Quantification of Exosomal Survivin in the Tumour Microenvironment with Enzyme-Linked Immunosorbent Assay (ELISA)

Wynne Wijaya

Exeter College

A thesis submitted for the degree of *MSc by Research*

Funded by the Jardine Foundation

Department of Oncology

University of Oxford

Hilary 2025

Table of Contents

Abstract

Exosomes facilitate intercellular communication within the tumour microenvironment (TME), affecting cancer progression and immune interactions. Survivin, an inhibitor of apoptosis (IAP), is highly expressed in cancer and linked to unfavourable clinicopathologic parameters. Survivin has been shown to be released via exosomes by cancer cells, making serum survivin levels insufficient to reflect its true amount. While exosomal survivin can be measured with Western blot and flow cytometry, these methods are unsuitable for high-throughput clinical use. Moreover, practical consensus on exosome isolation remains lacking. This study aimed to develop a method to quantify exosomal survivin in biological samples using ELISA, combining polyethylene glycol (PEG) precipitation to concentrate exosomes and a non-ionic detergent-based lysis of exosomal membranes. We optimised ELISA to detect survivin in cancer biological samples, including cancer cell culture conditioned media and patient sera. Colorectal carcinoma (HCT116) and breast adenocarcinoma (MDA-MB-231) cell lines were used to generate conditioned media, and serum samples were collected from 15 cancer patients (ovarian and prostate) and 5 healthy controls. Two methods of exosome concentration were performed and compared i.e., centrifugal filtering and PEG precipitation. 1% NP-40 was identified as the optimal detergent for exosome lysis. ELISA revealed significantly higher survivin levels in cancer biological samples compared to controls. In serum, survivin detection was significantly improved after PEG precipitation and NP-40 treatment. Results were validated by Western blot. This study demonstrates a practical ELISA-based method for quantifying exosomal survivin by combining PEG precipitation with NP-40 lysis.

This method provides a potential clinical applications for exosomal biomarkers, particularly but not limited to survivin, in cancer diagnostics and monitoring.

Disclaimer

I confirm that the work presented in this thesis is entirely my own work, with the exception of Nanoparticle Tracking Analysis results which was conducted by Dr Helen Sheldon, a post-doctoral researcher from the Buffa's lab and production of survivin standard was conducted by Dr Kory Wang from the Centre for Medicines Discovery, University of Oxford for Sichen Liu, a DPhil student in the Jiang group, who passed it down to me.

List of Figures

List of Tables

List of Abbreviations

APS – ammonium persulfate

BCA – bicinchoninic acid

BIR – baculoviral IAP repeat

BIRC – baculoviral IAP repeat-containing protein

BSA – bovine serum albumin

CCC – circulating cancer cell

CDK4 – cyclin-dependent kinase 4

CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CM – conditioned medium/media

CMC – critical micelle concentration

CPC – chromosomal passenger complex

DMEM – Dulbecco's Modified Eagle Medium

DNA – deoxyribonucleic acid

ECL – enhanced chemiluminescence

EGFR – epidermal growth factor receptor

ELISA – enzyme-linked immunosorbent assay

EMT – epithelial mesenchymal transition

ESCRT – endosomal sorting complex required for transport

EV – extracellular vesicle

FBS – fetal bovine serum

GBM – glioblastoma

GTEx – Genotype-Tissue Expression

HBIXP – hepatitis B X-linked interacting protein

HIF – hypoxia-inducible factor

HRP – horse radish peroxidase

Hsp – heat shock protein

HUVEC – human umbilical vein endothelial cell

IAP – inhibitor of apoptosis protein

IEF – isoelectric focusing

ILV – intraluminal vesicle

INCENP – inner centromere protein

IP – immunoprecipitation

miRNA – microRNA

MVB – multivesicular body

NP-40 – Nonidet P-40

NTA – nanoparticle tracking analysis

OD – optical density

PBS – phosphate buffered saline

PCa – prostate cancer

PD-L1 – programmed cell death ligand 1

PDAC – pancreatic ductal adenocarcinoma

PEG – polyethylene glycol

PFS – progression-free survival

PS – phosphatidylserine

RNA – ribonucleic acid

SNARE – soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SOD1 – superoxide dismutase type 1

TCGA – The Cancer Genome Atlas

TEMED – N,N,N′,N′-tetramethylethylenediamine

TGF-β – transforming growth factor beta

TMB – tetramethylbenzidine

TME – tumour microenvironment

TRAIL – tumour necrosis factor-related apoptosis-inducing ligand

TX-100 – Triton X-100

VEGF – vascular endothelial growth factor

WHO – World Health Organization

XIAP – X-linked inhibitor of apoptosis protein

I. Introduction

I.1 Resistance to apoptosis as a hallmark of cancer

Apoptosis or programmed cell death is a pathway of cell death in which cells activate enzymes that degrade the cells' own nuclear DNA and nuclear and cytoplasmic proteins. Apoptosis might occur in both physiological and pathologic situations. In pathologic conditions, it serves to eliminate cells that are genetically altered or injured beyond repair and does so without triggering a severe host reaction, thereby keeping the extent of tissue damage to a minimum (Kumar, Abbas and Aster, 2017). Dysregulation of apoptosis results in numerous diseases such as cancer, autoimmune and immunodeficiency diseases, and neurodegenerative disorders (Su *et al.*, 2015).

To date, two apoptotic pathways have been identified, namely intrinsic and extrinsic pathways. These pathways trigger apoptosis through the activation of caspases, which comprise two types: initiator caspases (caspase-2, 8, 9, and 10) and effector caspases (caspase-3, 6, and 7) (Troy and Jean, 2015). Initiator caspases principally process and activate pro-forms of effector caspases in response to different signals. Meanwhile, effector caspases cleave intracellular protein substrates to prompt the apoptotic process (McIlwain, Berger and Mak, 2013).

Resistance to apoptosis is one of the hallmarks of cancer and represents a major obstacle to successful cancer treatment. Various mechanisms are involved in the dysregulation of apoptotic pathways, including loss of tumour suppressor genes, increased expression of antiapoptotic regulators or survival signals, and downregulation of proapoptotic factors (Hanahan and Weinberg, 2011). Dysregulated apoptotic

processes are associated with tumour formation, progression, and resistance to anti-cancer therapies (Fulda, 2009; Rebucci and Michiels, 2013).

I.2 Inihibitor of apoptosis (IAP) proteins

Aberrant expression of IAP proteins is one of the mechanisms contributing to the resistance to apoptosis in human cancers. The IAP family is a group of apoptosis negative regulators characterized by the presence of at least one copy of the baculovirus IAP repeats (BIR) domain (containing 70 amino acids) at their N-terminus. The human IAP family consists of eight members: NAIP/BIRC1, cIAP1/BIRC2, cIAP2/BIRC3, XIAP/BIRC4, Survivin/BIRC5, BRUCE/Apollon/BIRC6, ML-IAP/Livin/BIRC7 and ILP-2/BIRC8 (**Figure 1**) (Silke and Vaux, 2001). IAPs block both intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptotic processes by directly binding to caspases. The initiator caspases of the intrinsic and extrinsic pathways are caspase-9 and -8, respectively (Brady *et al.*, 2015). BIRC1 (NAIP) inhibits apoptosis by binding and suppressing caspase-3 and caspase-7, although its anti-apoptotic role is secondary to its immune functions. BIRC2 (cIAP1) and BIRC3 (cIAP2) prevent death receptor–mediated apoptosis by ubiquitinating key signalling intermediates, thereby blocking caspase-8 activation. BIRC4 (XIAP) is the most potent direct caspase inhibitor, binding and suppressing executioner caspases-3 and -7 as well as initiator caspase-9, thus regulating both intrinsic and extrinsic pathways. BIRC5 (survivin) indirectly inhibits caspases by stabilising XIAP and forms part of complexes that prevent caspase activation, while also coordinating cell division. BIRC6 (BRUCE/apollon) suppresses apoptosis by ubiquitinating and promoting the degradation of active caspases-3 and -7. BIRC7 (Livin)

and BIRC8 (ILP-2) inhibit caspases-3, -7, and -9, although they are less well characterised. Beyond apoptosis regulation, several IAPs, particularly NAIP, cIAP1, cIAP2, and XIAP, also modulate cytokine signalling through pathways such as NF-κB activation, inflammasome assembly, and NOD2 signalling, thereby linking cell death control with immune and inflammatory responses (Marivin *et al.*, 2012).

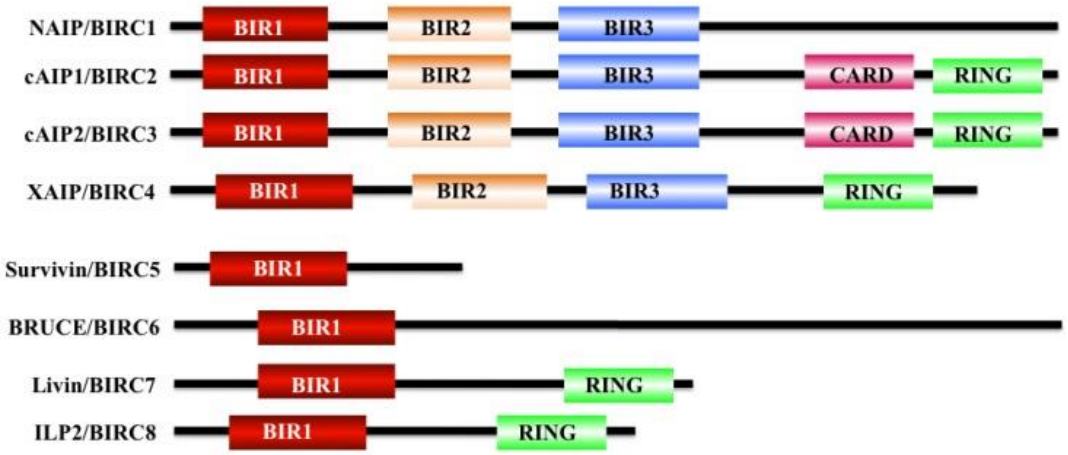

Figure 1. Structures of IAP proteins in mammals (Chen *et al.*, 2016).

I.3 Survivin

I.3.1 Structure and functions

Survivin is the smallest member of the IAP protein family containing only one of the characteristic N-terminal baculovirus IAP repeats (BIR) domains (Ambrosini, Adida and Altieri, 1997; Salvesen and Duckett, 2002). Wild-type human survivin gene, *BIRC5*, spans 14.7 kilobases at the telomeric end of chromosome 17 and consists of 4 exons and 3 introns. Human survivin is composed of 142 amino acids and has a molecular weight of 16.5 kDa. Unlike other IAP proteins, survivin is expressed during embryonic and fetal development and is undetectable in normal adult tissues (Ambrosini, Adida and Altieri, 1997).

Survivin contains two functional domains, a baculoviral IAP repeat (BIR) at the N-terminus (amino acids 18-88) and α-helix at the C-terminus (amino acids 98-142) (**Figure 2**) (Sah and Seniya, 2015; Wheatley and Altieri, 2019). The N-terminal BIR domain contains a zinc-finger motif that stabilises its fold. Structurally, it consists of four α-helices and a three-stranded β-sheet typical of IAP proteins. Functionally, the BIR domain mediates survivin's anti-aopototic function by binding to and stabilising XIAP/BIRC4, sequestering pro-apoptotic factors like Smac/DIABLO, and inhibiting caspase activity. The C-terminal α-helix forms an elongated amphipathic helix that interacts with Borealin and INCENP to form the triple-helical bundle of the chromosomal passenger complex (CPC), a structure essential for accurate chromosome segregation and cytokinesis during mitosis. This region also contains microtubule-binding sites and contributed to survivin's dimerisation (Mace, Shirley and Day, 2010).

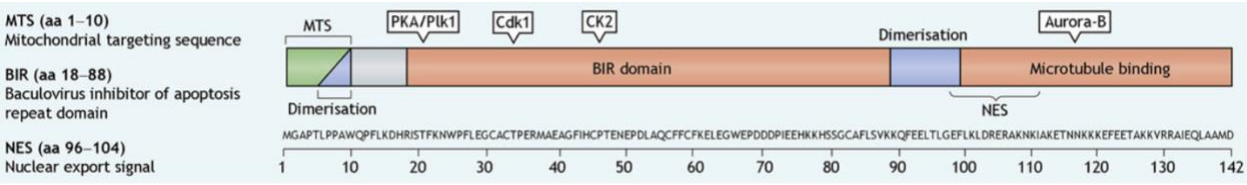

Figure 2. Structure and functions of survivin (Wheatley and Altieri, 2019).

Survivin gene locus also encodes 5 other genetic splice variants with unique properties and functions: survivin-2α, survivin-3α, survivin-ΔEx3, survivin-2β, and survivin-3β (**Figure 3**). Survivin-2α is generated by the inclusion of a short intronic sequence after exon 2, leading to a truncated protein lacking the α-helical C-terminus. This isoform localises predominantly in the nucleus and has been shown to attenuate the anti-apoptotic activity of the wild-type survivin (Caldas, Honsey and Altura, 2005).

Survivin-2β contains an additional 23 amino acids from a retained intron between exons 2 and 3 (Exon 2B), which alters the BIR domain and is thought to antagonise the anti-apoptotic function of wild-type survivin, acting in a pro-apoptotic or dominant-negative manner in some contexts; however, it has also exhibited pro-survival and anti-apoptotic functions in others (Li, 2005). Survivin-ΔEx3 lacks exon 3, resulting in a C-terminal truncation that disrupts the α-helical domain and impairs CPC incorporation, but it retains anti-apoptotic activity and is often upregulated in cancer (Waligórska-Stachura *et al.*, 2014). Survivin-3α and survivin-3β arise from alternative splicing at the 5′ end, producing N-terminally extended proteins whose precise functions are less well defined but may include altered subcellular localisation and binding partner specificity (Pavlidou, Kroupis and Dimas, 2014).

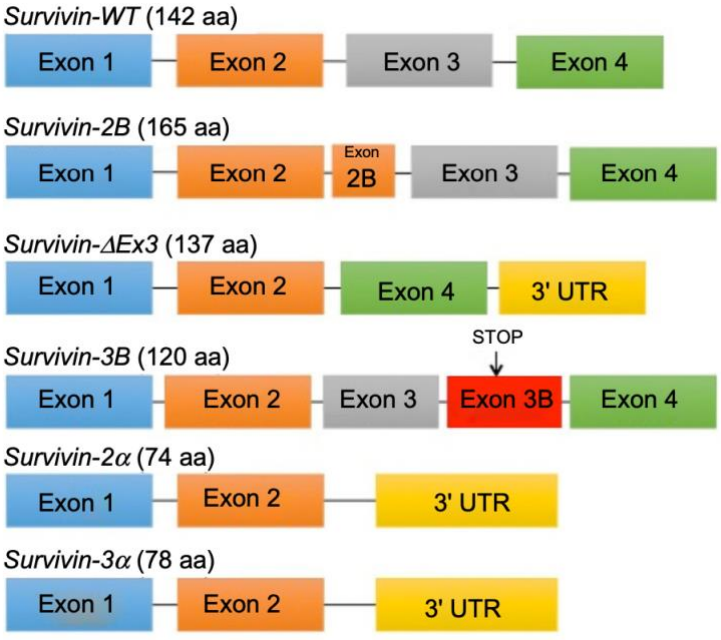

Figure 3. Survivin splice variants (Khan *et al.*, 2015).

Survivin plays essential roles in the regulation of mitosis, inhibition of apoptosis, and angiogenesis (**Figure 4**) (Altieri, 2003). Survivin is a component of the chromosomal CPC and interacts with Borealin, the Aurora B kinase and the inner centromere protein (INCENP) to carry out its roles in cell division (Jeyaprakash *et al.*, 2007). Survivin has been suggested to inhibit cell death induced by extrinsic and intrinsic apoptotic pathways and causes resistance to apoptosis by directly suppressing caspase activity (Song, Yao and Wu, 2003). Survivin prevents and regulates caspase-9 activation by its interaction with hepatitis B X-linked interacting protein (HBXIP) and X-linked IAP (XIAP). Similarly, the anti-apoptotic effects of survivin are also regulated by the pro-apoptotic Smac molecule, which is released from mitochondria as cells undergo apoptosis. Smac promotes apoptosis by binding to the baculoviral IAP repeat (BIR) domains of IAP family members, particularly XIAP, thereby displacing bound caspases and relieving IAP-mediated caspase inhibition. Although survivin does not directly bind caspases, it stabilises and cooperates with XIAP to prolong its inhibitory effects on caspase activity. Smac can indirectly antagonise survivin's anti-apoptotic function by disrupting the survivin-XIAP complex, reducing the stability of XIAP, and allowing caspase activation to proceed (Du *et al.*, 2000). Survivin has also been shown to induce tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and BCL2-associated X, apoptosis regulator (Bax) (Li, Hu and Li, 2018). In addition, survivin can correspondingly accelerate cellular mobility via activating AKT and increasing the expression of α5 integrin (Pavlidou, Kroupis and Dimas, 2014). In angiogenesis, survivin is expressed in vascular endothelial cells during neovascularisation and promotes endothelial cell proliferation, migration, and

survival, via the activation of vascular endothelial growth factor receptor (VEGFR) and angiopoietin-1/Tie-2 (Pavlidou, Kroupis and Dimas, 2014; Shakeel *et al.*, 2017).

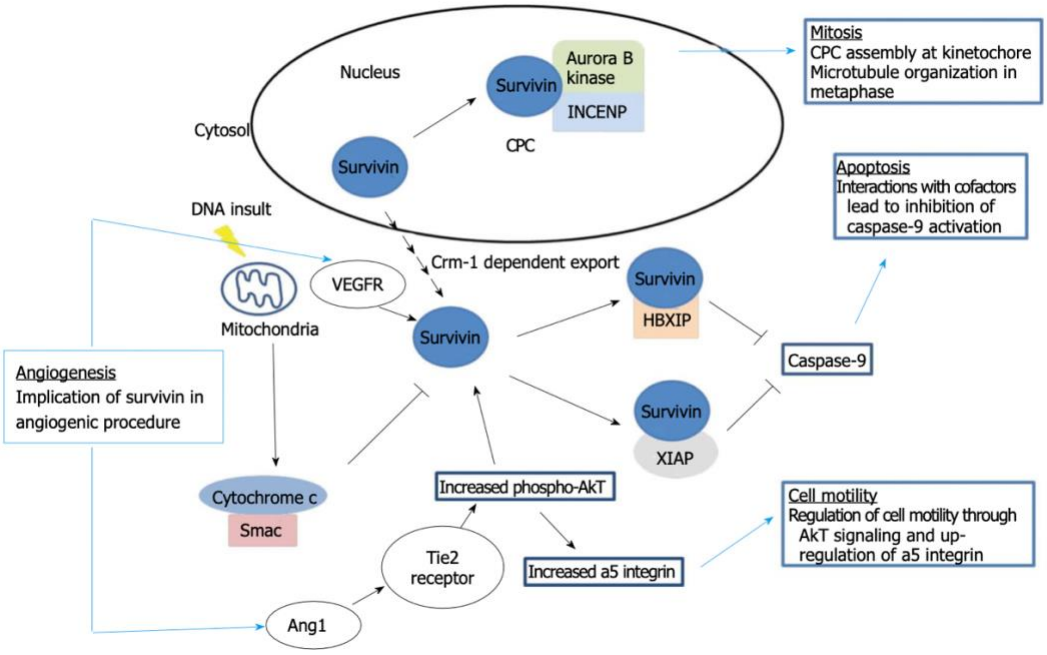

Figure 4. Survivin physiological functions (Pavlidou, Kroupis and Dimas, 2014).

In normal physiology, survivin expression is almost undetectable in most differentiated adult tissues but is abundant during embryonic and foetal development, where rapid cell proliferation occurs. In adults, survivin is re-expressed under conditions requiring high proliferative activity, including wound healing (Shojaei-Ghahrizjani *et al.*, 2020) and in certain proliferative organs or cells such as the basal layer of the skin, colonic crypt epithelium (Martini *et al.*, 2016), and hematopoietic progenitors (Small *et al.*, 2010).

I.3.2 The role of survivin in cancer

Survivin is involved in tumorigenesis through a variety of mechanisms: inhibition of apoptosis pathways, regulation of cytokinesis and cell cycle progression, and participation

in numerous pathways, including p53, Wnt, hypoxia, TGF-β, and Notch signalling pathways (**Figure 5**) (Chen *et al.*, 2016).

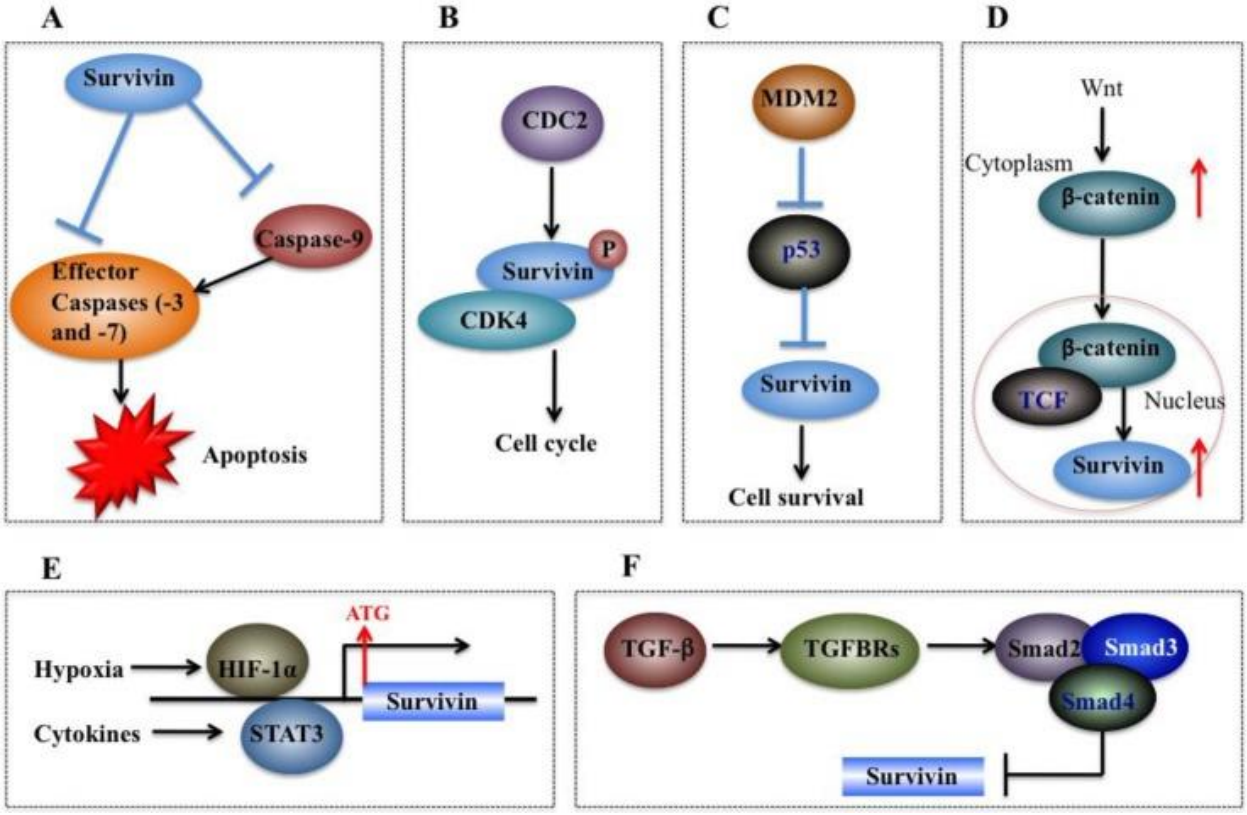

Figure 5. Survivin mechanisms of action in tumorigenesis.

