINICAL DATA

A comprehensive cohort description analysis based on demographic, clinical and biological data was performed. Categorical variables were summarized through frequencies and percentages and quantitative variables using means and standard errors or medians and interquartile ranges.

Different endpoints were analysed for each tumour: (i) Time to progression (TTP) was defined as the time from date of cohort treatment initiation to date of progression, (ii) Overall survival (OS) was defined as the time from date of cohort treatment initiation to date of death resulting from any cause, (iii) disease-free survival (DFS) was defined as the time from curative therapy (surgery) to distance relapse, (iv) overall survival since cancer diagnosis (OSCD) was defined as the time from cancer diagnosis to date of death resulting from any cause, (v) progression-free survival (PFS) specifically in ovarian cancer was defined as the time between first treatment and progression or death, whatever occurs first. Patients who were alive (for OS) or disease free (for TTP) will be censored at the date of last follow-up. Median times for TTP, OS, DFS, and OSCD will be estimated using the method of Kaplan-Meier and reported with their confidence intervals (CI) at the 95% level. Log-Rank was used to compare Kaplan-Meier Curves. ORR will be reported as the proportion of patients who have a partial (PR) or complete response (CR) to therapy.

CELL LINES AND CELL CULTURE

Cell lines and cell culture

The main cell lines and culture conditions used in the present work can be found in Table 4. If not otherwise specified, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) or RPMI (ThermoFisher) supplemented with 10% heat-inactivated fetal bovine serum (FBS, ThermoFisher) and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin (Life Technologies). All cultures were maintained at 37°C in a 5% CO_2 incubator.

Table 4. Cell lines and culture conditions used.

Cell line	Tissue of origin	Provider	Culture method
T47D cells	Breast cancer	Sigma-Aldrich-ECACC (European Collection of Authenticated cell cultures, 85102201-1VL)	DMEM +10% FBS + (P/S)
SKOV3 cells	Ovarian cancer	American Type Culture Collection (ATCC)	Gibco™ McCoy's 5A (Modified) Medium (ThermoFisher) +10% FBS + (P/S)
OVCAR3 cells	Ovarian cancer	American Type Culture Collection (ATCC)	RPMI +10% FBS + (P/S) + insulin solution human (0.01 mg/mL) (Sigma-Aldrich)
MCF7 cells	Breast cancer	Sigma-Aldrich-ECACC (European Collection of Authenticated cell cultures, 85102201-1VL)	DMEM +10% FBS + (P/S)

Primary cells

Peripheral blood mononuclear cells (PBMCs) from blood of healthy donors were obtained from buffy coats, that were purchased from the *Catalan Banc de Sang i Teixits*. The buffy coats received were totally anonymous and untraceable and the information given was whether they have been tested for diseases. Blood from cancer patients was collected in EDTA tubes. All PBMCs (both from healthy donors and from cancer patients) were obtained by Ficoll-Paque density gradient centrifugation. Once obtained, total PBMCs were resuspended and cultured in complete RPMI media: RPMI supplemented with 10% heat-inactivated foetal bovine serum (FBS, ThermoFisher) and 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies). When appropriate, PBMCs were stimulated with hIL-2 (6.5 IU/mL, Roche) and PHA (4 µg/ml, Sigma-Aldrich) for three days. Monocytes isolation was performed from total PBMCs using negative selection antibody cocktail (EasySep™ Human Monocyte Isolation Kit, #19359, StemCell Technologies), following the manufacturer protocol. Isolated monocytes were cultured in complete RPMI culture medium and differentiated to monocyte derived macrophages (MDM) for 4 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech) at 100 ng/ml.

Generation of SAMHD1 *knock-out* cells.

For the generation of *knock-out* (KO) cells, T47D cells were transfected with a plasmid expressing a CRISPR-Cas9 construct designed to disrupt the sequence corresponding to exon 5 of SAMHD1 gene that encodes for HD domain (CRISPR-SAMHD1), as described previously (141). Briefly, $1.5x10^5$ cells were seeded in 24-well plates. After overnight culture, 0.5µg of CRISPR-SAMHD1 plasmid were mixed with Lipofectamine 2000 reagent (Invitrogen) in serum-free medium OptiMEM (Invitrogen) and then added to previously washed cells. Media was replaced by complete DMEM four hours after transfection and left in the incubator for 3 days. Cells were then selected by treating with puromycin (1 µg/mL) for 24h. After puromycin selection, single cell clones were obtained by limiting dilution in 96-well plates. Once grown, SAMHD1-KO clones were identified by confirming SAMHD1 protein depletion by western blot. Control cells (WT) were generated in parallel and used in all experiments.

Generation of SAMHD1 *knock-down* cells by RNA interference (RNAi)

For SAMHD1 *knock-down* in the ovarian cancer cell lines OVCAR3 and SKOV3 and the breast cancer cells MCF7, siRNAs targeting *SAMHD1* gene (siSAMHD1) and non-targeting control (siNT) (siGENOME SMARTpool, Dharmacon, Cultek) were transfected following standard procedures. In brief, 100 nM of the corresponding siRNA were mixed with Lipofectamine 3000 reagent (ThermoFisher) and added to $1.6×10^5$ ovarian cancer cells or $2×10^5$ MCF7 cells seeded in 24-well

plates in Optimem medium without FBS. After 24h, the corresponding complete culture medium was added and SAMHD1 depletion evaluated by RNA and protein expression 48h later.

DRUGS

1-beta-D-arabinofuranosylcytosine (AraC), difluorodeoxycytidine (gemcitabine), 5-chloropyrimidine-2, 4(1H,3H)-dione (fluorouracil), pemetrexed, doxorubicin, paclitaxel, cisplatin, carboplatin, and 3-Azido-3-deoxythymidine (zidovudine, AZT), an HIV reverse transcription inhibitor were purchased from Sigma-Aldrich. The CDK4/6i palbociclib, ribociclib and abemaciclib were purchased from Selleckchem.

CELLULAR BIOLOGY

Cell Proliferation Assays

Cell proliferation capacity was measured by colony formation assays and growth curves. For colony formation assays, cells were trypsinized and plated in 6-well dishes at a density of 100 cells/well. Fifteen days later, cells were washed with PBS 1× and covered with fixing/staining solution (crystal violet solution + 20% ethanol + 2% formaldehyde 1×). Cells were washed several times with water and the number of colonies were counted. For growth curves, cells were plated in 96-well plates at 5.000 cells/well and at indicated time points, cells were lysed with CellTiter-96® AQueous One Solution Reagent (Promega) and luminescence was read using the Envision plate reader. All measurements were performed in triplicates.

Evaluation of Cytotoxicity

Cells were treated at indicated doses of the test compounds for 4 days and the number of viable cells was quantified by a tetrazolium-based colorimetric method (MTT method) as described elsewhere (142,143). MTT assay measures metabolic activity of cells, resulting in a very sensitive procedure to evaluate cell viability and cell proliferation, including the effect of cytostatic agents that slow or stop cell growth. Sensitivity to neoadjuvant chemotherapy in SAMHD1-KO cells was also measured by MTT method.

Cell Cycle Analysis

Approximately 100.000 cells were harvested and washed with PBS. Cells were resuspended in 250 µL of Solution 10-Lysis buffer (ChemoMetec 910-3010) supplemented with 10 µg/mL of Solution 12–500 µg/mL DAPI (ChemoMetec 910-3012) and incubated at 37°C for 5 min. Then, 250 µL of Solution 11-Stabilization buffer (ChemoMetec 910-3011) was added. Moreover, 10 µL of the cell suspension was load into the chambers of the NC-Slide A8. Cell cycle analysis was run in the NucleoCounter® NC-3000™.

Evaluation of DNA Damage and Apoptosis

Cells were treated with the corresponding drug concentrations or left untreated as a control for 24h before evaluation of γH2AX expression by flow cytometry and western blot and PARP cleaved and cleaved caspase 3 by western blot, as described above. For flow cytometry staining, cells were permeabilized using Intracellular Staining Permeabilization Wash Buffer (BioLegend) diluted in water prior to labeling with the Alexa Fluor® 647 Mouse anti-γH2AX (pS139) antibody (BD Biosciences) for 30 min at 4°C in dark. After incubation, cells were washed with Intracellular Staining Permeabilization Wash Buffer and fixed with 1% formaldehyde (FA) prior to acquisition in the flow cytometer. Flow cytometry was performed in a FACS LSRII flow cytometer (BD Biosciences). Data were analyzed using the FlowJo software (Single Cell Analysis Software).

MOLECULAR BIOLOGY

Quantitative RT-polymerase chain reaction (qRT-PCR) and network PCR arrays

For relative mRNA quantification, RNA was extracted using the NucleoSpin RNA II kit (Magerey-Nagel), as recommended by the manufacturer, including the DNase I treatment step. Reverse transcriptase was performed using the PrimeScript™ RT-PCR Kit (Takara). Expression of genes related with molecular mechanisms of cancer was evaluated by using the commercial TaqMan™Array Human Molecular Mechanisms of Cancer (4414161, ThermoFisher), which included primers and probes for 96 genes. A comprehensive set of 28 cytokine associated genes were evaluated using the commercial TaqMan Human Cytokine Network array (4414124, ThermoFisher). Other mRNA relative levels of genes were measured by two-step quantitative RT-PCR and normalized to GAPDH mRNA expression using the DDCt method. Primers and DNA probes were purchased from Life Technologies TaqMan gene expression assays as indicated in Table 5.

RNA from tumour samples was extracted from six 10 µm slides per tumour using the RNeasy FFPE Kit from Qiagen (Venlo, Netherlands), following manufacturer recommendations. The protocol included deparaffinization with xylene and enzymatic processing with proteinase K and DNAase. The resulting RNA was quantified using the Nanodrop and only samples with a minimum concentration of 50 µg/µL and a 260/280 absorbance ratio between 1.7 and 2.3 were used.

Table 5. List of TaqMan gene expression assays

Gene	Reference
IL6	Hs00174131_m1
SAMHD1	Hs00174103_m1
IL8	Hs00174103_m1
IL1α	Hs00174092_m1
CRLF1	Hs00191064_m1
CNN2	Hs04377468_g1
TCP1	Hs01053946_g1
TYK2	Hs01105947_g1
GAPDH	Hs00266705_g1
TNF	Hs00174128_m1
CXCL10	Hs00171042
ISG15	Hs00192713_m1
IL18	Hs01038788_m1

Western Blot

Cells were rinsed in ice-cold PBS, extracts were prepared in lysis buffer (50 mM TrisHCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1mM NaV_3O_4, 10 mM sodium-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 270 mM sucrose and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche) and 1mM phenylmethylsulfonyl fluoride. Samples were electrophoresed in SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Blocked membranes were incubated overnight at 4°C with the antibodies described in table 6:

Table 6. List of antibodies used in this thesis.

Antibody	Dilution	Company	Reference
anti-human Hsp90	1:1000	BD Biosciences	610418
anti-SAMHD1	1:1000	ab67820	Abcam
anti-phospho-SAMHD1 (Thr592) (D7O2M)	1:1000	89930	Cell Signalling
anti-Cleaved PARP1 (E51)	1:1000	ab32064	Abcam
anti-GAPDH	1:10.000	ab9485	Abcam
anti-γH2AX (Ser139)	1:1000	ab2577	Cell Signalling
anti-cleaved caspase 3 (Asp175)	1:1000	ab9661	Cell Signalling
anti-MDA5	1:1000	5321	Cell Signalling
anti-RIG-I	1:1000	3743	Cell Signalling
anti-IRF7	1:1000	4920	Cell Signalling
anti-phosphoSTAT1 (Y701)	1:1000	9167	Cell Signalling
anti-IFITM2	1:1000	13530	Cell Signalling
anti-β-Actin	1:1000	A5441	Sigma

After washing, the membranes were incubated with the corresponding secondary conjugated horseradish peroxidase antibody anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000, Pierce) for 1h at room temperature and then revealed with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical).

Quantification of IL-6 and IL-8 proteins

IL-6 and IL-8 quantification in culture supernatants were determined using high-sensitivity ProQuantum immunoassays, following manufacturer's protocol (ThermoFisher A35573 and A35575, respectively). Each sample was assayed in duplicate. The limit of detection (LOD) for IL-6 and IL-8 was 0.064 and 0.0128 pg/mL, respectively.

Nucleic acid extraction from tumour samples and sequencing

RNA from tumour samples was extracted from six 10 µm slides per tumour using the RNeasy FFPE Kit from Qiagen (Venlo, Netherlands), following manufacturer recommendations. The protocol included deparaffinization with xylene and enzymatic processing with proteinase K and DNAase. The resulting RNA was quantified using the Nanodrop and only samples with a minimum concentration of 50ug/µL and a 260/280 absorbance ratio between 1.7 and 2.3 were used. For relative cell mRNA quantification, RNA was extracted using the NucleoSpin RNA II kit (Magerey-Nagel), as described above.

DNA was extracted from paraffin-embedded breast tumour samples. Six 10 µm slides of each tumour were used for DNA extraction using the E.Z.N.A. FFPE DNA Kit (Omega BIO-TEK) according to the manufacturer's protocol.

Primers were designed to amplify the 16 coding exons of SAMHD1 using Primer3 software (Table 7) and 400 ng of DNA were PCR-amplified using the following conditions: 95°C for 5 min hot start, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min final extension. PCR products were run on a 2% agarose gel to confirm band size for each exon, prior to purification (Illustra™ ExoProStar™, Sigma) and sequencing in a ABI3730XL system (Applied Biosystems, Macrogen). Identification of mutations was performed by direct comparison of genome sequencing data from sequenced regions and wild-type sequences.

Table 7. List of primers for SAMHD1 exons

Exon	bp	Sequence forward	Sequence reverse
1	460	(a) GCCAATAGGCTGCCAATACT (b) ATGCAGCGAGCCGATTC	(a) TGTCTTGTAGTCGGGATGGA (b) GCTACCTCGGATGTTCTTCAG
2	182	TGGATCTGGGTAAATGTTGG	GCTTTGTCCCTGAAAGATGG
3	192	TGCAGGGATTATTATTAAGC	TCACTGAGAAGCAGATTTCCTC
4	272	ATGGCTGCACACAAATTCA	CCATGCCTGGCCTAAGATAA
5	218	TCACTCCTCTTGCAAACAGA	TCCATATTCTCTTGGTTGATCTGA
6	184	TGTTCTAAGGCTGCTTTTGT	GCACCCTGGACACTGTAATG
7	221	GCTCCCAATGGGCTAGAATC	AAGGCTAGATGAAAAGCAACCA
8	235	AAATAGATTTGGTGCCTATCCT	ACAAGGAAGCTGTACCTTAAAT
9	195	AGGTACAGCTTCCTTGTTGAA	TTCTTCTTATTGCCTCCTCTGG
10	205	CCCTTTTCCTTCCTTGTCCT	GGGAAATGACAATCAAGTTTCTTAC
11	377	(a) ACGGTGGAGAAGCAGTTGTC (b) AGGATTACAGATGCTTTCCTCAA	(a) CCTCCAGCACCTGTAATCTCTATG (b) CAATTCAGGGACTTCTTACAGTTTATC
12	379	(a) CATTTGCGAACTGCCTGTTA (b) CCAAATTGAAAGACGCACGAGAG	(a) CGTCTCACCCACATACTTGA (b) GGTCTCCTCTTGGAGGACAGA
13	166	TGTGGCTCAAAGACTTGATGA	TGGGTGCTTTATCTTTAAAACG
14	296	(a) TGCTCCTACAGCCCTGAGTT (b) CAACATGGATTATGGAATGCAAGA	(a) GGCAGTCTTACAATAGAAGCTAACA (b) CTATAAAGATTTGCTACATGCCACT
15	353	(a) GGACCAGCTGATATCTCCAATG (b) CAGAGCAGCTGATTCGAGTA	(a) CTTGCGGCATACAAACTCTTTC (b) AAATGGGAACTTTTCAGCAGAT
16	308	(a) CTCAGAAATAAGATGATGGAAACTGG (b) CCCACTCATAACACCTCAAA	(a) CGGAGGCGAGTTGGATTT (b) TGCAGGAGAGGGAGTTT

DEVELOPMENT AND CHARACTERIZATION OF 3D *IN VITRO* MODEL INCLUDING TUMOUR AND IMMUNE CELLS.

Generation of breast cancer spheroids and establishment of immune cell-tumour cocultures

T47D or MCF7 spheroids were generated by seeding $5x10^3$ cells per well on Nunclon Sphera round bottom plates (ThermoFisher) in RPMI 50 uL of medium. The following day 150 µL of complete RPMI medium was added and spheroids were allowed to grow for 3 days. Immune cell-tumour co-cultures were established 4 days after spheroid formation by adding $5x10^5$ PBMCs per spheroid and well (Figure 12).

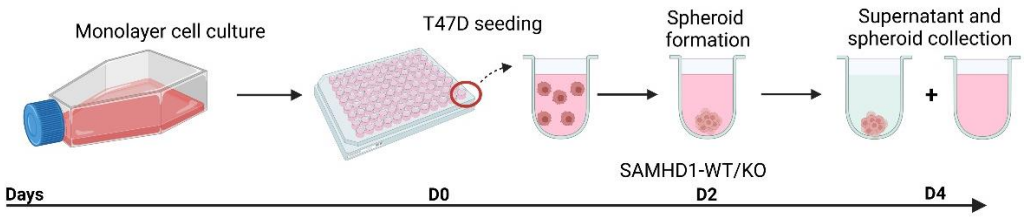


Figure 12. Generation of T47D tumour spheroids. Workflow of wild-type (WT) and SAMHD1-knockout (KO) T47D spheroid generation. Spheroids were generated by seeding T47D cells on low adherent plates. On day 2, spheroids were already formed. On day 4, T47D spheroids were matured. At this point, co-cultures could be established. Created with BioRender.

Imaging

T47D cells were pre-stained with CellTracker™ Red CMTPX Dye (ThermoFisher) before spheroid formation and PBMCs were stained with CellTracker™ Green CMFDA Dye (ThermoFisher) prior to co-culture establishment, according to manufacturer procedure. Immune cells-tumour cocultures were left for 3 days, pooled, and washed as described above, prior to being embedded in Epredia™ HistoGel™ Specimen Processing Gel (HG-4000-012, ThermoFisher), following standardized procedures. Afterwards, embedded spheroids were included in paraffin and then sliced in 5 µm-thick sections. The slides were mounted with DAPI-containing Fluoromount-G (ThermoFisher) and imaged using a fluorescence microscope (Nikon Ts2R-FL). Quantification of CFSE+ cells infiltrating the spheroids was done using the ImageJ software.

Immunophenotypic characterization of PBMCs by flow cytometry

For the immunophenotypic characterization of PBMCs infiltrated into the spheroids, three days after co-culture establishment, spheroids were isolated by first pooling 6 cocultures in eppendorf tubes. Spheroids were washed 3x with PBS, gently resuspended and left to sediment to the bottom of the eppendorf to remove the non-infiltrated PBMCs and then trypsinized to obtain a cell suspension that was washed again to eliminate Trypsin. Then, Fc receptors were blocked with Human TruStain FcX™ (Fc Receptor Blocking Solution, BioLegend) for 10 minutes at RT. After incubation, cells were labelled with: CD3-APC-Cy7, CD4-APC, CD14-BV510, CD15-BV650, CD16-PerCP-Cy5.5, CD56-Pe-Cy7 and CD86-BV786 (all of them from BD Biosciences and BioLegend) for 30 minutes at 4°C in dark. Cells were washed and fixed in 1% formaldehyde. Prior to analysing in the flow cytometer (FACS LSR II, BD Biosciences), Perfect-Count™ Microspheres (PE) (CYT-PCM-50-R) were added. Immunophenotyping of different populations was performed based on the following gating strategy (Figure 13): different cell subsets were defined based on the following expression combinations gated on the live singlet lymphocytes, monocytic cells (CD14+), high cytotoxic natural killer cells (NKs) (CD16+CD56-), low cytotoxic NKs (CD16+ CD56+), myeloid antigen presenting cells (APCs) (CD86+), T cells (CD3+), cytotoxic T cells (CD3+ CD8+) and helper T cells (CD3+ CD4+). Data was analysed using the FlowJo software (BD Biosciences). In all cases, before applying the gating strategy, cell doublets were removed from the analysis (FSC-A vs FSC-H) and lymphocytes were gated by using the forward and side scatter areas (FSC and SSC).

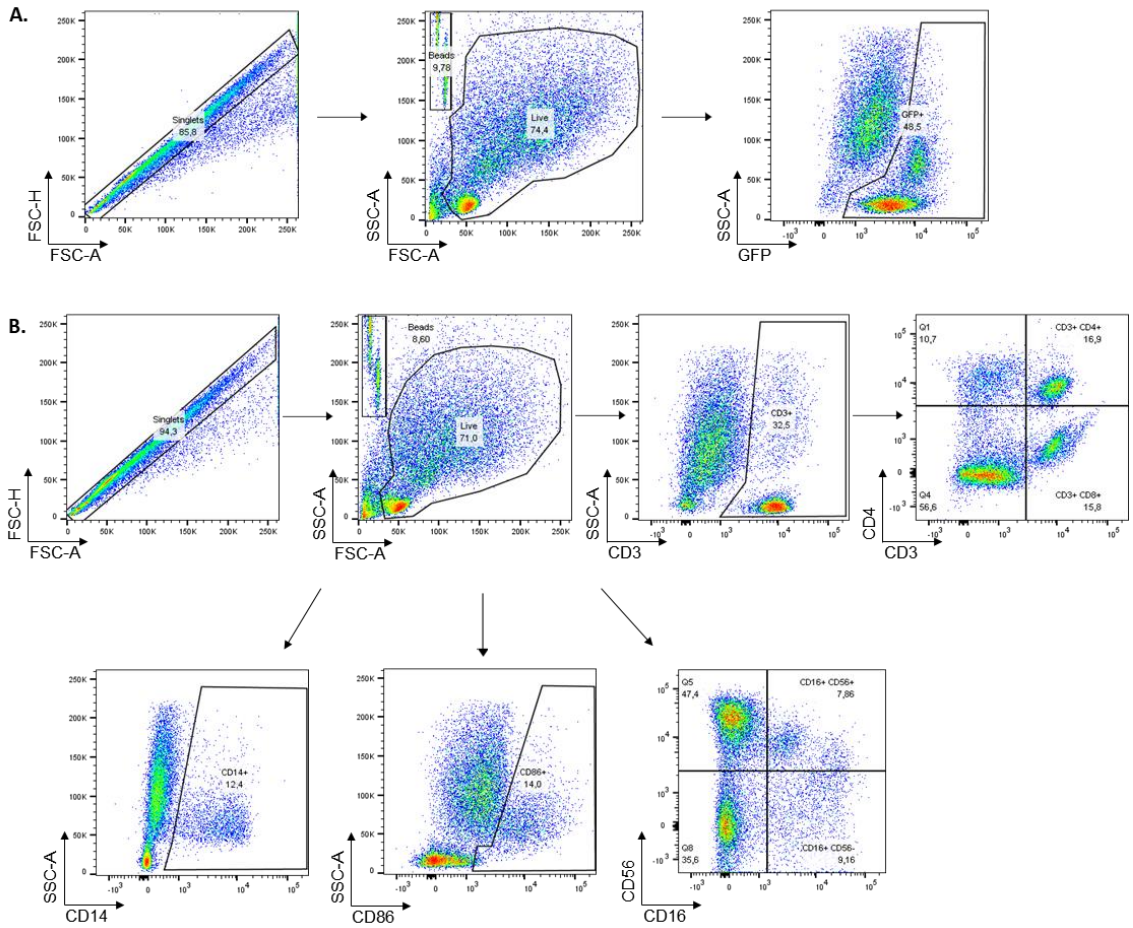


Figure 13. Gating strategy for the immunophenotypic characterization of PBMCs infiltrated into the spheroids. Different cell populations were defined based on the following expression combinations gated on the live singlet lymphocytes: monocytic cells (CD14+), high cytotoxic natural killer cells (NKs) (CD16+CD56-), low cytotoxic NKs (CD16+ CD56+), myeloid antigen presenting cells (APCs) (CD86+), T cells (CD3+), cytotoxic T cells (CD3+ CD8+) and helper T cells (CD3+ CD4+).