(A) Binding and suppression of effector caspases (caspase-3 and 7) and caspase-9. (B) Phosphorylation by CDC2 and interaction with CDK4 resulting in nuclear translocation and cell cycle progression. (C) Survivin suppression by p53 at the transcriptional level. (D) Activation of Wnt signalling pathway. (E) HIF-1α and STAT3 directly binding to survivin promoter. (F) Downregulation of survivin by TGF-β signalling pathway (Chen *et al.*, 2016).

Survivin is found to be highly expressed in cancer cells relative to normal and terminally differentiated cells (Ambrosini, Adida and Altieri, 1997; Salvesen and Duckett,

2002). Overexpression of survivin has been reported in almost all human cancers, including breast, lung, colon, pancreatic, prostate, bladder, gastric, oesophageal, , hepatocellular, ovarian, cervical cancer, melanoma, diffuse large B-cell lymphoma, and acute myeloid leukaemia (Fukuda and Pelus, 2006; Huang *et al.*, 2015; Zhang *et al.*, 2015; Jiang *et al.*, 2018; Samarkos *et al.*, 2018; Shao *et al.*, 2018; Ye *et al.*, 2019; M. Brown *et al.*, 2020). It has been clinically correlated with tumour progression, resistance to therapy, and worse prognosis (Jaiswal, Goel and Mittal, 2015; Garg *et al.*, 2016). Microarray studies have described that molecular profiles with higher survivin expression are associated with aggressive and invasive tumour behaviour, inadequate response to treatment, poor prognosis, higher relapse rate, and short survival (Li, Aljahdali and Ling, 2019; Frazzi, 2021). Consequently, survivin is potentially valuable as a molecular marker for diagnosis and prognosis as well as a therapeutic target of cancer (Garg *et al.*, 2016; Li *et al.*, 2021).

I.3.3 Survivin as a cancer biomarker

Numerous clinical studies have shown that increased levels of survivin in tumour tissues and bodily fluids, such as blood and urine, can help distinguish malignant from benign conditions and are associated with poor prognosis and unfavourable clinicopathologic parameters (Weston, Glantz and Connor, 2011; Garg *et al.*, 2016; Zhou, Hu and Xiao, 2021). However, some studies demonstrated no prognostic or diagnostic significance of survivin mRNA (Warnecke-Eberz *et al.*, 2008), cytoplasmic expression (Atikcan *et al.*, 2006), and serum levels (Jakubowska *et al.*, 2016) (**Table 1**).

Table 1. Summaries of studies evaluating survivin as a cancer biomarker.

Cancer Type	First Author, Year	Sample	Patients No. and Conditions	Assay	Conclusions
General/ mixed types of cancer	Gunaldi, 2018	Blood	Breast cancer (49.3%), colon cancer (25.4%), ovarian cancer (14.9%), and others (10.4%)	ELISA	Serum levels were significantly higher in cancer patients than healthy subjects. High survivin levels indicate a 4-fold increased risk of cancer.
	Yie, 2008	CCCs in peripheral blood	55 gastric cancer patients, 86 colorectal cancer patients	RT-PCR ELISA	Detection of mRNA in CCCs accurately identifies gastric and colorectal cancer patients at high risk of relapse.
Lung cancer	Puskas, 2021	Blood	118 advanced-stage lung cancer patients before and after chemotherapy	ELISA	Serum levels positively correlate with metastasis and the number of organ sites, but do not predict survival.
	El-Magd, 2012	Pleural fluid	65 specimens	RT-PCR	mRNA expression differentiates benign from malignant pleural effusion.
	Warnecke-Eberz, 2008	Tissue	64 NSCLC patients (stages I-IIIA, R0 resections)	RT-PCR	mRNA overexpression may aid in non-invasive tumour detection but has no prognostic importance.
	Atikcan, 2006	Tissue	58 NSCLC patients	IHC	Nuclear expression predicts prognosis,

					while cytoplasmic expression is not significant.
	Lu, 2004	Tissue	48 NSCLC patients	IHC	Nuclear staining correlates with worse survival, suggesting its role as a biomarker.
Colon cancer	Jakubowska, 2016	Tissue and serum	55 CRC patients	IHC for tissue, ELISA for serum	Tissue expression has diagnostic potential, but serum levels are diagnostically ineffective.
	Hernandez, 2011	Tissue	168 primary CRC patients	RT-PCR, IHC	mRNA upregulation correlates with higher tumour grade and apoptosis-related genes, providing potential therapeutic targets.
	Shen, 2008	CCCs in peripheral blood	156 CRC patients	qRT-PCR	Immunomagnetic bead enrichment combined with qRT-PCR detects circulating CRC cells sensitively and non-invasively.
	Chen, 2004	Tissue	52 CRC patients	RT-PCR, IHC	Expression is significantly higher in CRC tissues and is associated with apoptosis inhibition and proliferative activity.
Pancreatic cancer	Demirci, 2023	Serum	41 newly diagnosed metastatic pancreatic cancer patients	ELISA	High baseline serum levels are linked to treatment resistance and poor prognosis.

	Vay, 2022	Tissue	236 PDAC patients	IHC	Expression correlates with advanced disease stages and poor prognosis.
	Dong, 2015	Tissue and serum	80 PDAC patients	IHC for tissue, ELISA for serum	High serum levels and tissue expression predict poor outcomes in PDAC patients.
	Ekeblad, 2012	Tissue	111 pancreatic endocrine tumour patients	IHC	High nuclear expression is a marker for poor prognosis.
	Lee, 2005	Tissue	49 pancreatic cancer patients	IHC	Tissue expression correlates with venous and perineural invasion and may predict chemotherapy response.
	Qiao, 2004	Tissue	42 pancreatic cancer patients, 10 normal pancreas	IHC	Overexpression correlates with tumour progression and Bcl-2 expression, serving as a prognostic marker.
	Kami, 2004	Tissue	52 PDAC patients	IHC	Tissue expression may act as a useful prognostic marker in pancreatic cancer.
Breast cancer	Khan, 2014	Serum and tissue	40 (serum), 23 (tissue)	ELISA for serum, IHC for tissue	Serum levels were significantly higher in breast cancer samples. Exosomal survivin variants (Survivin-2B) show potential as diagnostic/prognostic markers.
Prostate cancer	Hennigs, 2020	Tissue	900 prostate cancer samples	IHC	Tissue expression increases in metastatic

					prostate cancer. Cytoplasmic, but not nuclear, survivin links to aggressiveness.
	Eslami, 2016	Tissue	94 prostate adenocarcinoma samples	IHC	High expression correlates with higher Gleason scores.
	Khan, 2012	Plasma	39 samples	ELISA	Tumour-derived serum levels are significantly elevated compared to benign prostatic hyperplasia (BPH) and controls.
	Zhang, 2009	Tissue	456 locally advanced prostate cancer patients	IHC	Nuclear overexpression associates with better survival, while cytoplasmic survivin links to local progression.
Bladder cancer	Zhou, 2024 (SRMA)	Tissue	2285 bladder cancer patients (40 studies)	IHC, ELISA, RT-PCR	mRNA shows greater diagnostic significance than protein.
Glioblastoma	Tong, 2019	Tissue	144 patients	RNA sequencing	mRNA expression negatively correlates with overall survival in glioblastoma patients.
	Zhang, 2018 (SRMA)	Tissue	1260 patients from 16 studies	IHC, RT-PCR, Western blot	Higher expression is associated with worse overall survival in glioma patients.

I.4 Exosomes in cancer

I.4.1 Biogenesis and role of exosomes

Extracellular vesicles (EVs) encompass a heterogeneous group of membrane-bound structures, including exosomes, microvesicles, and apoptotic bodies, which can be distinguished by their size, origin, and molecular composition (**Table 2**, **Figure 6**). Exosomes, the smallest type of EVs, typically range from 30 to 200 nm in size. They are secreted by various cell types and have attracted significant attention in recent years due to their various biological functions and promising clinical application potential. They are enriched in tetraspanin markers (CD63, CD9, and CD81) and generated within the endosomal system through the formation of multivesicular bodies. Their biogenesis can proceed via ESCRT-dependent and ESCRT-independent mechanisms, which will be explained further in the upcoming paragraphs. Microvesicles are larger EVs (100-1000 nm) that bud directly from the plasma membrane through outward blebbing, and frequently express markers such as Annexin A1 and ARF6. Their release is Ca2+-dependent and often triggered by specific cellular stimuli, making their cargo content highly context-dependent. Apoptotic bodies are the largest EV subtype (1000-2000 nm), produced during the late stages of apoptosis via plasma membrane blebbing, and typically contain cellular fragments, including organelles and chromatin. They are characterised by the externalisation of phosphatidylserine (PS), detectable by Annexin V binding, and other apoptosis-related markers. Together, these vesicle types differ not only in size and composition but also in their formation pathways, which influence their biological roles and diagnostic potential. Similar to other extracellular vesicles, exosomes

carry a variety of cargo, including lipids, nucleic acids, and proteins (Colombo, Raposo and Théry, 2014; Yáñez-Mó *et al.*, 2015; Kalluri and LeBleu, 2020).

Table 2. Extracellular vesicle categories (Hu *et al.*, 2020; Sheta *et al.*, 2023).

Categories	**Size**	**Markers**	**Formation**	**Pathways**
Exosomes	30-200 nm	CD63, CD9, CD81	Fusion of multivesicular bodies with the plasma membrane	ESCRT-dependent ESCRT-independent: tetraspanin, lipids
Microvesicles	100-1000 nm	Annexin A1, ARF6	Outward blebbing of the plasma membrane	Ca^{2+}-dependent Stimuli- and cell-dependent
Apoptotic bodies	1-2 μm	Annexin V, PS	Plasma membrane budding of apoptotic cells	Apoptosis-related

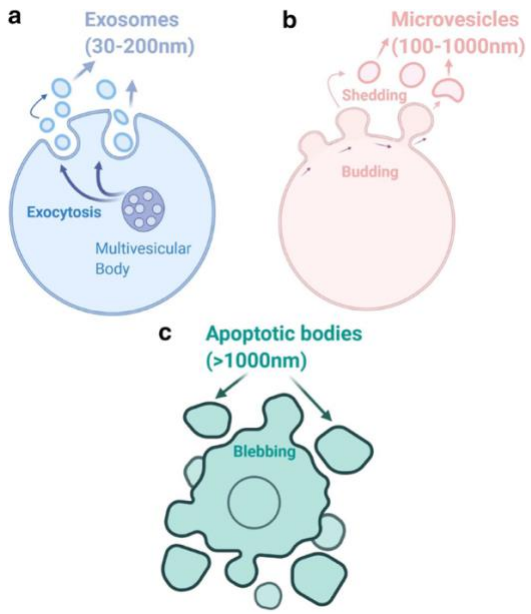

Figure 6. Extracellular vesicles (Exosomes) categories:

(a) exosomes, (b) microvesicles, and (c) apoptotic bodies (Gurung *et al.*, 2021).

The biogenesis of exosomes (**Figure 7**) is a highly regulated intricate process involving several cellular pathways (Kalluri and LeBleu, 2020). It occurs via the endosomal pathway, which starts with the cargo internalization by the invagination of plasma membrane (endocytosis). These internalized cargoes are sorted to form early endosomes, which seubsequently mature into late endosomes, or also known as multivesicular bodies (MVBs), through the inward budding of the endosomal membrane. This process leads to the accumulation of intraluminal vesicles (ILVs) – small vesicles within the lumen of MVBs (Colombo, Raposo and Théry, 2014). ILVs carry a range of molecules, including proteins, lipids, nucleic acids, and other biomolecules, that reflect the content of the originating cell. These cargoes are delivered from the trans-Golgi network and the cytosol (Stoorvogel *et al.*, 1991; Grant and Donaldson, 2009; Abels and Breakefield, 2016).

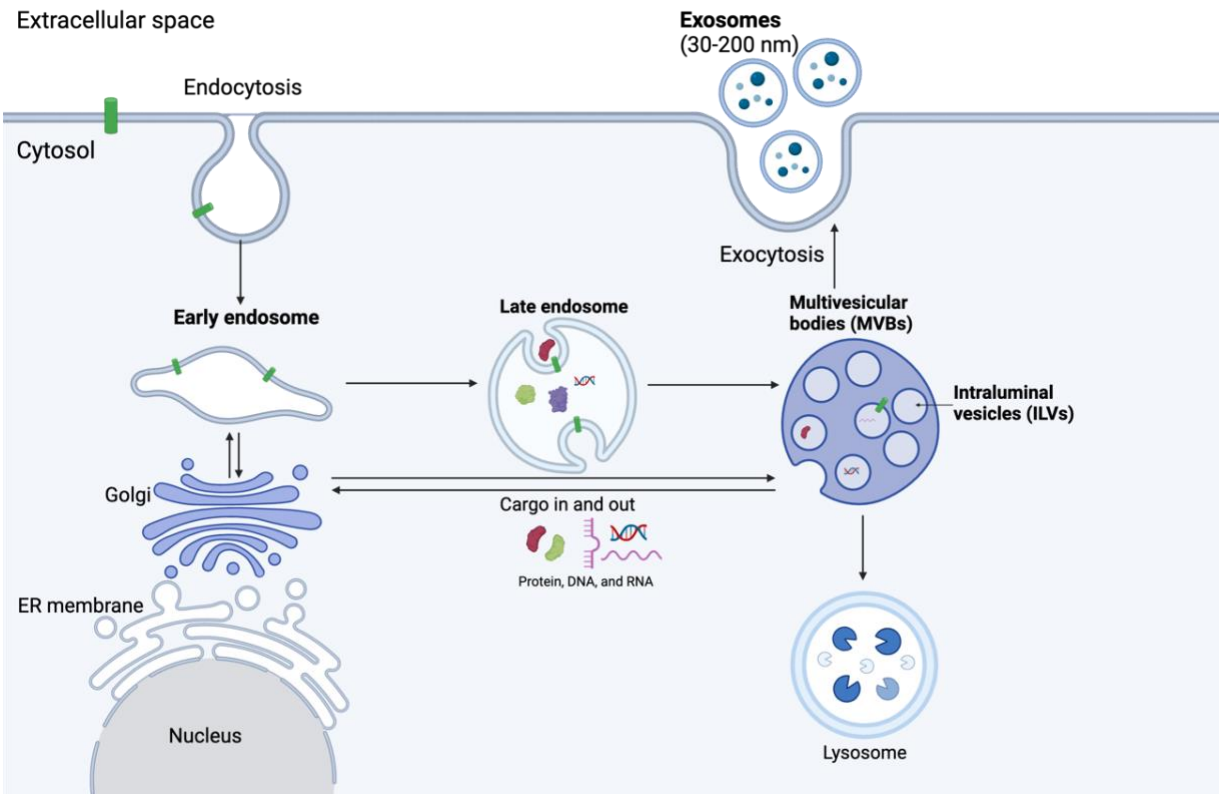

Figure 7. Exosome biogenesis

(created with BioRender.com)

The sorting of cargo into the ILVs during exosome biogenesis is regulated by several molecular mechanisms, with the endosomal sorting complexes required for transport (ESCRT) machinery playing the primary role (Raiborg and Stenmark, 2009). This machinery consists of four protein complexes (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) that work sequentially to identify and sort ubiquitinated cargo proteins into ILVs. Initially, ubiquitinated proteins destined for packaging in exosomes are recognized and gathered into endosomal membranes. This process begins with ESCRT-0 (hepatocyte growth factor-regulated tyrosine kinase substrate [Hrs] and signal transducing adaptor molecule [STAM]), which recognise and cluster ubiquitinated cargo. ESCRT-I and ESCRT-II complexes are then recruited to further concentrate the cargo

and initiate the membrane budding which forms vesicle precursors. Finally, ESCRT-III is recruited to the budding site, where it creates filaments that constrict the neck of the emerging vesicle, leading to its separation from the endosomal membrane (Wollert and Hurley, 2010; Hurley, 2015).

In addition to the ESCRT-dependent pathways, several ESCRT-independent mechanisms have been implicated in cargo sorting and ILV formation. Certain complex lipids, such as ceramide, can accumulate in localised regions of the membrane, leading to the formation of lipid rafts. These rafts are more ordered and tightly packed than the surrounding membrane, leading to inward budding and vesicle formation (Trajkovic *et al.*, 2008). Tetraspanins (CD63, CD81, CD9) can induce membrane curvature through clustering, contributing to vesicle formation as well (Andreu and Yáñez-Mó, 2014). Additional ESCRT-independent pathways include syndecan-syntenin-ALIX pathway (Baietti *et al.*, 2012) and accumulation of sphingolipids (Kajimoto *et al.*, 2013).

Following their formation, MVBs can take one of the two primary routes: fusion with lysosomes for degradation or with the plasma membrane for exosome release. The trafficking and fusion of MVBs are precisely regulated by a number of molecular mechanisms, including Rab GTPases (such as Rab27a and Rab27b), soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), and lipid signalling pathways. Upon fusion with the plasma membrane, MVBs release their ILVs into the extracellular space as exosomes (Ostrowski *et al.*, 2010).

Exosome release is influenced by both physiological and pathological conditions. This regulation is crucial to maintain cellular homeostasis and facilitate intercellular communication. However, dysregulation of this process can contribute to disease

development and progression, including cancer, neurodegenerative diseases, and inflammatory conditions. Exosome release is often upregulated under stress-related conditions, such as hypoxia (King, Michael and Gleadle, 2012), acidic microenvironmental pH (Parolini *et al.*, 2009), and cellular senescence (Lehmann *et al.*, 2008). In cancer, tumour cells exploit exosome release for intercellular communication.

Various factors such as cellular stress, inflammation, and oncogenic transformation can influence exosome release and its cargo composition. Recent studies have revealed that intercellular communication plays an essential role in regulating exosome release, suggesting that exosomes may serve as important mediators of cell-to-cell signalling under both normal and pathological conditions. In cancer, tumour cells actively release exosomes to promote tumour growth, angiogenesis, and metastasis. The altered microenvironment and oncogenic signalling in tumour cells significantly increase exosome secretion (Melo *et al.*, 2014). A more detailed discussion on the role of exosomes in cancer will follow in the next section. In neurodegenerative diseases like Alzheimer's and Parkinson's, dysregulated release of exosome contributes to the spread of neurotoxic proteins, such as amyloid-β and α-synuclein, accelerating disease progression (Saman *et al.*, 2012). Similarly, in inflammatory conditions, exosome release is enhanced and they serve as carriers of inflammatory cytokines and mediators (Buzas *et al.*, 2014).

I.4.2 The role of exosomes in cancer and the tumour microenvironment (TME)

Exosomes play a crucial role in intercellular communication, including interactions between cancer cells and non-cancerous cells within the tumour microenvironment (TME).

Exosomes transport a cargo of proteins, lipids, and nucleic acids that are capable of altering signalling pathways and cellular behaviours both locally or at distant sites, resulting in drug resistance and cancer metastasis (**Figure 8**) (Dai *et al.*, 2020).

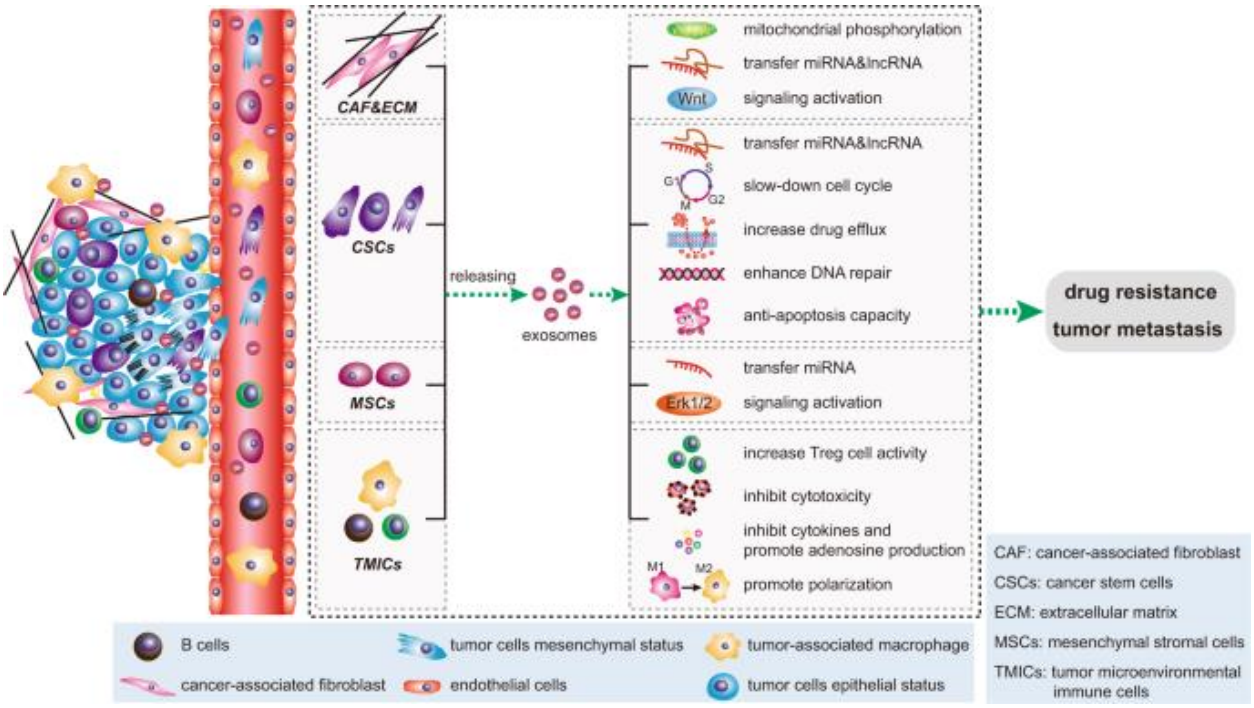

Figure 8. Signal transduction pathway of exosome in the tumour microenvironment (Dai *et al.*, 2020).

One of the key roles of exosomes in cancer is to modulate the TME to favour tumour progression. Cancer-derived exosomes can reprogram stromal cells, such as fibroblasts and endothelial cells, to support tumour growth and angiogenesis. For example, exosomes released by A431 epidermoid carcinoma cells were found to contain the epidermal growth factor receptor (EGFR). When human umbilical vein endothelial cells (HUVECs) were treated with these exosomes, the EGFR was transported to the recipient cells and stimulated signalling pathways that increased VEGF production and promoted angiogenesis (Al-Nedawi *et al.*, 2009).

Cancer-derived exosomes also contribute to immune evasion. They carry immunosuppressive molecules that inhibit the activity of T cells and NK cells, and promote the differentiation of regulatory T cells (Treg) and myeloid-derived suppressor cells, further suppressing anti-tumour immunity (Chang, Cerione and Antonyak, 2021). PD-L1 has been demonstrated to be secreted in tumour-derived exosomes, suppressing T cell activation in draining lymph nodes. Exosomal PD-L1 has also been shown to resist blockade by anti-PD-L1 antibody, whereas its suppression inhibits tumour growth, even in models resistant to anti-PD-L1 antibodies (Poggio *et al.*, 2019). This property of exosomes not only supports tumour growth but also poses a major obstacle to effective immunotherapy.

Exosomes also have potential implications in therapeutic resistance. They can transfer drug resistance molecules from drug-resistant cancer cells to sensitive ones. Examples include P-glycoprotein, an efflux pump associated with multidrug resistance, and specific microRNAs (miRNAs) that can supress the expression of drug targets or activate survival pathways. Furthermore, cancer cell-derived exosomes can sequester chemotherapeutic agents, reducing the effective drug concentration available to target cells (Musi and Bongiovanni, 2023). Thus, exosomes do not only facilitate the spread of resistance among cancer cells but also significantly hinder the success of cancer treatment.