RNA-SEQ AND ANALYSIS

RNA sequencing and library preparation

Cellular RNA was extracted from the tumour T47D spheroids using the NucleoSpin RNA II kit (Magerey-Nagel), as recommended by the manufacturer, including the DNase I treatment step. RNA-sequencing samples were prepared in biological duplicates. Determination of RNA integrity (RIN) was performed using Agilent RNA 6000 Nano Kit (Cat: 5067-1511) and Agilent 2100 Bioanalyzer System. After quality control check, RNA library was constructed using Illumina TruSeq Stranded mRNA LT Sample Prep Kit and sequencing was performed using NovaSeq 6000 System with 150 bp paired-ends reads (Macrogen).

Transcriptomic analysis

Transcriptomic analysis was performed as implemented in the computational workflow for the detection of differentially expressed genes and pathways from RNA-seq data (144). Reads were

aligned to the human GRCh38 (annotation NCBI_109.20200522) using HISAT2. Low-expression genes with at least one zero counts were filtered out and the remaining reads normalized with Relative Log Expression (RLE) method as implemented in DESeq2 R library. Differential gene expression between the control and treatment groups was estimated with the DESeq2 Wald test. Sequencing files can be accessed on gene expression omnibus repository (GSE215309).

Gene Set Enrichment Analysis (GSEA) was performed on a pre-ranked GSEA list based on Log 2FC values of differentially expressed genes (DEGs: Log2FC >1, *p*-value < 0.05), against Molecular Signatures Database (MsigDB v7.4) "Reactome" gene-set. Weighted enrichment statistics were based on 1000 permutations. Significantly enriched gene-sets with FDR adjusted *q*-value<0.1 were selected for Enrichment map visualization as previously described. Briefly, Enrichment files were inputted into the Enrichment Map app within the Cytoscape program for visualization. Parameters were set at default values (node cutoff FDR Q value 0.1, Jaccard Overlap combined coefficient cutoff 0.375, k-constant 0.5). Nodes were manually laid out and combined into a common biological process for clarity using the AutoAnnotate app.

STATISTICAL ANALYSIS OF *IN VITRO* DATA

Experimental data were analysed with the PRISM statistical package. If not stated otherwise, all data were normally distributed and expressed as mean ± SD. *p*-values were calculated using an unpaired, two-tailed, t-student test. Statistical significance for *in vitro* and *ex vivo* experiments was calculated using appropriate t-test in GraphPad Prism (v9.3.0). All experiments were performed in at least three independent replicates and n values are provided in the figure legends. Plots were drawn using GraphPad Prism and R software.

RESULTS

CHAPTER 1. MODULATION OF DNA DAMAGE RESPONSE BY SAM AND HD DOMAIN CONTAINING DEOXYNUCLEOSIDE TRIPHOSPHATE (SAMHD1) DETERMINES PROGNOSIS AND TREATMENT EFFICACY IN DIFFERENT SOLID TUMOUR TYPES

Summary

In this first chapter of results, we aimed at evaluating the prognostic and predictive value of SAMHD1 expression in different solid tumours treated with platinum derivatives and/or antimetabolites. Moreover, we developed novel *in vitro* models to explore the mechanisms driving SAMHD1 function in cancer development and treatment response.

To do so, we performed the first in-depth study of SAMHD1's role in advanced solid tumours, by analysing samples of 128 patients treated with chemotherapy agents based on platinum derivatives and/or antimetabolites, developing novel *in vitro knock-out* models to explore the mechanisms driving SAMHD1 function in cancer. Low (or no) expression of SAMHD1 was associated with a positive prognosis in breast, ovarian, and non-small cell lung cancer (NSCLC) cancer patients. A predictive value was associated with low-SAMHD1 expression in NSCLC and ovarian patients treated with antimetabolites in combination with platinum derivatives. *In vitro*, SAMHD1 *knock-out* cells showed increased γ-H2AX and apoptosis, suggesting that SAMHD1 depletion induces DNA damage leading to cell death. *In vitro* treatment with platinum-derived drugs significantly enhanced γ-H2AX and apoptotic markers expression in *knock-out* cells, indicating a synergic effect of SAMHD1 depletion and platinum-based treatment. We conclude that SAMHD1 expression represents a new strong prognostic and predictive biomarker in solid tumours and, thus, modulation of the SAMHD1 function may constitute a promising target for the improvement of cancer therapy.

1.1 SAMHD1 Is Differentially Expressed in Solid Tumours and Correlates with Tumour Differentiation or Grade

To determine the contribution of SAMHD1 expression to cancer progression and/or treatment efficacy, available primary or metastatic tumour biopsies were retrospectively collected for all patients included in the study. Expression of SAMHD1 across different tumour types was evaluated by immunohistochemistry (Figure 14 and samples were stratified according to SAMHD1 expression. SAMHD1 expression varied significantly across tumour types, ranging from high percentage of positivity in rectal to low expression in pancreatic tumours, whereas values of 50–60% positivity were obtained for ovarian, NSCLC and breast cancer cases (Table 8). Our data do not differ from that available in public databases (Figure 15), except for rectal cancer, where in our series SAMHD1 was strongly expressed in all neoplastic cells. Considering the lack of variability in SAMHD1 expression levels among rectal and pancreatic cancer cases, these two cancer types were excluded from further analysis, as the absence of a control arm made it impossible to evaluate SAMHD1 function in these tumour types.

As SAMHD1 function might influence cell proliferation, we first investigated whether SAMHD1 expression was associated with tumour differentiation grade or histologic type as a surrogate measure of tumour cell proliferation. SAMHD1 positivity correlated with poorly differentiated histology (p=0.024) and high grade (p=0.011) in NSCLC. In ovarian carcinoma samples, those with high grade serous papillary ovarian carcinoma were the most positive for SAMHD1 (p=0.028). For breast cancer patients, SAMHD1 positivity was correlated with high grade breast tumours (p=0.017) (Table 9).

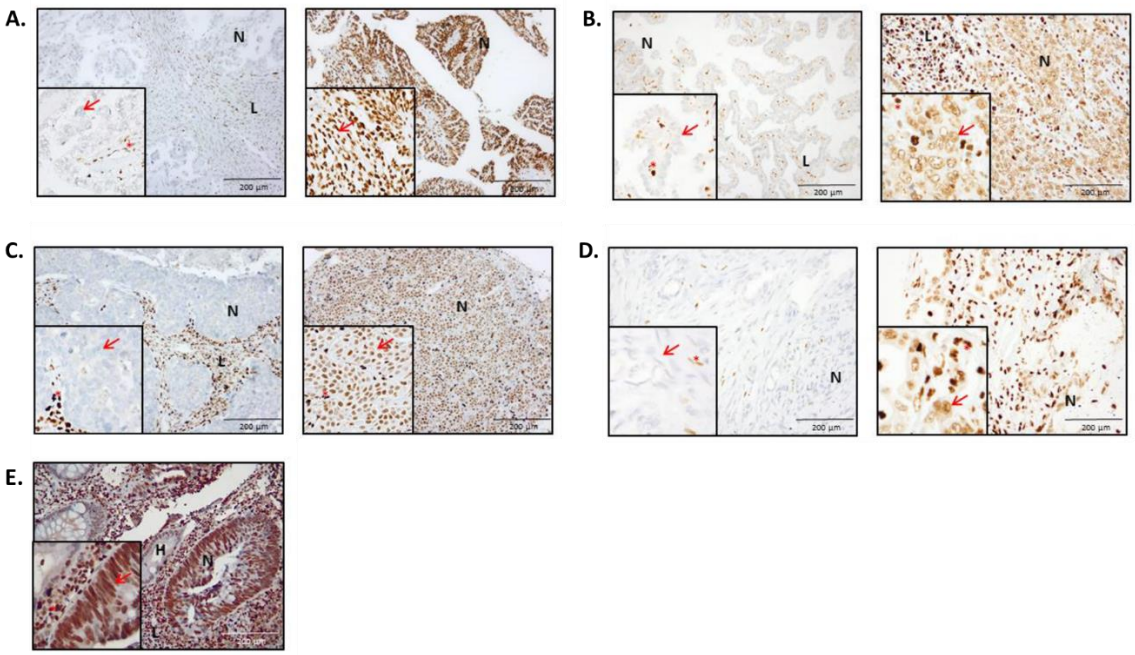


Figure 14. Expression of SAMHD1 protein by immunochemistry in tumour samples. (A–E) Representative microscopy images of SAMHD1 expression in paraffin-embedded tumour biopsies for the different tumour types included in the study, from ovarian (A), NSCLC (B), breast (C), pancreas (D), and rectal (E). Images on the left represent negative SAMHD1 expressing tumours and on the right positive expressing tumours in all cases except in rectum (E), where all analysed tumours presented extremely high levels of SAMHD1 expression. High expression of SAMHD1 observed in lymphocytic cells infiltrating in the tumours was used as a positive control of immunohistochemistry for negative or low expressing biopsies. In case of negative or low expressing biopsies, high expression of SAMHD1 was observed in lymphocytic cells.

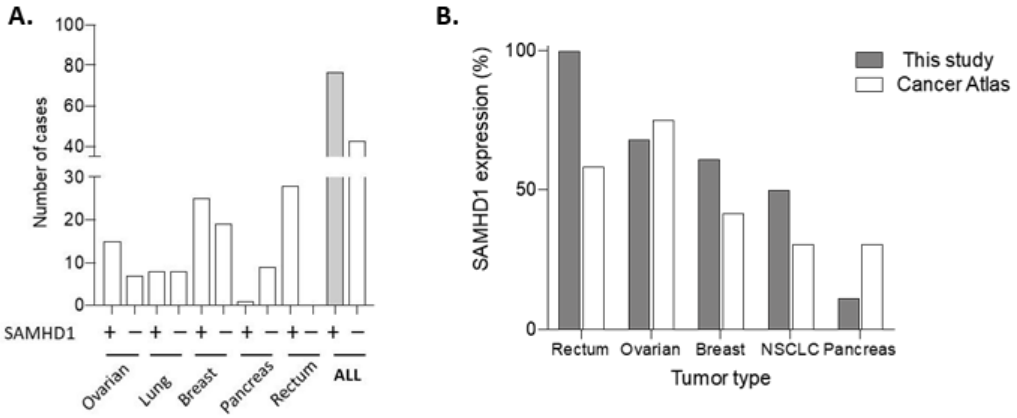


Figure 15. (A) Summary of all analysed biopsies stratified based on SAMHD1 expression and (B) comparative analysis of SAMHD1 positivity in distinct cancer cohorts, depending on tumour type. (A) SAMHD1 expression was determined as positive or negative using a cut-off of 25% of tumour cells. (B) Percentage of positive SAMHD1 tumour biopsies reported in our cohort (grey bars) and in cancer atlas (white bars) in the tumour types tested. No major difference was detected except for rectal tumours (**means significant with a $p<0.005$).

Table 8. Expression of SAMHD1 by immunohistochemistry in patient biopsies across tumour types

Tumour type	SAMHD1 Expression		
	% Expression, mean (IQR)	Positivity ratio evaluable patients, n (%)	Non evaluable, n
Rectal (n=30)	64.64 (25-90)	28 (100%)	2
Ovarian (n=22)	55.82 (0-100)	15 (68.2%)	0
Lung (n=16)	34.06 (0-100)	8 (50%)	0
Breast (n=46)	27.88 (0-80)	25 (61%)	5
Pancreas (n=14)	16.11 (0-75)	1 (11.1%)	5

*Positivity ratio, SAMHD1 positive vs. negative tumours. SAMHD1 was considered positive if ≥25% of positive immunohistochemistry evaluated tumour cells.

Table 9. SAMHD1 expression and tumour histology and grade of ovarian, lung and breast cancer patients.

Tumour	Variable	SAMHD1 positivity ratio		*p*-value*
		Positive (≥25%)	Negative (<25%)	
Ovarian	**Histology**			
	High grade serous papillary, n (%)	14 (82.3%)	3 (17.7%)	.028*
	Clear cell carcinoma, n (%)	1 (25%)	3 (75%)	
	Low grade serous papillary, n (%)	0 (0%)	1 (100%)	
Lung	**Histology**			
	Squamous, n (%)	2 (33.4%)	4 (66.6%)	.024*
	Adenocarcinoma, n (%)	1 (20%)	4 (80%)	
	Poorly differentiated, n (%)	5 (100%)	0 (0%)	
	Tumour grade			
	I, n (%)	1 (100%)	0 (0%)	.011*
	II, n (%)	1 (14.3%)	6 (85.7%)	
	III, n (%)	7 (87.5%)	1 (12.5%)	
Breast	**Tumour grade**[£]			
	I, n (%)	0 (%)	0 (0%)	.017*
	II, n (%)	8 (40%)	12 (60%)	
	III, n (%)	16 (84.2%)	3 (15. 8%)	

*Pearson Chi-Square, [£]Tumour grade was not evaluable in 2 of 41 patients

1.2 SAMHD1 Expression as a Negative Prognostic Factor in Breast, Ovarian, and NSCLC Patients

The contribution of SAMHD1 in cancer onset and progression is controversial, with strong evidence demonstrating both its role as a tumour suppressor and as an oncogene (145). Thus, to gain insight into the putative role of SAMHD1 as a prognostic factor in solid tumours, we first evaluated disease-free survival (DFS) in ovarian, NSCLC, and breast cohorts. SAMHD1 positive patients presented shorter DFS than SAMHD1 negative patients in all three cohorts of patients (Figure 16A).

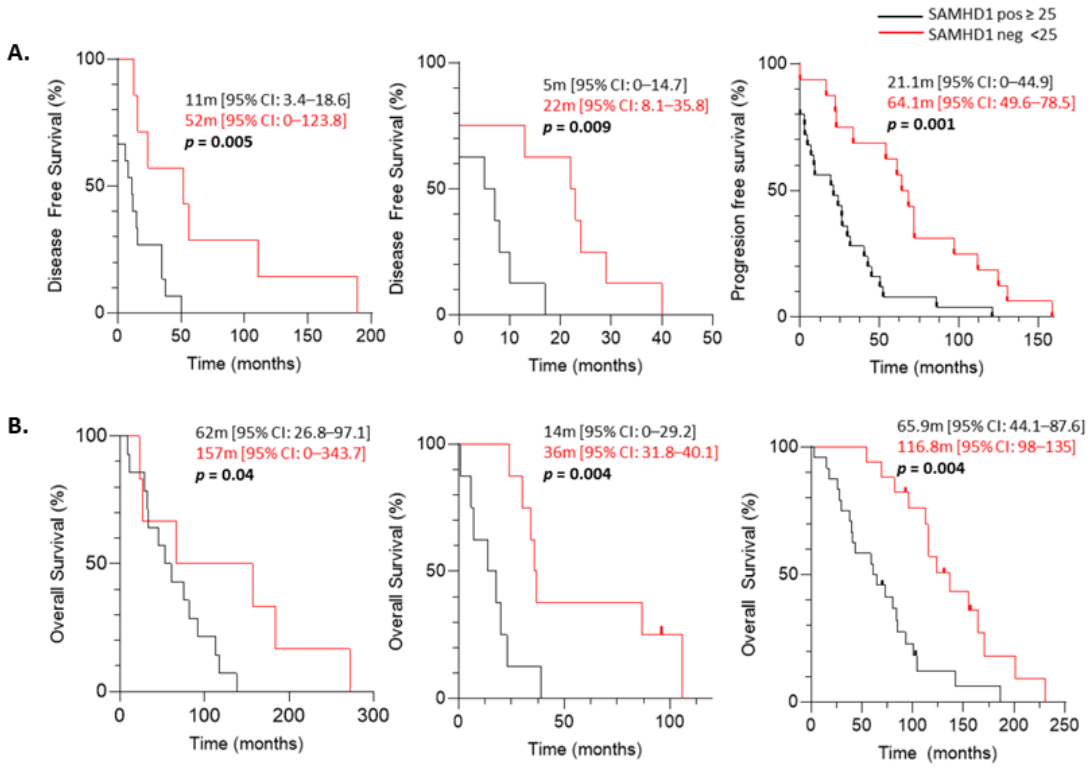


Figure 16. Prognostic value of SAMHD1 in ovarian, lung and breast cancer cohorts. Kaplan–Meier curves of disease-free survival (DFS) (A) and OS since cancer diagnostic (B) according to SAMHD1 status for different tumour types. From left to right: ovarian, NSCLC and breast. Kaplan–Meier curves are represented. SAMHD1 expression below 25% in cancer cells was considered as negative SAMHD1 (red lines) and equal or above 25% was considered as positive SAMHD1 tumours (black lines). Median survival times with CI 95% of both groups are showed. Log rank test was used to test the significance and censored patients are indicated by vertical line.

Ovarian carcinoma patients showed a median DFS of 52 months for SAMHD1 negative patients in front of 11 months for SAMHD1 positive patients (p=0.005). Median DFS in NSCLC cancer patients was 22 months for SAMHD1 negative patients compared to 5 months for SAMHD1 positive patients (p=0.009). In breast cancer patients, median DFS was 64 months for SAMHD1 negative patients compared to 21 months for SAMHD1 positive patients (p=0.001). Moreover, when we exclude patients diagnosed with advanced disease that did not receive any type of local therapy, SAMHD1 positivity continued to be associated with shorter DFS in all the cohorts (Figure 17).

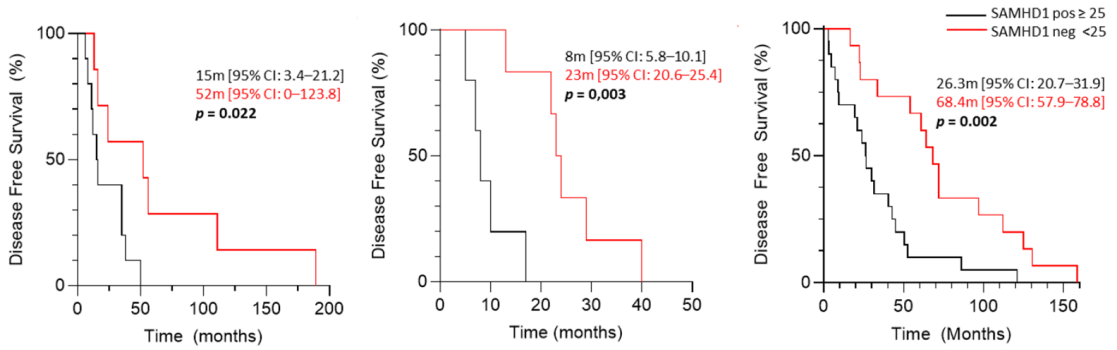


Figure 17. Kaplan-Meier curves of disease-free survival (DFS) of ovarian (left), NSCLC (middle) and breast (right) cancer cohorts stratified by SAMHD1 status. Patients who were diagnosed with advanced disease or received any type of local therapy were excluded from the analysis. SAMHD1 expression below 25% in cancer cells was considered as negative SAMHD1 (red lines) and equal or above 25% was considered as positive SAMHD1 tumours (black lines). Median survival times with CI 95% of both groups are showed. Log rank test was used to test the significance and censored patients are indicated by vertical line.

The multivariate analysis for breast and NSCLC patients showed that negative SAMHD1 status was the only factor significantly associated with longer DFS (p=0.005 and p=0.04, respectively) and a similar trend was observed in ovarian cancer patients (longer DFS associated to SAMHD1 negativity, p=0.09). Data can be found on (138).

Accordingly, when we evaluated overall survival since cancer diagnosis (OSCD) patients with SAMHD1 positive tumours presented shorter OSCD than negative patients in all three cohorts (Figure 16B). Median OSCD was 157 months in SAMHD1 negative in front of 62 months in SAMHD1 positive, for ovarian cancer patients (log rank function, p=0.040). NSCLC patients with SAMHD1 negative presented a median OSCD of 36 months in front of 14 months in SAMHD1 positive patients, (log rank function, p=0.004). Finally, the median OSCD for SAMHD1 negative breast cancer patients was 116.7 months in front of 65.9 months for SAMHD1 positive (log rank function, p=0.004).

Overall, these data indicate that SAMHD1 expression is a strong independent negative prognostic factor in ovarian, breast and NSCLC patients.

1.3 Predictive Significance of SAMHD1 Expression in Cancer Patients Treated with Antimetabolite- and/or Platin-Containing Regimens

SAMHD1 function has been clearly associated with the efficacy of several nucleoside analogues used as antivirals or chemotherapeutic agents. Thus, we determined the value of SAMHD1 as a predictive factor in ovarian, NSCLC and breast cancer treated with corresponding antimetabolite-containing regimens. As above, tumour biopsies were stratified as positive or negative and overall response rate (ORR) was evaluated for each tumour. Interestingly, ovarian and NSCLC cancer patients treated with antimetabolite plus platinum-based chemotherapeutic

regimens presented lower ORR in case of SAMHD1 positivity (p=0.04 and p=0.016, respectively, Tables 11). On the contrary, no association was found between SAMHD1 expression and treatment efficacy in breast cancer patients treated with capecitabine (p=0.232, Table 10).

In line with ORR data, when time to progression since therapy initiation was evaluated, SAMHD1 positive patients presented shorter TTP than SAMHD1 negative patients, for ovarian and NSCLC tumours (log rank function, p=0.003 and p=0.005, respectively). In contrast, no differences were observed in TTP related to SAMHD1 status for breast cancer patients (log rank function, p=0.511) (Figure 18A). Similar results were obtained with OS since therapy initiation was evaluated, a shorter OS was observed in SAMHD1 positive patients than in SAMHD1 negative patients, only for ovarian and NSCLC tumours (log rank function, p=0.060 and p=0.014, respectively). Still, no differences in OS related to SAMHD1 status for breast cancer patients were observed (log rank function, p=0.676) (Figure 18B). These data suggest that SAMHD1 may serve as a predictive factor only in NSCLC and ovarian cancer but not in breast cancer. Interestingly, NSCLC and ovarian patients have been treated with antimetabolites in combination with platinum-containing agents in contrast with breast patients that had been treated with capecitabine alone.

Table 10. Response to treatment in ovarian, lung, and breast cancer patients depending on SAMHD1 positivity in the corresponding tumour biopsies.

Variable	Ovarian (n=22)		Lung (n=16)		Breast (n=46)	
	SAMHD1 Pos (≥25)	**SAMHD1 Neg (<25)**	**SAMHD1 Pos (≥25)**	**SAMHD1 Neg (<25)**	**SAMHD1 Pos (≥25)**	**SAMHD1 Neg (<25)**
Clinical response rate						
Complete response (CR), n (%)	2 (66.6%)	1 (33.4%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)
Partial response (PR), n (%)	4 (50%)	4 (50%)	0 (0%)	5 (100%)	11 (68.8%)	5 (31.2%)
Stable disease (SD), n (%)	3 (100%)	0 (0%)	4 (60%)	2 (40%)	2 (16.8%)	10 (83.2%)
Progressive disease (PD), n (%)	5 (100%)	0 (0%)	2 (100%)	0 (0%)	11 (91.6%)	1 (8.4%)
Overall response rate (ORR)[√]						
Yes, n (%)	6 (54.5.5%)	5 (45.5%)	0 (0%)	5 (100%)	12 (70.5%)	5 (29.5%)
No, n (%)	8 (100%)	0 (0%)	6 (75%)	2 (25%)	13 (54.2%)	11 (45.8%)
p-value*	.04*		.016*		.232	

*Pearson Chi-Square, [√]Overall Response rate: CR+PR, Pos=Positive, Neg=Negative

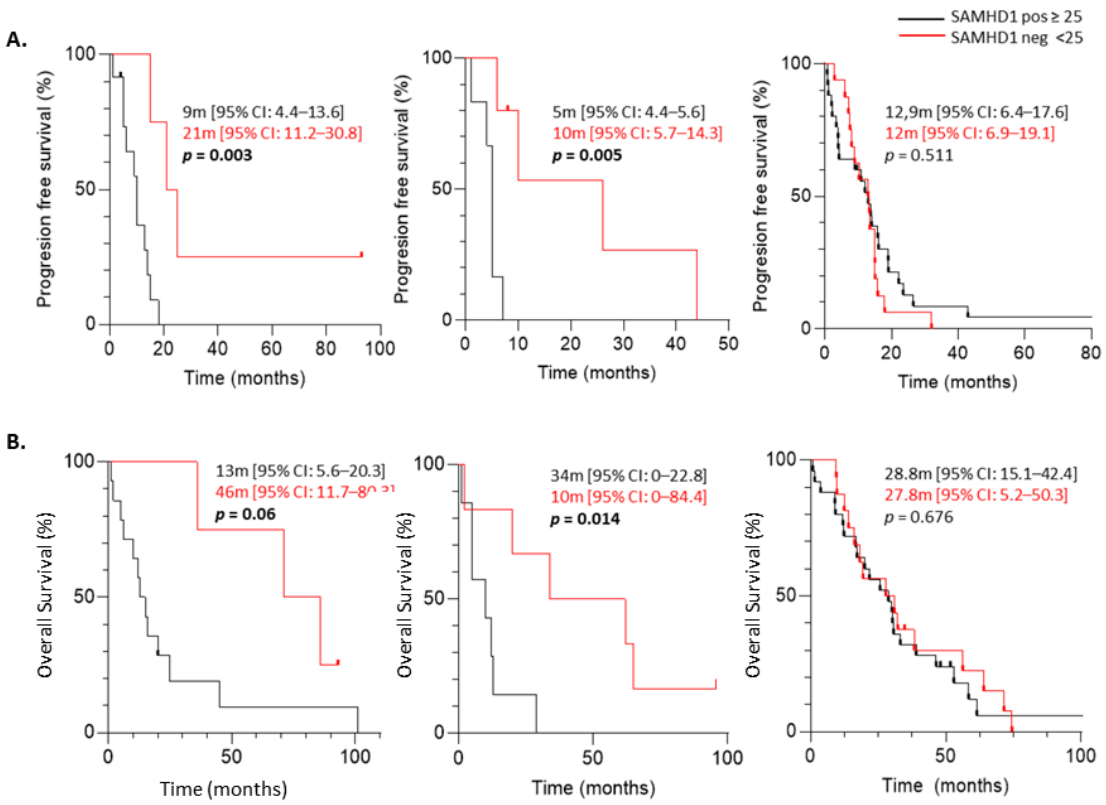


Figure 18. Predictive value of SAMHD1 in ovarian, lung and breast cancer cohorts treated with antimetabolite-containing regimens. Kaplan–Meier curves of time to progression (TTP) (A) and overall survival since cohort treatment initiation (OS) (B) for each tumour according to SAMHD1 status for different tumour types. From left to right: ovarian, NSCLC and breast. SAMHD1 expression below 25% in cancer cells was considered as negative SAMHD1 (red lines) and equal or above 25% was considered as positive SAMHD1 tumours (black lines). Median survival times with CI 95% of both groups are showed. Log rank test was used to test the significance and censored patients are indicated by vertical line.

1.4. Loss of SAMHD1 Induced Cellular Apoptosis by Enhanced Genomic Instability and DNA Damage Response

To unravel the molecular mechanisms associated to the prognostic and predictive value of SAMHD1 expression in the clinic, *in vitro* models of SAMHD1 depletion were developed in breast and ovarian cell lines. Initial evaluation of SAMHD1 KO breast cancer cells did not show any difference in cell proliferation capacity compared to wild type cells, neither when cell growth was evaluated (Figure 19A) nor in colony formation assays. Similarly, no relevant differences were observed between wild type and KO cells in cell cycle profile and expression of main genes and pathways associated to molecular mechanisms of cancer, except for the different expression of SAMHD1 protein (Figure 19B and C).

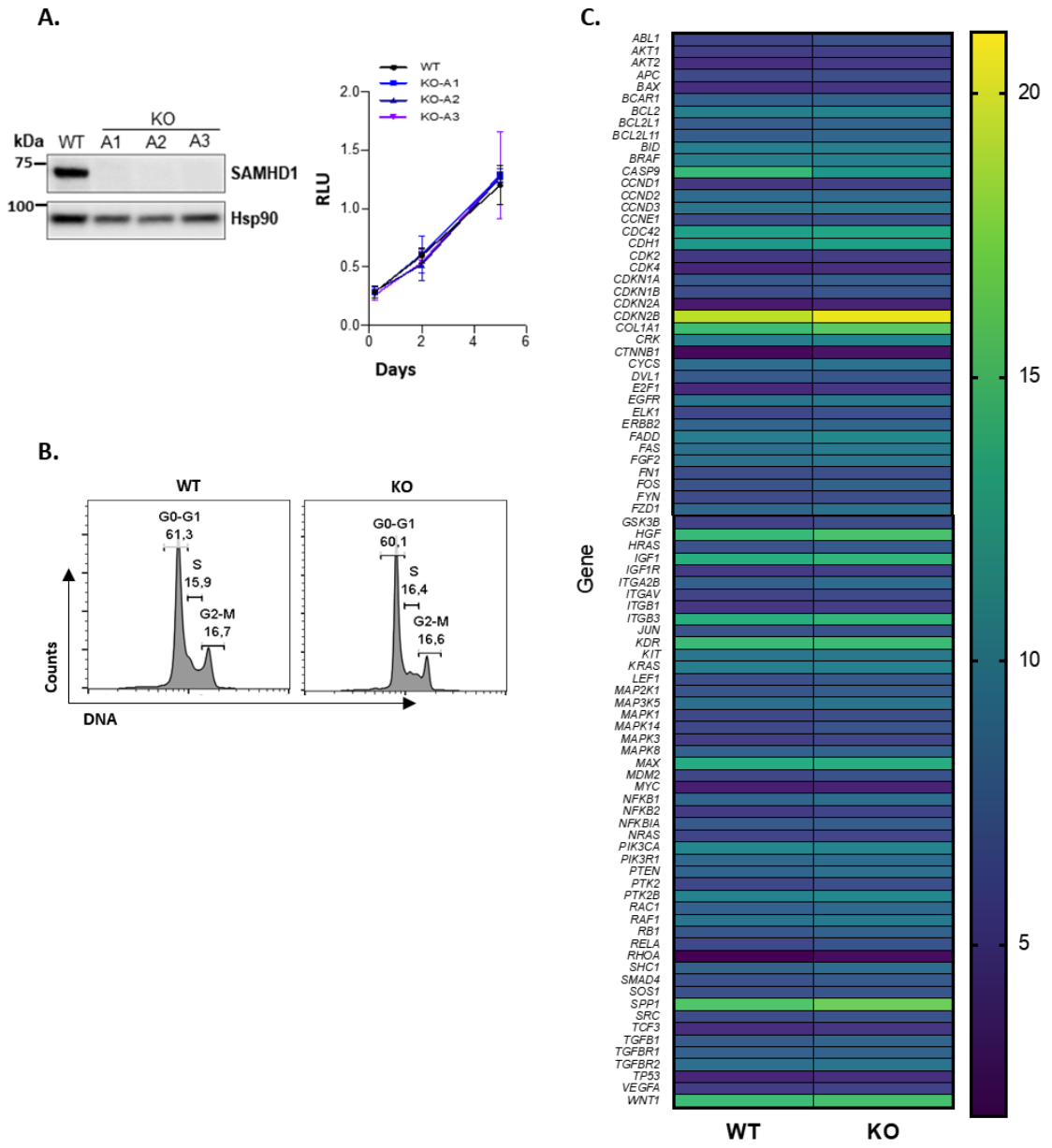


Figure 19. Characterization of SAMHD1 knockout T47D cells. (A) Representative western blot (left) showing depletion of SAMHD1 in WT and three different clones of SAMHD1-KO T47D cells. Growth curves (right) of wild type (WT, black) and three different clones of SAMHD1 knockout (KO, blue) T47D cells. Cell growth was measured at different timepoints and expressed as relative light units (RLU). Values represent mean ± SD from three different experiments. In WB, Hsp90 was used as a loading control. (B) Cell cycle analysis of WT and SAMHD1 knockout T47D cells. Representative histograms showing relative quantity ratios of G1/G0 phase, S phase and G2-M phase are shown in the figure. (B) Gene expression profiling in wild-type and SAMHD1 knockout T47D cells. No major differences in main cancer pathways were observed.

As SAMHD1 has been shown to significantly affect antiviral and cytotoxic efficacy of several antimetabolites, cell proliferation capacity was also measured in SAMHD1 KO cells treated with the chemotherapeutic drugs used in the clinical cohorts, i.e., gemcitabine, pemetrexed and 5-fluorouracil, the pharmacologically active drug of capecitabine and the platinum-based drugs, cisplatin, and carboplatin. Ara-C was added as a positive control, as it has been previously shown that SAMHD1 significantly impairs Ara-C efficacy *in vitro* and *in vivo*, by directly hydrolyzing the triphosphorylated form of the drug (146). Again, no differences were observed in the proliferation capacity of SAMHD1-KO cells in the presence of drugs except for Ara-C, used as a control (Figure 20), suggesting that SAMHD1 expression is not directly affecting cell proliferation capacity irrespective of the treatment.

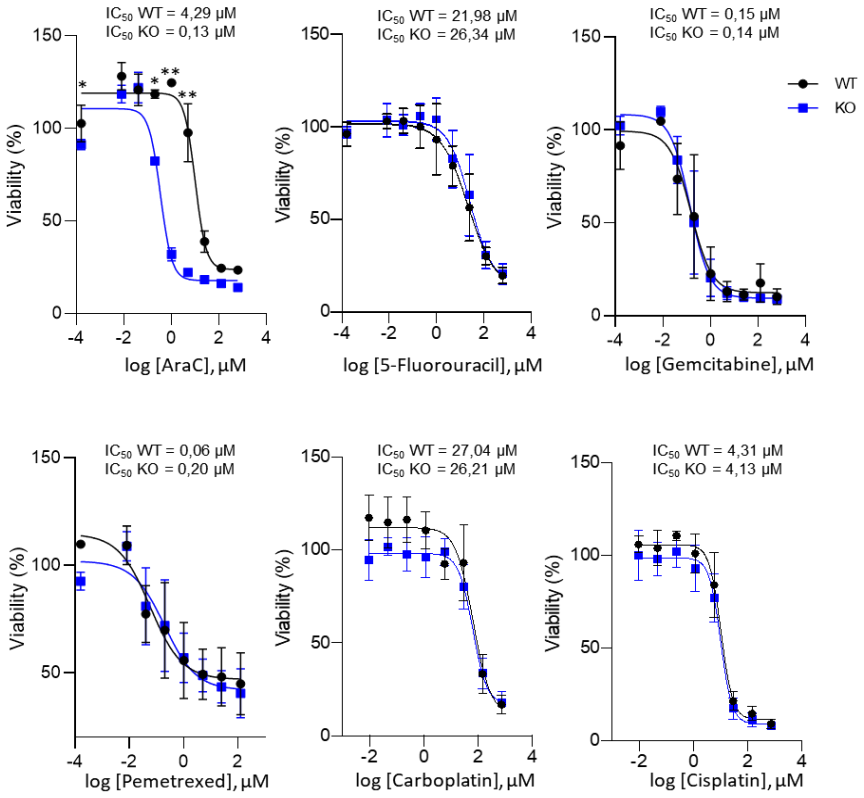
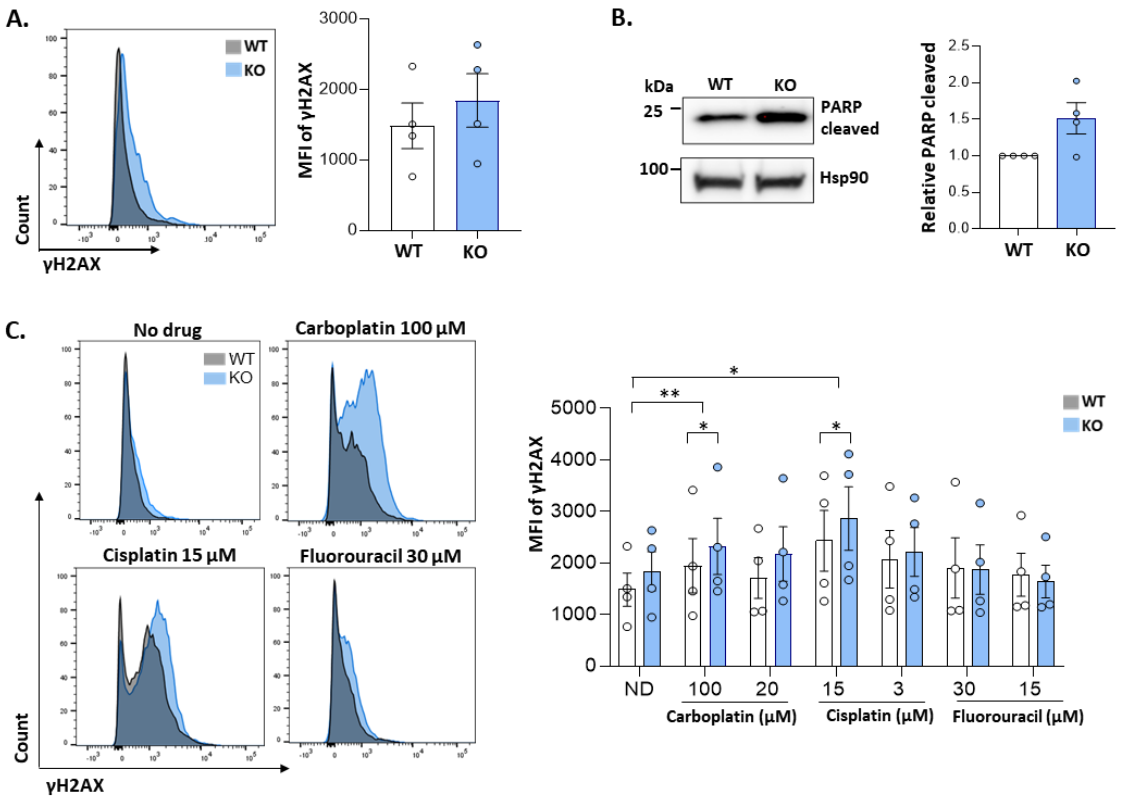


Figure 20. Dose-response curves showing cell viability of WT (•) and SAMHD1 knockout (■) T47D cells after 4 days of treatment with 5-fluorouracil, gemcitabine, pemetrexed, carboplatin and cisplatin. Cytarabine (AraC) was used as a control. The IC50 values were determined by curve fitting with four parameter non-linear regression analysis. Values represent mean ± SD from three different experiments.

Independently of SAMHD1 canonical dNTP hydrolase activity, numerous evidence indicates that SAMHD1 is also involved in the DNA damage response (DDR) (114), a process that may also contribute to cancer onset, disease progression and certain therapies affecting cell proliferation and survival following DNA damage induction (147). Thus, we evaluated DNA damage induction and survival in wild type and KO *in vitro* model by assessing γH2AX expression—a well-known marker for DNA double stranded breaks (DSBs)—by flow cytometry, which has been widely used

as a sensitive and reliable method for quantification of the DNA damage response (148–150). SAMHD1 KO cells tend to show higher levels of γH2AX than wild type cells (Figure 21A), both when measured and quantified by flow cytometry and confirmed by western blot (Figure 21D). To test whether this increased in DNA damage in KO cells is translated into increased apoptosis levels, we evaluated cleaved caspase 3 and cleaved PARP protein expression by western blot. Our results showed increased amounts of PARP cleaved and cleaved caspase 3 in two SAMHD1 KO cell lines, suggesting increased apoptosis in SAMHD1-depleted cells (Figure 21B).

Then, DNA damage induction and apoptosis were evaluated after treatment with the DNA damage inducers cisplatin and carboplatin, or the antimetabolite fluorouracil, used in the study cohorts in combination with antimetabolites. Interestingly, SAMHD1 KO cells showed a trend to increased expression of both DNA damage and apoptotic markers compared to wild type cells when treated with platinum derivatives (Figure 21C, D and E). In contrast, no effect was seen when cells were treated with fluorouracil, a nucleotide analogue that blocks thymidylate synthase impeding DNA replication. Similar results were obtained in ovarian cancer cells after effective know-down of SAMHD1 by RNA interference (Figure 22A), showing increased DNA damage and apoptosis upon SAMHD1 depletion an effect that was further enhanced upon treatment with platinum derivatives (Figures 22B and C). Overall, *in vitro* data support the idea that SAMHD1 depletion is enhancing susceptibility to DNA damage and, therefore, providing biological basis for the observed SAMHD1 predictive value in patient cohorts.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WT
KO
Count
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MFI of γH2AX
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B.
kDa
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PARP cleaved
100
Hsp90
Relative PARP cleaved
2.5
2.0
1.5
1.0
0.5
0.0
WT
KO
C.
No drug
Carboplatin 100 µM
Cisplatin 15 µM
Fluorouracil 30 µM
WT
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Count
γH2AX
MFI of γH2AX
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Carboplatin (µM)
Cisplatin (µM)
Fluorouracil (µM)

Figure 21. SAMHD1-depletion induces DNA damage and apoptosis after treatment with platinum derivatives. (A) The DNA damage marker γH2AX expression in WT and SAMHD1-KO cell lines. Representative flow cytometry histogram (left) with overlay of WT (grey) and SAMHD1-KO (blue) T47D cells comparing γH2AX expression. Histogram has been normalized to the modal values. Bar graph (right) showing mean fluorescence intensity (MFI) of γH2AX in WT and SAMHD1-KO T47D cells. Mean ± SEM of four independent experiments is shown. (B) Cell apoptosis measured as PARP cleaved and cleaved caspase 3 in WT and SAMHD1-KO cell lines. Representative western blot (left) and quantification (right) showing differential expression of PARP cleaved and cleaved caspase 3 in T47D WT and SAMHD1-KO cells. Mean ± SEM of four independent experiments is shown. (C) Left panel, representative flow cytometry histograms measuring γH2AX expression in WT (grey) and SAMHD1-KO (blue) T47D cells treated with carboplatin, cisplatin, and fluorouracil for 24 h. Right panel, bar graphs representing MFI of γH2AX expression in WT and KO T47D cells after treatment with different concentrations of carboplatin, cisplatin, and fluorouracil. Mean ± SEM of four different experiments is shown. (D) Representative western blots (upper panel) and quantification (bottom panel) showing γH2AX expression in WT and SAMHD1-KO T47D cells treated with carboplatin (left) and cisplatin (right) for 24h. Mean ± SEM of three different experiments is shown. (E) Representative western blots (upper panel) and quantification (bottom panel) showing PARP cleaved and cleaved caspase 3 expression in WT and SAMHD1-KO T47D cells treated with carboplatin, cisplatin and fluorouracil for 24 h. Mean ± SEM of four different experiments is shown. *, p < 0.05, **, p < 0.01.

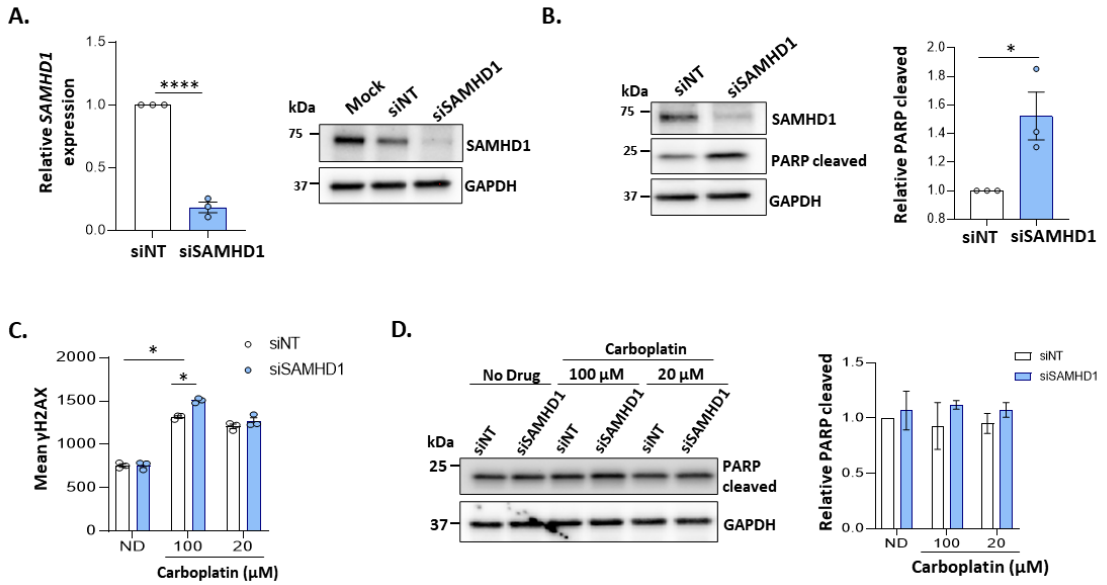


Figure 22. SAMHD1-depletion induces DNA damage and apoptosis after treatment with platinum derivatives in OVCAR3 cells. (A) SAMHD1 mRNA (left) and protein expression (right) showing specific siRNA-mediated inhibition of SAMHD1 in OVCAR-3 cells. ****, *p*<0.0001 (B) Representative western blot (left) and quantification (right) showing depletion of SAMHD1 and increased PARP cleaved in SAMHD1-depleted cells. *, *p*<0.05 (C) Bar graph representing MFI of γH2AX expression in siNT and siSAMHD1 OVCAR-3 cells after treatment with different concentrations of carboplatin. Mean ± SEM of three different experiments is shown. *, *p*<0.05.

CHAPTER 2. SAMHD1 EXPRESSION IN RESIDUAL DISEASE IS A SURROGATE MARKER OF IMMUNE INFILTRATION AND DETERMINES PROGNOSIS AFTER NEOADJUVANT CHEMOTHERAPY IN EARLY BREAST CANCER.

Summary

In this chapter, we focused on the characterization of the use of SAMHD1 as a prognostic biomarker in early-stage breast cancer patients.