I.5 Exosomal survivin in cancer

I.5.1 Cancer cells release survivin via exosomes

Survivin has been shown to localise within various intracellular compartments i.e., mitochondria, cytosol, and nucleus, where it regulates apoptosis and mitosis (**Figure 2**) (Fortugno *et al.*, 2002; Dohi *et al.*, 2004; Li *et al.*, 2021). Recently, survivin has also been detected in the extracellular space, encapsulated within extracellular vesicles, secreted by various cancer cell lines (Skog *et al.*, 2008; Hong *et al.*, 2009; Khan *et al.*, 2009, 2011; Hurwitz *et al.*, 2016; Li *et al.*, 2020; Chang *et al.*, 2021; Zhang *et al.*, 2023; Vesiclepedia, 2024) (**Figure 9**, **Table 3**). However, mechanisms by which survivin is released via exosomes have not been fully understood. Evidence suggests that survivin may associate with heat shock proteins (HSP, including Hsp70 and Hsp90, in the conditioned medium of serum-starved HeLa cells (Khan *et al.*, 2011), indicating a stress-induced release mechanism. Exosomal survivin has been found to be enriched in breast cancer cells treated with paclitaxel and these exosomes significantly enhance cell survival and chemoresistance (Kreger *et al.*, 2016). Additionally, sublethal proton irradiation (3 Gy) resulted in a significant accumulation of survivin in the exosomal fraction taken from conditioned medium of HeLa cells 24 hours post irradiation (Khan *et al.*, 2011). These findings suggest that exosomal survivin release is stress-responsive, though the specific pathways involved remain to be elucidated.

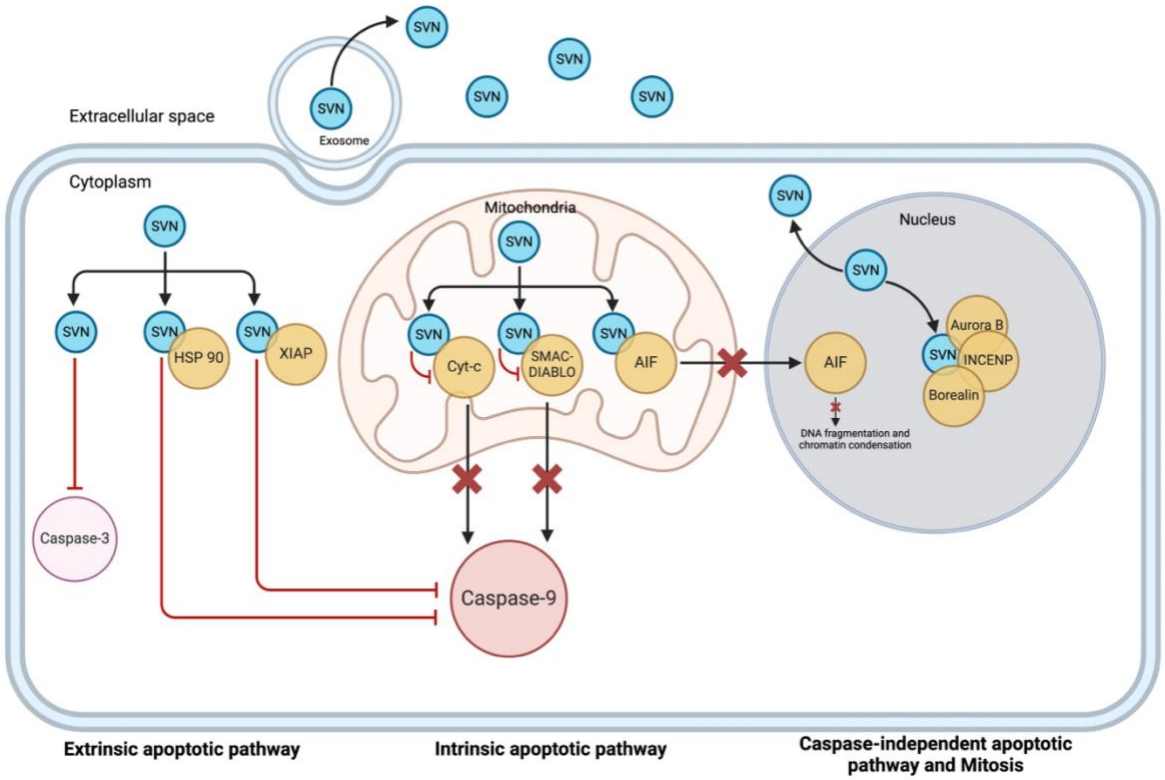

Figure 9. Survivin cellular distribution and functions

(reproduced from Li et al., 2021 using BioRender.com) (Li *et al.*, 2021)

Table 3. Summary of findings of survivin-containing extracellular vesicles in various cancer cell lines (Skog *et al.*, 2008; Hong *et al.*, 2009; Khan *et al.*, 2011, 2012; Aspe, 2014; Hurwitz *et al.*, 2016; Kreger *et al.*, 2016; Gonda *et al.*, 2018; Li *et al.*, 2020; Chang *et al.*, 2021; Figel *et al.*, 2021).

Cancer site	Cancer type	Cell line	Vesicle type	Studies
Brain	Glioblastoma	SNB-19	EV	Hurwitz et al., 2016
	Glioblastoma	U251	EV	Hurwitz et al., 2016
	Glioblastoma	Obtained from	Exosome	Skog et al., 2008

		surgical resections		
	Glioblastoma	U87	Exosome	Kreger et al., 2016
	Glioblastoma	A-1207	Exosome	Figel et al., 2021
Breast	Breast adenocarcinoma	MDA-MB-468	EV	Hurwitz et al., 2016
	Breast adenocarcinoma	MDA-MB-231	Exosome	Li et al., 2020; Kreger et al., 2016; Chang et al., 2021;
	Ductal carcinoma	BT-549	Exosome	Li et al., 2020
	Breast adenocarcinoma	SKBR3	Exosome	Kreger et al., 2016
Colon	Colorectal adenocarcinoma	HCT-116	EV	Hurwitz et al., 2016
	Colorectal adenocarcinoma	HCT-15	EV	Hurwitz et al., 2016
	Colorectal adenocarcinoma	KM12	EV	Hurwitz et al., 2016
	Colorectal adenocarcinoma	SW620	EV	Hurwitz et al., 2016
	Colorectal adenocarcinoma	SW840	Exosome	Hong et al., 2009
Leukemia	Acute lymphoblastic leukemia (ALL)	CCRF-CEM	EV	Hurwitz et al., 2016
Lung	Squamous cell carcinoma	NCI-H226	EV	Hurwitz et al., 2016
	Large cell carcinoma	NCI-H460	EV	Hurwitz et al., 2016
	Lung adenocarcinoma	NCI-H23	Exosome	Chang et al., 2021
	Alveolar cell carcinoma	A549	Exosome	Chang et al., 2021
Melanoma	Melanoma	UACC-62	EV	Hurwitz et al., 2016
	Melanoma	YUSAC 2	Exosome	Aspe, 2014

Ovary	High-grade ovarian serous adenocarcinoma	OVCAR-5	EV	Hurwitz et al., 2016
	High-grade ovarian serous adenocarcinoma	OVCAR-8	EV	Hurwitz et al., 2016
	High-grade ovarian serous adenocarcinoma	NCI-ADR-RES	EV	Hurwitz et al., 2016
Cervix	Cervical adenocarcinoma	HeLa	Exosomes	Khan et al., 2011; Gonda et al., 2018
Prostate	Prostate carcinoma	DU145	EV	Hurwitz et al., 2016
	Prostate carcinoma	PC3	Exosomes	Khan et al., 2011
Pancreas	Epithelioid adenocarcinoma	PANC-1	Exosomes	Khan et al., 2011; Chang et al., 2021
	Pancreatic adenocarcinoma	MIA PaCa-2	Exosomes	Aspe, 2014; Chang et al., 2021

Exosomes carrying survivin can be internalised by surrounding cancer cells and induce pro-survival field effect that promotes proliferation and survival in recipient cells (Mulcahy, Pink and Carter, 2014). This uptake process begins when exosomes are taken up by recipient cells via endocytosis. Exosomes can fuse with the endosomal membranes of recipient cells, releasing their cargo, including survivin, directly into the cytoplasm (Becker *et al.*, 2016). For instance, survivin-rich exosomes released by breast cancer cells have been shown to be internalized by fibroblasts, where survivin upregulates SOD1 expression and induces their transformation into myofibroblasts. In turn, these myofibroblasts promote cancer cell proliferation, epithelial-to-mesenchymal transition (EMT), and the stemness of breast cancer cells (Li *et al.*, 2020).

The release of survivin and other anti-apoptotic proteins via exosomes may represent a survival mechanism by which cancer cells attempt to counteract stress within

the TME (Khan *et al.*, 2012). The quantity and contents of exosomes vary according to the cancer cell's origin, developmental stage, and response to treatment (Akers *et al.*, 2013; Kreger *et al.*, 2016; Allenson *et al.*, 2017; Dejima *et al.*, 2017; Chen *et al.*, 2018; Keklikoglou *et al.*, 2019). The presence of survivin in exosomes has significant implications for its use as a diagnostic marker or therapeutic and informs strategies aimed at modulating TME in the treatment of solid tumours.

I.5.2 Exosomal survivin as a biomarker in cancer

Exosomes are present in human serum and represent their cell of origin. Meanwhile, survivin has been clinically detected in serum or plasma using commercially available enzyme-linked immunosorbent assay (ELISA) in patients with various malignancies. Elevated survivin levels have been associated with adverse clinicopathological features, including poor therapeutic response and metastasis (Tas *et al.*, 2004; Guney *et al.*, 2006; Goksel *et al.*, 2007; Derin *et al.*, 2008; Naumnik *et al.*, 2009; Fawzy *et al.*, 2012; Yahya *et al.*, 2012; Demirci *et al.*, 2023). Survivin has been shown to be a potential biomarker for early cancer detection (Gunaldi *et al.*, 2018; Mahmoudzadeh-Sagheb *et al.*, 2024). However, the presence of survivin inside exosomes suggests that measuring only free serum survivin may not accurately reflect its true amount in circulation. Exosomal survivin quantification may provide a more accurate picture of survivin abundance in cancer patients. Recent evidence, summarised in **Table 4**, demonstrates the potential of exosomal survivin as a valuable biomarker for early detection, diagnosis, prognosis, and monitoring of cancer progression (Khan *et al.*, 2012, 2014; Yıldırım *et al.*, 2022; Novais *et al.*, 2023).

Table 4. Exosomal survivin as a diagnostic/prognostic/monitoring biomarker (Khan *et al.*, 2012, 2014, 2017; Galbo *et al.*, 2017; Chang *et al.*, 2021; Yıldırım *et al.*, 2022).

Study	Cancer Type	Patients no.	Samples	Methods of exosome isolation	Methods of exosomal survivin mesurement	Findings
Khan et al., 2012	Prostate cancer (PCa) • Low-grade (Gleason 6) • High-grade (Gleason 9) • Advanced-disease, on a second-line chemotherapy trial (Chemoresistant)	39	• Plasma (20) • Serum (19)	• Plasma: Differential centrifugations, ultracentrifugation (110,000g, 18h, 4°C) and ultrafiltration (0.22 μm filter) • Serum: ExoQuick (SBI, USA)	Western blot with proportion analysis of survivin density to LAMP1 density	• Exosomes quantities were higher when purified from plasma than serum • Exosomal survivin was significantly higher in PCa patients compared to BPH patients and controls. • No significant difference in exosomal survivin content between Gleason 6 and Gleason 9 PCa. • Exosomal survivin is significantly higher in chemo-resistant PCa patients compared to healthy controls.
Khan et al., 2014	Breast cancer • Stage II-IV	40	• Serum (40) • Tissue (23)	• Serum: ExoQuick (SBI, USA)	• Serum: Western blot with proportion analysis of	• Exosome amounts were significantly higher in cancer patients' sera

				• Tissue: not applicable, does not require isolation	survivin density to LAMP1 density • Tissue: Staining intensity of immunohistochemistry slides, imaged using laser-scanning confocal microscopy	compared to controls. • Survivin splice variants (ΔEx3 and 2B) are also exosomally packaged in breast cancer patients' sera • Higher exosomal survivin and survivin ΔEx3 was associated with worse clinical staging. • Exosomal survivin-2B showed an inverse correlation with tumor grade and clinical staging. • Low or no survivin-2B expression was strongly correlated with HER2-negativity and triple negativity.
Khan et al., 2017 (Khan *et al.*, 2017)	Prostate cancer	41 African-American (AA) men and 31 European-American (EA) men	Plasma and serum	ExoQuick (SBI, USA)	Western blot with densitometric analysis of survivin to LAMP1	• Quantities of EV from plasma were significantly larger than from serum. • The amount of EV in AA patients plasma and serum was significantly higher than EA patients.

						• EVs from AA patients contain significantly higher amounts of survivin than EVs from EA patients.
Chang et al., 2021 (Chang *et al.*, 2021)	Pancreatic ductal adenocarcinoma (PDAC)	13	Serum	Differential centrifugations, ultracentrifugation (120,000g, 4h)	Western blot with densitometric analysis of survivin to Flotillin-2	Survivin expression was increased in the exosomes from 8 out of 13 PDAC patients and was significantly higher compared to non-PDAC patients.
Yildirim et al., 2022 (Yıldırım *et al.*, 2022)	Invasive ductal breast cancer	55	Serum	ExoQuick (SBI, USA)	• Disruption of membrane integrity with lysis buffer • Quantification of survivin with ELISA	• Exosomal survivin levels were significantly higher in breast cancer patients compared to controls. • No significant association between clinicopathological parameters and exosomal survivin level.
Galbo et al., 2017 (Galbo *et al.*, 2017)	Recurrent high-grade glioblastoma (GBM) (WHO grade III and IV), following survivin	8	Serum	Differential centrifugations and ultracentrifugation (100,000g, 80 min, 4°C)	Imaging flow cytometry as a percentage of all CD9+ events	• GBM patients have CD9+/GFAP+/SVN+ and CD9+/SVN+ exosomes released into the circulation • Early reductions in SVN+ exosomes following anti-survivin immunotherapy

	vaccination (SurVaxM)	were associated with longer PFS

I.6 Enrichment of exosomes in biological samples with polyethylene glycol (PEG)

Despite significant progress in the EV field over the past few years, there is still no consensus on the best methods for the enrichment, isolation, or purification of EVs. Differential ultracentrifugation is the standard method for harvesting EVs from tissue culture supernatants and primary body fluids (Clos-Sansalvador *et al.*, 2022). Other methods, such as immunoprecipitation (Clayton *et al.*, 2001), ultrafiltration (Lamparski *et al.*, 2002), and size-exclusion chromatography (Gámez-Valero *et al.*, 2016) are used to enrich EVs. However, these techniques are not convenient and often require specialised equipment and training. Recently, increasing numbers of commercially available polymeric precipitation reagents have emerged, allowing the precipitation of nanosized EVs at a low-speed centrifugation.

The most popular precipitation reagent is the highly hydrophilic polymer, polyethylene glycol (PEG) (Yakubovich, Polischouk and Evtushenko, 2022). PEG has long been used to precipitate viral particles, nucleic acids, and other biomolecules. However, the mechanism of precipitation in the presence of PEG is not fully understood. The most common theoretical models explaining this process are the theory of excluded volume and the theory of attractive depletion forces (Lohmann and Strube, 2020). The excluded volume model suggests that molecules precipitate due to decreased hydration in the presence of the polymer (Atha and Ingham, 1981). The attractive depletion forces model attributes precipitation to the attraction of molecules caused by the osmotic

pressure of the PEG solution (Marenduzzo, Finan and Cook, 2006). A schematic diagram for PEG precipitation mechanism is shown in **Figure 10**.

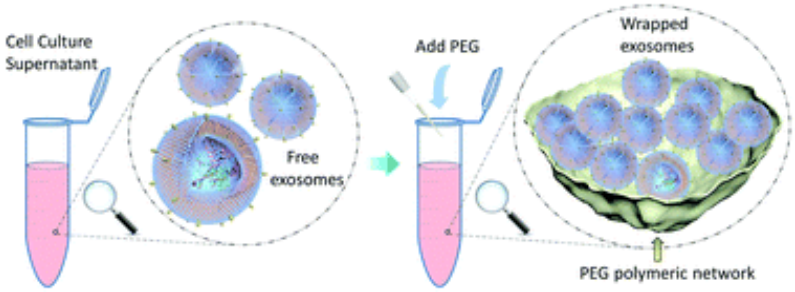

Figure 10. Mechanism of PEG precipitation to concentrate exosomes (Weng *et al.*, 2016).

The advantages of PEG precipitation are its simplicity, rapid processing, and the ability to analyse multiple samples simultaneously with minimal losses during extraction. Currently, PEG precipitation is the second most frequently used method after ultracentrifugation. Typically, 8–12% of 6-kDa PEG and 8–10% of 8-kDa PEG are used for exosome isolation (Rider, Hurwitz and Meckes, 2016; Ludwig *et al.*, 2018). Unlike ultracentrifugation and ultrafiltration, which can deform vesicle shapes, PEG precipitation preserves the high morphological and functional quality of exosomes. However, the main disadvantage is the low purity of the exosomal preparation, as the method concentrates the exosomal fraction without separating it from other sample components. The exosome precipitate contains free proteins such as lipoproteins, immunoglobulins, and viral particles (Weng *et al.*, 2016). Additionally, PEG impurities in the target product and the poor solubility of precipitated aggregates pose limitations for further analysis and application of the isolated exosomes. However, this limitation might be beneficial if the aim is to quantify the total amount of the protein of interest, which in this case is survivin.

In previous studies, exosomal survivin has been detected and measured with imaging flow cytometry or Western blot followed by densitometric analyses comparing the band density of survivin and a specific exosome marker (such as LAMP-1, Flotillin-2, CD9), which is not high-throughput and quantitative. In this project, we attempt to combine ELISA with a detergent to quantify both free soluble survivin and exosomal survivin. We will use the detergent to break the exosomal membrane and ELISA to measure the concentration to develop a high throughput and quantitative method. We will also attempt to concentrate exosomes in biological samples using PEG precipitation before measurement with ELISA.

II. Research Objectives

The objective of this study is:

To develop a practical and clinically applicable method for quantifying total and exosomal survivin in biological samples using ELISA, incorporating a non-ionic detergent-based lysis of exosomes and a practical polyethylene glycol (PEG)-based exosome concentration method.

III. Materials and Methods

III.1 Survivin ELISA kit development and optimisation

III.1.1 Determining the optimal dilutions of capture and detection antibodies with indirect ELISA

Survivin ELISA kit was developed by Changzhou Niujin Shisong Biotechnology Company Ltd (China) from a pair of survivin mouse monoclonal antibody (mAb): capture antibody

(concentration: 6.2 mg/mL) and detection antibody (concentration: 1.1 mg/mL) as detection antibody. First, we performed an indirect ELISA that detects the standard (survivin) using serial 2-fold dilutions of each antibody (from 1:1,000 down to 1:204,800). This assay was conducted to determine the optimal dilution of each antibody. These dilution factors were subsequently to be used in the sandwich ELISA.

Coating antigen to microplate

The survivin protein was obtained from our laboratory stock. It was previously purified from phage-resistant Rosetta cells that have been transformed with wild type 6His-tagged survivin construct (BIC5A-c001), using a combination of immobilised metal affinity chromatography and ion-exchange chromatography, followed by concentration in 3 kDa MWCO centrifugal filters. The purity was confirmed by SDS-PAGE and intact mass spectrometry. The purification procedure yielded 2 mL of survivin at 11.25 mg/mL (total yield: 22.5 mg), with storage at −80 °C in aliquots to prevent freeze–thaw degradation.

Survivin was diluted to a final concentration of 0.5 µg/mL in coating buffer. Each well of the PVC microtitre plate was coated with 100 µL of the antigen dilution at 4^{o}C overnight. On the next day, the coating solution was removed and the plate was washed three times with 200 µL Phosphate-buffered saline 0.05% Tween20 (PBST). The solutions were removed by flicking the plate over a sink. The remaining drops were removed by patting the plate on a paper towel.

Blocking

The remaining protein-binding sites in the coated wells were blocked by adding 200 µL of blocking buffer (2.5% BSA in PBS). The plate was covered with an adhesive plastic and incubated for 2 hours at room temperature. The blocking solution was removed and the plate was washed three times with PBST 0.05%. The washing solution was removed in the same manner as described in the coating step.

Incubation with primary and secondary antibody

100 µL of diluted primary antibodies (capture and detection antibodies) (1:1,000 down to 1:204,800) were added to each well. The plate was covered and incubated for 1 hour at room temperature. The plate was washed three times with 200 µL PBS. 100 µL of conjugated secondary antibody (Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP, Agilent, P0260) diluted at 1:10,0000 according to the manufacturer's instructions. The plate was covered with an adhesive plastic and incubated for 1 hour at room temperature. Afterwards, the plate was washed five times with PBST 0.05%.

Detection

50 µL of TMB (3,3',5,5'-tetramethylbenzidine) solution was added to each well and incubated for 15 minutes. Equal volume of stopping solution (2 M H_2SO_4) was added. The optical density (absorbance) was read at 450 nm on a microplate reader (Varioskan™ LUX Multimode Microplate Reader, Thermo Scientific, VL0000D0) with SkanIt™ Software (Thermo Scientific, 5187139).

Analysis of data

The curve was prepared from the absorbance values (y axis) of each antibody dilution (x axis, log scale) with GraphPad PRISM using sigmoidal, 4PL model. The optimal dilution was determined by considering the optical density and final antibody concentration.

III.1.2 Developing a standard curve

Coating with capture antibody

The wells of a microtitre plate were coated with the capture antibody (Biotin-Ab-4217) at the optimal dilution obtained from the prior indirect ELISA. The capture antibody was diluted with coating buffer. The plate was covered and incubated overnight at 4^{o}C. The coating solution was removed and the plate was washed three times with 200 µL of PBST for each well.

Blocking

The remaining protein-binding sites in the coated wells were blocked by adding 200 µL of blocking buffer (2.5% BSA in PBS). The plate was covered with an adhesive plastic and incubated for 2 hours at room temperature. The blocking solution was removed and the plate was washed three times with PBST.

Preparing and adding standards and blanks

Recombinant survivin with the concentration of 50 ng/mL was used as standard and serially diluted 2-fold down to 0.78 ng/mL into several clean vials using blocking buffer (1% BSA in PBS) as the diluent (**Figure 11**). 50 µL of diluted standards, samples, and blanks were added to each well and incubated for 2 hours at room temperature. After that, all

solutions were removed and the plate was washed three times by filling the wells with 200 µL PBS.

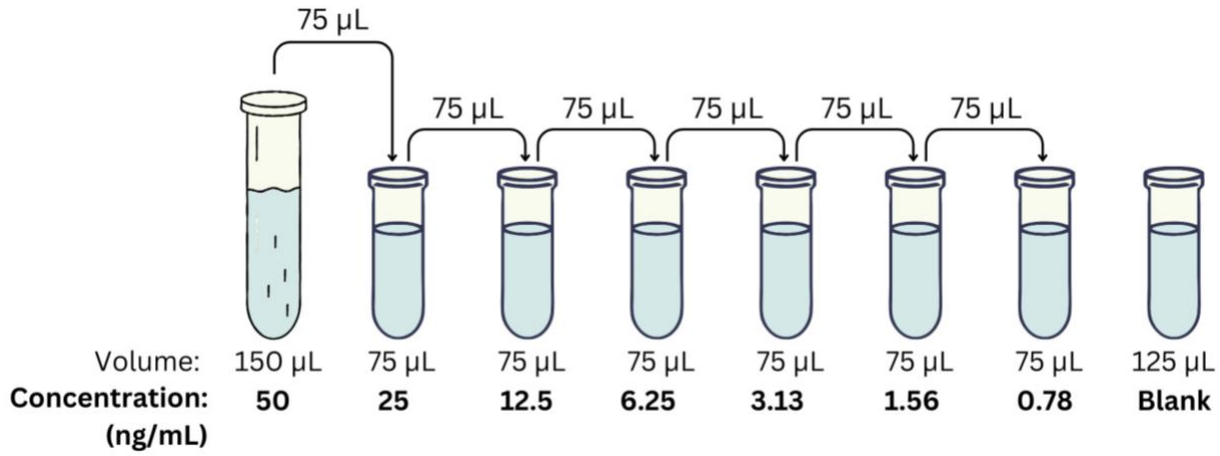

Figure 11. Preparation of diluted standards.

Incubation with detection antibody and secondary antibody

100 µL of diluted detection antibody was added to each well. The plate was covered with an adhesive plastic and incubated for 1 hour at room temperature. After incubation, the plate was washed three times with PBST. 100 µL of conjugated secondary antibody (HRP Streptavidin, BioLegend, 405210) was diluted at 1:10,000 in blocking buffer immediately before use. The plate was covered and incubated for 1 hour at room temperature. After that, the plate was washed five times with 200 µL PBST for each well.

Detection

One hundred (100) µL of TMB solution was added to each well. The plate was incubated for 15 minutes in a dark condition before adding an equal volume of stop solution (2M H_2SO_4). The optical density was read at 450 nm on a microplate reader (Varioskan™ LUX

Multimode Microplate Reader, Thermo Scientific, VL0000D0) with SkanIt™ Software (Thermo Scientific, 5187139).

Analysis of data

The standard curve was prepared from the absorbance values (y axis) of each sample dilution (x axis, log scale) with GraphPad PRISM using sigmoidal, 4PL model. The concentration of future unknown samples will be interpolated from this standard curve. The sensitivity of the assay was also inferred from this standard curve.

III.1.3 Testing lysis buffer (non-ionic detergents) interference and compatibility with survivin ELISA kit

Non-ionic detergents contain uncharged hydrophilic head groups and are considered to be mild and relatively non-denaturing. Two common non-ionic detergents, NP-40 (NP-40 Surfact-Amps™ Detergent Solution, Thermo Scientific, #85124) and Triton X-100 (Triton™ X-100, Sigma-Aldrich, #X100-500ML), were tested at different concentrations to assess interference and ensure compatibility with the ELISA. Concentrations tested are 0.1%, 0.25%, 0.5%, 1%, and 2%.

III.2 Cell lines and cultures

Colorectal carcinoma (HCT116), breast carcinoma (MDA-MB-231), and human fibroblasts (Hs68) cell lines were obtained from the American Type Culture Collection (ATCC). Cells were maintained in DMEM supplemented with 10% foetal bovine serum (FBS), 100 units of penicillin, 100 µg/mL of streptomycin. Cells were grown at 37°C in a

humidified atmosphere of 95% air and 5% CO_2 until 80-90% confluent (Khan *et al.*, 2011; Gonda *et al.*, 2018).

Preparation of an aseptic environment

All cell culture hood surface areas were sprayed with 70% ethanol before use. All media, supplement, reagents, and additional supplies were also sprayed with 70% ethanol before being placed inside the hood.

Thawing cells

Cryovials of cells were taken out from liquid nitrogen storage and transported on dry ice to the water bath. Vial was thawed by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, the cap and O-ring was kept out of the water. The vial was removed from the water bath as soon as the contents were thawed with a remaining small ice chunk. Vial was decontaminated by spraying with 70% ethanol before being placed inside the hood. The vial contents were transferred to a 15-mL centrifuge tube. 10 mL of pre-warmed medium were added to the tube drop by drop, then gradually on an increasing rate to avoid osmotic shock. The tube was centrifuged at 300 x g for 5 minutes to form cell pellet remove the freezing medium that contains dimethyl sulfoxide (DMSO). The medium was discarded to waste container. The cell pellet was resuspended in 10 mL of pre-warmed medium before being transferred to a T75 flask. The flask was moved with a north-south or east-west rocking motion as appropriate to ensure even distribution of the cells. The flask was placed inside CO_2 37°C incubator. The cells were checked the next day for good adhesion and proper morphology.

Changing media

Fresh culture media were warmed up in a 37°C water bath for at least 30 minutes before use. The media from the flask were aspirated and discarded to a waste pot containing some disinfectant. The flask was added with appropriate volume of media in replacement of the old media and returned to the CO_2 37°C incubator to continue growth.

Sub-culturing/Passaging

Culture medium was removed from the flask by aspirating and discarding into a waste pot containing 100 mL of 10% sodium hypochlorite. Cells were rinsed once with 5 mL of sterile PBS. 5 mL of cell dissociation reagent (Trypsin EDTA/TrypLE) was added into the flask to cover all the cells. The flask was moved with a north-south or east-west rocking motion gently. The flask was placed in the incubator for 3 minutes before being looked upon under the microscope to check the dissociation. 15 mL (3 times the volume of cell dissociation reagent) of culture media containing FBS was added into the flask to inactivate trypsin. The cells were transferred to a 15-mL conical tube and centrifuged at 300 x g for 5 minutes to form cell pellet. The medium was removed and discarded into a waste pot. The cell pellet was resuspended with appropriate volume of warm medium and pipetted up and down gently to mix. A small sample (0.5 mL) was removed and placed in an Eppendorf tube for cell counting. The number of cells acquired from cell counting was used to determine the volume of additional fresh medium to add for optimal seeding density. After adding appropriate volume of medium, cell suspension was transferred to

fresh flasks at the required split ratio. The flasks were placed back in the incubator to allow replication and growth.

Counting cells with haemocytometer

10 µL of cell suspension was transferred into a new Eppendorf tube and mixed with 10 µL of 0.4% Trypan Blue. 10 µL of Trypan blue-treated cell suspension were taken and applied on a haemocytometer. The haemocytometer was observed under the microscope with 10x objective. Using a hand tally counter, live, unstained cells and dead stained cells were both counted separately in four corner sets of 16 squares (**Figure 12**). Cells were only counted when they were set within a square on the right-hand or bottom boundary line.

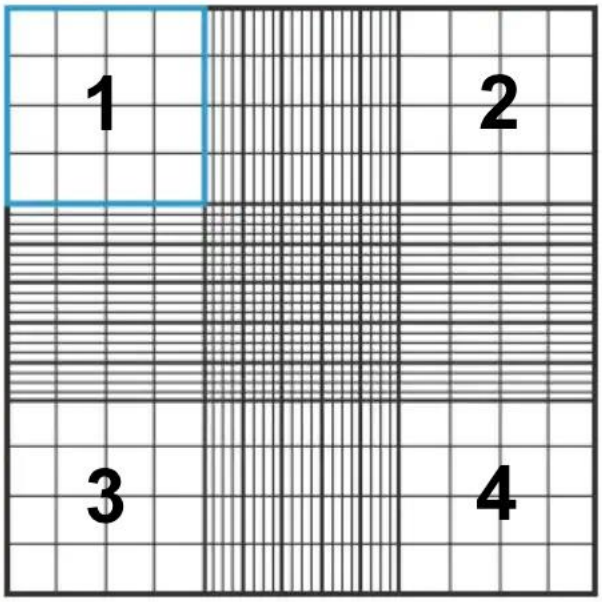

Figure 12. Haemocytometer squares.

Number of viable cells per mL was calculated by taking the average cell count from each of the sets of 16 corner squares, multiplied by 10,000 (10^4), and then multiplied by 2 to

correct for the 1:1 dilution from the Trypan blue addition. The final value is the number of viable cells/mL in the original cell suspension. Viability is a measure of the proportion of live, healthy cells within the cell suspension. It is calculated by dividing the live cell count by the total cell count and multiplying by 100% to get the percentage viability.

Freezing cells

A portion of cells were also frozen as stocks for future experiments. Cryovials were first labelled with cell type, passage number, and date. Cryogenic containers (Corning CoolCell LX) were prepared beforehand. An appropriate volume of dimethyl sulfoxide (DMSO) was added to culture medium making up a concentration of 5-10% according to the manufacturer's guideline for cell freezing. Cells were resuspended in culture medium containing 5-10% DMSO by pipetting gently up and down. 1 mL of cell suspension was transferred to each cryovial. Vials were stored at -80^{o}C for 1-2 days before being moved to vapor phase liquid nitrogen for long-term storage.

III.3 Assessment of lysis efficacy of different non-ionic detergent concentrations

The lysis efficacy of non-ionic detergents, both NP-40 and Triton X-100, at different concentrations (0.25%, 0.5%, and 1%) was assessed by using these detergents to lyse 1.5x10^{7} MDA-MB-231 cells, also detecting the presence of intracellular survivin. Four T175 flasks of MDA-MB-231 cells were grown until 80% confluent before the medium was removed and substituted with serum-free medium (DMEM not supplemented with FBS). Cells were left to grow for 48 hours more for MDA-MB-231 and 24 hours more for HCT116.

These procedures were conducted to ensure the same treatment with future samples for detection of exosomal survivin.

Cell detachment

The culture media (18 mL/flask), which made up a total of 72 mL of conditioned medium (CM), was removed by aspirating and discarding into fresh 50 mL conical tubes. CM was kept at 4^{o}C or on ice at all times to avoid exosomes and protein degradation. Enough sterile PBS was pipetted into the flask to give cells a wash and get rid of any residual culture media or detached dead cells. 5 mL of trypsin EDTA was pipetted into the bottom of each flask to detach the cells. The flask was tapped and rolled gently to ensure trypsin contact with all cells. The flask was placed in 37^{o}C incubator for 5 minutes. Afterwards, it was checked under the microscope to ensure complete detachment. 5 mL of culture media containing FBS was added to the flask to inactivate the trypsin. Cell suspension was transferred to a fresh 50-mL conical tube.

Cell lysis

Cells were pelleted by centrifugation at 400 x g at 25^{o}C for 5 minutes. Cell pellet was rinsed with 10 mL of ice-cold PBS. PBS was discarded and cell pellet was resuspended with 3.5 mL of ice-cold PBS. Cell suspension was divided into 7 different tubes (500 µL/tube) corresponding to different concentrations of non-ionic detergents (0%; NP-40: 0.25%, 0.5%, and 1%; Triton X-100: 0.25%, 0.5%, and 1%). Cells were lysed by adding 500 µL of appropriate non-ionic detergent, previously made at 2X concentration to account for the 1:2 dilution. Cell suspensions were pipetted up and down several times

and incubated at 4°C or on ice for 30 minutes, with vortexing or pipetting up and down every 10 minutes. Lysates were centrifuged at 18,000 x g for 20 minutes at 4°C. The supernatant (500 µL) was placed in different cold 1.5-mL tubes based on their downstream analysis, including ELISA, BCA assay, and Western blot.

III.4 Protein gel electrophoresis

Preparation of separating and stacking PAGE gel

A 14% gel was used to accommodate proteins in the molecular weight range of 10-80 kDa. Two 14% 1.5 mm thick gels were prepared by mixing the ingredients in **Table 5** in order. One separating gel was made with 6.5 mL of the final mixture. The mixture was poured, leaving 2 cm below the bottom of the comb for the stacking gel. Bubbles were removed by layering the top of the gel with isopropanol. Isopropanol also helps keep the polymerized gel from drying out. Gels were completely polymerized in 20-30 minutes.

Table 5. Separating and stacking PAGE gel recipe.

14% Separating PAGE gel (7 mL/gel)	
Deionized water (ddH_2O)	4.5 mL
4x separating buffer	4 mL
30% acrylamide	7.5 mL
10% APS*	80 µL
TEMED*	10.7 µL
4% Stacking PAGE gel (2 mL/gel)	
Deionized water (ddH_2O)	3.25 mL
4x separating buffer	1.33 mL
30% acrylamide	0.71 mL
10% APS*	26.7 µL
TEMED*	5.3 µL

*APS and TEMED were added when ready to pour, since gel will polymerise quickly upon adding them.

Two 4% 10-well, 1.5 mm thick, 66 µL/well gels were prepared by mixing the ingredients in **Table 5** in order. One stacking gel was made with 2 mL of the final mixture. The mixture was poured on top of the separating gels. Combs were put in to create the wells and left for 20-30 minutes to polymerise. Gels were subsequently submerged in running buffer. The gels were stored moist with running buffer at 4°C up to several days until ready for use.

Measurement of samples protein concentration with BCA assay

Diluted albumin standards were prepared with Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, #23225) (*Pierce™ BCA Protein Assay Kits*, no date) (**Table 6**). The diluent used was deionized water (ddH_2O).

Table 6. Preparation of diluted albumin (BSA) standards.

Vial	Volume of Diluent (ddH_2O) (µL)	Volume and Source of BSA (µL)	Final BSA concentration (µL/mL)
A	50	150	1500
B	100	100	1000
C	100	100 of vial A dilution	750
D	100	100 of vial B dilution	500
E	100	100 of vial D dilution	250
F	100	100 of vial E dilution	125
G	160	40 of vial F dilution	25
H	200	0	0

Working reagent (WR) was prepared by mixing 50 parts of BCA Reagent A and 1 part of BCA Reagent B (50:1 of Reagent A:B). The total volume of WR required was determined using the following formula: Total volume of WR = (# standards + # unknowns) x (# replicated) x (volume of WR per sample). When reagent B is first added to reagent A, turbidity was observed but quickly disappeared upon mixing yielded a clear, green colour.

25 µL of standards and unknowns were pipetted into the microplate wells. 200 µL of WR was added to each well. Microplate was placed on a plate shaker for 30 seconds. The plate was then covered and incubated at 37°C for 30 minutes. The plate was cooled to room temperature before being placed on a plate reader. The absorbance was measured at or near 562 nm.

Sample preparation

After determining the protein concentration of each sample, samples were prepared by adding 6x Laemmli SDS sample buffer, non-reducing (Thermo Scientific, J60660-AD) to the desired protein concentration (Laemmli buffer volume is 1/6 part of the total sample volume). DTT 10% was also added if a reducing condition was required for the antibody detection. Samples were heated at 95°C for 10 minutes and cooled down to 4°C. Samples were stored at -20°C if not analysed immediately.

Protein separation by gel electrophoresis

Equal amounts of samples (40 µg) were loaded into the wells of the PAGE gel along with molecular weight markers (protein ladders) using P20 pipette slowly. The lid for the electrophoresis box was placed to match the terminal colours and plugged into the power

pack. The gel was initially run at 80V for 20 minutes before increasing the voltage to 120V to finish the run in about 1 hour. Upon finishing, the gel cassette was removed from the gel box and was opened by inserting the edge of the comb in the slot opposite the sample wells and twisting. The top plate was removed from the gel cassette. Unwanted parts of the gel were trimmed with the comb.

For Coomassie blue staining, the cut gel was incubated in QuickBlue Protein Stain (Lubio Science, # L001000) solution at room temperature for 1 hour on a shaker. The gel was subsequently washed three times in ddH_2O on a shaker at room temperature for 5 minute. The gel was imaged on BioRad ChemiDoc imaging reader with appropriate settings. The image was saved for later use and analysis.

III.5 Western blot

Protein transfer from the gel to the membrane

The gel was immersed in 1x transfer buffer for 10 minutes. The transfer sandwich was assembled on the transfer cassette in the following order from the anode to the cathode (**Figure 13**). 0.2 µm nitrocellulose membranes (Bio-Rad,#1620112) were used (Bhatt *et al.*, 2022). Air bubbles between, if any, were removed by using the roller. The cassette was closed with locks secured on both sides and placed in the transfer tank along with an ice block. The transfer was run in a cold room at a constant current of 300 mA for 120 minutes. Afterwards, the stack was disassembled and the gel was discarded. The membrane was cut to a desirable size.

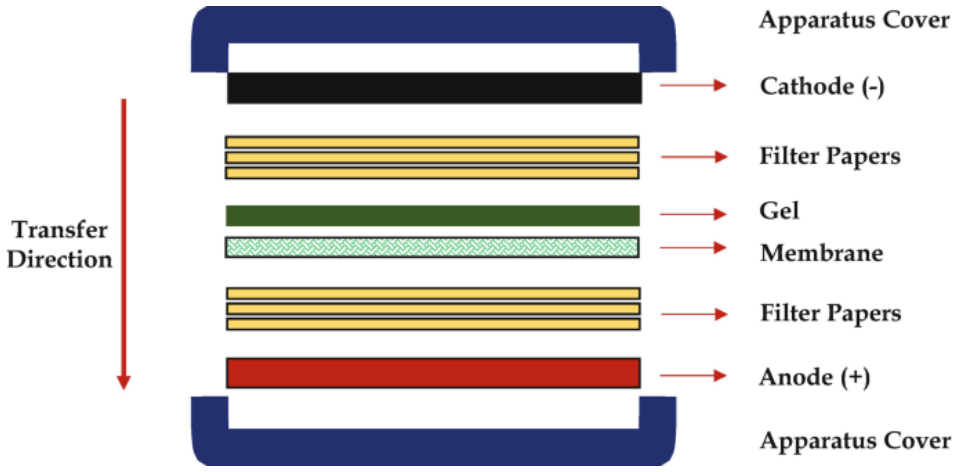

Figure 13. Transfer stack for Western blot (Bhatt *et al.*, 2022).

Ponceau S staining

Ponceau S staining was performed to evaluate the efficiency and quality of transfer. The membrane was first washed in deionized water with gentle agitation to remove all transfer buffer. The membrane was subsequently immersed in Ponceau S Staining Solution (Thermo Scientific, #A40000279) on a shaker for 5 minutes. After that, the membrane was washed again in deionized water for 30-90 seconds until the desired staining intensity was achieved. The membrane was washed with PBST 0.1% three times for 2 minutes each to rinse off the Ponceau S stain.

Antibody incubation

The blot was immersed in a sufficient volume of 5% skimmed milk in PBS-0.1% Tween20 at room temperature for 1 hour with gentle agitation (on a shaker). After that, the blot was incubated overnight in the primary antibody solution at 4°C. The primary antibodies used in this experiment included antibodies against: Syntenin-1 (32 kDa) (cytosolic marker for

exosomes) (Cell Signaling Technology® Syntenin-1/MDA9 (E2I9L) Rabbit mAb #27964), CD63 (25-60 kDa, under a non-reducing condition) (transmembrane marker for exosomes) (Cell Signaling Technology® CD63 (E1W3T) Rabbit mAb #52090), β-actin (42 kDa) (loading control) (BioLegend® Direct-Blot™ HRP anti-β-actin Antibody, #664803), and survivin (16 kDa) (Cell Signalling Technology ® Survivin (71G4B7) Rabbit mAb, #2808). The primary antibodies were diluted in 1% BSA in PBS-0.1%Tween20 according to the manufacturer's recommended ratio. The blot was rinsed 3 times for 5 minutes each with PBS-0.1% Tween20. The blot was incubated in HRP-conjugated secondary antibody solution for 1 hour at room temperature. The secondary antibody was also diluted with 1% BSA in PBST according to the manufacturer's recommended ratio.The blot was rinsed 3 times for 5 minutes each with PBS-0.1%Tween20.

Development

SuperSignal™ West Dura Extended Duration Substrate (Thermo Scientific, #34075) was used as the enhanced chemiluminescence (ECL) HRP substrate. The substrate working solution was prepared by mixing equal parts of the substrate and stable peroxide components. Sufficient volume was used to ensure that the blot was completely wetted with the substrate and did not become dry (0.1 mL/cm^2). The blot was incubated with the substrate working solution for 5 minutes. The working solution was removed and the blot was placed in a clear plastic sheet before imaging.

Imaging

The blot was imaged with the ChemiDoc Imaging Systems (Bio-Rad, Hercules, CA, #12003153) and analysis with the Image Lab Touch Software with the chemiluminescence application for the band reading and the colorimetric application for the ladder reading. The images from the two readings were merged to generate a Western blot image.

III.6 Conditioned medium preparation

Serum starvation of cells

Eight (8) T175 flasks of MDA-MB-231 and HCT116 cell lines were grown until 80-90% confluent. The medium was substituted with 7-10 mL of serum-free medium per flask 48-72 hours (for MDA-MB-231) and 24-48 hours (for HCT116) before collection.

Serial centrifugations

A total of 56 mL of conditioned medium (CM) collected from cell cultures was centrifuged at 500 x g for 10 minutes at 4°C to remove intact cells. The supernatant was carefully removed using a 25 mL serological pipette and transferred to a new 50 mL conical tube. The CM was centrifuged again at 2,000 x g for 20 minutes at 4°C to remove non-cellular debris. The supernatant was taken and further centrifuged at 10,000 x g for 30-40 minutes at 4°C to remove larger extracellular vesicles and insoluble materials.

Exosome concentration

a. Centrifugal filtering

CM was concentrated by centrifugal filtering using Amicon® Ultra-15K 3K filter devices at 4,000 x g (swing bucket) or 5,000 x g (fixed angle) for 60 minutes at 4°C (Merck, no date). This process was repeated several times until the volume went down to the dead-stop volume of the device (50 µL). Afterwards the sample was diluted to 326 µL with cold PBS and appropriate volume of protease inhibitor. The sample was transferred into fresh 500 µL tubes. Sample was stored at -80°C for later analyses.

b. Polyethylene glycol (PEG) 6000 precipitation

56 mL of 20% PEG 6000 in 0.3 M NaCl and 56 mL of conditioned medium were mixed well together in a tube, making up a final concentration of 10% PEG 6000, and subsequently left overnight (14 hours) at 4°C. This choice of PEG molecular weight and concentration was based on the findings in previous studies (Rider, Hurwitz and Meckes, 2016; Ludwig *et al.*, 2018). The mixture was centrifuged at 5,250 g for 1 hour at 4°C. Supernatants were discarded completely leaving only the precipitate (pellet) at the bottom of the tube. The pellet of the protein was resuspended in the appropriate volume of cold PBS based on the downstream analysis i.e., a total of 100 µL for Western blot and 400 µL for ELISA.

III.7 Exosome lysis

Sample was divided into two different tubes as follows (**Table 7**). The solution in each tube was pipetted up and down or vortexed and incubated on ice for 30 minutes with frequent vortexing every 10 minutes.

Table 7. Volumes of lysed and non-lysed samples.

Components	Volume (µL)	
	Tube 1 (Non-lysed)	Tube 2 (Lysed)
Sample	154	154
10% NP-40 or ddH_2O	18 (ddH_2O)	18 (10% NP-40)
Protease inhibitor (25x)	8	8
Total volume (µL)	180	180

III.8 Exosomal survivin quantification

ELISA was performed on both samples by using 100 µL each. Exosomal survivin could be quantified by subtracting the survivin concentration of non-lysed sample from lysed sample. Survivin presence was validated by performing BCA assay (20 µL) and consequently Western blot (50 µL) on both samples.

III.9 Exosomes characterization by nanoparticle tracking analysis (NTA)

The particle concentration and size of exosomes was determined using nanoparticle tracking analysis with NanoSight NS300 (Malvern, UK) following the manufacturer's protocols. 1 mL of collected CM was spared before being concentrated and loaded onto the machine with a syringe pump. Machine settings were as follows: software-NanoSight NTA 3.2, camera level-13-14, detection threshold-5, capture time-60sec, captures-5, flow rate-30.