Neoadjuvant chemotherapy (NACT) has become part of the standard of-care treatment of these patients. Achieving pathological complete response (pCR) is an essential prognostic factor with favourable long-term outcomes. However, the lack of validated surrogate biomarkers and more efficient alternative treatment options for patients who do not achieve a pCR constitutes still an unmet need for breast cancer clinical management. Based on this, we describe and validate the use of SAMHD1 expression as a prognostic biomarker in residual disease *in vivo* and *in vitro*, by specifically delineating the immunomodulatory consequences of SAMHD1 depletion, through the dysfunction of specific interleukin signalling pathways. As described below, we confirm SAMHD1 can act as a novel prognostic biomarker in early breast cancer with residual disease after NACT that impacts immune-mediated signalling and differentially regulates inflammatory intra-tumoural response.

2.1 SAMHD1 expression is associated to increased risk of recurrence in post-NACT tumour biopsies.

To evaluate the contribution of SAMHD1 expression in cancer progression and/or treatment, a retrospective study including all breast cancer patients treated in ICO Badalona during a 10-year period (2002-2012) was performed (n=2470). Within the whole cohort, 11% of cases received NACT (n=228) and SAMHD1 expression was evaluated in all infiltrating ductal carcinoma cases with follow-up (n=182) that do not achieve pCR (n=151, Fig 10). No significant clinical differences were found between the entire NACT cohort and the subgroup of patients without pCR.

Expression of SAMHD1 was evaluated by immunohistochemistry in available retrospectively collected post-NACT tumour biopsies and samples were stratified according to SAMHD1 expression (Figure 23A). Residual tumours were SAMHD1 positive in 30% of evaluable patients (31/101 evaluable tumours). Given the high percentage of SAMHD1 negative tumours, a group of SAMHD1-high expressing tumours (over 75%, n=6) and SAMHD1-negative tumours (n=5) were selected for analysing putative gene mutations in *SAMHD1* coding region that might influence protein expression levels. No non-synonymous or frameshift mutations were identified in any of the cases, suggesting that expression differences are not linked to sequence variations in *SAMHD1* coding region (Figure 24).

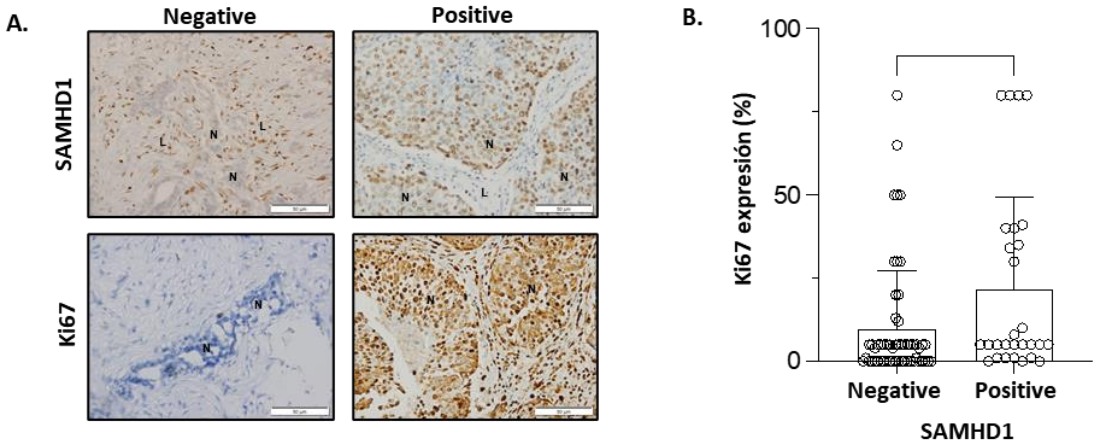
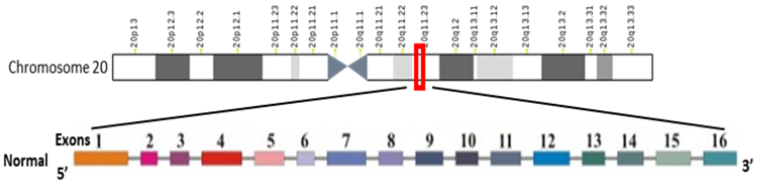


Figure 23. SAMHD1 is variably expressed in BC tumours and correlates with Ki67 expression. (A) Expression of SAMHD1 and Ki67 by immunochemistry in tumour samples. Representative microscopy images of SAMHD1 (upper panel) and Ki67 (lower panel) expression in paraffin-embedded tumour biopsies. High expression of SAMHD1 was observed in lymphocytic cells infiltrating in the tumours, and it was used as a positive control of immunohistochemistry for negative biopsies. N, neoplastic cells, L, lymphocytes. SAMHD1 expression was exclusively nuclear. (B) Percentage of Ki67 expression was significantly lower in SAMHD1-negative tumours post-NACT.



	Exon / Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
High SAMHD1 expression	4	NM	c.782A>C Leu-Phe	NM	ND	ND	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
	6	ND	NM	NM	NM	ND	NM	ND	c.36G>A Gly289Glu	NM	NM	ND	ND	ND	NM	ND	NM
	11	NM	NM	NM	NM	ND	NM	NM	ND	NM	NM	NM	NM	ND	NM	NM	NM
	15	NM	NM	NM	ND	ND	NM	ND	ND	NM	NM	NM	ND	NM	NM	ND	NM
	17	NM	NM	NM	NM	ND	NM	NM	NM	NM	NM	ND	NM	NM	ND	NM	ND
	19	NM	NM	NM	c.141T>A	ND	NM	NM	ND	NM	NM	NM	NM	NM	NM	ND	NM
Low SAMHD1 expression	2	NM	NM	NM	NM	NM	NM	NM	ND	NM	NM	NM	NM	NM	NM	NM	NM
	3	NM	NM	NM	ND	NM	NM	NM	ND	NM	NM	NM	NM	NM	NM	NM	NM
	5	NM	NM	NM	ND	NM	NM	ND	ND	NM	NM	NM	NM	NM	NM	ND	ND
	7	NM	NM	NM	ND	NM	NM	NM	ND	NM	NM	NM	NM	NM	NM	ND	ND
	13	NM	NM	NM	NM	NM	NM	NM	ND	NM	NM	NM	ND	NM	ND	NM	NM

Figure 24. Evaluation of SAMHD1 mutations in breast cancer tumours. Top panel: Representation of SAMHD1 gene location in chromosome 20 and its exons. Bottom panel: Table of mutations found in breast cancer tumours expressing high (n=6, orange) or low (n=5, green) SAMHD1 levels. Only three missense mutations were identified. NM=No Mutations, ND=No Determined

Then, a comprehensive clinical description of the post-NACT cohort, stratified according SAMHD1 expression was performed (Data not shown). No major differences were observed between SAMHD1 positive and negative tumours post-NACT, except for histological grade, Ki67 expression and recurrence. Interestingly, SAMHD1 positivity was associated with grade III tumours (p=0.025), higher levels of Ki67 after NACT (p=0.017) and increased risk of recurrence (p=0.005), suggesting that SAMHD1 expression might represent a relevant prognostic biomarker for breast cancer post-NACT (Figure 23B).

2.2 SAMHD1 tumour positivity determines shorter TTP and OS after NACT.

To gain insight into the putative role of SAMHD1 as a prognostic factor in BC, TTP was evaluated. SAMHD1-expressing tumours presented shorter TTP than SAMHD1 negative cases (log-rank function, p=0.002), with a median TTP of 4.58 years for 318 SAMHD1-positive patients, whereas it was not reached for SAMHD1 negative patients (Figure 25A).

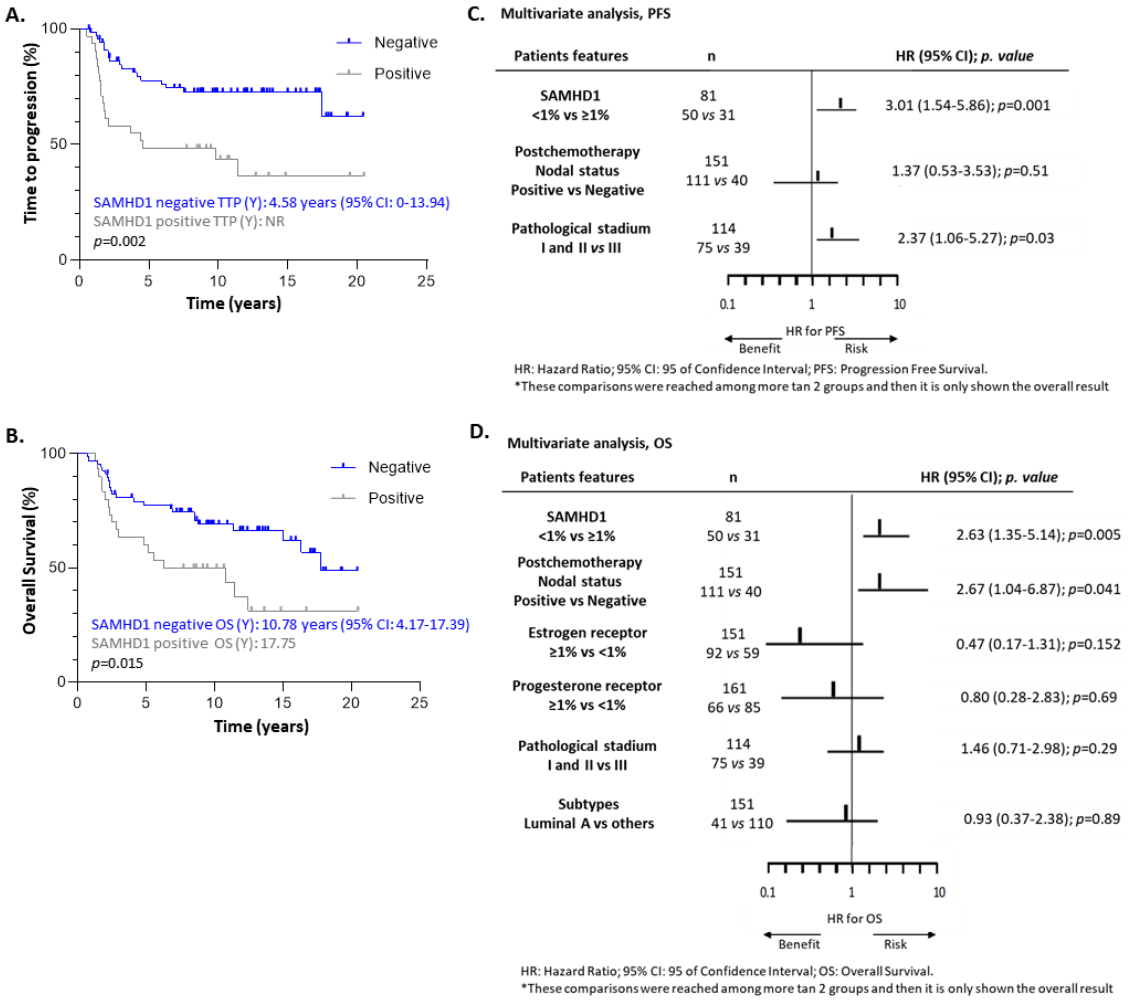


Figure 25. Prognostic value of SAMHD1 in breast cancer cohorts. Kaplan–Meier curves of time to progression (TTP) (A) and overall survival (OS) since treatment initiation/cancer diagnosis (B) according to SAMHD1 status. SAMHD1 negative patients after neoadjuvant chemotherapy have significantly better TTP and OS. SAMHD1 expression below 1% in cancer cells was considered as negative SAMHD1 (blue lines) and equal or above 1% was considered as positive SAMHD1 tumours (grey lines). Median survival times with CI 95% of both groups are shown. Log-rank test was used to compare survival curves between groups. Hazard ratios (HRs) and 95% confidence intervals (CIs) were obtained from the Cox multivariate model to analyse the risk of progression (C) and death (D). SAMHD1 negativity and pathological stadium I and II versus III after NACT, are associated with increased progression free survival, and SAMHD1 negativity and negative nodal affection are associated with overall survival improvement.

Similar results were obtained for OS, where median OS was significantly shorter for SAMHD1 positive tumours (10.78 years, 95% CI, 4.17-17.39 in SAMHD1 positive *vs*. 17.75 years, 95% CI, 0 to 14.6 for SAMHD1 negative cases, p=0.016) (Figure 25B). In the univariate analysis for PFS and OS, SAMHD1-negative cases were associated with longer TTP (p=0.003) and prolonged OS (p=0.014), like other well-characterized prognostic factors as are negative nodal status post NACT, pathological stage (I and II versus III) or adjuvant chemotherapy and adjuvant hormonal therapy among others (Data not shown). More importantly, multivariate analysis for TTP showed also that SAMHD1-negative status and pathological stage (I and II *versus* III) were

associated with prolonged TTP after NACT with anthracyclines-based regimens (p=0.001 and p=0.03, respectively) (Figure 25C). Similar data were obtained on multivariate analysis for OS, where SAMHD1-negative status and negative post chemotherapy nodal status were the only variables associated with prolonged OS after NACT with anthracyclines-based regimens (p=0.005 and p=0.041, respectively) (Figure 25D). Overall, our clinical data suggest that SAMHD1 expression is a relevant prognostic factor in breast cancer in the neoadjuvant setting.

2.3 SAMHD1-KO tumour spheroids downregulate metabolic and immune signalling pathways. To explore the functional mechanism underlying SAMHD1 impact on BC prognosis, we used our previously described T47D SAMHD1 *knock-out* (KO) cell model (138) to investigate putative effects of SAMHD1 expression in the response to neoadjuvant treatment, i.e., anthracyclines and taxanes. Contrary to the reported effect upon platinum-derived drugs exposure, no significant differences were observed between SAMHD1 wild-type or KO cells treated with doxorubicin or paclitaxel in cell proliferation assays, as well as no changes in expression of DNA damage induction or apoptotic markers (Figure 26).

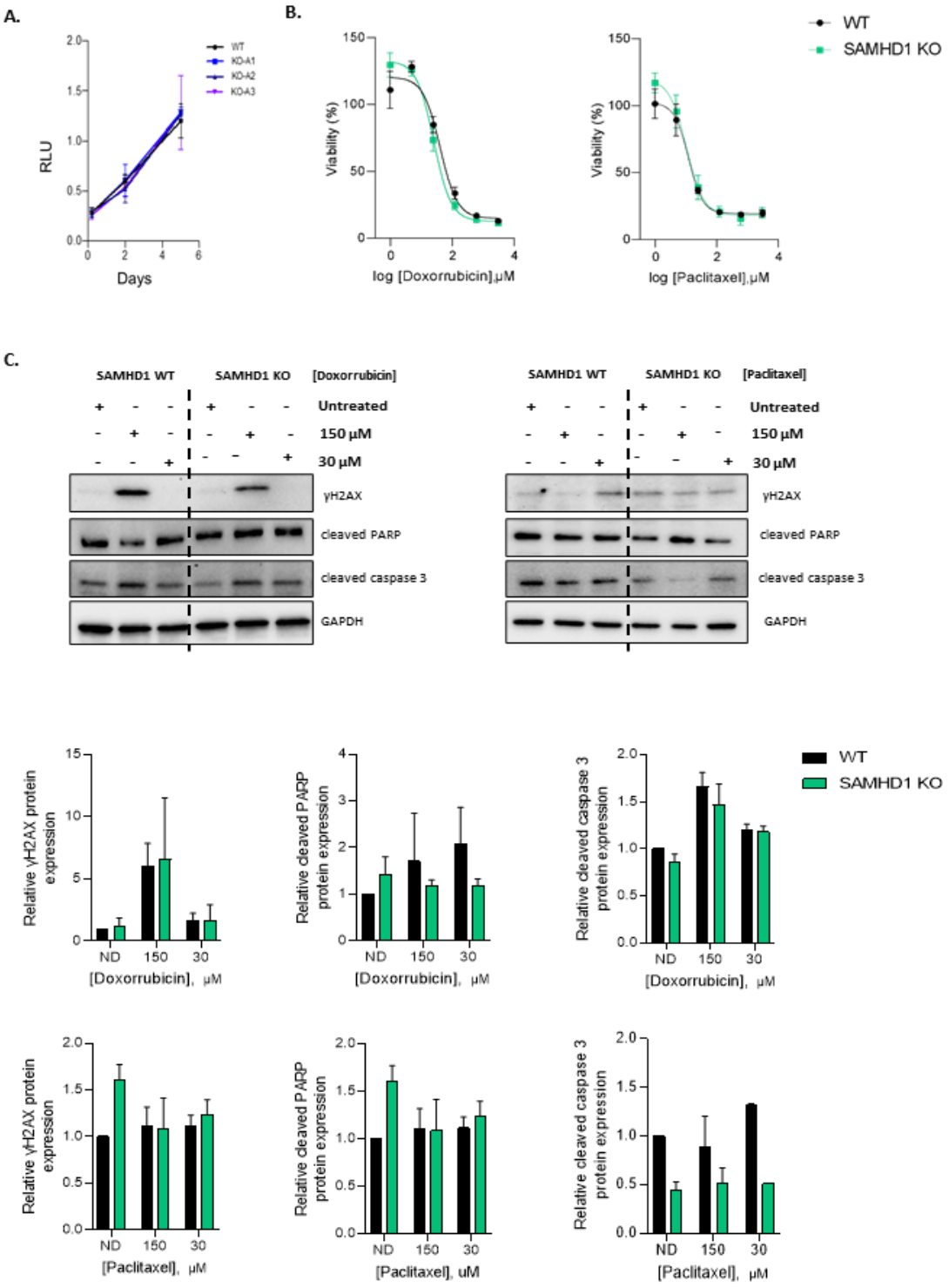


Figure 26. (A) Growth curves of wild type (WT, black) and three different clones of SAMHD1 knockout (KO, blue) T47D cells. SAMHD1-KO did not show any difference in growth rate compared to WT when cultured in monolayer. Cell growth was measured using CellTiter-96® Aqueous One Solution Reagent and expressed as relative light units (RLU). Values represent mean ± SD from three different experiments. All measurements were performed in triplicates. (B) Dose-response curves showing cell viability of WT (black) and SAMHD1-knockout (green) T47D cells after 4 days of treatment with doxorubicin and paclitaxel. Values represent mean ± SD from three different experiments. (C) Representative western blots (upper panels) and quantification (bottom panels) showing γH2AX, cleaved PARP and cleaved caspase 3 expression in WT and SAMHD1-KO T47D cells treated with doxorubicin and paclitaxel for 24h. Mean ± SEM of three different experiments is shown.

Proliferation assays of monolayer cancer cells fail to model three-dimensional (3D) solid tumours, whereas cells cultured in 3D aggregates adopt the proper shape and experience cell-cell contacts and nutrient diffusion in all directions, representing more accurately the natural microenvironment of tumours and tissues (151). Thus, we set up a 3D cell culture model based on T47D spheroids, with the aim to better understand the functional consequences of SAMHD1 depletion (Figure 12). Wild-type and SAMHD1-KO spheroids showed a tight spherical shape and stable appearance with an average size of 645 +/- 30µm and no significant differences were observed between wild-type *vs*. SAMHD1-KO spheroids (Figures 27A and 27B). Similarly, both wild-type and SAMHD1-KO spheroids presented the typical oxygen-gradient structure with a dense cell core that corresponds with the low-oxygen necrotic zone as described before (152) (Figure 27A).

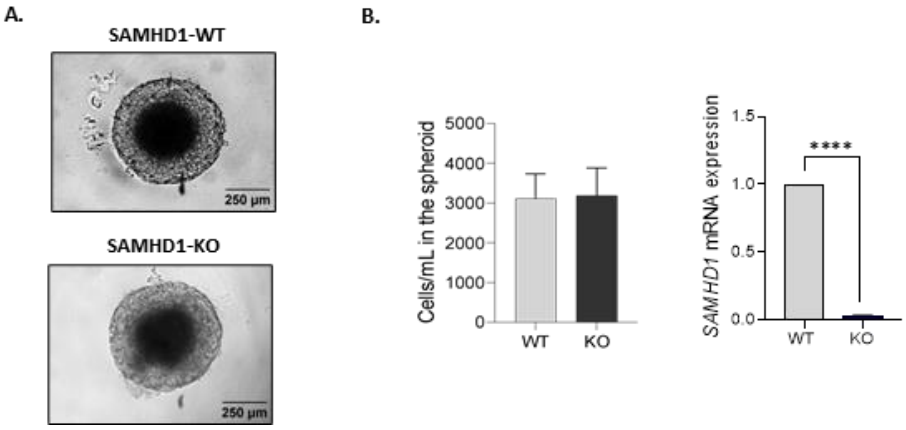


Figure 27. T47D spheroids formation. (A) Brightfield microscope images of SAMHD1 wild-type (WT) (top) and SAMHD1-knockout (KO) (bottom) T47D tumour spheroids showing no differences in spheroid structure or integrity. Images were taken 4 days after cells were seeding. (B) Left graph shows total cell count of WT (grey) and SAMHD1-KO (black) spheroids. Cells were counted after spheroid disaggregation at day 4. All measurements were performed in triplicates. Right graph shows the lack of SAMHD1 mRNA expression in SAMHD1-KO spheroids. All measurements were performed in triplicates.

To further explore the mechanisms underlying SAMHD1 role, whole transcriptome profiling was performed on wild-type and SAMHD1-KO breast cancer spheroids. Hierarchical clustering of wild-type and SAMHD1-KO spheroids using the union of all differentially expressed genes (DEGs) revealed distinct genetic signatures among them, while KO spheroids derived from different clones presented more similar signatures, as expected (Figure 28A and 28B). To identify pathways specifically affected by the downregulation of SAMHD1 in spheroids, we performed gene-set enrichment analysis (GSEA) using the Reactome gene-sets (Figure 28C). Overall, depletion of SAMHD1 in spheroids induced a global downregulation of several signalling pathways, especially at transcription and RNA processing level. More interestingly, downregulation of the IL-12 family was also observed in SAMHD1-KO spheroids, suggesting that

downregulation of SAMHD1 may influence immune signalling and response in tumours, in accordance with previous data demonstrating a link between SAMHD1 and IL-12 (153).

***2.4 In vitro* modeling of SAMHD1 knockout tumours exhibits reduced interleukin signalling.**

To characterize the putative effect of differential interleukin signalling of SAMHD1-KO tumour spheroids, we conducted a more detailed evaluation of the IL-12 downregulated pathway. Examination of the leading-edge genes of the IL-12 family, indicated that the gene subsets that contributed the most were *CNN2*, *CRLF1*, *TYK2* and *TCP1* among others (Figure 28D), whose downregulation was further confirmed in additional spheroid samples by quantitative PCR analysis (Figure 28E). The differentially expressed genes are mostly involved in the regulation of immune mediated signalling, thus, we further evaluated the expression of other interleukins in SAMHD1-KO tumour spheroids. Interestingly, reduced production of IL6, IL8 and IL1α were also observed, both at mRNA level (Figure 29A) and protein level, measured as IL6 and IL8 production in spheroid culture supernatant (Figure 29B).