III.10 Serum sample preparation

Serum samples belonged to 15 cancer patients (9 ovarian, 6 prostate) and 5 healthy controls. Serum samples of cancer patients were obtained from OVM-200 vaccine clinical

trial (NCT05104515) ('A Phase 1, Multicentre, Open-label, Nonrandomised, First-in-human Study of OVM-200 as a Therapeutic Vaccine in Patients With Locally Advanced or Metastatic Non-Small Cell Lung Cancer, Ovarian Cancer, and Prostate Cancer', 2021) and were collected at baseline i.e., before OVM-200 vaccine administration. Serum samples of healthy controls, a kind gift from Professor Paul Leeson, Dr Adam Lewandowski, and Prenali Dwisthi Sattwika, were obtained from CLARITY trial at the Cardiovascular Clinical Research Facility, Radcliffe Department of Medicine, University of Oxford.

Blood samples were drawn into a red-top tube (no anticoagulant) and left in an upright position for 30-60 minutes at room temperature until a clot formed. Within 2 hours from time of collection, the samples were centrifuged at 1000 x g for 10-20 minutes at room temperature. Serum was recovered with transfer pipette. 0.5 to 1 mL of the serum was placed into a cryovial tube. Each tube was clearly labelled with appropriate study code and date of collection. Serum was stored at -80^{o}C until use.

200 µL of serum was divided into two different tubes, one of which was mixed with NP-40 as in **Table 8**, resulting in a final total volume of 100 µL each. Both samples were incubated on ice or at 4^{o}C for 30 minutes with vortexing/pipetting up and down every 10 minutes. Afterwards, they were stored at 4^{o}C overnight before survivin concentration measurement with ELISA along with PEG-precipitated samples.

Table 8. Volumes of non-lysed and lysed serum.

Sample	**Serum/Suspension volume (µL)**	**Diluent**	**Total volume (µL)**
0% NP-40	90	10 µL (ddH_2O)	100

1% NP-40	90	10 µL (10% NP-40)	100

To prepare the PEG-precipitated samples, equal volumes of serum (200 µL) and 20% PEG 6000 in 0.3 M NaCl (200 µL) were mixed well together in a tube and left overnight (14 hours) at 4°C. The next day, the mixture was centrifuged at 5,250 g for 1 hour at 4°C. Supernatants were discarded completely leaving only the precipitate (pellet) at the bottom of the tube. The pellet was resuspended in the 200 µL of cold PBS and divided into two 1.5-mL Eppendorf tubes as in Table 7 above. They were then also incubated on ice or 4°C for 30 minutes with vortexing/pipetting up and down every 10 minutes. Afterwards, all samples were analysed with ELISA.

III.11 Human serum survivin ELISA

Survivin concentration in cancer patients' serum was measured with Survivin Quantikine® ELISA Kit (R&D Systems, #DSV00) according to the manufacturer's protocol (R&D Systems, no date) with a modification in the standard, sample, and assay diluent volumes i.e., reduced from 100 µL to 50 µL, due to limited volume of serum samples. All samples were analysed in duplicates.

III.12 Statistical analysis

All data were repeated in two to three independent experiments. Data analysis was performed with GraphPad PRISM 9 and R (version 4.3.2). Quantitative data are presented as means ± standard deviation. Normality of the data distribution was tested with Shapiro-Wilk test. Comparisons between two groups were performed using the independent t test or Mann Whitney U test in accordance with the normality of the data

distribution. Comparisons between more than 2 groups were performed with One-way ANOVA or Kruskal Wallis test in accordance with the normality of the data distribution. The cut-off value for survivin concentration was determined using Receiver Operating Characteristic (ROC) curve analysis and Youden's index. Results were considered statistically significant if p value is <0.05.

IV. Results

IV.1 Survivin ELISA optimisation

IV.1.1 Determining the optimal dilutions of capture and detection antibodies

Indirect ELISA produced absorbance curves for the capture antibody (**Figure 14a**) and the detection antibody (biotinylated) (**Figure 14b**). For the capture antibody (6.2 mg/mL), an OD between 1.0 to 1.5 was achieved with dilution factors ranging from $1:2^{16}$ (~1:60,000) to $1:2^{9}$ (~1:500), corresponding to antibody concentrations of 0.1-12.4 µg/mL. Similarly, for the detection antibody (1.1 mg/mL), the same OD range was observed with dilutions between $1:2^{11}$(~1:2,000) to $1:2^{7}$ (~1:250), corresponding to concentrations of 0.5-4.4 µg/mL. Based on these results, the optimal working dilutions were determined to be 1:1,250 for the capture antibody (4.96 µg/mL) and 1:1,000 for the detection antibody (1.1 µg/mL).

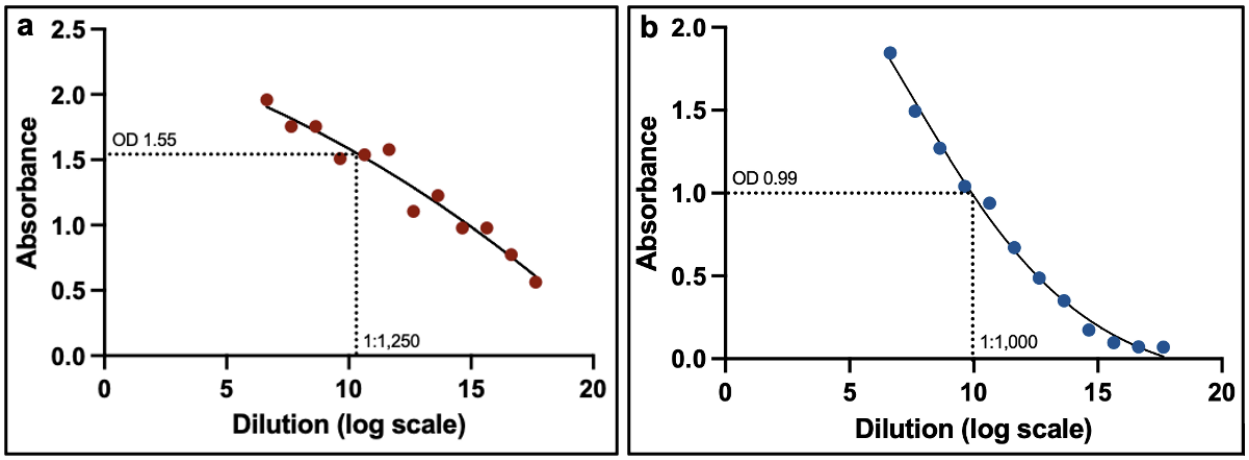

Figure 14. Absorbance curves produced from serial dilutions

(a) capture antibody and (b) detection antibody.

IV.1.2 Standard curve development

A standard curve was established using sandwich ELISA with the capture antibody and the detection antibody, applying the optimal dilution factors determined previously. The curve was generated starting from a highest standard concentration of 50 ng/mL, followed by a series of 2-fold serial dilutions down to 0.78 ng/mL (**Figure 15**). The linear range of the curve was determined to be 6.25-50 ng/mL.

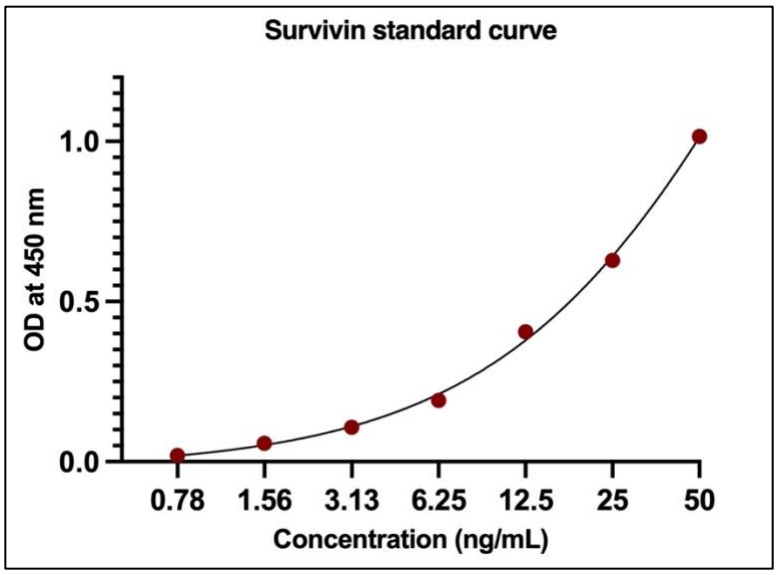

Figure 15. Standard curve of survivin ELISA kit with a labelling of 50 ng/mL down to 0.78 ng/mL.

IV.1.3 Non-ionic detergents interference towards survivin ELISA

Figure 16a shows that at lower NP-40 concentrations (0.1%, 0.25%, 0.5%, and 1%), the OD readings remained comparable to the control (0% NP-40) across the tested survivin concentrations. This indicates that NP-40 at concentrations up to 1% does not significantly interfere with antibody-survivin binding in the ELISA. However, at 2% NP-40, a marked reduction in OD values is observed, particularly at higher survivin concentrations. This reduction suggests that higher NP-40 levels may disrupt ELISA performance, either by interfering with the enzymatic reaction responsible for signal development or by impairing antigen-antibody binding.

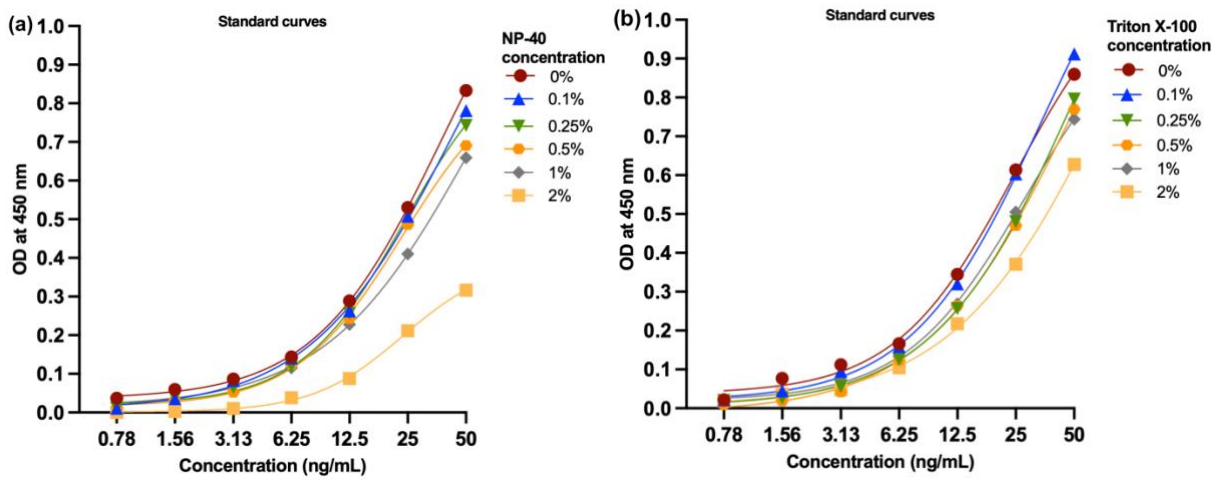

Figure 16. Survivin ELISA standard curves using different concentrations of (a) NP-40 and (b) Triton X-100 (2 independent experiments each).

For Triton X-100 (**Figure 16b**), a similar pattern was observed. At lower concentrations (0.1% to 1%), the OD readings were relatively consistent, indicating minimal interference with the assay. However, at 2%, Triton X-100 caused a substantial reduction in OD values, suggesting significant disruption of ELISA at this concentration.

Overall, both non-ionic detergents (NP-40 and Triton X-100) had little impact on OD readings at concentrations up to 1%. Therefore, concentrations at or below 1% were deemed suitable for further testing. However, statistical analysis was not performed as the independent experiments were repeated only twice.

IV.2 Non-ionic detergent lysis protocol optimisation

Both NP-40 and Triton X-100 were tested at three concentrations (0.25%, 0.5%, and 1%) to lyse MDA-MB-231 cells to identify the optimal detergent and condition to use for

disrupting the lipid bilayer membranes of exosomes. The resulting survivin concentrations from each condition are presented in **Table 9** and **Figure 17**. For NP-40, a dose-dependent lysis effect was observed, with higher concentrations of NP-40 yielding greater survivin release. The highest survivin concentration, 16.08 ng/mL, was obtained using 1% NP-40. In contrast, Triton X-100 produced a non-linear pattern: survivin concentrations increased at 0.25% but then decreased at 0.5% before rising again at 1%.

Table 9. Survivin concentrations of MDA-MB-231 cell lysates at different concentrations of NP-40 and Triton X-100.

Detergent concentration	**Survivin concentration (ng/mL) (n=2)**	
	NP-40	**Triton X-100**
0%	0	0
0.25%	7.17	7.12
0.5%	9.09	6.23
1%	16.08	13.58

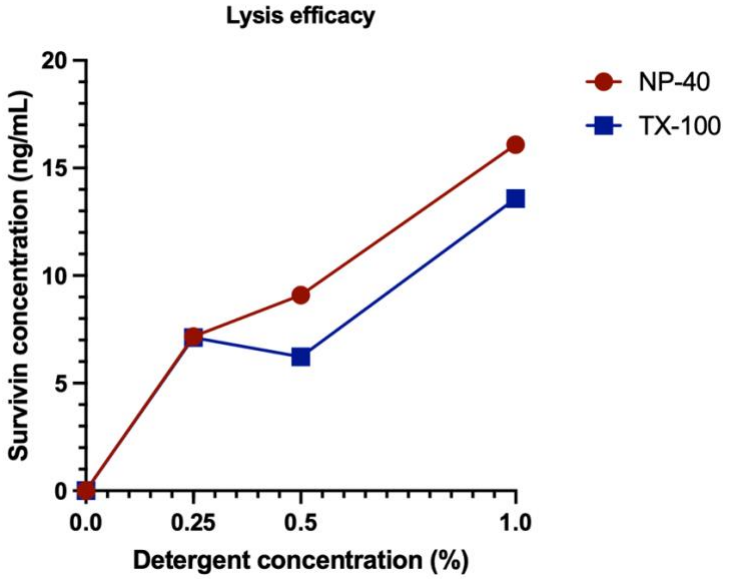

Figure 17. Survivin concentrations of MDA-MB-231 cell lysates at different non-ionic detergent concentrations (n=2).

Total protein concentrations of each sample were also measured prior to Western blot analysis (**Table 10**, **Figure 18**). For both non-ionic detergents, total protein levels increased with rising detergent concentrations, as expected: higher concentrations should lyse more cells and release greater amounts of intracellular protein into the solution. The trends in total protein concentration were similar with those observed in the survivin ELISA results.

Table 10. Total protein concentration across different concentrations of NP-40 and Triton X-100.

Detergent concentration	**Total protein concentration (µg/mL)**	
	NP-40	**Triton X-100**
0%	388.35	388.35
0.25%	3522.58	3313.63
0.5%	3625.49	4142.68
1%	4081.49	4350.81

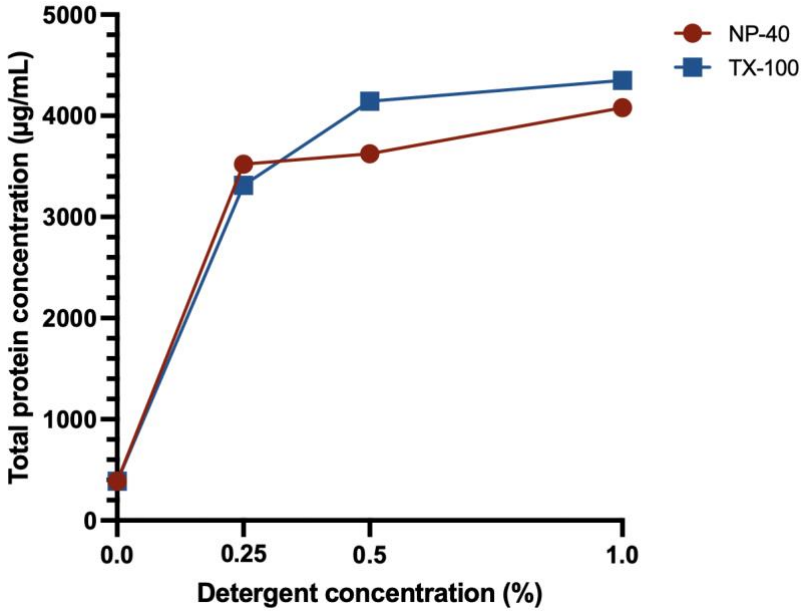

Figure 18. Total protein concentrations of MDA-MB-231 cell lysates, lysed with different non-ionic detergent (NP-40 and Triton X-100) concentrations.

Western blotting was then performed using equal amounts of protein (40 µg) from each sample (**Figure 19**). In the absence of detergent, survivin band was almost undetectable. The addition of NP-40 or Triton X-100 resulted in a dose-dependent increase of survivin bands density. NP-40 appeared slightly more effective than Triton X-100, with the strongest survivin signal observed at 1% NP-40. β-actin levels were consistent across samples, confirming equal protein loading. As expected, survivin expression was negligible in normal fibroblast (Hs68) lysates despite detergent treatment, implying that survivin detection was specific to cancer cells.

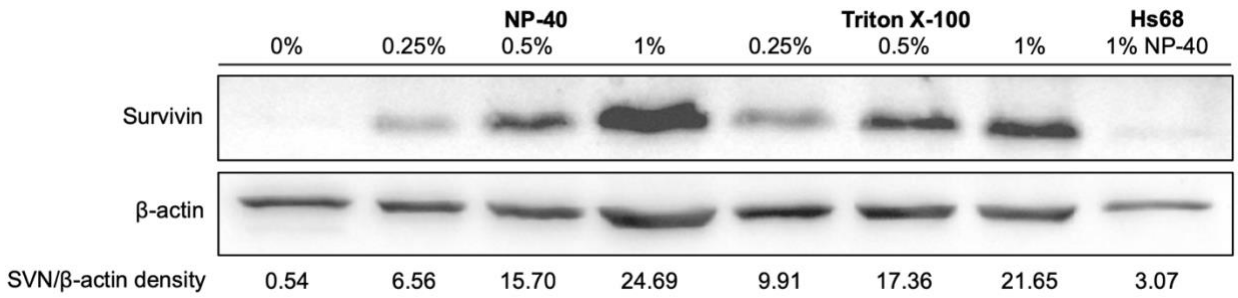

Figure 19. Western blot of MDA-MB-231 whole cell lysates using different concentrations of non-ionic detergents (NP-40 and Triton X-100). Hs68 whole cell lysate serving as the biological negative control demonstrated no survivin band.

These results suggest that NP-40 provided a more consistent lysis across the tested concentrations, with that 1% NP-40 emerging as the optimal non-ionic detergent condition for use in combination with the survivin ELISA. In addition, Hs68 cells may serve as a suitable biological negative control for survivin detection and quantification.

IV.3 Cancer cell lines express higher survivin level compared to a non-cancer cell line

Equal number of cells of MDA-MB-231, HCT116, and Hs68 (each $2x10^7$ cells) were lysed with 1% NP-40. Both MDA-MB-231 and HCT116 yield significantly higher survivin concentration than Hs68 (**Table 11**, **Figure 20**).

Table 11. Survivin expression of MDA-MB-231, HCT116, and Hs68 cell lysate.

Cell lines	**Survivin concentration (ng/mL) (n=3)**	**p value (compared to Hs68)**
MDA-MB-231	5.19 ± 0.69	0.0052
HCT116	9.04 ± 1.47	0.0017
Hs68*	1.18 ± 1.05	NA

*Hs68 (human fibroblasts) serves as a biological negative control.

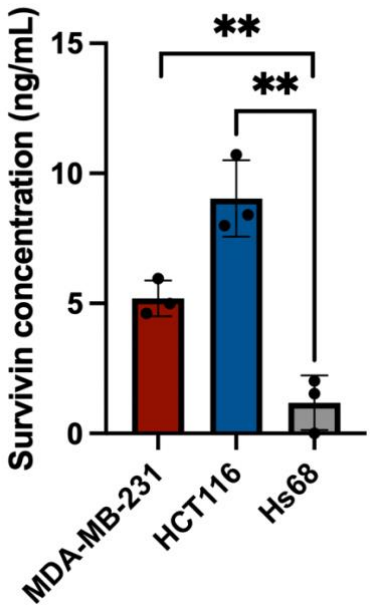

Figure 20. Cell lysates from cancer cell lines (MDA-MB-231 and HCT116) yield significantly higher concentration of survivin compared to a non-cancer cell line (Hs68).

IV.4 Survivin quantification in conditioned media of cancer cell line cultures

IV.4.1 Exosome concentration with centrifugal filtering

ELISA

Conditioned media collected from both MDA-MB-231 and HCT116 cells yielded significantly higher survivin concentrations following the addition of 1% NP-40, reflecting the combined concentration of free soluble and exosomal survivin (**Table 12**, **Figure 21**). In contrast, cell lysates from Hs68 cells, used as a biological negative control showed very low survivin level (1.18 ± 1.05 ng/mL).

Table 12. Survivin concentration in non-lysed and lysed samples.

Cell lines	Survivin concentration (ng/mL) (n=3)	p value

	Non-lysed CM (0%)	Lysed CM (1% NP-40)	
MDA-MB-231	4.45 ± 0.41	5.77 ± 0.61	0.0348
HCT116	8.30 ± 0.31	14.55 ± 2.21	0.0083

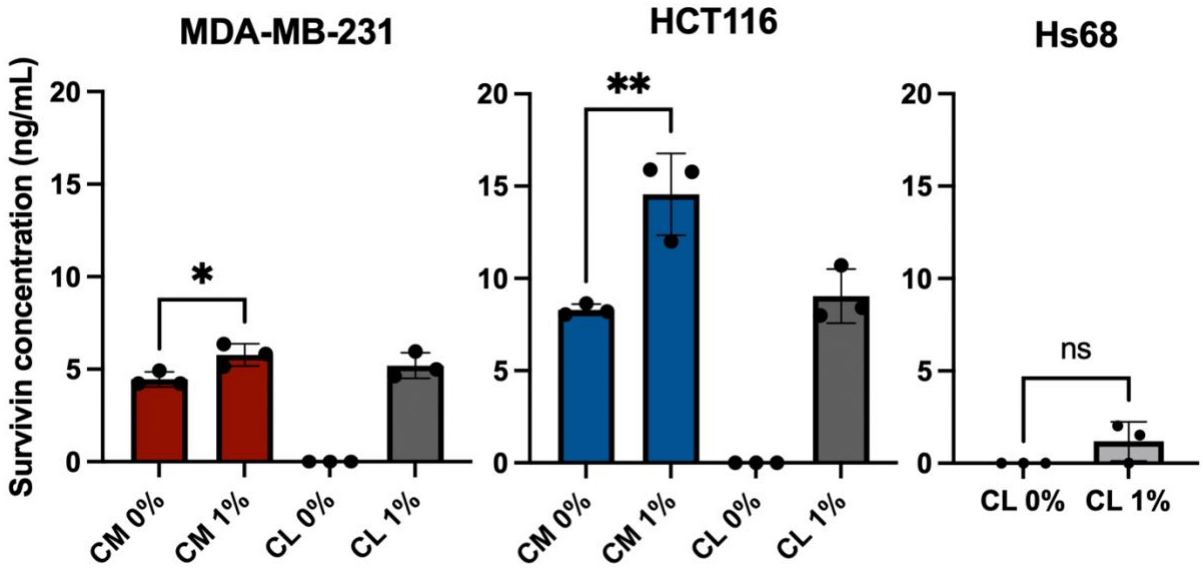

Figure 21. Survivin concentration of conditioned media and cell lysates obtained from three different cell lines (MDA-MB-231, HCT116, and Hs68).