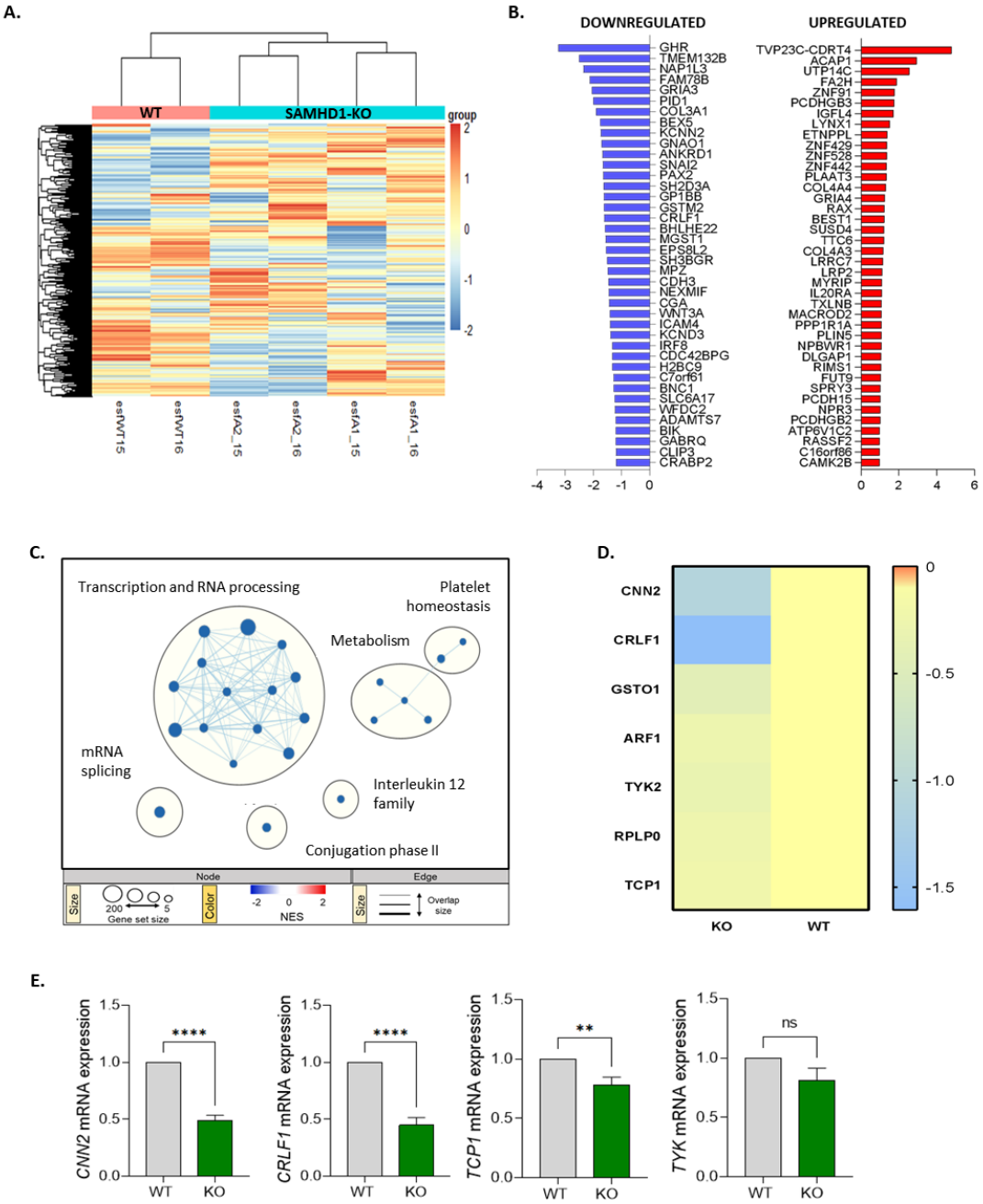


Figure 28. Gene expression changes in SAMHD1-KO and SAMHD1-WT breast cancer spheroids. (A) Heatmap representation of gene expression changes in SAMHD1-KO and SAMHD1-WT breast cancer spheroids. Heatmap was generated by unsupervised hierarchical clustering of significantly expressed genes ($p < 0.05$). (B) Bar plots of top 30 differentially enriched genes (DEG) for SAMHD1-KO breast cancer spheroids relative to SAMHD1-WT spheroids, based on Log2 gene expression (Log2FC) and $p<0.05$. Significantly down- or up-regulated DEG are highlighted in blue or red, respectively. (C) Reactome Gene set enrichment map of significantly enriched pathways for SAMHD1-KO spheroids. Reactome Gene set clusters are annotated, and nodes manually laid out for clarity. Single gene sets are highlighted and annotated in-text, underneath the cytoscape enrichment map. Node size represents number of genes, node colour represents significance (NES), and edge thickness represents number of shared genes. (D) Gene expression of leading-edge genes from the Reactome *IL-12* family gene set. SAMHD-WT spheroids were used as a reference set for leading-edge genes selection and ranking. Significantly down-regulated gene sets and genes are highlighted in blue. (E) mRNA expression of *CNN2*, *CRLF1*, *TCP1* and *TYK2* genes in T47D spheroids showing decreased expression in SAMHD1-depleted spheroids compared to SAMHD1-WT spheroids (p=0.0001, 0.0001, 0.0057 and 0.0944, respectively).

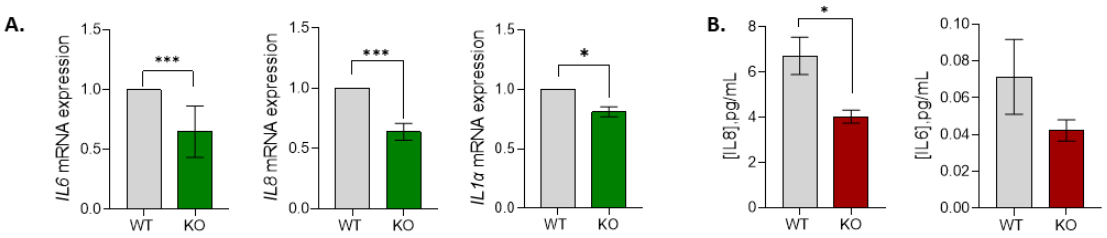


Figure 29. SAMHD1-KO breast cancer spheroids presented decreased pro-inflammatory cytokine expression compared to SAMHD1-WT spheroids. (A) Gene expression of distinct proinflammatory cytokines (IL6, IL8, IL1α) in breast tumour spheroids. IL6, IL8, IL1α were significantly decreased in SAMHD1-KO spheroids (*p*=.001, <.0001 and .0475, respectively). (B) Evaluation of protein expression in supernatants from spheroids cultured for 4 days. SAMHD1-KO spheroids present decreased IL8 and IL6 (*p*=0.0277 for IL8), further confirming the RNA expression results. All measurements were performed at least in triplicates.

In addition, transient downregulation of SAMHD1 by RNAi in MCF7 cell line showed no differences in spheroid integrity or proliferation but a reduction of *IL6* expression (Figure 30). This was similar to what had been previously observed in T47D spheroids, further confirming the specificity of the effect.

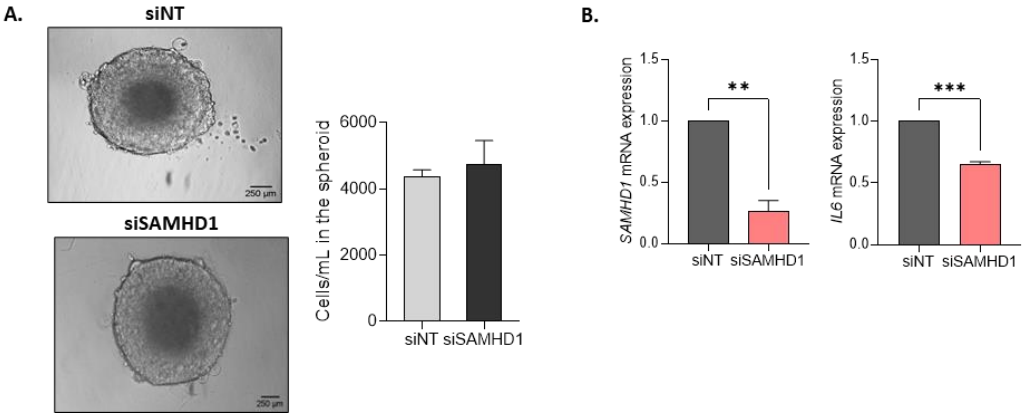


Figure 30. SAMHD1-depleted MCF-7 spheroids show a decrease in IL6 expression but not differences in integrity or spheroid structure. (A) Brightfield microscope images of SAMHD1-WT (up) and SAMHD1-depleted (bottom) MCF7 tumour spheroids showing no differences in spheroid structure or integrity. Images were taken 4 days after cells were seeding. Right graph shows the total cell count of WT (grey) and SAMHD1-KO (black) spheroids. Cells were counted after spheroid disaggregation at day 4. All measurements were performed in triplicates. (B) *SAMHD1* (left) and *IL6* (right) mRNA showing specific siRNA-mediated inhibition of SAMHD1 and the subsequent decrease in *IL6* in MCF-7 spheroids (*p*=0.0036 and 0.0003, respectively).

To validate the involvement of the IL-12 family pathway *in vivo*, we selected a group of patients (n=47) with available biopsies from primary tumours to quantify gene expression of key genes, i.e., *SAMHD1* and IL-12 family leading-edge genes. mRNA expression of *SAMHD1*, *CNN2, CRLF1* and *TCP1* genes was measured by qRT-PCR in 42 patients. Correlation analysis was performed in samples with detectable expression for all genes tested, except *CRLF1,* whose expression was undetectable in most samples. No significant correlation was observed between *SAMHD1* and

CNN2 gene (data not shown). However, a positive correlation between *SAMHD1* and *TCP1* expression was identified (p=.0081), confirming the transcriptomic data and pointing towards TCP1 as an additional factor determining SAMHD1 effect in breast cancer (Figure 31A). Interestingly, TCP1 is part of chaperonin multi subunit protein-folding complex (TRiC or CCT) that has been previously described to interact with many oncoproteins and mutant tumour suppressors. To this end, using TCGA data on survival probability in BC, we confirmed that *TCP1* low expressing tumours had better prognosis, similar to the data obtained with SAMHD1 expression in our cohort, while *CNN2*, *TYK2* and *CRLF1* expression do not affect survival, in agreement with our data from RNA expression in tumour biopsies (Figure 31B).

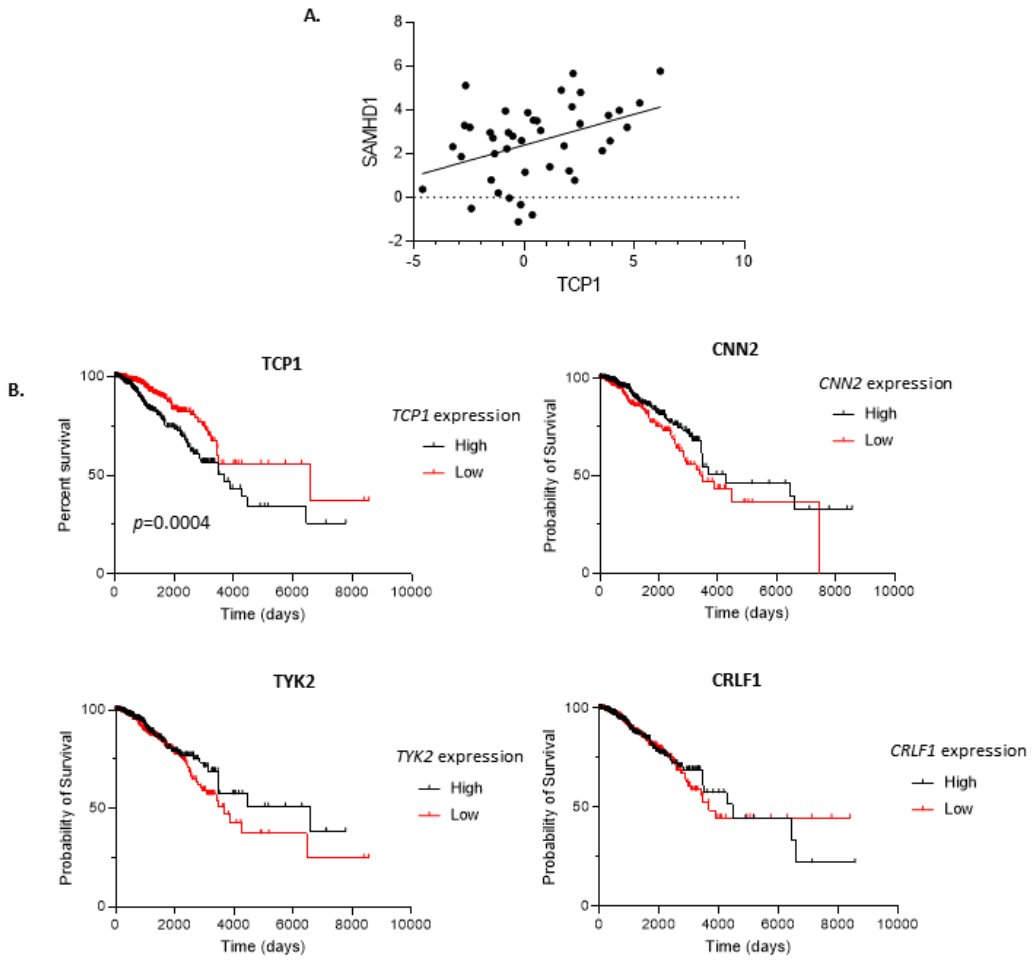


Figure 31. (A) Correlation between SAMHD1 and TCP1 expression levels in RNA extracted from tumour biopsies. (B) Kaplan-Meier curves of overall survival in breast cancer patients from the Human Protein Atlas (HPA) datasets for *TCP1, CNN2, TYK2* and *CRLF1* genes divided by high (black line) or low (red line) expression levels. According to HPA, *TCP1* is a prognostic factor in breast cancer (p=0.0004). Cut off for determining high or low expression level was calculated using medium FPKM (FPKM=36.85). Data from men and stages I and IV was excluded for a better representation of the cohort described in this paper. In contrast, *CNN2, TYK2* and *CRLF1* are not prognostic factors in breast cancer (log-rank test p=0.0215, 0.14, 0.779 respectively). Cut off for determining high or low expression level was calculated using medium FPKM that was 37.2, 9.62 and 1.01 respectively for each gene. Data from men and stages I and IV was excluded for a better representation of the cohort described.

2.5 SAMHD1-KO spheroids are less susceptible to myeloid and neutrophil infiltration

Accumulating evidence have shown that the interaction between cancer cells and the TME, specifically the immune microenvironment, is a vital factor in tumour progression and therapy (154). Thus, as interleukins and their signalling pathways identified above, are potent chemoattractant for immune cells and may determine tumour immune infiltration, we evaluated the capacity of immune cells to infiltrate in SAMHD1 KO and WT spheroids. We developed an *in vitro* coculture system including T47D BC spheroids and peripheral blood mononuclear cells (PBMCs) derived from healthy donors (Figure 32A). No significant changes in spheroid integrity were observed between WT and KO-SAMHD1 cocultures (Figure 32B). Then, the number of immune cells infiltrated into the tumour spheroids was quantified by flow cytometry and immunofluorescence. Interestingly, a significant reduction in the number of infiltrating PBMCs was observed in SAMHD1-KO spheroids compared to WT spheroids, presumably due to of the reduced IL-mediated signalling upon SAMHD1 depletion (Figure 32C and 32D). Next, the type of infiltrated PBMCs was evaluated by immunophenotypic characterization through flow cytometry, identifying major changes in myeloid antigen presenting cells, monocytic cells and high cytotoxic NK cells (p=0.0156, 0.0053 and 0.0025, respectively), whereas CD4+ and CD8+ T lymphocytes did not differ (Figure 32E). Overall, our data suggest that impaired IL-mediated signalling observed in SAMHD1-KO spheroids have an impact on preferentially myeloid and NK cell subsets. Hence, we determined whether SAMHD1 expression correlated with the immune infiltration level in BC patients by calculating the coefficient of SAMHD1 expression and the distinct immune cell types evaluated experimentally in TIMER (155) (Figure 32F). SAMHD1 expression positively correlated with monocytes cells and NK cells, i.e., the lower SAMHD1 expression the lesser infiltrated cells. These results agree with the data described above in the *in vitro* models (Figure 32E) and confirm the role of the SAMHD1 as a regulator of BC prognosis through the induction of changes in immune response and TME.

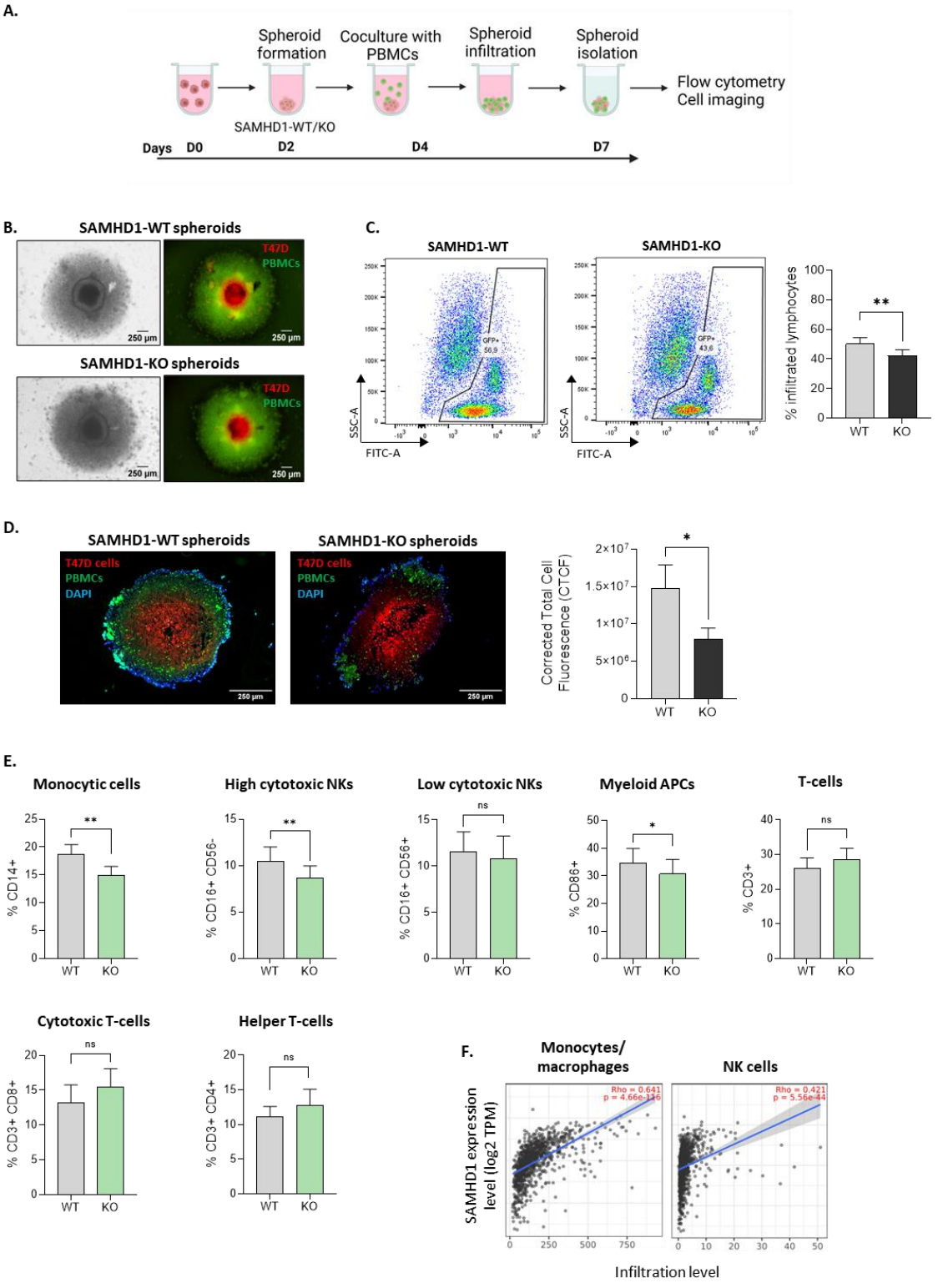

A.
Spheroid formation
Coculture with PBMCs
Spheroid infiltration
Spheroid isolation
Flow cytometry
Cell imaging
SAMHD1-WT/KO
Days D0 D2 D4 D7
B.
SAMHD1-WT spheroids
T47D
PBMCs
250 µm
SAMHD1-KO spheroids
C.
SAMHD1-WT
SAMHD1-KO
SSC-A
FITC-A
% infiltrated lymphocytes
WT
KO
D.
SAMHD1-WT spheroids
SAMHD1-KO spheroids
T47D cells
PBMCs
DAPI
Corrected Total Cell Fluorescence (CTCF)
E.
Monocytic cells
% CD14+
High cytotoxic NKs
% CD16+ CD56-
Low cytotoxic NKs
% CD16+ CD56+
Myeloid APCs
% CD86+
T-cells
% CD3+
Cytotoxic T-cells
% CD3+ CD8+
Helper T-cells
% CD3+ CD4+
F.
Monocytes/ macrophages
NK cells
SAMHD1 expression level (log2 TPM)
Infiltration level

Figure 32. SAMHD1-KO breast cancer spheroids presented decreased immune infiltration than SAMHD1-WT spheroids. (A) Workflow of coculture generation of breast tumour spheroids and primary PBMCs. At day 4, when spheroids were complete formed, cocultures were established by adding peripheral blood mononuclear cells (PBMCs) from healthy donors. After three days, infiltration into the spheroids was evaluated by multipanel flow cytometry. Imaging of spheroid composition and cell invasion was performed by microscopy. Created with BioRender. (B) Representative brightfield (left) and fluorescence microscopy (right) images from cocultures of SAMHD1-WT (top) and SAMHD1-KO (bottom) T47D spheroids and PBMCs 3 days after cocultures were established. T47D cells were pre-stained with CellTracker™ Red CMPTX Dye. PBMCs were stained with CellTracker™ Green CMFDA Dye. (C) Representative cytometry plots (left) and quantification (right) of immune cells infiltrated into the spheroids. SAMHD1-KO spheroids presented decreased % of infiltrated lymphocytes (p=.0014) compared to SAMHD1-WT. All measurements were performed at least in triplicates. (D) Representative fluorescence images (left) and CTCF quantification (right) of sections of paraffin embedded PBMCs and spheroid cocultures. Quantification of corrected total cell fluorescence (CTCF) showing decreased infiltration into the SAMHD1-KO spheroids (p=. 0484). SAMHD1-KO spheroids presented decreased immune infiltration than SAMHD1-WT spheroids. (E) Immunophenotypic characterization of infiltrated cells into the spheroids. Infiltrated immune cells into spheroids were evaluated by multipanel flow cytometry. SAMHD1-KO spheroids presented significant decreased of myeloid APCs (CD86+), monocytic cells (CD14+) and high cytotoxic NKs (CD16+CD56+) (p=.0156, .0053 and .0025, respectively), whereas helper T cells (CD3+ CD4+), cytotoxic T cells (CD3+ CD8+) and low cytotoxic NKs (CD16+ CD56-) did not differ. All measurements were performed at least in triplicates. (F) Correlation of SAMHD1 expression levels with monocyte/macrophage and NK cell infiltration in BC, as implemented in the TIMER resource.

CHAPTER 3. SAMHD1 EXPRESSION MODULATES INNATE IMMUNE ACTIVATION AND CORRELATES WITH OVARIAN CANCER PROGNOSIS.

Summary

Considering the key role of SAMHD1 in the induction of IFN-mediated immune activation derived from its role in DNA damage repair, together with reported deficiencies in nucleic acid sensing and subsequent loss of innate immune activation in ovarian cancer (156), we focused our attention on innate immune response and cytosolic pattern recognition receptors (PRRs).

This chapter of the thesis was dedicated to evaluating the role of SAMHD1 expression and function in ovarian cancer, both *in vitro* and in ovarian cancer patient cohorts. We found that SAMHD1 depletion correlates with increased innate immune cell signalling in ovarian cancer cells. In clinical samples, SAMHD1-low expressing tumours showed increased progression free survival and overall survival irrespective of *BRCA* mutation status. Therefore, our results point towards SAMHD1 modulation as a new therapeutic strategy, able to enhance innate immune activation directly in tumour cells, leading to improved prognosis in ovarian cancer.

3.1 Depletion of SAMHD1 enhances apoptosis and regulates innate immune response.

To determine the contribution of SAMHD1 in ovarian cancer, we effectively downregulated SAMHD1 expression in the ovarian cancer cell lines OVCAR3 and SKOV3, leading to a 70-80% reduction in SAMHD1 RNA and protein expression levels (Figure 33A, B). We have previously shown that SAMHD1 knockout breast cancer cells presented increased DNA damage and apoptosis, an effect that was enhanced upon platinum-based treatment (138). In SKOV3 and OVCAR3 ovarian cancer cell lines, SAMHD1-depleted cells also showed increased expression of the apoptotic markers, cleaved PARP and cleaved caspase-3 expression, although differences were not statistically significant (Figure 33B), indicating the existence of additional mechanisms in contrast to previous data (138).

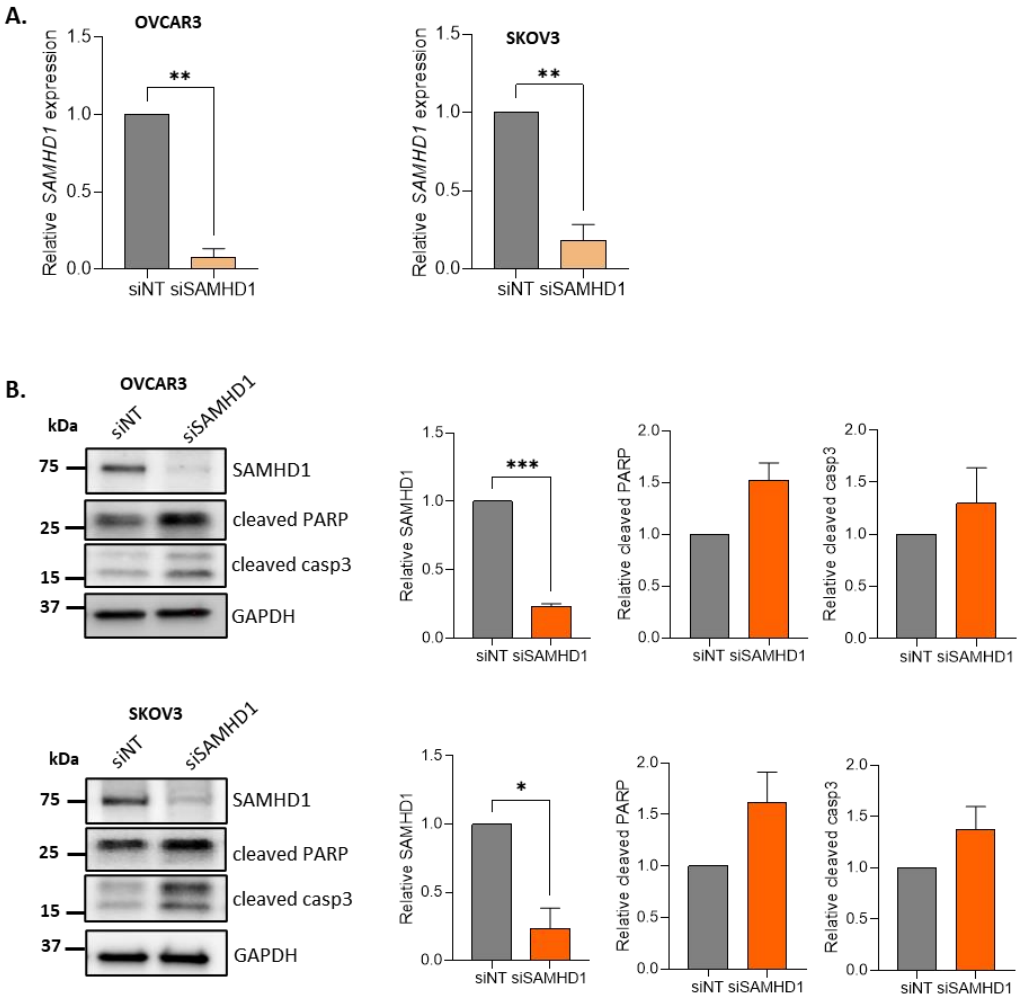


Figure 33. Depletion of SAMHD1 enhances apoptosis and regulates innate immune response. (A) Effective SAMHD1 downregulation by RNA interference in ovarian cancer cell lines. siRNAs targeting SAMHD1 gene (siSAMHD1) were transfected into OVCAR-3 (left) and SKOV3 cells (right). Gene expression was evaluated by RT-qPCR. (B) SAMHD1 knockdown induces apoptosis in ovarian cancer cells. Expression of apoptotic markers, cleaved PARP and cleaved Caspase 3 proteins, was measured by western blot in siRNA treated OVCAR3 (upper panel) and SKOV3 (bottom panel) cell lines. Representative western blot (left) and quantification (right) showing specific siRNA-mediated inhibition of SAMHD1 and increased cleaved PARP and cleaved caspase 3 expression. *p<0.05, **p<0.005, ***p<0.0005.

3.2 SAMHD1 modulates RLR (RIG-I like receptor) expression and innate immune signalling.

Considering the key role of SAMHD1 in the induction of IFN-mediated immune activation derived from its role in DNA damage repair (118,147), together with reported deficiencies in nucleic acid sensing and subsequent loss of innate immune activation in ovarian cancer (156), we focused our attention on innate immune response and cytosolic pattern recognition receptors (PRRs). First, we evaluated changes in the most common RNA and DNA PRR (Figure 33A). Interestingly, significant upregulation of both RNA sensors RIG-I, encoded by the *DDX58* gene and MDA5, encoded by the *IFIH1* gene was observed in siSAMHD1 cells, whereas DNA sensors cGAS (*MB21D1*) and STING (*TMEM173*) expression did not change upon SAMHD1 downregulation (Figure 34A). In addition, when ovarian cancer cells were exposed to LPS, known to recognize and activate TLR, or Poly(I:C), known to activate the cytosolic RNA helicases as RIG-I and MDA-5 (157), induction of IFN-stimulated genes (ISG) was only observed with poly(I:C) treatment, further supporting the more prominent role of RNA sensors in IFN-mediated response in ovarian cancer cells (Figure 35). Then, we evaluated a comprehensive set of 28 cytokine associated genes included in the commercial TaqMan Human Cytokine Network array, finding significant transcriptional changes associated to SAMHD1 depletion in *IFNA7*, *IFNB1*, *IL16*, *IL18*, *IL4*, *IL6*, *IL8*, *LTA* and *TNF* (Figure 34B). These findings were confirmed in independent experiments, indicating an increased IFN-induced signalling upon SAMHD1 downregulation (*IL6* p=0.0259, *IL8* p=0.0173 and *TNF* p=0.023, respectively) (Figure 34C, upper panel). Interestingly, further evaluation of additional innate immune activation pathways showed similar results, i.e., expression of IFN-stimulated genes (ISG) as *CXCL10* (p=0.0327) and *ISG15* were also upregulated in SAMHD1-depleted cells (Figure 34C, bottom panel) and SAMHD1 knockdown also induced increased protein expression of the PRR, MDA5 and RIG-I, IRF7 transcription factor and IFN-induced transmembrane protein IFITM2 as well as enhanced phosphorylation of STAT1 (Figure 34D), all suggestive of enhanced IFN-mediated inflammation.

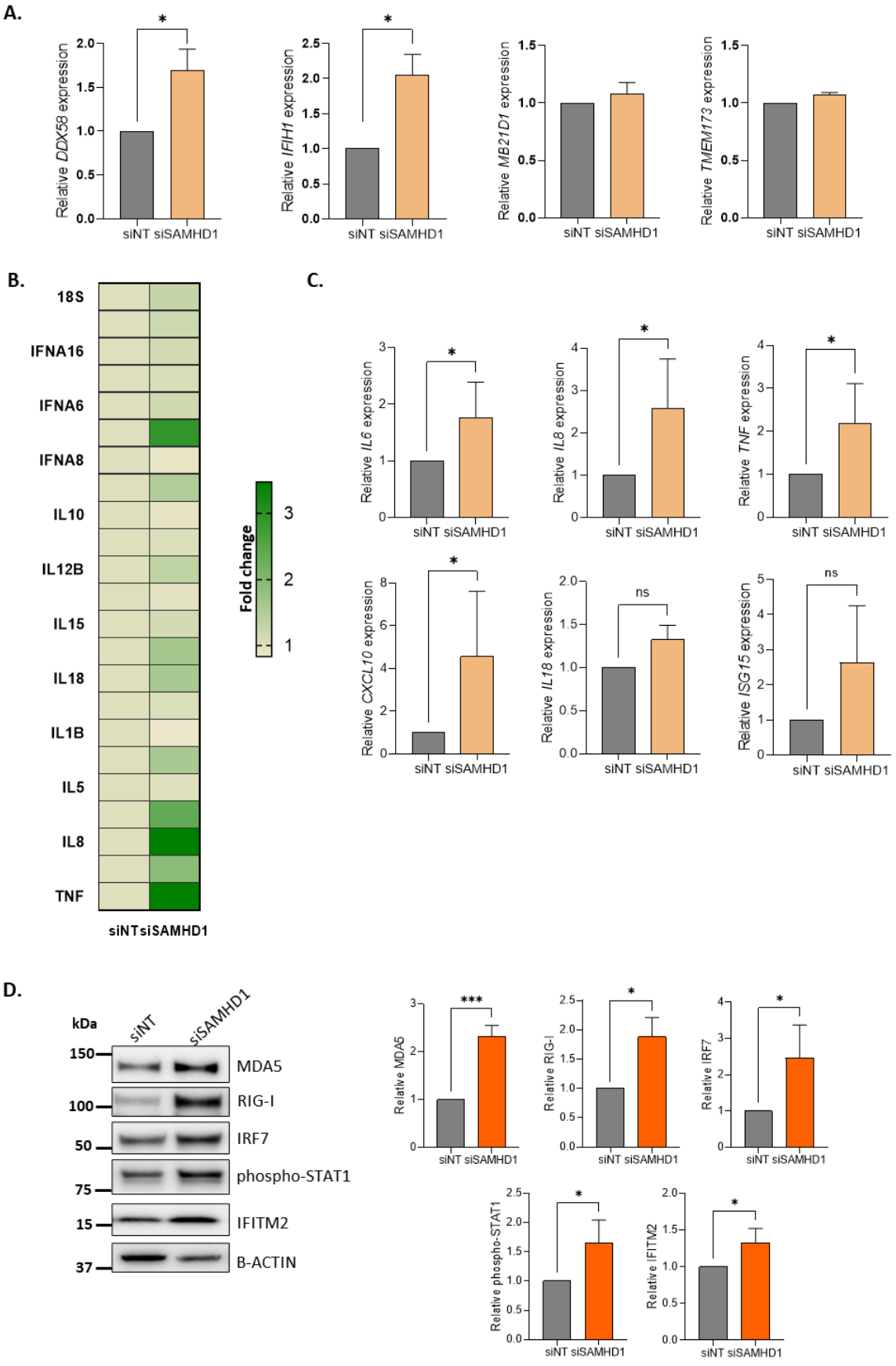

A.
Relative DDX58 expression
Relative IFIH1 expression
Relative MB21D1 expression
Relative TMEM173 expression
siNT siSAMHD1
B.
18S
IFNA16
IFNA6
IFNA8
IL10
IL12B
IL15
IL18
IL1B
IL5
IL8
TNF
siNTsiSAMHD1
Fold change
C.
Relative IL6 expression
Relative IL8 expression
Relative TNF expression
Relative CXCL10 expression
Relative IL18 expression
Relative ISG15 expression
ns
D.
kDa
siNT
siSAMHD1
150
100
50
75
15
37
MDA5
RIG-I
IRF7
phospho-STAT1
IFITM2
B-ACTIN
Relative MDA5
Relative RIG-I
Relative IRF7
Relative phospho-STAT1
Relative IFITM2

Figure 34. SAMHD1 knockdown modulates RLR (RIG-I like receptor) expression and innate immune signalling. (A) Gene expression of DNA sensors (*MB21D1* encoding cGAS protein, *TMEM173* encoding STING protein) and RNA sensors (*DDX58* encoding RIG-I protein, *IFIH1* encoding MDA5 protein), upon SAMHD1 *knock-down*. (B) SAMHD1 knockdown induces proinflammatory cytokine expression. Heatmap showing fold change increase expression in siSAMHD1 cells compared to non-targeting control, evaluated using the TaqMan Human Cytokine Network array. (C) Gene expression of distinct IFN-stimulated genes (ISG) in SAMHD1 knockdown cells. Increased *IL6*, *IL8*, *TNF*, *IL18*, *CXCL10* and *ISG15* expression upon SAMHD1 depletion was confirmed in additional experiments. (D) Representative western blot (left) and quantification (right) showing increased protein expression of distinct IFN-stimulated proteins in SAMHD1 knockdown cells. Protein expression of RNA sensors MDA5 and RIG-I, transcription factor IRF7, phosphorylation of STAT1 and IFITM2 was determined by western blot. Mean +/- SD of at least three independent experiments is shown. **p*<0.05, ****p*<0.0001. siNT, non-targeting siRNA used as control, siSAMHD1, siRNA specifically targeting SAMHD1. ns, non-significant.

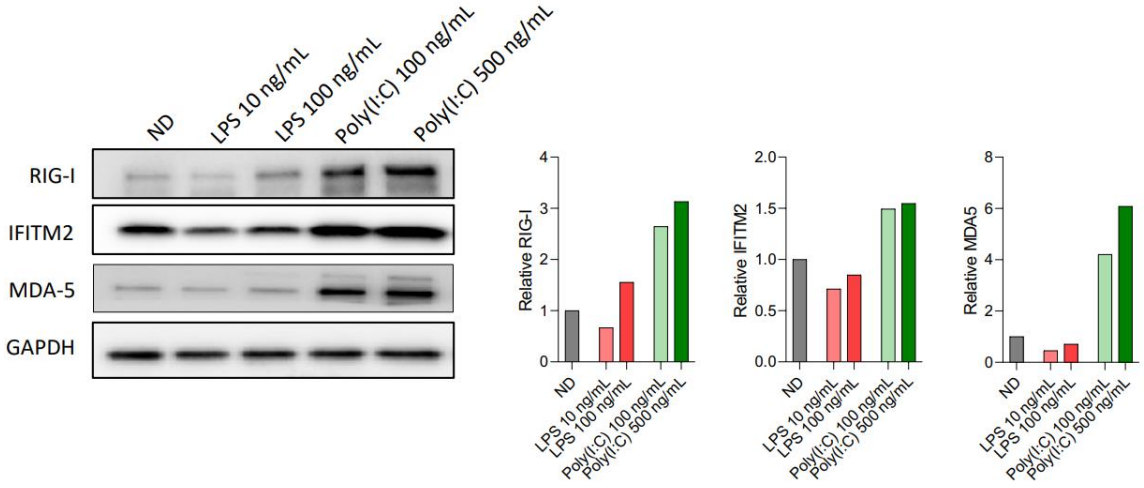


Figure 35. Expression of RNA sensors and ISG upon exposure to LPS and polyI:C in OVCAR cells. PolyI:C, but not LPS is reported to specifically activate the cytosolic RNA helicases retinoic acid-inducible protein I (RIG-I) and melanoma differentiation-associate gene 5 (MDA-5) (21). Indeed, in OVCAR cells, only polyI:C treatment, was able to induce expression of RNA sensors and IFN-stimulated genes as IFITM2, further supporting the role of RNA sensors in IFN-mediated response. A representative western blot (left) and associated quantification is shown (right).

3.3 SAMHD1 expression is a prognostic factor in ovarian cancer patients.

In view of these findings, the role of SAMHD1 was also evaluated in a cohort of 22 ovarian cancer patients. Median age at diagnosis was 63.00 years (min-max 51-82 years), the most frequent histology was high-grade serous subtype (n=17, 77.3%), and 4 patients were known to harbour germline pathologic *BRCA1/2* mutations (18.2%, all of them with high-grade serous histology). Median progression free survival (PFS) and median overall survival (OS) of the whole sample were 16.00 months (95% CI 5.66-26.34), and 66.00 months (95% CI 33.03-98.96), respectively.

SAMHD1 expression was evaluated by immunohistochemistry in ovarian cancer biopsies (Figure 36A), that were classified as SAMHD1 positive or negative depending on the percentage of

SAMHD1 stained tumoural cells [positivity was arbitrarily defined as those with cellular positivity ≥25%, based on previous reported thresholds (138)]. SAMHD1 positivity correlated with high-grade serous histology (p=0.007), but not with *BRCA1/2* status (p=0.144). More importantly, SAMHD1 expression showed a statistically significant effect on survival outcomes. Median progression free survival (PFS) of the SAMHD1-high expression subgroup was statistically significantly shorter than those of the SAMHD1-low expression subgroup (15.00 [95% CI 9.95-20.05] *vs.* 52.00 [95% CI 0.00-123.86], p=0.010) (Figure 36B, upper panel). Median overall survival (OS) of the SAMHD1-high expression subgroup was also shorter than those of the SAMHD1-low expression subgroup (62.00 [95% CI 26.83-97.17] *vs.* 157.00 [95% CI 0.00-343.66], p=0.040) (Figure 36B, bottom panel). Hazard ratio for PFS was 4.54 (95% CI 1.27-16.23, p=0.020), and hazard ratio for OS was 3.564 (95% CI 0.99-12.56, p=0.052), favouring the low-expression subgroup. These differences remained statistically significant when individually analysing the *BRCA* wild type or unknown subgroup (Figure 36B, lower panel) (Table 11).

Table 11. Baseline characteristics of the clinical sample and survival outcomes.

	Descriptive statistics (%)	**Median PFS (months, 95% CI)**	***p-value***	**Median OS (Months, 95% CI)**	***p-value***
Age at diagnosis (years)	63 (51-82 years)	NA		NA	--
Histologic subtype High grade serous Others*	 17 (77.30%) 5 (22.70%)	 16.00 (13.02-18.98) 52.00 (0.00-112.12)	0.040	 66.00 (37.59-94.40) 157.00 (0-359.77)	0.144
BRCA status Mut** Wild type/UK***	 4 (18.20%) 18 (81.80%)	 12.00 (4.16-19.84) 23.00 (6.37-39.63)	0.372	 113.00 (0-238.75) 62.00 (34.97-89.03)	0.857
SAMHD1 <25 >25	 7 (31.80%) 15 (68.20%)	 52.00 (0.00-123.86) 15.00 (9.95-20.05)	0.010	 157.00 (0.00-343.66) 62.00 (26.83-97.17)	0.040
Total	22	16.00 (5.66-26.34)		66.00 (33.03-98.96)	--

*4 clear cells and 1 low grade serous tumour, **Pathologically mutated, *** UK: unknown

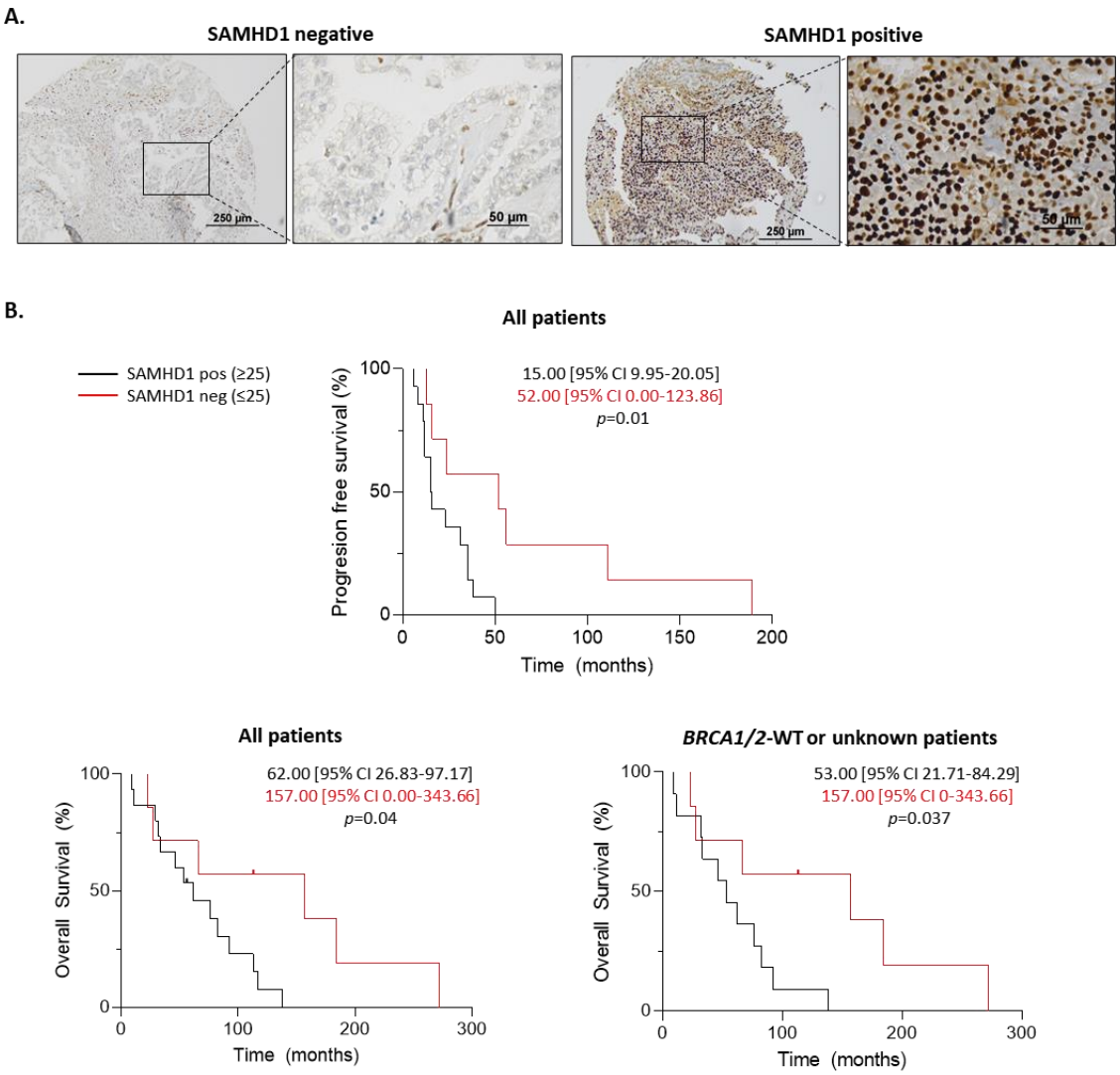


Figure 36. SAMHD1 expression is a prognostic factor in ovarian cancer patients. (A) Representative microscopy images of SAMHD1 expression in paraffin-embedded ovarian tumour biopsies. Images on the left represent SAMHD1-low expressing tumours and on the right positive expressing tumours. SAMHD1 expression was evaluated by immunohistochemistry in tumour samples. High expression of SAMHD1 observed in lymphocytic cells infiltrating in the tumours was used as a positive control of immunohistochemistry for negative or low expressing biopsies. (B) Kaplan-Meier curves of progression-free survival (PFS, top) and overall survival (OS, bottom) stratified according to SAMHD1 status, i.e, SAMHD1-low (SAMHD1 expression below 25% in cancer cells, red lines) or SAMHD1-high (SAMHD1 expression equal or above 25%, black lines). Median survival times with 95% CI of both groups are shown. Log rank test was used to test the significance and censored patients are indicated by a vertical line.