Conditioned media of Hs68 were not measured since the cell lysates failed to show a significant expression of survivin.

Western blot

The survivin concentrations measured by ELISA were validated by Western blot analysis. Both MDA-MB-231 and HCT116 cell samples showed a thicker survivin band following the addition of 1% NP-40 (**Figure 22**). Syntenin-1 was used as the exosome marker and loading control.

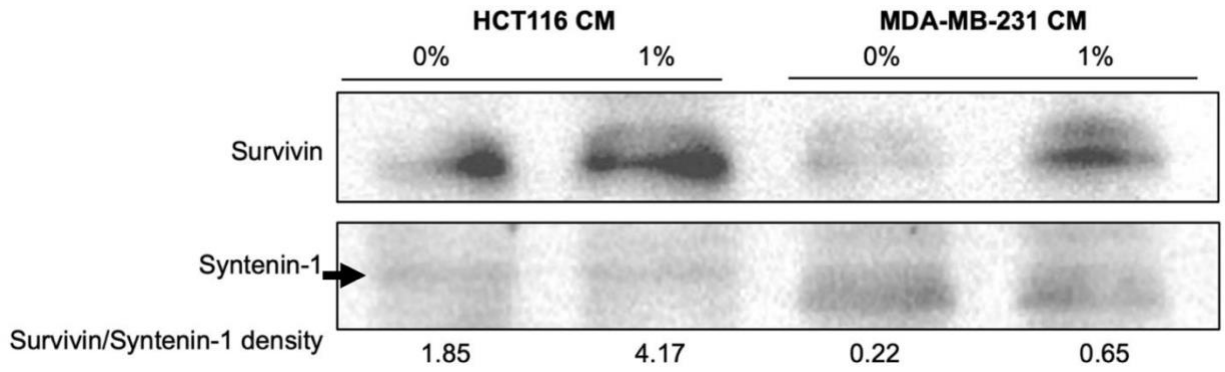

Figure 22. Survivin (16 kDa) Western blot of conditioned medium containing exosomes from HCT116 and MDA-MB-231 cells without and with the addition of 1% NP-40. Syntenin-1 (MW 32 kDa) (arrow) is used as the exosome marker and loading control.

Nanoparticle tracking analysis (NTA) for exosome characterisation

1 mL of conditioned medium was set aside after serial centrifugations and prior to exosome concentration, and subsequently analysed using the NTA with the NanoSight NS3000 (Malvern Panalytical, Malvern, United Kingdom). The mean vesicle size was 119.0 ± 49.5 nm, consistent with expected size range of exosomes (30-200 nm). The mean particle concentration was $1.39 \times 10^9 \pm 2.77 \times 10^7$ particles/mL. These results confirmed the presence of small EVs in the collected conditioned media (**Figure 23**).

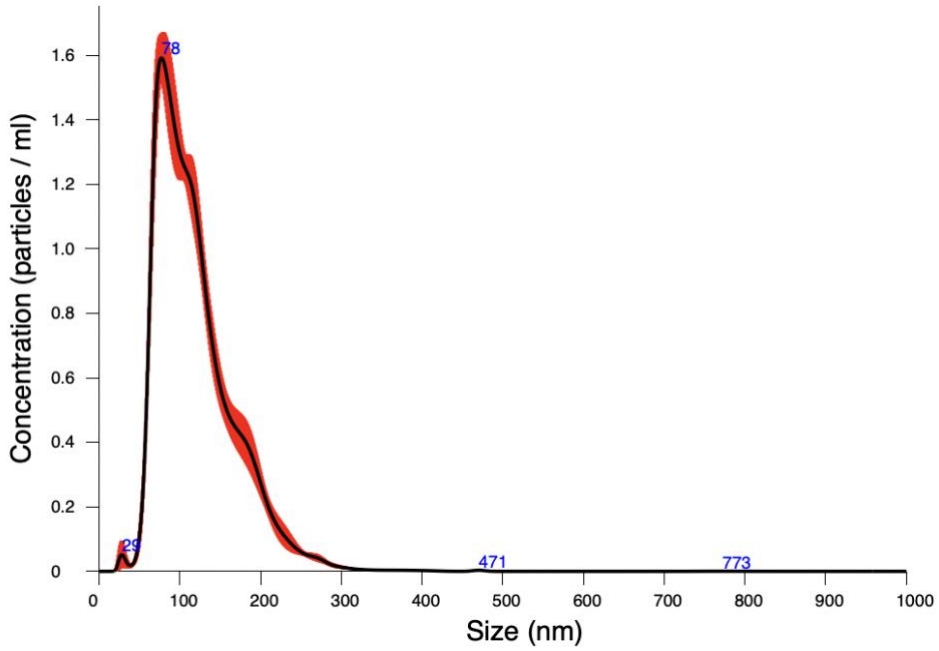

Figure 23. Extracellular vesicles concentration and size in HCT116 conditioned medium determined with nanoparticle tracking analysis (NanoSight NS3000).

IV.4.2 Exosome with polyethylene glycol (PEG) precipitation

Conditioned medium was mixed in equal volumes with 20% PEG 6000 to achieve a final concentration of 10% PEG 6,000, and incubated overnight (minimum of 8 hours) at 4°C. The mixture was then centrifuged at 5,250 x g for 1 hour at 4°C, resulting in the formation of a white precipitate (pellet) at the bottom of the tube. This pellet was presumed to contain survivin, survivin-enriched exosomes, and other proteins. It was resuspended in an appropriate volume of PBS and 25x protease inhibitor cocktail. The sample was divided into two treatment conditions: one with 1% NP-40 and one without, prior to analysis by Western blot and ELISA.

Western blot

Survivin bands were visibly stronger in conditioned medium (CM) treated 1% NP-40 compared to untreated controls (0% NP-40) for both HCT116 and MDA-MB-231 (**Figure 24**). Cell lysate (CL) samples were used as negative (0% NP-40) and positive (1% NP-40) controls. Syntenin-1 was used as an exosome marker and a loading control. CM concentrated with PEG precipitation (**Figure 24**) exhibited higher syntenin-1 expression than those concentrated with centrifugal filtration (**Figure 22**), suggesting greater exosome enrichment with PEG precipitation.

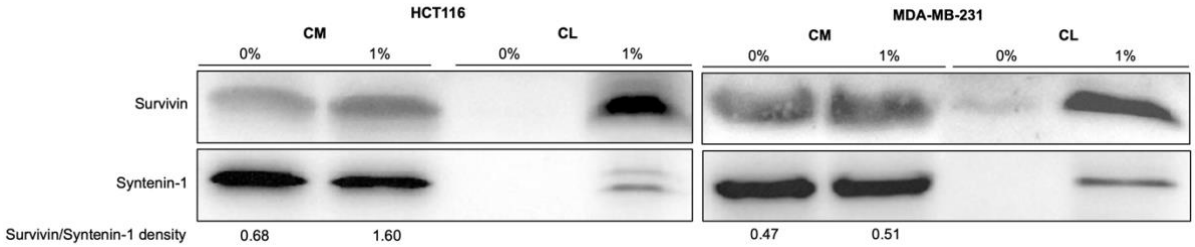

Figure 24. Survivin (16 kDa) western blot of conditioned medium containing exosomes from HCT116 and MDA-MB-231 cells without and with the addition of 1% NP-40. Syntenin-1 (MW 32 kDa) is used as the exosome marker and loading control.

IV.5 Survivin quantification in human serum

IV.5.1 Interference of NP-40 towards Survivin Quantikine® standard curve

Consistent with the findings in subsection IV.1.3, the OD readings of the standard curve prepared with 1% NP-40 were only slightly reduced compared to those without NP-40 (**Figure 25**). This suggests that 1% NP-40 does not significantly interfere with the survivin-antibody binding in the commercial Survivin Quantikine® ELISA kit.

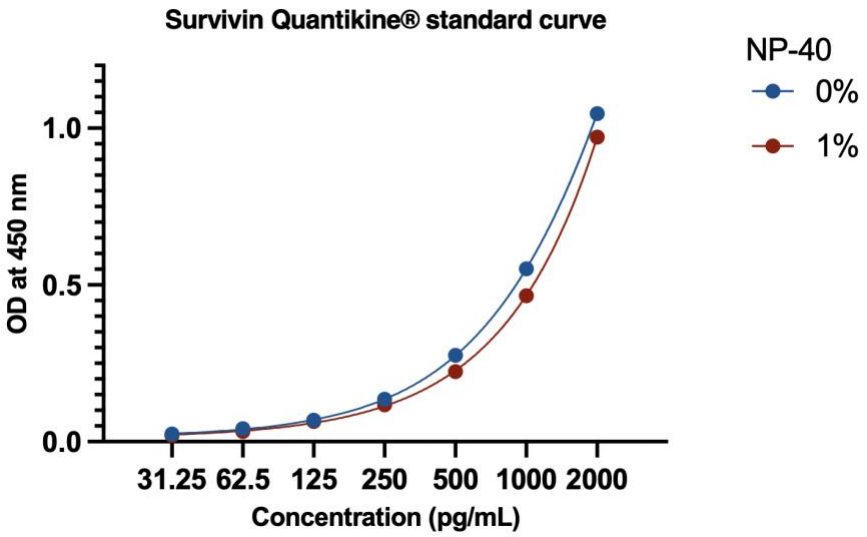

Figure 25. Survivin Quantikine® standard curve

with 1% NP-40 (red) and without NP-40 (blue) (n=2).

IV.5.2 Survivin quantification in plain serum and PEG-precipitated serum

IV.5.2.1 Characteristics of subjects

The characteristics of subjects providing serum samples are summarised in **Table 13**. The average age of cancer patients (63.1 ± 7.7) and healthy controls were significantly different (32.6 ± 0.6) ($p<0.001$). Meanwhile, the sex distribution was similar between these two groups.

Table 13. Characteristics of subjects.

Variables	**Cancer patients (n=15)**	**Healthy controls (n=5)**	**p-value**
Age, years	63.1 ± 7.7	32.6 ± 0.6	**<0.001**
Sex, n (%)			
Male	6 (40%)	2 (40%)	>0.999
Female	9 (60%)	3 (60%)	

IV.5.2.2 PEG precipitation and the addition of 1% NP-40 increases survivin concentrations only in cancer patients' serum

Table 14 and **Figure 26** present the comparison of survivin concentrations in plain serum and PEG-precipitated serum, with and without the addition of 1% NP-40. In cancer patients, the addition of 1% NP-40 to plain serum resulted in a significant increase in survivin levels (0%: 90.03 ± 9.35 vs 1%: 102.4 ± 7.63 pg/mL, p=0.0309), whereas no significant change was observed in healthy controls. A similar pattern was seen in PEG-precipitated serum, where survivin concentration significantly increased in cancer patients with the addition of 1% NP-40 (0%: 102.7 ± 25.35 vs 1%: 132.7 ± 42.13 pg/mL, p=0.0068).

Table 14. Survivin concentration in plain serum and PEG-precipitated serum.

Conditions[a]	**Survivin concentration (pg/mL), Mean ± SD**	**p-value**[b]
Cancer patients		
A: 0%	90.03 ± 9.35	**A vs B: 0.0309 (*)**
B: 1%	102.4 ± 7.63	A vs C: 0.5071 (ns)
C: PEG + 0%	102.7 ± 25.35	B vs D: 0.1780 (ns)
D: PEG + 1%	132.7 ± 42.13	**C vs D: 0.0068 (**)**
		A vs D: <0.0001 (****)
Healthy controls		
A: 0%	85.99 ± 9.90	A vs B: >0.9999 (ns)
B: 1%	85.32 ± 19.01	A vs C: >0.9999 (ns)
C: PEG + 0%	79.14 ± 12.79	B vs D: >0.9999 (ns)
D: PEG + 1%	94.71 ± 17.45	C vs D: 0.8069 (ns)
		A vs D: >0.9999 (ns)

[a]A and B are plain serum, C and D are PEG-precipitated serum; 0% indicates no NP-40, 1% indicates the addition of 1% NP-40 into the sample.

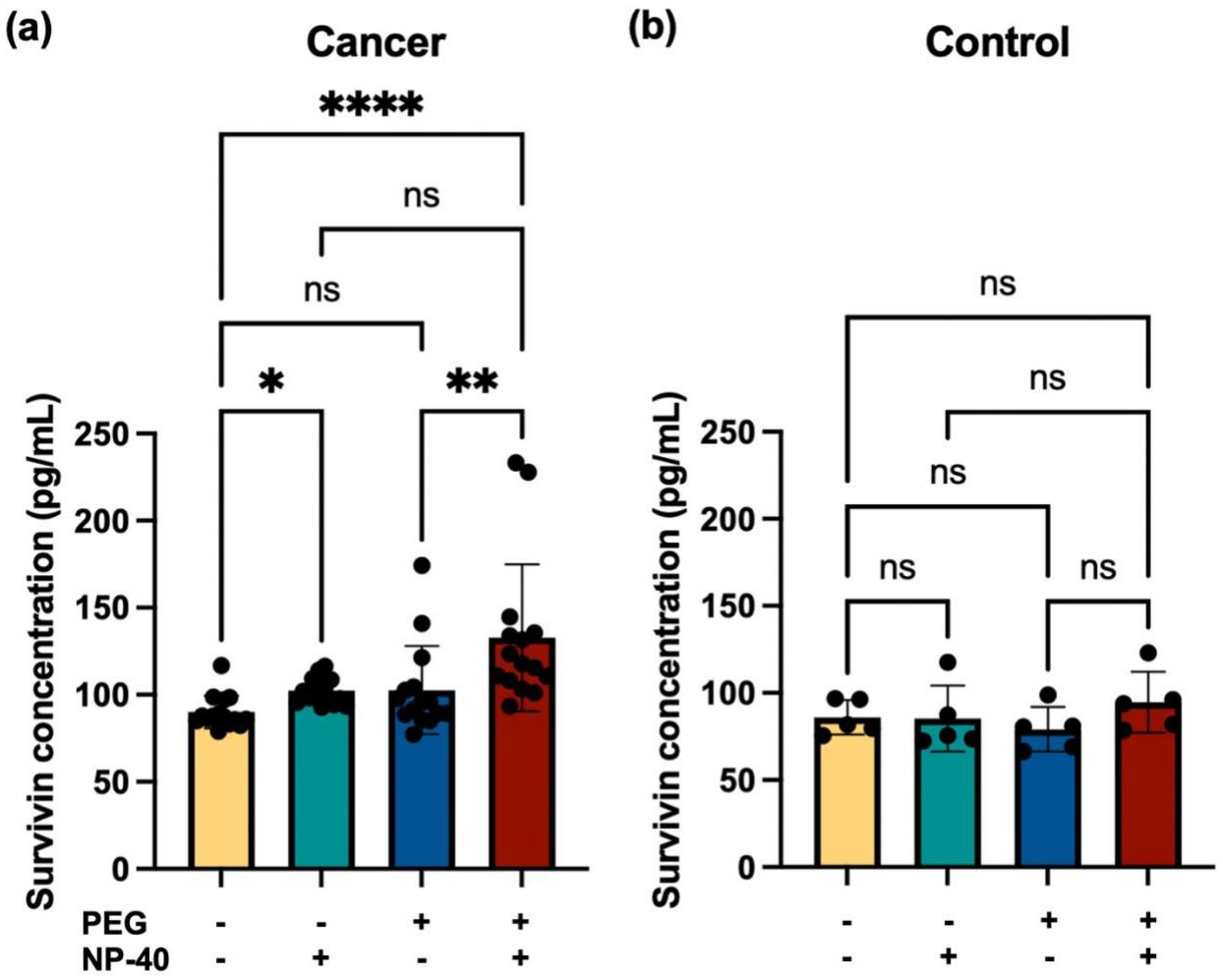

Figure 26. Survivin concentration in plain and PEG-precipitated serum of (a) cancer patients and (b) healthy controls.

Without the addition of NP-40, survivin concentrations remained comparable between plain serum (90.03 ± 9.35 pg/mL) and PEG-precipitated serum (102.7 ± 25.35 pg/mL) (p=0.1780). However, with 1% NP-40, survivin concentrations were significantly higher in PEG-precipitated samples (132.7 ± 42.13 pg/mL) compared to the plain serum samples (102.4 ± 7.63 pg/mL). In contrast, no significant differences were observed between conditions in healthy controls.

In summary, the combination of PEG precipitation and 1% NP-40 treatment resulted in significantly higher survivin concentrations compared to plain serum only in cancer patients.

IV.5.2.3 Comparison of survivin concentrations in cancer patients and healthy controls

Across all conditions, survivin concentrations were consistently higher in cancer patients compared to healthy controls (**Table 15**, **Figure 27**). However, statistically significant differences between the two groups were only observed in PEG-precipitated samples, both without (C: PEG + 0%) and with (D: PEG + 1%) the addition of NP-40.

Table 15. Comparison of survivin concentrations in the serum of cancer patients and healthy controls under different conditions.

Conditions[a]	**Survivin concentration (pg/mL)**		**p-value**[b]
	Cancer patients (n=15)	**Healthy controls (n=5)**	
0%	90.03 ± 9.35	85.99 ± 9.90	0.2661 (ns)
1%	102.4 ± 7.63	85.32 ± 19.01	0.0526 (ns)
PEG + 0%	102.7 ± 25.35	79.14 ± 12.79	**0.0290 (*)**
PEG + 1%	132.7 ± 42.13	94.71 ± 17.45	**0.0146 (*)**

[a]0% indicates no NP-40, 1% indicates the addition of 1% NP-40 into the sample.

[b]Mann-Whitney U test, statistically significant if $p<0.05$ (typed in bold)

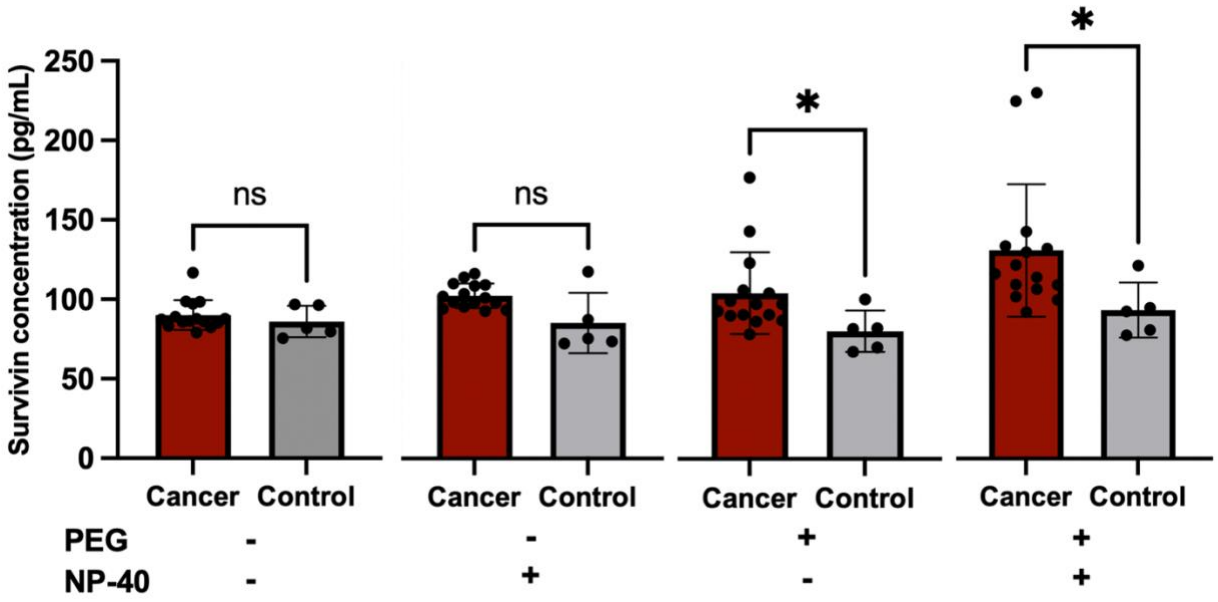

Figure 27. Comparison of survivin concentrations in the serum of cancer patients and healthy controls under different conditions.

Survivin optimal cut-off

Receiver operating characteristic (ROC) curve analysis was performed to determine the optimal cut-off value for survivin concentration in distinguishing cancer patients from healthy controls (**Figure 28**). The optimal cut-off value was identified as 98.7 pg/mL, yielding a sensitivity of 93% and specificity of 80%.

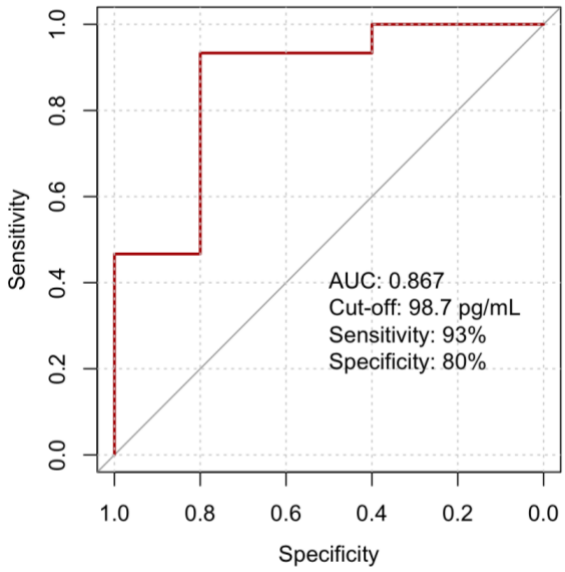

Figure 28. ROC curve analysis for survivin cut-off.

IV.5.3 Serum survivin concentrations in different cancer types

Serum samples from cancer patients in this study were obtained from 15 subjects, including 9 with advanced-stage (III and IV) ovarian cancer and 6 with advanced stage prostate cancer. The mean serum survivin concentrations for each cancer type are presented in **Table 16**. No significant differences in survivin concentrations were observed between ovarian cancer and prostate cancer patients under all conditions.

Table 16. Serum survivin concentration comparison in ovarian cancer and prostate cancer.

Conditions[a]	**Survivin concentration (pg/mL)**		**p-value**[b]
	Ovarian cancer (n=9)	**Prostate cancer (n=6)**	
0%	88.2 ± 6.6	92.8 ± 12.6	0.434
1%	103.7 ± 7.8	100.3 ± 7.6	0.418
PEG 0%	101.1 ± 28.5	105.0 ± 22.2	0.768
PEG 1%	139.0 ± 52.2	123.4 ± 20.8	0.437

[a]0% indicates no NP-40, 1% indicates the addition of 1% NP-40 into the sample.

[b]Mann-Whitney U test, statistically significant if $p<0.05$ (typed in bold)

V. Discussion

V.1 Survivin ELISA optimisation

V.1.1 Optimal dilutions of capture and detection antibodies

ELISA is a highly sensitive immunoassay designed to detect and quantify specific proteins or antibodies. In this study, the capture antibody binds to survivin immobilised on the ELISA plate, while the biotinylated detection antibody binds to the survivin-capture antibody complex, forming a “sandwich” structure that facilitates detection. An HRP-conjugated enzyme reacts with the TMB substrate to produce a measurable colour change, whose intensity (measured as optical density) is directly proportional to the amount of bound detection antibody, and thus to the survivin concentration (ThermoFisher Scientific, 2024b).

The linear portion of the absorbance curve is essential for accurate quantification, as this is where OD increases proportionally with antigen concentration. Selecting antibody dilutions within this linear range ensures reliable, reproducible, and accurate results (Crowther, 2000). As shown in **Figure 13**, the ELISA curves exhibit a typical sigmoidal dose-response, reflecting saturation of binding sites at higher concentrations and diminished binding at lower concentrations.

Optimal dilutions were determined to be 1:1,250 for the capture antibody and 1:1,000 for the detection antibody. These dilutions fall within the linear range of the standard curve, providing a strong and stable signal while minimising reagent usage, potential issues like the hook effect (Ekka, 2023), or non-specific binding (Crowther, 2000). The hook effect refers to the phenomenon in which at very high antibody concentrations, excess unbound antibody interferes with antigen binding, reducing the signal (Ekka,

2023). The final concentrations of 4.96 µg/mL for the capture antibody and 1.1 µg/mL for the detection antibody were also selected based on their ability to achieve an optimal optical density (OD) (1.0 to 1.5) that provides the best balance between sensitivity and specificity (Lequin, 2005).

V.1.2 Standard curve development

The survivin standard curve showed a typical sigmoidal dose-response pattern characteristic of a well-performing sandwich ELISA, indicating robust antibody-antigen interactions and an adequate dynamic range for quantifying varying concentrations of survivin. The linear portion of the curve (where the optical density correlates proportionally with antigen concentration) was defined between 6.25 ng/mL and 50 ng/mL. This range is critical for quantitative analysis, offering the highest accuracy, sensitivity, and reproducibility.

Below 6.25 ng/mL, the curve plateaued, suggesting reduced sensitivity and limited reliability for detecting low antigen levels. This is likely due to assay background noise and decreased antibody binding efficiency at very low concentrations. As a result, samples with concentration below this threshold may yield less reliable quantitative data.

In practice, the survivin standard curve serves as a reference to determine the concentration of survivin in experimental samples. To ensure accurate results, samples with expected concentrations outside the linear range should be appropriately diluted or concentrated to fall within this range. Including assay replicates and appropriate controls (e.g., blanks and standards) helps minimise variability and validate measurement accuracy (Crowther, 2000; Lequin, 2005).

The survivin standard protein used in this ELISA was a recombinant 6His-tagged survivin produced in *E. coli* Rosetta cells and purified using a combination of immobilised metal affinity chromatography (IMAC) and ion-exchange chromatography, followed by concentration in 3kDa MWCO centrifugal filters. This process generated a high-purity protein preparation, confirmed by SDS-PAGE and intact mass spectrometry. The final preparation was stored in aliquots at −80°C to minimise freeze-thaw degradation. Although such recombinant preparations offer a reliable and reproducible survivin standard, they may differ from survivin in biological samples in tertiary structure, post-translational modifications, and association with other proteins. This could affect antibody binding in ELISA. Moreover, even under optimal storage, prolonged freezing can result in subtle conformational changes or aggregation that may influence assay calibration. For greater accuracy and specificity, future studies should perform periodic evaluation of the stability of the survivin standard over time with appropriate methods, such as SDS-PAGE or Western blot to monitor purity, degradation, and aggregation, and protein quantification techniques (e.g. BCA assay, mass spectrometry) to detect changes in concentration.

V.1.3 Interference of non-ionic detergents with survivin ELISA

This experiment aimed to evaluate whether non-ionic detergents at various concentrations interfered with ELISA performance. These detergents were intended for lysing exosomal membranes and releasing survivin to facilitate its detection. Non-ionic detergents are preferred over ionic detergents in ELISA for several reasons. First, non-ionic detergents are milder compared to ionic detergents and are less likely to denature proteins – they do not disrupt protein structures as aggressively as ionic detergents, which

can alter protein folding due to their strong ionic interactions with protein surfaces. They are also less likely to interfere with the specific binding interactions between antibodies and antigens, whereas ionic detergents can disrupt these interactions by altering the charge environment around the proteins, which is critical for antibody-antigen binding. Non-ionic detergents help reduce nonspecific binding and background noise in ELISA (Kenny and Dunsmoor, 1987). Non-ionic detergents, like NP-40 and Triton-X, are preferred in ELISA applications due to their mildness and less denaturing nature. They are able to solubilize membrane proteins when it is critical to preserve the function and native structure as well as retain native protein-protein interactions (ThermoFisher Scientific, 2024a).

Both NP-40 and Triton X-100 were tested across different concentrations of 0.25%, 0.5%, and 1%. The differences in properties and main applications between NP-40 and Triton X-100 are summarised in **Table 17** (Johnson, 2023).

Table 17. Properties and main applications of NP-40 and Triton X-100 (Johnson, 2023).

Properties	**NP-40**	**Triton X-100**
MW (Da) monomer	680	625
MW (Da) micelle	90,000	90,000
CMC (mM) 25ºC	0.059	0.2-0.9
Aggregation no.	-	100-155
Cloud point (ºC)	45-50	65
Average micellar weight	-	80,000
Strength	Mild	Mild
Dialysable	No	No
Applications	Enzyme immunoassays, IP, Membrane solubilization	IEF

CMC: critical micelle concentration; IP: immunoprecipitation; IEF: isoelectric focusing;

Impact of detergent concentration

Both non-ionic detergents, NP-40 and Triton X-100, show minimal interference with ELISA at concentrations up to 1%. This suggests that membrane lysis using either detergent at or below this concentration does not compromise survivin ELISA performance. These findings are consistent with several previous studies (Qualtiere, Anderson and Meyers, 1977; Dimitriadis, 1979). At 2% NP-40, there was a marked reduction in OD readings, particularly at higher protein concentrations, whereas 2% Triton X-100 caused only a modest downward shift. This suggests am inhibitory effect, due to interference with antibody-antigen binding or of the enzyme-substrate reaction essential for the colorimetric signal. Moreover, this interference appears to be detergent-specific, likely due to the impact of their distinct chemical properties on assay performance.

Mechanisms of interference

Higher concentrations of non-ionic detergents may alter the conformation of antibodies or antigens, or displace pre-bound antigen, thereby reducing binding efficiency (McCabe, Fletcher and Jones, 1988). The hydrophobic interactions facilitated by these detergents may also mask epitopes or change the native state of the protein involved in the assay (Wong *et al.*, 1985; Charles A Janeway *et al.*, 2001). In addition, higher detergent concentrations may also interfere with HRP, the enzyme linked to the secondary antibody, either through direct inhibition or by disrupting the enzyme-substrate interaction, affecting

ELISA's ability to produce a detectable signal. However, this study lacks the mechanistic details on how detergents interfere with the antibody-antigen binding at molecular levels.

These results suggest that 1% or lower concentration of both non-ionic detergents tested will minimize interference with ELISA. The practical implications are: (1) For sample preparations containing non-ionic detergents for downstream analyses with ELISA, maintaining the concentrations at or below 1% is recommended to avoid negative impacts on the assay results - this information is vital for researchers designing experiments where buffers containing non-ionic detergents are used, (2) When faced with unexpected ELISA results, researchers need to consider the composition of the sample preparation, especially the detergent concentrations, as a potential source of variability.

Potential alternative detergents

Alternative detergents that may be effective at lysing the lipid bilayer membranes of EVs while remining compatible with ELISA include sterol-binding detergents (digitonin, saponin) and zwitterionic detergents (CHAPS). Digitonin and saponin offer cholesterol-dependent permeabilisation that is more selective, making them suitable for mild release of intravesicular proteins while preserving the epitopes (Bio-Techne, 2025). However, the reversible action of saponin may require its continuous presence during detection to prevent vesicle re-sealing. Zwitterionic detergents such as CHAPS can disrupt vesicle membranes with minimal protein denaturation (Hjelmeland, 1980). Ultimately, the choice of detergent requires balancing lysis efficiency with downstream compatibility with ELISA.

Future studies should explore the compatibility of these potential alternative detergents with ELISA.

V.2 Non-ionic detergent lysis protocol optimisation

This experiment aimed to determine the optimal conditions of NP-40 and Triton X-100 for the lysis of lipid bilayer membrane by testing three concentrations (0.25%, 0.5%, and 1 %) and assessing their ability to release intracellular survivin from MDA-MB-231 cells, a human breast adenocarcinoma cell line. The effectiveness of each detergent across the three concentrations was evaluated by measuring the survivin concentration in whole cell lysates with ELISA, total protein concentration with BCA assay, and detecting survivin bands with Western blot.

NP-40 demonstrated a clear dose-dependent increase in lysis efficacy. Survivin concentration peaked at 16.08 ng/mL with 1% NP-40, a trend that was consistent with BCA assay results, where the total protein concentration reached 4081.49.3 µg/mL at the same detergent concentration (**Figure 17**). In contrast, Triton X-100 showed a less consistent pattern, with a decrease in total protein concentration and survivin concentration at 0.5% Triton X-100.

To assess survivin expression in non-cancerous cells, 1% NP-40 was also applied to Hs68 cells, a human fibroblast line. Western blot analysis revealed only faint survivin bands in Hs68 whole cell lysates, supporting its use as a biological negative control for survivin detection. Human fibroblast cell lines as the appropriate healthy/negative control for survivin expression has been reported in a previous study (Adinolfi, Pellegrino and Baldini, 2015). Although survivin is also expressed in normal cells, its expression is

developmentally regulated and very low in most terminally differentiated adult tissues (Fukuda and Pelus, 2006).

V.3 Survivin expression in cancer cells

V.3.1 Cancer cell lines express higher survivin levels compared to non-cancer cell lines

Both MDA-MB-231 and HCT116 cell lines showed significantly higher survivin concentrations compared to the non-cancerous Hs68 fibroblast line when lysed with 1% NP-40. This result is consistent with previous findings demonstrating survivin overexpression in a wide range of cancers relative to normal tissues. Survivin is typically expressed in proliferating cells during the G2 and M phase of the cell cycle, whereas in cancer cells its expression persists throughout the entire cell cycle, contributing to uncontrolled proliferation and resistance to apoptosis (Wuttke *et al.*, 2009). Survivin expression is especially very low in terminally differentiated cells, such as Hs68. Hs68 is terminally differentiated human fibroblasts isolated from the foreskin of an aspartoacylase deficiency White male patient (American Type Culture Collection, 2024). The elevated survivin levels in cancer cell lines support its role in promoting cancer cell survival and its potential as a therapeutic target and a biomarker.

V.3.2 Differential survivin expression across cancer types

In this study, HCT116 cells showed higher intracellular survivin levels than MDA-MB-231 cells. This observation aligns with previous findings that survivin expression varies across cancer types. A bioinformatics analysis of The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases revealed differential expression of the

BIRC5 gene (which encodes survivin) across 33 types of human cancers (**Figure 29**) (Zhang *et al.*, 2023). Our findings align with the analysis results that survivin expression in colorectal adenocarcinoma (COAD) is slightly higher than breast invasive carcinoma (BRCA). However, this analysis did not explore the differential expression of survivin in different subtypes or classification of each cancer type. As widely known, different subtypes of breast cancer demonstrate different characteristics and prognosis, suggesting a difference in the biology of each subtype. For example, low or negligible survivin-2B expression was shown to be strongly correlated with HER-negativity and triple negativity (Khan *et al.*, 2014). In the future, it will be interesting to explore the differential expression of survivin across cancer subtypes, especially in cancers known to have high survivin expression.

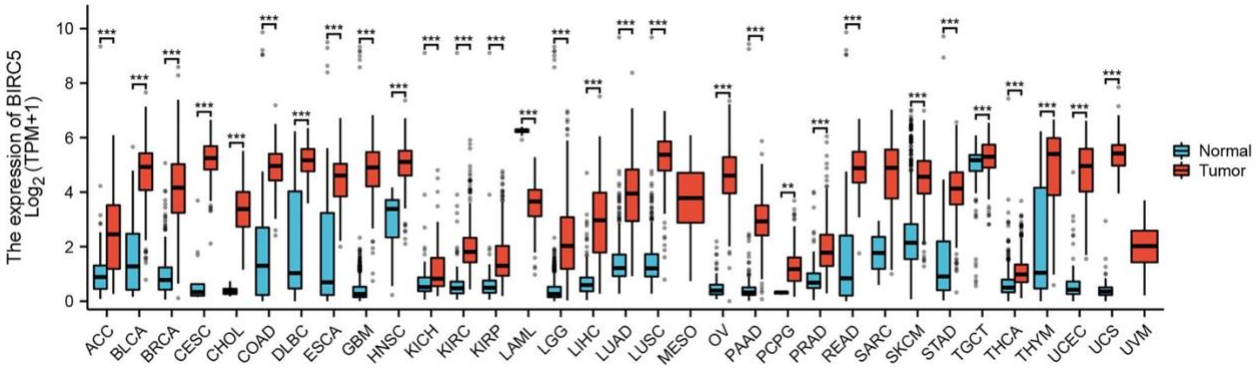

Figure 29. Differential survivin (BIRC5) expression across various types of cancer (Zhang *et al.*, 2023).

BIRC5 expression level in 33 cancer types from TCGA datasets, compared to the corresponding normal tissues from GTEx datasets. *p < 0.05; **p < 0.01; ***p < 0.001. ACC: adrenocortical carcinoma (n = 77; 122 for normal); BLCA: bladder urothelial carcinoma (n = 407; 28 for normal); BRCA: breast invasive carcinoma (n = 1099; 292 for normal); CESC: cervical squamous cell carcinoma (n = 306; 13 for normal); CHOL: cholangiocarcinoma (n = 36; 9 for normal); COAD: colon adenocarcinoma (n = 290; 349 for normal); DLBC: lymphoid neoplasm diffuse large B cell lymphoma (n = 47; 444 for normal); ESCA:

esophageal carcinoma (n = 182; 666 for normal); GBM: glioblastoma (n = 166; 1157 for normal); HNSC: head and neck squamous cell carcinoma (n = 520; 44 for normal); KICH: kidney chromophobe (n = 66; 53 for normal); KIRC: kidney renal clear cell carcinoma (n = 531; 100 for normal); KIRP: kidney renal papillary cell carcinoma (n = 289; 60 for normal); LAML: acute myeloid leukemia (n = 173; 70 for normal); LGG: brain lower grade glioma (n = 523; 1152 for normal); LIHC: liver hepatocellular carcinoma (n = 371; 160 for normal); LUAD: lung adenocarcinoma (n = 515; 347 for normal); LUSC: lung squamous cell carcinoma (n = 498; 338 for normal); MESO: mesothelioma (n = 87); OV: ovarian serous cystadenocarcinoma (n = 427; 88 for normal); PAAD: pancreatic adenocarcinoma (n = 179; 171 for normal); PCPG: pheochromocytoma and paraganglioma (n = 182; 3 for normal); PRAD: prostate adenocarcinoma (n = 496; 152 for normal); READ: rectum adenocarcinoma (n = 93; 318 for normal); SARC: sarcoma (n = 262; 2 for normal); SKCM: skin cutaneous melanoma (n = 469; 813 for normal); STAD: stomach adenocarcinoma (n = 414; 210 for normal); TGCT: testicular germ cell tumors (n = 154; 165 for normal); THCA: thyroid carcinoma (n = 338; 512 for normal); THYM: thymoma (n = 119; 446 for normal); UCEC: uterine corpus endometrial carcinoma (n = 181; 101 for normal); UCS: uterine carcinosarcoma (n = 57; 78 for normal); UVM: uveal melanoma (n = 79).

V.4 Survivin quantification in conditioned media of cancer cell cultures

V.4.1 Exosome concentration via centrifugal filtering

Exosomes in conditioned media were concentrated using centrifugal filtration with Amicon® Ultra-15K 3K filter devices. This method is commonly used to concentrate extracellular vesicles (EVs) in biological fluids by applying centrifugal force to drive liquid through a membrane with a defined molecular weight (MW) cut-off. Molecules larger than 3 kDa, including survivin (MW ~16 kDa) and exosomes (MW ~10 to 1200 MDa) (B. A. Brown *et al.*, 2020), are retained above the membrane, while smaller molecules and solvents pass through into the filtrate (Sartorius, 2025).

Conditioned media from both MDA-MB-231 and HCT116 cell cultures showed significantly increased survivin concentrations following treatment with 1% NP-40, consistent with the disruption of exosomal membranes and release of their contents. Western blot analysis supported these findings: lysed CM samples exhibited stronger survivin bands compared to non-lysed controls. Syntenin-1, which serves as the exosome marker and loading control, remained relatively constant but appeared broad and smeared across all lanes. This may be attributed to several mechanisms during the centrifugal filtering process. Prolonged spinning during the centrifugal filtering through filtration membranes can apply shear stress to exosomes. If exosomes rupture during this process, their contents, including syntenin-1, are released and may be more susceptible to aggregation and misfolding. Syntenin-1 may also interact with or adsorb to the filter membrane, or aggregate at the membrane interface, particularly if the concentration is too high or the filter clogs. Due to these mechanisms, syntenin-1 may not migrate properly on gels during electrophoresis, resulting in smeared and diffuse bands (Vergauwen *et al.*, 2017).

Nanoparticle tracking analysis confirmed the presence of small vesicles in the CM, with a mean size of 119.0 ± 49.5 nm, consistent with the expected size of small EVs or exosomes (30-200 nm) and a particle concentration of $1.39 \times 10^9 \pm 2.77 \times 10^7$ particles/mL. Consistent with the whole cell lysate findings (section V.3), HCT116 CM demonstrate higher survivin expression than MDA-MB-231 CM in both ELISA and Western blot.

These findings suggest that exosomes play a crucial role in the secretion and distribution of survivin in the tumour microenvironment. How cancer cells rely on exosome-mediated survivin transport also suggests that targeting exosome biogenesis,

release, or uptake could be a potential therapeutic approach. Inhibiting these pathways may reduce survivin levels in the extracellular environment, sensitizing cancer cells to apoptosis and improving treatment efficacy. The differential survivin levels in lysed (1% NP-40) versus non-lysed (0%) conditioned medium versus normal cells imply that the addition of 1% NP-40 increases the sensitivity of survivin ELISA and allows for the quantification of total (exosomal and free) survivin in biological fluids such as human plasma or serum.

V.4.2 Exosome concentration with polyethylene glycol (PEG) precipitation

In HCT116 and MDA-MB-231 cell lines, treatment of conditioned medium (CM) with 1% NP-40 resulted in higher survivin expression compared to untreated CM. This increase supports the hypothesis that survivin release is associated with exosomes, and that NP-40 effectively disrupts exosomal membranes, releasing survivin into the solution. Syntenin-1 served as an exosome marker and loading control. Western blot analysis revealed denser syntenin-1 bands in CM concentrating using PEG precipitation (Figure 23) than in samples processed by centrifugal filtration (**Figure 22**), suggesting superior exosome enrichment with the PEG method. This observation is consistent with the results of previous studies (Rider, Hurwitz and Meckes, 2016; Weng *et al.*, 2016; Ludwig *et al.*, 2018).

PEG precipitation is commonly used for exosome concentration due to its simplicity, cost-effectiveness, and compatibility with diverse biological fluids. PEG acts by reducing the solubility of exosomes in solution, causing them to aggregate and precipitate out of suspension when centrifuged (**Figure 30**) (Liu, Ma and Kang, 2022). The most common

theoretical models explaining this process are the theory of excluded volume and attractive depletion forces (Lohmann and Strube, 2020). The excluded volume model suggests that molecules precipitate due to decreased hydration in the presence of the polymer (Atha and Ingham, 1981). Meanwhile, the attractive depletion forces model proposes that the precipitation occurs due to the attraction of molecules caused by the osmotic pressure of the PEG solution (Marenduzzo, Finan and Cook, 2006)

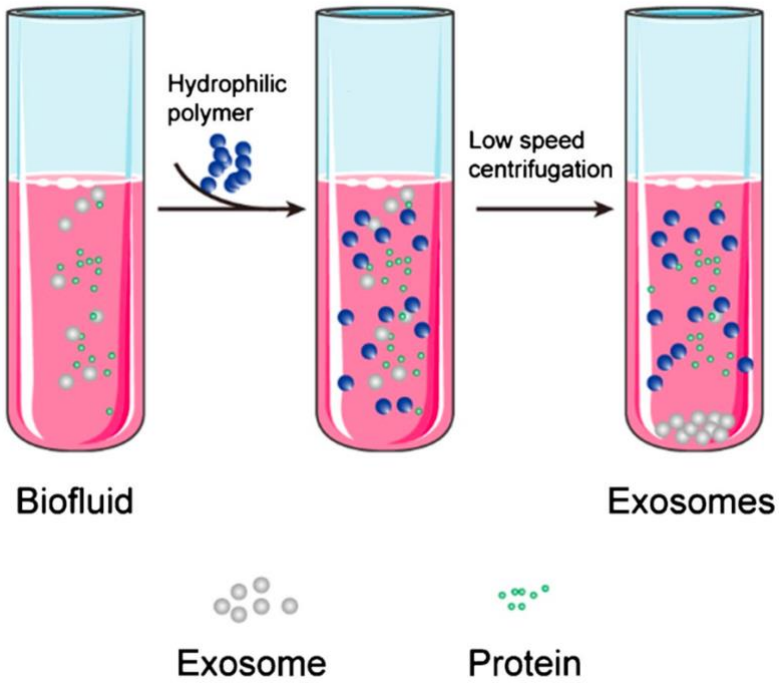

Figure 30. PEG precipitation-based exosome concentration (Liu, Ma and Kang, 2022).

PEG precipitation offers several advantages that make it an attractive method for exosome concentration, particularly in clinical research settings. It is highly scalable and yields a relatively high amount of exosomes without causing deformation or damage to vesicle structure. Unlike methods such as ultracentrifugation and size-exclusion chromatography, PEG precipitation is simple, cost-effective, and does not require specialised equipment or technical expertise. Furthermore, it is well-suited for processing

large sample volumes and can be easily integrated with downstream detection platforms for analysing protein and genetic material content (Coumans *et al.*, 2017; Liu, Ma and Kang, 2022). These advantages support its potential utility in translational research and wider clinical applications.

However, PEG precipitation also presents several limitations, primarily concerning purity and specificity. This method frequently co-precipitates non-exosomal contaminants including soluble proteins, lipoproteins, protein aggregates, virions, and immune complexes (Li *et al.*, 2017; Konoshenko *et al.*, 2018). As a result, PEG precipitation is not suitable for detailed descriptive or functional analyses of exosomes (Gámez-Valero *et al.*, 2016). In addition, the efficiency of precipitation depends on numerous factors such as PEG concentration, molecular weight, and incubation time, all of which require optimisation to ensure consistent and reproducible results. Despite these drawbacks, PEG precipitation remains a popular and promising method for exosome concentration due to its accessibility and practicality. Researchers continuously attempt to optimise PEG-based protocols and improve their efficiency and reproducibility across various biological samples to achieve better exosome isolation (Rider, Hurwitz and Meckes, 2016; Ludwig *et al.*, 2018; Singh *et al.*, 2023; Wang *et al.*, 2023; Yu *et al.*, 2024).

V.5 Survivin quantification in human serum

In this study, the average age of cancer patients and healthy controls were significantly different. Age-matching was not plausible due to the limited availability of serum samples from healthy individuals of similar age i.e., people around the age of 60 are most likely to have comorbid conditions, such as degenerative diseases (Lechler *et al.*, 2011),

inflammatory diseases (Gravina *et al.*, 2017), or diabetes mellitus (Liu *et al.*, 2015; Xu *et al.*, 2018), that may influence survivin levels.

PEG precipitation and 1% NP-40 treatment increases survivin yield only in the serum of cancer patients

A significant increase in serum survivin concentration, both in plain and PEG-precipitated samples, was observed only in cancer patients upon the addition of 1% NP-40, but not in healthy controls. This suggests that survivin is released in an exosome-associated form by cancer cells and is largely absent from non-cancerous samples. These findings also support the use of exosomal survivin as a potential cancer-specific biomarker.