The contribution of the observed differences in PFS and OS depending on SAMHD1 expression might be partially affected by previous platinum-based chemotherapy which was common to all patient cohort. However, *in vitro* exposure to carboplatin did not induce a differential response in SAMHD1 *knock-down* cells, compared to non-targeting control (Data not shown), suggesting that SAMHD1 expression might be the main contributor to the observed correlation with low-SAMHD1 and better survival outcomes.

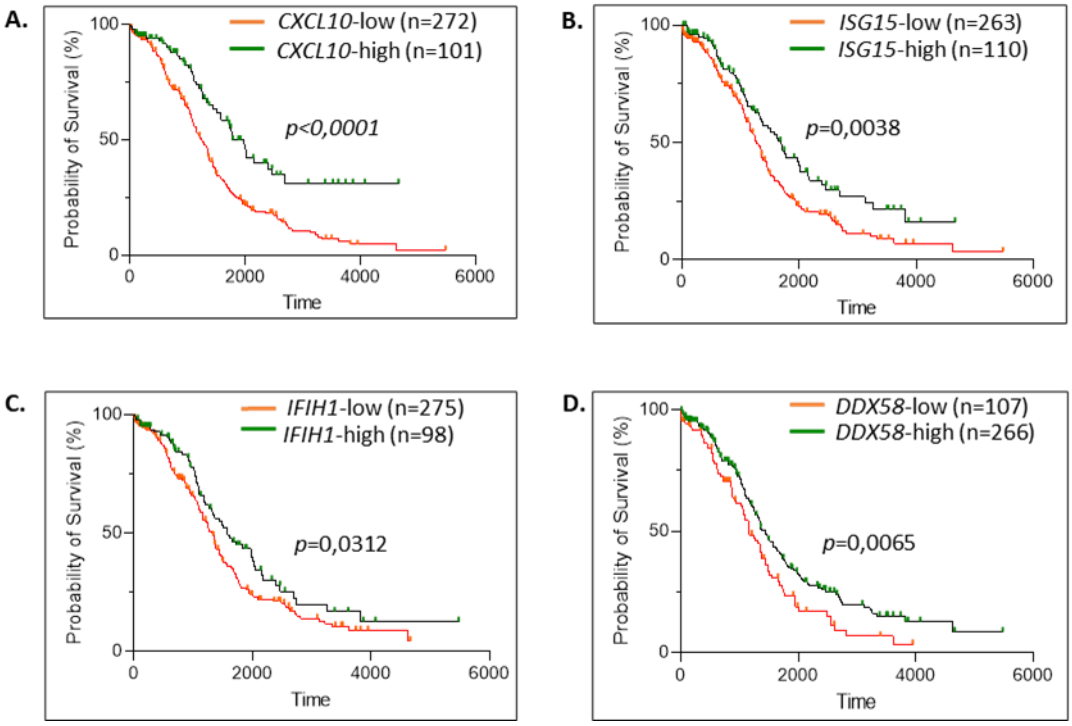


Figure 37. High expression of innate immune genes was associated with improved ovarian cancer survival. Kaplan-Meier curves of probability of survival from the human protein atlas datasets for ovarian cancer according to *CXCL10* (A), *ISG15* (B), *IFIH1* (C) and *DDX58* (D) divided by high (green) or low (orange) expression level.

Overall, clinical data allowed us to propose SAMHD1 as a prognostic marker in ovarian cancer, whose function might putatively induce antitumoural innate immune response, as demonstrated *in vitro* in cell lines. Interestingly, exploring the ovarian cancer proteome using TCGA transcriptomics data obtained from Human Protein Atlas database (www.proteinatlas.org) (158), we observed that high expression of several innate immune activation hallmark genes was associated to improved ovarian cancer survival, supporting the idea that upregulation of innate immune response is linked to better prognosis in ovarian cancer, a mechanism that is regulated by SAMHD1, as demonstrated *in vitro* (Figure 37).

DISCUSSION AND PERSPECTIVES

Cancer is a major burden of disease worldwide. According to World Health Organization (53), in 2020 there were an estimated 20 million new cases and 10 million deaths from cancer. Specifically, breast cancer accounts for 11.7% of cases, while ovarian cancer encompasses 1.6%. Although cancer incidence and mortality vary according to the tumour type, advanced-stage tumours are mostly incurable. To determine the best action for cancer patient care, an early diagnosis is essential. However, variability between and within tumours and specific characteristics of each patient significantly hampers the success of a universal approach for cancer treatment, resulting in increased importance of better-targeted therapies and paving the way towards personalized medicine. Personalized medicine aims to maximize benefit of the treatment, while minimizing harm for the patient. It takes into account therapy-induced toxicities, inter- and intra- tumour variability, and tumour-immune microenvironment of each person diagnosed with cancer (159). Due to cancer heterogeneity, although patients may be diagnosed histologically with the same cancer type, their tumours can comprise varying tumour microenvironments and molecular characteristics that can impact treatment response and prognosis. In this regard, identification of biomarkers that can predict disease evolution soon after diagnosis or guide targeted treatment is key.

Over the years, growing evidence pointed towards SAMHD1 as one of these putatively valuable biomarkers. It is clear that SAMHD1 plays multifaceted roles in cancer, immunity, cell cycle regulation, and DNA damage response, contributing to the complex interplay between these processes in cellular homeostasis and disease-associated pathogenesis. However, SAMHD1 role in solid tumours is still a matter of intense debate, as most of research has been focused on haematological cancers. In this context, additional studies are needed to further delineate how SAMHD1 function might influence onset and evolution of cancer disease and whether SAMHD1 might be able to predict clinical outcomes and in which cases (160). Consequently, the present PhD thesis focused on describing the role of SAMHD1 as a predictive and prognostic biomarker in solid tumours, while trying to elucidate the underlying mechanisms of SAMHD1 in the induction and regulation of tumourigenesis, bearing in mind its putative immunomodulatory function.

Clinical evaluation of SAMHD1 in advanced solid tumours

First of all, we studied the role of SAMHD1 in different solid tumours. Our first clinical analysis included a set of advanced cancer cases belonging to high incident and/or high mortality tumours such as NSCLC, breast, ovarian, and pancreatic cancers, all of them treated with antimetabolites, specific chemotherapeutic drugs interfering with nucleot(s)ide metabolism.

Then, we performed an extended evaluation in breast and ovarian cancer cohorts, to gain insight into SAMHD1 prognostic value.

When we first evaluated SAMHD1 expression, we found significant heterogeneity across different tumour types (from 100% positivity for rectal cancer, through 68% in ovary, 61% in breast, 50% in NSCLC and 11% in pancreatic cancer), in accordance with available data from public databases (161). In addition, we found that SAMHD1 expression was significantly associated with tumour histology and tumour grade, being poorly differentiated high-grade tumours those presenting the highest proportion of SAMHD1 positive cases. Accordingly, SAMHD1 expression was significantly associated with the cell proliferation marker Ki67 in early cases of breast cancer after NACT. These associations correlate very well with SAMHD1 dNTPase function: in those cancers where SAMHD1 is highly expressed, dNTP pool is controlled and cells grow slowly, while in cancers with low SAMHD1 expression, dNTPs are not degraded, dNTP pool is higher and therefore, cells grow faster. These results point towards a relevant role of SAMHD1 in cell proliferation as previously suggested (145), albeit contrarywise to existing evidences derived mainly from the study of haematological cancers where it was assumed that SAMHD1 depletion led to an increase in dNTP availability, thereby favouring cell proliferation (162,163). However, the reduction of dNTP levels can lead to stalling of replication forks. In this line, decreased dNTP pools have been proposed to be a source of genome instability in early stages of cancer development (164). Other studies also performed in haematological cancers, for example in classical Hodgkin lymphoma, found no association between SAMHD1 expression and histological subtype (165).

While the initial understanding and majority of research of SAMHD1 role has focused on hematological cancers through its involvement in dNTP regulation, its function in solid tumors is still unclear and an area of active ongoing research. Indeed, the clinical impact of SAMHD1 in advanced solid tumours has been evaluated in a limited number of studies. Here, we showed that SAMHD1 expression was significantly associated with poorer prognostic clinical outcomes, including disease free survival (DFS) and overall survival after cancer diagnosis (OSCD) in all tumour types analysed, and shorter progression free survival (PFS) specifically in ovarian cancer. In addition, SAMHD1 positivity was found to be an independent prognostic factor of worst DFS in breast and NSCLC cancer. Albeit similar results have also been reported by others (128,129,165,166), several studies also suggest that SAMHD1 might function as a tumour suppressor in many cancers, pointing towards an association between high expression of SAMHD1 and improved prognosis, and identifying loss-of-function mutations in SAMHD1 in patients with more aggressive disease (163,167,168).

When we compare our results of SAMHD1 as a negative prognostic factor with those already present in scientific literature, no harmonious conclusions emerged easily. In contrast to our results, data derived from TCGA or ICGC databases points towards SAMHD1 tumour promoter function in ovarian carcinoma, but not in lung and breast carcinoma. Nevertheless, our data in breast cancer is in consonance with other published data from The Cancer Genome Atlas database, where high SAMHD1 expression was associated with reduced OS in adult white cancer patients (169).

If we focus in lung cancer, conflicting results have been reported. Low expression of SAMHD1 was associated with advanced lung disease (170). Additionally, overexpression of SAMHD1 has been described to inhibit the proliferation of lung tumour cells and appeared to be downregulated by methylation in lung adenocarcinoma compared to adjacent normal tissue (168). However, in line with our results, other studies report that SAMHD1 levels increase in the serum of lung cancer patients upon progression (129). The differences in the results could be attributed to several factors: on one hand, the specific methodologies and experimental approaches used may contribute to the divergent findings. Some authors determine SAMHD1 expression in tumour samples by qPCR including both tumour cells but also other cell types from the TME that can introduce important biases in expression. In contrast, we evaluated SAMHD1 expression by immumohistohemistry exclusively in tumoural cells, representing a more accurate measure without confounding factors. Others measured SAMHD1 in the serum of lung cancer patients, impeding the direct comparison with our results. On the other hand, the specific patient populations and sample sizes analysed may also contribute to the differences in results. Variations in the genetic backgrounds, disease stages, and treatment regimens among patients not considered here can influence the prognostic significance of SAMHD1.

In breast cancer, our initial approach revealed a negative prognostic value in advanced BC. In line with this data, we also identified SAMHD1 expression as a biomarker determining prognosis in early cases of BC who did not achieve pCR after NACT, being SAMHD1 expression linked to shorter TTP and OS. Neoadjuvant chemotherapy in BC has been used to reduce tumour burden prior to surgery. However, until now, the impact on prognosis depends on the establishment of pCR. Due to the lack of validated surrogate biomarkers in patients not achieving a pCR, this constitutes an important finding and represent an opportunity to test novel adjuvant treatments enabling personalized therapy. Finally, when we investigated SAMHD1 role in ovarian cancer, we confirmed that SAMHD1 positivity was significantly associated with poorer prognostic clinical outcomes, but in addition we found no significant association between SAMHD1 and *BRCA1/2*

status, although all *BRCA1/2* mutated patients tend to show high expression of SAMHD1, suggesting a correlation between these two variables.

Moreover, our data are in accordance with other results including (i) colorectal cancer where high SAMHD1 expression level in tumours correlated to increased risk of metastases (128), (ii) untreated classical Hodgkin lymphoma, where SAMHD1 was an independent adverse prognostic factor (165), (iii) NSCLC EGFR mutated cancer patients where SAMHD1 serum levels were significantly increased when compared with normal controls upon cancer progression (129) and (iv) in mantle cell lymphoma patients treated with chemotherapy (166).

The apparent contradictory results can in part be explained by differences in methods for determining SAMHD1 status, the heterogeneity of the clinical series evaluated, and the possible different treatments that patients receive. Conversely, although our cohorts were not very homogeneous either, they were evaluated using the same criteria across tumour types and samples. Michaelis *et al*. highlight the fact that most of the available scientific articles focus on a potential role of SAMHD1 as a tumour suppressor and point towards a putative confirmation and publication bias as one of the reasons to perpetuate this idea (169). Major differences in SAMHD1 function appeared when comparing hematological cancers and solid tumours. Indeed, the initial function of SAMHD1 related to the regulation of dNTP availability was confirmed in hematological cancers, whose characteristics, behavior, progression, and treatment are very different from solid tumours. Moreover, the higher expression of SAMHD1 in blood cells supports the notion of a distinct function or impact in hematological cancers than in solid tumours (161,171).

Overall, our results, and that of others, point toward a tumour type-dependent function of SAMHD1 in cancer onset and/or progression, reflecting once more the great heterogeneity of cancer biology, which deeply challenges the drive for personalized treatment. These findings also show that our understanding of the processes underlying cancer needs to improve further.

On the other hand, the first evidence of SAMHD1 influencing cancer treatment and outcome came from its capacity to influence nucleos(t)ide analogue efficacy, pointing towards a putative predictive role. In this sense, previous studies have demonstrated that SAMHD1 also determines tumour evolution by regulating the therapeutic efficacy of nucleotide analogues used as antineoplastic agents, including cytarabine and decitabine, commonly used for AML treatment and identified as SAMHD1 substrates, i. e., SAMHD1 hydrolases the TTP of the active drug, limiting its activity (136,172). Additional evidence pointed towards a similar effect for other antiproliferative drugs such as fludarabine, decitabine, vidarabine, and clofarabine, all

considered substrates of SAMHD1 (135), or forodesine an inhibitor of dGTP synthesis (173), an important cofactor for SAMHD1 tetramerization and function. Thus, we evaluated the predictive role of SAMHD1 in the response to antimetabolite-containing treatment regimens. Antimetabolites are commonly used in chemotherapeutic regimens to target rapidly dividing cancer cells, due to their function by mimicking the structure of natural compounds involved in DNA synthesis, RNA synthesis, or other vital metabolic pathways, including dNTPs and/or its precursors, leading to impaired cell division, either by incorporation of chemically altered nucleotides or by depleting the supply of nucleotides needed for replication and cell proliferation. Examples of currently used antimetabolites are (i) nucleot(s)ide analogues (that can be purine or pyrimidine antagonists) including 5-Fluorouracil (5-FU), Capecitabine (Xeloda®), Fludarabine, Gemcitabine (Gemzar®), or Cytarabine (Ara-C®) and (ii) antifolates or folic acid antagonists, including pemetrexed or metothrexate. Soon after the first evidences of SAMHD1 triphosphohydrolase activity, its role on antiviral and anticancer nucleotide analogue efficacy was tested. Nowadays, overwhelming evidences demonstrate the key role of SAMHD1 in Ara-C efficacy both *in vitro* and *in vivo*. On one hand, SAMHD1 exhibits Ara-CTPase activity *in vitro* (174) and degradation or inactivation of SAMHD1 potentiates the cytotoxicity of Ara-C in AML cells. Moreover, SAMHD1 expression levels negatively correlate with Ara-C treatment success in individuals with AML (134,136,175). However, *in vitro* results with other antimetabolites that are structurally similar to Ara-C (e. g. Gemcitabine) yielded opposite results (174,176). In this sense, previous results from our group demonstrated that *in vitro*, SAMHD1 can either enhance or limit the efficacy of several chemotherapeutic drugs (137), allowing the classification of antimetabolites in SAMHD1 substrates, i. e., have enhanced activity in the absence of SAMHD1, or dNTP competitors, have reduced activity in the absence of SAMHD1. However, the predictive role of SAMHD1 function in cohorts of cancer patients treated with antimetabolites has not been explored before this work.

Thus, we designed a multi-cohort study to unravel SAMHD1 predictive role in distinct solid tumours treated with antimetabolites, either nuclet(s)ide analogues or antifolate drugs. Indeed, in the case of NSCLC patients treated with platinum in combination with gemcitabine or pemetrexed, or with gemcitabine in monotherapy, and ovarian cancer patients treated with cisplatin or carboplatin in combination with gemcitabine or gemcitabine in monotherapy, SAMHD1 positivity was associated with lower ORR and lower TTP, following the same trend observed for prognostic value and suggesting a role of SAMHD1 as a predictor of poorer outcome in these subsets of patients. On the contrary, for BC patients treated with capecitabine, we did not find any correlation between SAMHD1 status and ORR or TTP. Thus, although we

observe some degree of impact on predictive response, SAMHD1 tends to have a greater influence as a prognostic factor in cancer. The slight degree of impact on predictive response in some cancers but not in others could be explained by the heterogeneity among tumours and our limited sample size, that made challenging to establish definitive predictive correlations.

Overall, our data provide strong evidence of the involvement of SAMHD1 function in advanced cancer. However, the underlying mechanisms of SAMHD1 in the induction and regulation of tumourigenesis remain unknown, although it is hypothesized that SAMHD1 may mediate cell proliferation via both dNTPase-dependent or -independent mechanisms. Thus, we aimed to explore the mechanisms driving SAMHD1 function in cancer development, by modelling SAMHD1 role *in vitro*, through the generation of *knock-out* cell models. Overall, the study of SAMHD1-KO cells does not support the idea that SAMHD1 depletion provides transformed cells with a growth advantage simply due to elevated dNTP levels. From a functional point of view, the cellular functions of SAMHD1 are far beyond the regulation of intracellular dNTP pool. Several studies have reported that SAMHD1 interacts with cyclin/CDK complexes (176), USP18 and S-phase kinase-associated protein 2 (177,178), which are involved in the regulation of cell proliferation (176,178). Additionally, the role of SAMHD1 in cancer may relate to its function in DNA repair and DNA replication, as SAMHD1 is also recruited to DNA repair foci in response to DNA damage (114,115,121). Consistent with this hypothesis, we observed that SAMHD1 KO breast cancer cells present enhanced susceptibility to DNA damage leading to increased apoptosis, an effect that is significantly enhanced upon *in vitro* treatment with DNA damaging agents, such as platinum derivatives. Indeed, SAMHD1 is known to play a direct role in genome maintenance by promoting DNA end resection to facilitate DNA DSB repair by homologous recombination (114), and participates in the degradation of nascent DNA at stalk replication forks in human cell lines, allowing the forks to restart replication and promoting cell survival (115). Both shreds of evidence support our results, allowing to conclude that SAMHD1 plays a critical role in the response of cancer cells to DSB-inducing agents as platinum derivatives. In ovarian cancer cell lines, although depletion of SAMHD1 also lead to apoptosis and it was enhanced after platinum-based treatment, those differences were not statistically significant, indicating the existence of additional mechanisms. Furthermore, we show that SAMHD1 does not affect doxorubicin or paclitaxel-based treatment, as drug efficacy was similar in wild-type or SAMHD1-KO breast cancer cells and no changes in expression of DNA damage and/or apoptotic markers were observed. Herein, we aimed to explore deeply the mechanisms underlying SAMHD1 effect on breast and ovarian cancer as well as its impact in antitumour immune response, taking advantage of data highlighting the importance of SAMHD1 in regulating

immune response and inflammation. To do so, we opted to use three-dimensional (3D) breast cancer cell cultures, named spheroids, that better mimic cell-to-cell tumour interactions.

Modelling the role of SAMHD1 in cancer development *in vitro*

To date, current *in vitro* studies rely mostly on two-dimensional (2D) cell monolayers, which provide an easy-to-use, fast, and cost-effective tool. However, as argued by several authors, these 2D cell cultures do not adequately reproduce the natural three-dimensional (3D) cell microenvironment (179). Given unique features such as the existence of hypoxic areas, production of extracellular matrix, intercellular interactions, and growth factor exchange, the tumour microenvironment is particularly important in cancer research (179). In this regard, 3D tumour models should not be composed only by cancer cells that would only reproduce the complex cellular homeostasis within the tumour, but by a mix of tumour cell and non-tumoural cells that encompass better all the interactions occurred within the global tumour microenvironment. Some benefits of using spheroids in cancer research are:

1. Spheroids allow us to study cancer cells in a 3D environment that may mimic the complex structure and signalling of tumours in the body.
2. Improved drug testing: Spheroids can provide more accurate predictions of how cancer cells will respond to drugs than 2D cell culture models. This is important for identifying new drug candidates or improve the effectiveness of existing therapies.
3. Enhanced understanding of tumour biology: Spheroids are a key tool to study tumour cell behaviour, such as cell migration and invasion. Moreover, they can be used to establish cocultures with other types of relevant cells from the tumour micronenvironment, leading to a better understanding of tumour biology and the development of new treatments.
4. Personalized medicine: 3D cultures can be generated from a patient's own cancer cells, allowing for personalized drug screening and identification of optimal treatment options.

In this thesis, we generated individual spheroids in matrix- and growth factors-free medium that were cocultured with primary PBMCs, allowing a rapid, precise, and reproducible manipulation of coculture settings, including effector to target cells ratio and treatments conditions. Coculture of breast-tumour spheroid with immune cells allowed us to measure novel readouts such as immune infiltration both assessing number and type of infiltrated cells, which cannot be achieved through classical monolayer culture models.

To better elucidate the role of SAMHD1 we take advantage of both SAMHD1 KO cells and 3D spheroid model to evaluate by transcriptional profiling the putative signalling pathways involved. As expected, in SAMHD1-KO spheroids we observed a downregulation of transcription and RNA processing pathways together with changes in mRNA splicing, but more interestingly, changes in IL12 family were also observed. IL12 plays essential roles in shaping immune responses by promoting cell-mediated immunity and by regulating functions of a variety of effector cells (180,181). Therefore, we further explored the IL12 family signalling pathway and the putative immune-related function.

In our transcriptomics analysis, IL12-family signalling was mainly mediated by a series of genes involved in the regulation of the innate immune system and transcription of associated genes, including *CNN2* (Calponin), *CRLF1* (Cytokine receptor like factor 1), *TYK2*, a member of the Janus kinases (JAKs) family, and *TCP1*, a molecular chaperone that is a member of the chaperonin containing TCP1 complex (CCT or TRiC). Although we confirmed the downregulation of all these genes in additional *in vitro* experiments, only *TCP1* could be confirmed in patients. We found that TCP1-low-expressing tumours showed also low-levels of SAMHD1 expression by qRT-PCR on tumour biopsies. The chaperonin containing TCP1 complex (CCT) participates in the folding, stability, maturation, or assembly of many proteins essential for cancer cells. Different studies have previously linked expression levels of different CCT subunits in various cancers, such as CCT2 in prostate, breast and lung cancers (182,183) and CCT3 in hepatocellular carcinoma (HCC) (184). Specifically in BC, increased expression of the CCT complex has been linked to enhanced *in vitro* growth/survival (185) and worse prognosis in patients (186) and specifically, elevated expression of *TCP1* was associated with poor clinical outcomes in BC, in line with our results.

Overall, our own data confirm that SAMHD1 role in cancer may be also linked to tumoural immune function. Thus, a more profound understanding of the interplay between SAMHD1 activity and antitumoral immunity can provide valuable insights. Therefore, given the importance of interleukins and associated cytokines in cancer development and progression, we aimed to evaluate if the depletion of SAMHD1 in tumour spheroids also impairs the expression of other pro-inflammatory ILs, demonstrating that SAMHD1 depletion leads to reduced expression IL-6 and IL-8. This observation is intriguing considering that previous studies in immune cells showed increased cytokine and IFN production when SAMHD1 is depleted (187). Our findings suggest a complex and context-dependent role of SAMHD1 in modulating the expression of these inflammatory mediators.

IL-6 is a vital player involved in chronic inflammation and tumourigenesis, creating a feed-forward loop promoting inflammation (188), and allowing the breast tumour to proliferate, to

increase angiogenesis and also to inhibit apoptosis, which benefits breast cancer cell survival (189,190). In addition, IL-6 enhance monocyte infiltration at the inflammatory site, and also plays a vital role in the differentiation of different T cell subsets (188). On the other hand, IL-8 also presents a tumour-promoting role, by increasing invasiveness and metastatic potential of breast cancer cells (191). Interestingly, it has also been reported the significant potential of these ILs as a prognostic and/or predictive cancer biomarkers. Basal low levels of IL-6 and IL-8 correlate with improved OS in metastatic BC (192) and other trials have reported the individual prognostic role of IL-6 (193,194) and IL8 (195,196) in BC, observations that are in accordance with our data. i.e., SAMHD1 low expressing tumours show better survival and *in vitro* we show that the loss of SAMHD1 is linked to decreased IL production.

Furthermore, the role of cytokines, including IL-6 and IL-8, as potent chemoattractants for different immune cells is well described (197). Thus, we hypothesized that the above-described functional consequences of SAMHD1 depletion in tumour spheroids *in vitro* may also affect immune infiltration capacity, i.e., by limiting either the number or inducing a selective migration of a specific type of immune cells in tumour spheroids. Tumour-infiltrating lymphocytes (TILs) are indispensable components of the tumour microenvironment and have been used for the prediction of prognosis and treatment in cancer patients, although with inconsistent results. In some publications, tumour lymphocyte infiltration in breast cancer has no effect on prognosis or is associated with poor prognosis, although in other studies, it is considered to be a good prognostic marker (198). Increasing evidence suggests that the number of TILs are not as relevant as the specific immune phenotypes (199). Nevertheless, even the identification of specific immune populations within the tumour is throwing uncertain results. In our heterotypic model of tumour spheroids and primary PBMCs, we found that SAMHD1-KO condition was associated with less infiltration of immune cells, as should be expected by the reduced expression of ILs in tumour spheroids. We identified cells from the monocytic lineage and NK cells as those subtypes to which SAMHD1 depletion exerted a more significant effect, whereas CD4+ and CD8+ T lymphocytes did not differ. Circulating monocytes are precursors for the majority of tumour associated macrophages (TAMs), that are the most common and functionally active innate immune cells in the tumour microenvironment. TAMs positively correlated with tumour growth and metastasis in breast cancer but also in other cancers such as lung and prostate cancer (200,201). The exact mechanism of tumour promoting action of monocytes is not completely dilucidated but it has been shown that monocytes secrete CXCL7, an important immune cytokine that stimulate cancer cell migration, invasion, and metastasis, contributing therefore to the promotion of breast cancer progression (202). The NK cells are known to

interact with a variety of surface receptors on target cells. Upon activation, NK cells release cytotoxic granules to kill the target cells and inflammatory cytokines that enables a crosstalk with T-cells and dendritic cells to continue the immune response (203). However, the role of NK cells within breast cancer has not been fully elucidated and some studies suggest that NK cells are active during initial breast tumour development (204). Moreover, the ambiguous results obtained until now may be explained by the lack of identification of specific cell subsets and the general approach made. For example, CD68+ is used as a prototype macrophage marker, however, it does discriminate between M1 and M2 macrophages, which has been shown to present opposite properties (proinflammatory and anti-inflammatory, respectively) (205). Specific CD4+ and CD8+ T cell subpopulations such as Treg cells should be further evaluated.

Overall, our results indicate that SAMHD1 expression exerts a pro-tumourigenic effect in breast cancer, through a process that involves the interaction between tumours cells and TME putatively through the differential regulation of inflammatory intra-tumoural response (Figure 38).

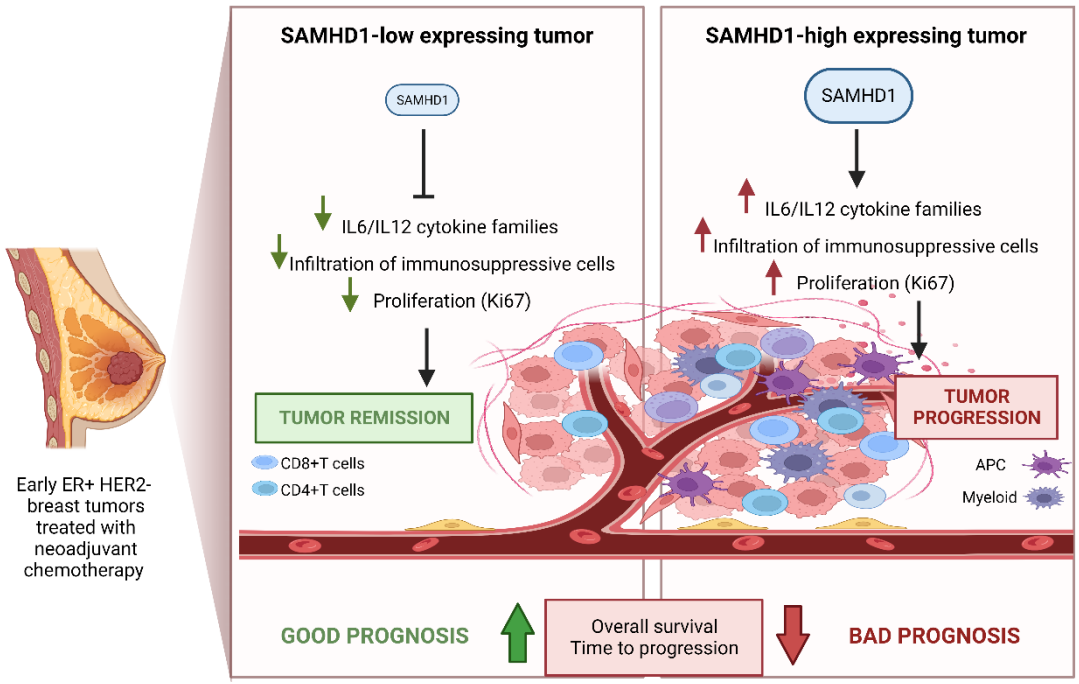


Figure 38. Model of SAMHD1 function in early ER+ HER2- breast tumours. SAMHD1-low expressing tumours present better prognosis due to decreased cytokines and less infiltration of immunosuppressive cells. On the contrary, SAMHD1-high expressing tumours present worse prognosis. Figure created with BioRender.

Based on the results in breast cancer, we then aimed to provide evidence of the involvement of SAMHD1 in the induction and modulation of anti-tumoural immunity in ovarian cancer. Although inflammation and cancer onset and progression are closely interrelated, in ovarian tissue, inflammation is a double-edge sword that has been associated with either tumour

progression or suppression (206,207), highlighting the importance of characterizing specific inflammatory pathways. Several evidence indicate the capability of tumour cells to generate inflammatory factors, representing key signals that determine the crosstalk between tumour and immune cells and ultimately affect the mechanisms of immunosuppression by which tumour cells circumvent innate and adaptive immune responses (208). In our study, we show that depletion of SAMHD1 in ovarian cancer cells leads to upregulation of RNA helicases and several IFN-stimulated genes, as cytokines and chemokines, suggesting an activation of innate immune signalling pathways that could trigger an inflammatory response in the tumour site (Figure 39) and may ultimately affect patient prognosis. The evaluation of innate immune activation pathways revealed an increased IFN-induced signalling upon SAMHD1 downregulation, concomitant with an upregulation of MDA5 and RIG-I RNA sensors. Interestingly, RIG-I expression has already been associated with OC outcome, through the induction of local immunosuppression in the tumour bed (91). Moreover, therapeutic targeting of RLRs have also been proposed as inducers of anticancer immunity or to sensitize "immune-cold" tumours to immune checkpoint blockade, by their capacity to not only recognize RNA intermediates from viruses and bacteria, but also interact with endogenous RNA such as the mislocalized mitochondrial RNA, the aberrantly reactivated repetitive or transposable elements in the human genome (209). Indeed, in line with this observations and in agreement with our data, SAMHD1-deficiency and subsequent accumulation of endogenous RNA substrates is a cause of type I interferonopathies, characterized by an upregulation of distinct IFN-stimulated genes (ISGs) (156,210), overall pointing towards SAMHD1 as an interesting therapeutic target.

To a large extent, we demonstrated the dual consequence of SAMHD1 depletion in innate immune response depending on cancer type. In ovarian cancer cells, SAMHD1 depletion led to increased immune activation, while in breast cancer cells, downregulation of SAMHD1 reduces innate immune activation. However, in both cancer types, reduction of SAMHD1 showed an anti-tumoural effect. The reason for the different effects of SAMHD1 depletion on innate immune activation in ovarian cancer cells compared to breast cancer cells is not entirely clear and may involve complex interactions between the tumour microenvironment, the immune system, and SAMHD1-regulated pathways.

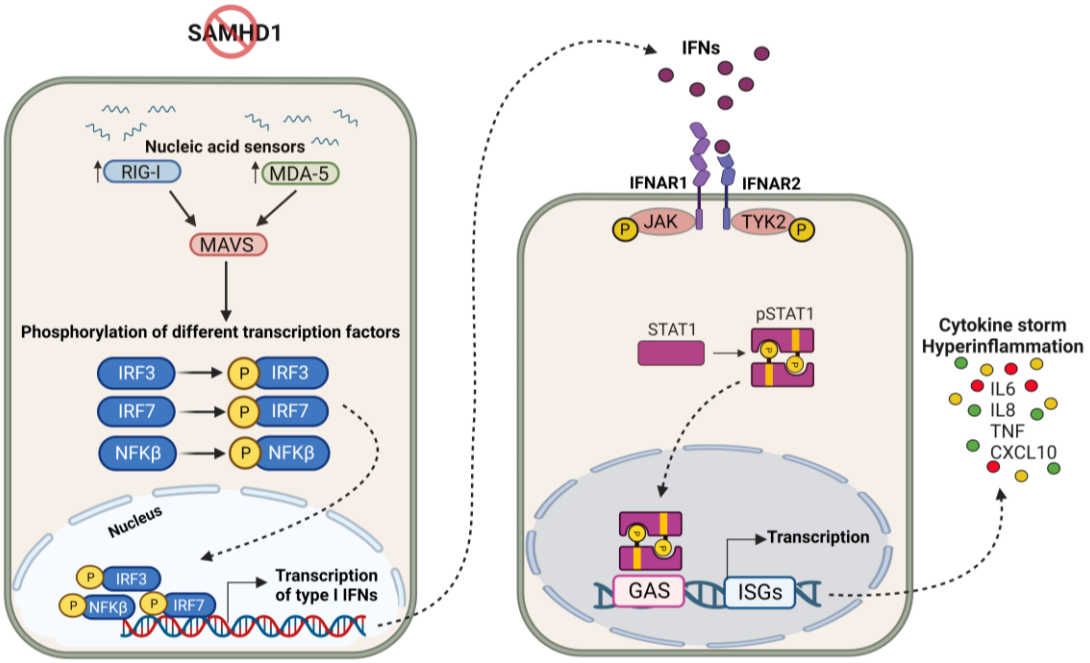


Figure 39. Working model of the innate immune signalling pathways triggered by the absence or the low expression of SAMHD1 in ovarian cancer. Low SAMHD1 expression induces increased levels of distinct IFN-stimulated genes in ovarian cancer cells, subsequently leading to increased antitumoural immunity and better prognosis in patients. Abbreviations are as follows: MDA5, melanoma differentiation-associated protein 5, RIG-I, retinoic acid-inducible gene-I, IFN, interferon, IFNAR, interferon a/b receptor 1, JAK, Janus kinase 1, TYK2, tyrosine kinase 2, STAT, signal transducer and activator of transcription, P, phosphoryl group, ISG, interferon-stimulated genes, IL6, Interleukin 6, IL8, Interleukin 8, TNF, Tumour Necrosis Factor, CXCL10, C-X-C motif chemokine ligand 10. Created with BioRender.

Due to its role in Aicardi–Goutières syndrome, where SAMHD1 depletion leads to excessive interferon (IFN) production, we may expect that SAMHD1 depletion induces an innate immune response, characterized by increased IL production, as seen in ovarian cancer cells, that induces an inflammatory environment. As chronic inflammation is implicated in tumourigenesis and tumour progression, it is important to take into consideration that this inflammatory state observed in ovarian cancer cells upon SAMHD1 depletion is transient and do not have a negative clinical impact. Moreover, SAMHD1 depletion has been found to activate the innate immune response by triggering the release of cytosolic DNA into the cytoplasm, which then activates the cGAS-STING pathway, resulting in the production of type I interferons (IFNs) and other pro-inflammatory cytokines (115,211). Specifically in ovarian cancer cells, SAMHD1 depletion induces a potent innate immune response, leading to increased expression of interferon-stimulated genes (ISGs) and pro-inflammatory cytokines. This could be in part explained to the high sensitivity of ovarian cancer cells to cytosolic DNA sensing and the cGAS-STING pathway, as reported elsewhere (156). However, in our case, in addition to the observed increased production of IFN and other cytokines, we also demonstrate an upregulation of the RNA-sensors,

RIG-I and MDA-5, instead of DNA-sensors cGAS and STING. In addition, in viral infections, where SAMHD1 role has been more extensively described, SAMHD1 has been suggested to down-regulate IFN and inflammatory responses by inhibiting nuclear factor-κB (NF-κB) activation and type I interferon (IFN-I) induction, as demonstrated in human monocytic cells or primary macrophages (187). These results are in accordance with our proposed working model for SAMHD1 in ovarian cancer.

On the other hand, in breast cancer cells, we found that SAMHD1 depletion reduces the innate immune response, leading to decreased expression of ISGs and pro-inflammatory cytokines. One possible explanation for this effect is that breast cancer cells may have developed mechanisms to evade or suppress innate immune activation, and that SAMHD1 plays a role in maintaining this immune evasion, although further investigations are needed in order to better explore this possibility.

Overall, the results suggest that the function of SAMHD1 in cancer cells is context-dependent and may vary depending on the specific cancer type and cell line. These different functions of SAMHD1 in innate immunity can be attributed to the cell-specific expression and regulation of SAMHD1 and its interacting proteins, as well as the activation of different signalling pathways in each cell type. For example, in ovarian cancer cell lines we have demonstrated that SAMHD1 regulates the RIG-I/MDA5 signalling pathway while in breast cancer, other receptors and signalling networks may be activated upon SAMHD1. Based on this, further experiments to characterise the complete pathway are needed.

Pharmacological modulation of SAMHD1

Based on both the clinical importance of SAMHD1 and the detailed knowledge of its functions and regulation mechanisms, SAMHD1 may become an interesting target for cancer treatment. Blocking SAMHD1 could have several potential benefits for cancer development and disease outcome. First, SAMHD1 inhibition may increase the sensitivity of cancer cells to chemotherapy. SAMHD1 depletion can enhance the cytotoxic effects of chemotherapy in some cancer cell lines, potentially improving treatment outcomes for patients. For example in AML, where SAMHD1 limits the efficacy of Ara-C, inhibitors of SAMHD1 will improve treatment efficacy (174). Second, SAMHD1 inhibition may affect anti-tumour immunity by regulating the production of different cytokines that could promote or inhibit tumour growth. This could be particularly beneficial for patients with immunologically "cold" tumours, which are typically unresponsive to immunotherapy. Third, SAMHD1 inhibition may have synergistic effects with other targeted therapies. For example, SAMHD1 depletion can enhance the cytotoxic effects of DNA-damaging

agents. As SAMHD1 plays a key role in DNA DSB repair by homologous recombination, depletion of SAMHD1 can disrupt DNA repair mechanisms (114). Therefore, in the absence of SAMHD1, the impaired DNA repair capacity leads to the accumulation of unrepaired DNA lesions, which may increase the cytotoxicity of DNA-damaging agents. Finally, although it is clear that SAMHD1 is present at the crossroads of several cellular processes, its involvement, clinical outcomes and potential clinical applications are not completely deciphered.

Biologically, HIV-2 and other simian viral strains such as SIVsm and SIVmac counteract SAMHD1 by the accessory protein Vpx, that targets SAMHD1 for proteosomal degradation by recruiting it to the E3 ligase complex (212,213). Herold *et al.* demonstrated *ex vivo* that cells in which SAMHD1 expression was transiently reduced by treatment with Vpx were dramatically more sensitive to ara-C-induced cytotoxicity (134). Thus, Vpx has become a valuable tool for research, although some challenges, mainly related with its delivery and target specificity, remain to be addressed (135).

Moreover, other studies have explored direct pharmacological inhibition of the dNTPase hydrolase activity of SAMHD1. Small molecules structurally similar to canonical dNTPs were designed to target SAMHD1 in a competitive manner, that will ultimately have the same effect as inhibit SAMHD1 directly (214). For example, ribonucleotide reductase (RNR) inhibitors reduced SAMHD1 ara-CTPase activity, thus enhancing cytarabine efficacy (174). In this context, although inhibition of SAMHD1 has been achieved, all molecules with apparent inhibitory activities presented some limitations, such as poor bioavailability, and no further studies have been performed (215,216). Current efforts targeting SAMHD1 function are moving towards the dNTP pool balance as it has been suggested that the intracellular nucleotide pool can be modified to suppress the dNTPase activity of SAMHD1 in various AML models (174). In fact, inhibitors of the key nucleotide biosynthetic enzyme ribonucleotide reductase (RNR) are currently being evaluated in a clinical trial (217).

Furthermore, as SAMHD1 presents a phospho-dependent regulation (it is active when dephosphorylated and inactive when phosphorylated), different pharmacologic agents aim to modify this (de)phosphorylation process, leading to SAMHD1 activation or inactivation. Different pharmacological agents block SAMHD1 phosphorylation, inducing SAMHD1 function and viral restriction. For example, the antiviral activity of the CDK4/6i palbociclib was originally demonstrated to be dependent on SAMHD1. Palbociclib blocks HIV-1 reverse transcription through the inhibition of CDK2-dependent SAMHD1 phosphorylation in human myeloid and lymphoid cells (218–220). Topoisomerase inhibitors, such as etoposide (ETO), regulate SAMHD1

function and show potent *in vitro* anti-HIV-1 activity (221). SAMHD1 phosphorylation is controlled by cyclin-dependent kinases (CDK), specially CDK2 and CDK6, and it is tightly regulated during cell cycle (220,222,223). A panel of FDA-approved tyrosine kinase inhibitors (TKIs) used in clinic to treat chronic myeloid leukemia (CML) are shown to potently induce SAMHD1 dephosphorylation, leading to its activation. Among them, dasatinib was determined to be the most potent and safe TKI that prevents SAMHD1 phosphorylation *in vitro* and *ex vivo*, preserving the antiviral function of SAMHD1 (224,225). In addition, SAMHD1 activation could also be modulated by CDK inhibitors (CDKi). It has been shown that selective CDK4/6 inhibitors, e. g. palbociclib, are pharmacological activators of SAMHD1 that act by inhibiting its inactivation by phosphorylation (137,218). As previously demonstrated in our group, pharmacological activation of SAMHD1 by CDK4/6i significantly enhanced the antiviral activity of several antimetabolites, such as pemetrexed. As those metabolites are also used as chemotherapeutic drugs, SAMHD1 activation may enhance efficacy of cancer treatments (137), further pointing towards the further study SAMHD1 as a therapeutic target.

Future perspectives

Medicine is moving towards a personalized treatment care for each patient. Specifically in the context of cancer, efforts are especially important due to the high variability among patients and cancer types. New tools to guide individualized treatment selection may include patient-derived spheroids to test drug panels of chemotherapy drugs to assess the viability and the sensitivity of drugs of each patient-derived spheroid. Indeed, several groups have already demonstrated the feasibility of this approach (226,227). In addition, a deeper understanding the immune system and how it interacts with different cancer types is useful for designing treatments to utilize the immune system and microenvironment to treat cancer. It can also enable engineering more predictive models for a better understanding of the cancer biology, as well as a more accurate prognosis and better treatment options for cancer patients.

By using our matrix-free medium technique to develop spheroids, we would be able to develop patient-derived organoids directly from patient tumours with the aim to use them as preclinical models to study tumour biology and test drug efficacy. Organoids derived from patients maintain the histological and genetic features of the original tumour and can be used to personalize cancer therapy by testing drug sensitivity and resistance in a patient-specific manner. This allows for more individualized treatment plans and can potentially improve patient outcomes.

On the other hand, several promising strategies are currently being evaluated, such as immunotherapy and targeted therapies, whose success is linked to a more precise knowledge

of the tumour molecular and genomic characteristics together with the characterization of the immune infiltration landscape. In this context, SAMHD1 may become an interesting target for cancer treatment due to its diverse functions in DNA damage response, cell cycle regulation, and innate immunity. However, it should be noted that SAMHD1 inhibition may not be effective or beneficial in all types of cancer. As mentioned earlier, the function of SAMHD1 in cancer cells is context-dependent and may vary depending on the specific cancer type and cell line. Therefore, personalized treatment approaches that take into account the individual patient's tumour biology and response to treatment may be necessary to maximize the potential benefits of SAMHD1 inhibition.

In conclusion, SAMHD1 inhibition represents a promising therapeutic strategy for cancer. However, further research is needed to fully understand the mechanisms of SAMHD1 to fully understand its role in cancer and to optimize treatment strategies. Additionally, potential side effects and long-term effects of SAMHD1 inhibition need to be carefully evaluated.

CONCLUSIONS

1. SAMHD1 is differentially expressed in all cancer types tested and its expression levels in tumour biopsies is correlated with tumour grade and cell proliferation markers as ki67.

2. SAMHD1 high expression tumors present lower disease-free survival (DFS) and overall survival after cancer diagnosis (OSCD) in colorectal, ovarian, non-small cell lung, breast, and pancreatic cancers, suggesting that SAMHD1 is a strong negative prognostic factor.

3. The predictive value of SAMHD1 expression in response to antimetabolite and platinum-based treatment regimens was only confirmed in NSCLC and ovarian cancer, where SAMHD1 positivity was associated with lower ORR and shorter TTP. On the contrary, no correlation was found between SAMHD1 status and treatment response in breast cancer patients receiving capecitabine.

4. *In vitro*, SAMHD1 depletion does not impair cell growth but leads to increased DNA damage in response to platinum-based chemotherapeutic agents. These data support a dNTP-independent role for SAMHD1 linked to differential susceptibility to DNA damage-induced cytotoxicity in solid tumors.

5. SAMHD1 expression does not influence 3D spheroid formation and growth of breast cancer *in vitro*, but results in differential expression of IL-mediated signaling pathways, subsequently affecting the number and type of tumour-infiltrated immune cells in a heterotypic tumor-immune cell culture system.

6. Depletion of SAMHD1 in ovarian cancer cells leads to upregulation of RIG-I-like receptors RNA helicases and IFN-stimulated genes, pointing towards a tumour-dependent role of SAMHD1 in solid tumours.

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