While the mechanisms underlying the release of exosomal survivin by cancer cells have not been fully elucidated, previous studies indicate that this process may be stress-induced. For instance, survivin has been found to be associated with heat shock proteins (Hsp), including Hsp70 and Hsp90, in the exosomes from serum-starved HeLa cells conditioned medium (Khan *et al.*, 2009), suggesting a response to cellular stress. These survivin-containing exosomes have been shown to re-enter surrounding cancer cells and cause therapeutic resistance, rapid proliferation, and increased metastatic potential *in vitro* (Khan *et al.*, 2009). Exosomal survivin is also enriched in breast cancer cells treated with paclitaxel, and these exosomes significantly enhance cancer cell survival and chemoresistance (Kreger *et al.*, 2016). Breast-cancer derived exosomal survivin has been shown to convert fibroblasts into myofibroblasts by up-regulating SOD1. This in turn promotes proliferation, epithelial-mesenchymal transition (EMT), and stemness of breast cancer (Li *et al.*, 2020). Similarly, sublethal proton irradiation (3 Gy) leads to a significant

accumulation of survivin in the exosomal fraction of the conditioned medium from serum-starved HeLa cells (Khan *et al.*, 2009). These findings suggest that cancer cells employ exosomal survivin in the tumour microenvironment to survive under stressful conditions. Further mechanistic studies are required to elucidate the pathways involved in the release of exosomal survivin.

In this study, 10% PEG 6000 was used to concentrate and precipitate exosomes. Without NP-40 treatment, survivin concentrations were not significantly different between plain serum (0%) and PEG-precipitated serum (PEG + 0%), indicating that PEG precipitation alone does not enhance survivin detection, likely because survivin remains encapsulated within intact exosomes. However, PEG-precipitated samples treated with 1% NP-40 (PEG + 1%) showed higher survivin concentrations than their non-precipitated counterparts (1%) in both cancer patients and healthy controls, although the increase was not statistically significant. One possible explanation is that PEG precipitation may also help remove unwanted interfering substances in the serum (**Table 18**, **Figure 31**) that could inhibit antibody binding or produce non-specific background signals (Tate and Ward, 2004; Schwickart *et al.*, 2014). PEG precipitation does so by leaving these substances soluble in the supernatant. Precipitation with PEG has been shown to remove human anti-animal antibodies (HAAA) interference (Tate and Ward, 2004).

Table 18. Immunoassay interferences (Tate and Ward, 2004).

Nature of Interferences	Examples
Interferences that alter the measurable analyte concentration in the sample	• Hormone binding proteins • Pre-analytical factors, e.g., anticoagulants, sample storage • Autoanalyte antibodies

Interferences that alter antibody binding	• Heterophile antibodies • Human anti-animal antibodies (HAAA) • High-dose hook effect

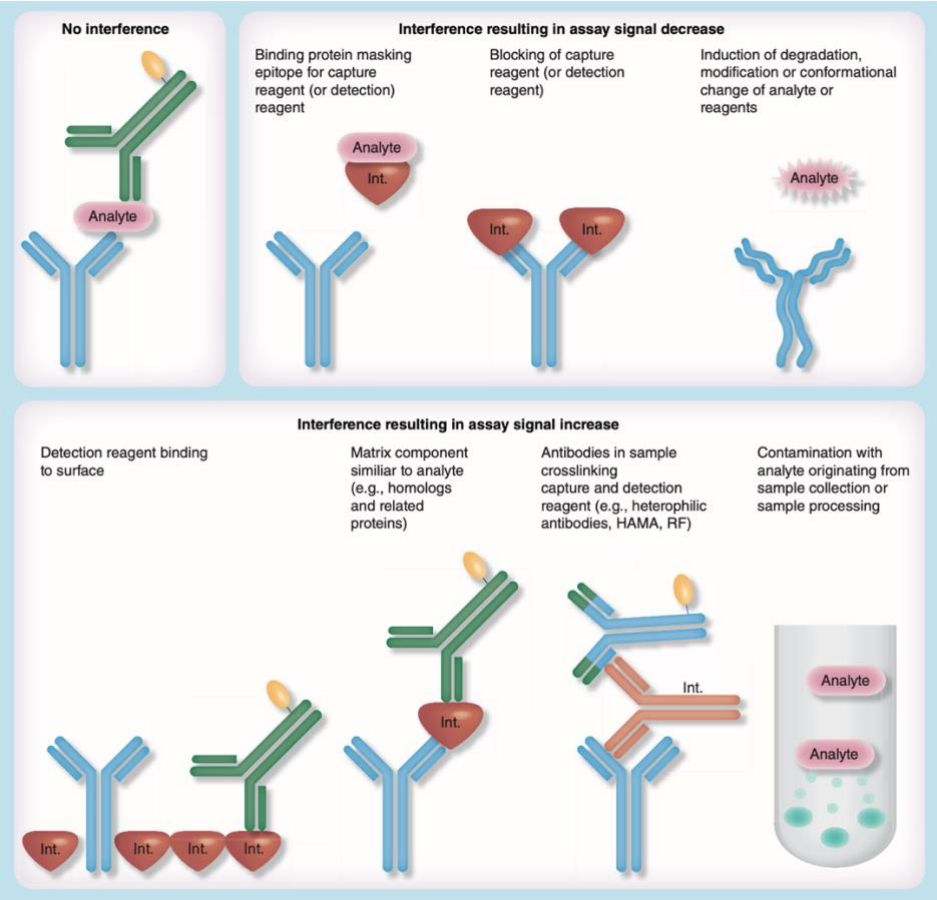

Figure 31. Interferences in immunoassay (Schwickart *et al.*, 2014).

By improving the quality and purity of the sample, PEG-precipitated serum produces clearer and stronger OD signals compared to plain serum. Therefore, the combined method of lysis with NP-40 and PEG precipitation may be an effective strategy to enhance the detection of survivin and potentially other exosome-encapsulated biomarkers in clinical and research settings.

Survivin concentration is significantly higher in cancer patients compared to healthy controls

In this study, survivin concentrations were consistently higher in cancer patients compared to healthy controls under all serum conditions, although statistical significance was observed only in PEG-precipitated samples. Serum survivin level has repeatedly been demonstrated to be higher in patients with various cancer types, including breast cancer, colon cancer, ovarian cancer, prostate cancer (Khan *et al.*, 2012), pancreatic cancer (Dong *et al.*, 2015), and many others, compared to healthy individuals, making it a potential diagnostic or screening biomarker. Meanwhile, in terms of prognosis, high serum survivin levels have been associated with unfavourable clinicopathologic parameters, including tumour invasion (Dong *et al.*, 2015), poorer survival (Demirci *et al.*, 2023), lower response rates (Demirci *et al.*, 2023). Interestingly, greater increase in survivin level after chemotherapy in malignant mesothelioma patients has been linked to longer survival outcomes i.e., both progression-free survival and overall survival (Goričar *et al.*, 2015). However, different studies have reported a very wide range of serum survivin levels among cancer patients with median values ranging from 4.1 to 176 pg/mL. This variability in serum survivin levels may be attributed to several factors, including differences in cancer types, stages of cancer, methods of sample collection, ELISA kits and protocols, which often lack standardisation. The potential surface location of survivin on exosomes and its release into the circulation through active secretion or passive shedding from dead cancer cells, may also contribute to the heterogeneity in the measured serum levels. Therefore, further research is needed to validate survivin as a

biomarker in clinical settings, along with the development of standardized assays and protocols to ensure its reliability and reproducibility.

Survivin cut-off for cancer detection

The ROC curve was used to evaluate the diagnostic performance of survivin concentration in PEG-precipitated serum samples treated with 1% NP-40 to differentiate between cancer patients and healthy controls. The resulting AUC was 0.867, indicating good discriminatory ability and is generally considered clinically useful (Çorbacıoğlu and Aksel, 2023). The optimal cut-off value of 98.7 pg/mL has a sensitivity of 93% and a specificity of 80%. This high sensitivity makes this cut-off suitable for screening purposes, although the specificity still needs to be improved to be acceptable for diagnostic purposes. Larger and more diverse cohorts are required to validate these findings.

This cut-off value is slightly lower than that reported in a previous study which also aimed to determine the optimal cut-off value of serum survivin in the early diagnosis of cancer (Gunaldi *et al.*, 2018). One possible explanation is that the serum samples of cancer patients analysed in this study were collected around 1-2 years prior to analysis and stored at -80^{o}C. This may lead to gradual degradation or loss of detectable survivin in the samples. In contrast, serum samples from healthy controls were collected only around 6 weeks prior to analysis. The difference in the sample age and handling may have influenced the comparability of survivin concentrations between groups and contributed to a lower cut-off value.

Survivin concentration in different types of cancer

In this study, survivin concentrations were not significantly different between the two cancer types analysed i.e., ovarian and prostate cancer. This finding contrasts with the bioinformatics analysis of *BIRC5* gene expression using TCGA and GTEx datasets, which shows that ovarian serous cystadenocarcinoma (OV) exhibits higher survivin (*BIRC5*) expression compared to prostate adenocarcinoma (PRAD) (**Figure 29**). This suggests that circulating survivin may not always represent or correspond to the intracellular expression levels.

There are several possible explanations to this. First, survivin is predominantly an intracellular protein (Wheatley and Altieri, 2019) and the extent to which it is secreted or released into the circulation may vary depending on cancer type, regardless of its intracellular gene expression level. Tumours with higher levels of necrosis or apoptosis, leading to cell lysis, may release more survivin into the circulation, such as found in patients previously receiving chemotherapy (Naumnik *et al.*, 2009; Goričar *et al.*, 2015). Secondly, like any other biomarkers, the amount of detectable survivin in serum may correlate more strongly with tumour burden (size and metastasis) (Kut, Mac Gabhann and Popel, 2007; Karnezis *et al.*, 2012) or vascular density (Ali, Sheta and El Mohsen, 2011) rather than gene expression. In addition, like any other proteins (Dutta and Jain, 2023), survivin also undergoes post-translational modifications and proteasomal degradation that could influence how much it is released into the circulation (Nogueira-Ferreira *et al.*, 2013).

In terms of technical factors, the variability in serum biomarker levels, including survivin, may be attributed to pre-analytical variables including sample collection, processing, handling and storage (Agrawal *et al.*, 2018). Pre-analytical variability is widely

acknowledged as a significant challenge in biomarker development. For instance, delay between blood collection and centrifugation or freezing can lead to protein degradation (Gottfried-Blackmore *et al.*, 2020; Zeng *et al.*, 2024) or release of the intracellular protein (Huang *et al.*, 2021), altering the biomarker concentration. Repeated freeze-thaw cycles can also degrade biomarkers, reducing their stability and thus measurement (Lee, Kim and Shin, 2015). Standardisation of pre-analytical and analytical protocols is required to ensure reproducibility and reliability in biomarker assays, including for serum survivin.

Further studies should attempt to perform paired analysis of tumour tissue and serum samples from the same patients to better understand the relationship between intratumoural survivin expression and its release into the circulation.

V.6 Strengths and limitations

This study has a number of strengths. It introduces a high-throughput, practical, clinically translatable method for the quantification of total survivin (including both free-soluble and exosomal survivin). By incorporating PEG precipitation, this study provides a cost-effective and simple alternative for exosome concentration, making it more accessible for research and clinical applications by eliminating the need for specialised equipment and expertise. The improved sensitivity of survivin measurement observed only in cancer patients provides clinical relevance by supporting its role as a potential biomarker in cancer.

On the other hand, some limitations in this study remain to be addressed: (1) small sample size and lack of generalisability – this study included only 15 cancer patients (with only two cancer types) and 5 healthy controls, limiting the statistical power and

generalisability of the findings; (2) lack of mechanistic studies – this study does not include mechanistic studies of how survivin is released via exosomes, limiting biological understanding; (3) single validation method – validation relies on only Western blot; (4) no longitudinal follow-up of patients – due to the constraints of time, the study does not assess survivin levels over time or in response to treatment, which would provide insights on its prognostic or therapeutic monitoring potential.

VI. Conclusions and Future Directions

This study presents a high-throughput, practical method for quantifying exosomal survivin in biological samples by combining ELISA with 1% NP-40 lysis and PEG 6000 precipitation. We demonstrate that 1% NP-40 is an optimal condition for lysing lipid-bilayer membrane, while PEG precipitation concentrates exosomes and may reduce assay interference, thereby improving detection sensitivity. The improvement in measurement was observed only in cancer patients, suggesting that exosomal survivin is specifically released by cancer cells, potentially as a survival mechanism within the tumour microenvironment.

These findings support the clinical potential of this method for assessing survivin levels in cancer, with applications in diagnosis, prognosis, and monitoring. The ability to measure both free and exosomal survivin could improve biomarker sensitivity and specificity, particularly in malignancies where survivin is associated with poor outcomes and therapeutic resistance.

Future research should focus on elucidating the mechanisms by which survivin is packaged into and released via exosomes by cancer cells, identifying the conditions and

factors that regulate this process, and exploring its functional roles in tumour progression, metastasis, and therapeutic resistance. This could reveal new targets for therapeutic intervention. This method should be tested on a larger set of clinical samples across various types and stages of cancer, evaluating its link with clinicopathologic parameters, such as treatment response, survival, metastasis, invasion, tumour burden, etc. This will help establish the clinical relevance and utility of exosomal survivin. To further prove the reliability of this method, it should be compared with other existing methods, such as flow cytometry or mass spectrometry, in terms of sensitivity, specificity, and reproducibility.

To further improve the accuracy and specificity of exosomal survivin detection, several experiments could be considered in future studies. Exosomal survivin should be combined with or incorporated into a multi-marker panel of exosomal proteins associated with or specific to cancer. This will improve the specificity of the assay, especially in heterogenous clinical samples. Mass spectrometry-based proteomic profiling could be used to validate the presence of survivin within preparations, identify co-enriched biomarkers, and detect potential post-translational modifications that may influence its detectability on other methods, including Western blot and ELISA. These approached would allow for a more comprehensive characterisation of exosomal cargo and support the development of a more accurate and clinically applicable diagnostic platform.

Paired analysis of survivin expression in tumour tissue and serum samples from the same patients should also be conducted to understand the relationship between survivin intratumoural expression and its release into the circulation. Given the broad expression of survivin across various cancer types, this method could be applied to different malignancies to evaluate its generalisability. These efforts will help define the

biological significance and clinical utility of survivin and its exosome-associated form as both a biomarker and a potential therapeutic target in cancer.

Acknowledgements

I would like to express my tremendous gratitude towards the following people, without whom I would not have been able to complete this research and degree:

- My supervisors: Professor Shisong Jiang and Dr Su Myat Phyu whose insights and knowledge into the project guided me through this research.
- All members of the Jiang Lab: Dr Wenshu Lu, Dr Demin Li, Yuqian Ou, Sichen Liu, Constantinos Demetriou, Hanjun Zhao, and Zachary Rawlinson who have provided me with practical help on experiments, insights and knowledge into the research, and mental support in the laboratory.
- The Jardine Foundation, which has provided me with a full-ride scholarship to study at the University of Oxford.
- My family (mum, dad, and brother) and beloved partner (Julius Alberto Liem) who, despite being in a different time zone and thousands of miles away from me (Indonesia and Australia), have always supported me along the way, provided me with tremendous mental support and encouragements, and who have always been there for me. No words can describe how thankful I am to have them in my life. They are also one of the reasons I had been so motivated in finishing this thesis – so I can finally go home and be reunited with them.

- My Indonesian friends aka peer support group in Oxford (Aji Wijaya Abadi, Nisrina Nur Fatiha, and Avinindita Nura Lestari) who have been keeping me sane in Oxford with fortnightly day trips to pretty towns in the UK.
- Prenali Dwisthi Sattwika, my amazingly generous and inspiring senior, who kindly and persistently helped me acquire the healthy controls serum samples from her research group's clinical trial despite all the obstacles and challenges.
- Last but not least, my annoying, absent-minded landlord (and, arguably, friend), Dexter J. Perrott, who has been kind enough to provide me with witty and, most of the time, boring conversations and petty rows during my stay in Oxford.

Appendices

Publications

Wijaya, W., Phyu, S.M., Jiang, S., 2024. Extracellular Vesicle (EV) Survivin for Cancer Diagnostics and Therapeutics: A Review. FBL 29, 302.

https://doi.org/10.31083/j.fbl2908302

References

‘A Phase 1, Multicentre, Open-label, Nonrandomised, First-in-human Study of OVM-200 as a Therapeutic Vaccine in Patients With Locally Advanced or Metastatic Non-Small Cell Lung Cancer, Ovarian Cancer, and Prostate Cancer’ (2021). Available at: https://clinicaltrials.gov/study/NCT05104515 (Accessed: 1 November 2021).

Abels, E.R. and Breakefield, X.O. (2016) ‘Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake’, *Cellular and molecular neurobiology*, 36(3), pp. 301–312. Available at: https://doi.org/10.1007/s10571-016-0366-z.

Adinolfi, B., Pellegrino, M. and Baldini, F. (2015) ‘Human dermal fibroblasts HDFa can be used as an appropriate healthy control for PMMA nanoparticles-survivin molecular beacon cellular uptake studies’, *Biomedicine & Pharmacotherapy*, 69, pp. 228–232. Available at: https://doi.org/10.1016/j.biopha.2014.12.003.

Agrawal, L. *et al.* (2018) ‘Understanding Preanalytical Variables and their Effects on Clinical Biomarkers of Oncology and Immunotherapy’, *Seminars in cancer biology*, 52(Pt 2), pp. 26–38. Available at: https://doi.org/10.1016/j.semcancer.2017.12.008.

Akers, J.C. *et al.* (2013) ‘MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development’, *PloS one*, 8(10), p. e78115.

Ali, E.M., Sheta, M. and El Mohsen, M.A. (2011) ‘Elevated serum and tissue VEGF associated with poor outcome in breast cancer patients’, *Alexandria Journal of Medicine*, 47(3), pp. 217–224. Available at: https://doi.org/10.1016/j.ajme.2011.07.003.

Allenson, K. *et al.* (2017) ‘High prevalence of mutantKRAS in circulating exosome-derived DNA from early-stage pancreatic cancer patients’, *Annals of Oncology*, 28(4), pp. 741–747.

Al-Nedawi, K. *et al.* (2009) ‘Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR’, *Proceedings of the National Academy of Sciences*, 106(10), pp. 3794–3799.

Altieri, D.C. (2003) ‘Survivin, versatile modulation of cell division and apoptosis in cancer’, *Oncogene*, 22(53 REV. ISS. 7), pp. 8581–8589. Available at: https://doi.org/10.1038/sj.onc.1207113.

Ambrosini, G., Adida, C. and Altieri, D.C. (1997) ‘A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma’, *Nature Medicine*, 3(8), pp. 917–921. Available at: https://doi.org/10.1038/nm0897-917.

American Type Culture Collection (2024) *Hs68 - CRL-1635 | ATCC*. Available at: https://www.atcc.org/products/crl-1635 (Accessed: 5 July 2024).

Andreu, Z. and Yáñez-Mó, M. (2014) ‘Tetraspanins in Extracellular Vesicle Formation and Function’, *Frontiers in Immunology*, 5, p. 442. Available at: https://doi.org/10.3389/fimmu.2014.00442.

Aspe, J.R. (2014) *Exosomal Survivin-T34A: A Novel, Potential Cancer Therapeutic*. Loma Linda University. Available at: https://scholarsrepository.llu.edu/etd/201 (Accessed: 28 February 2024).

Atha, D.H. and Ingham, K.C. (1981) ‘Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume.’, *Journal of Biological Chemistry*, 256(23), pp. 12108–12117. Available at: https://doi.org/10.1016/S0021-9258(18)43240-1.

Atikcan, S. *et al.* (2006) ‘Correlation between survivin expression and prognosis in non-small cell lung cancer’, *Respiratory Medicine*, 100(12), pp. 2220–2226. Available at: https://doi.org/10.1016/j.rmed.2006.02.031.

Baietti, M.F. *et al.* (2012) ‘Syndecan–syntenin–ALIX regulates the biogenesis of exosomes’, *Nature Cell Biology*, 14(7), pp. 677–685. Available at: https://doi.org/10.1038/ncb2502.

Becker, A. *et al.* (2016) ‘Extracellular vesicles in cancer: cell-to-cell mediators of metastasis’, *Cancer cell*, 30(6), pp. 836–848.

Bhatt, M. *et al.* (2022) ‘SDS-PAGE and Western Blotting: Basic Principles and Protocol’, in R. Deb et al. (eds) *Protocols for the Diagnosis of Pig Viral Diseases*. New York, NY: Springer US, pp. 313–328. Available at: https://doi.org/10.1007/978-1-0716-2043-4_23.

Bio-Techne (2025) *Fixation and Permeabilization in Immunocytochemistry/Immunofluorescence (ICC/IF) | Bio-Techne*. Available at: https://www.bio-techne.com/applications/imaging/immunocytochemistry/fixation-and-permeabilization-in-icc-if (Accessed: 11 August 2025).

Brady, S.W. *et al.* (2015) ‘PI3K-independent mTOR activation promotes lapatinib resistance and IAP expression that can be effectively reversed by mTOR and Hsp90 inhibition’, *Cancer biology & therapy*, 16(3), pp. 402–411.

Brown, B.A. *et al.* (2020) ‘Charge Detection Mass Spectrometry Measurements of Exosomes and other Extracellular Particles Enriched from Bovine Milk’, *Analytical chemistry*, 92(4), pp. 3285–3292. Available at: https://doi.org/10.1021/acs.analchem.9b05173.

Brown, M. *et al.* (2020) ‘The role of survivin in the progression of pancreatic ductal adenocarcinoma (PDAC) and a novel survivin-targeted therapeutic for PDAC’, *PLoS ONE*, 15(1), pp. 1–18. Available at: https://doi.org/10.1371/journal.pone.0226917.

Buzas, E.I. *et al.* (2014) ‘Emerging role of extracellular vesicles in inflammatory diseases’, *Nature Reviews. Rheumatology*, 10(6), pp. 356–364. Available at: https://doi.org/10.1038/nrrheum.2014.19.

Caldas, H., Honsey, L. and Altura, R. (2005) ‘Survivin 2alpha: A novel Survivin splice variant expressed in human malignancies’, *Molecular cancer*, 4, p. 11. Available at: https://doi.org/10.1186/1476-4598-4-11.

Chang, W.-H. *et al.* (2021) ‘KRAS-dependent cancer cells promote survival by producing exosomes enriched in Survivin’, *Cancer Letters*, 517, pp. 66–77. Available at: https://doi.org/10.1016/j.canlet.2021.05.031.

Chang, W.-H., Cerione, R.A. and Antonyak, M.A. (2021) ‘Extracellular Vesicles and Their Roles in Cancer Progression’, *Methods in molecular biology (Clifton, N.J.)*, 2174, pp. 143–170. Available at: https://doi.org/10.1007/978-1-0716-0759-6_10.

Charles A Janeway, J. *et al.* (2001) ‘The interaction of the antibody molecule with specific antigen’, in *Immunobiology: The Immune System in Health and Disease. 5th edition*. Garland Science. Available at: https://www.ncbi.nlm.nih.gov/books/NBK27160/ (Accessed: 8 May 2024).

Chen, G. *et al.* (2018) ‘Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response’, *Nature*, 560(7718), pp. 382–386.

Chen, X. *et al.* (2016) ‘Survivin and tumorigenesis: Molecular mechanisms and therapeutic strategies’, *Journal of Cancer*, 7(3), pp. 314–323. Available at: https://doi.org/10.7150/jca.13332.

Clayton, A. *et al.* (2001) ‘Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry’, *Journal of Immunological Methods*, 247(1–2), pp. 163–174. Available at: https://doi.org/10.1016/s0022-1759(00)00321-5.

Clos-Sansalvador, M. *et al.* (2022) ‘Commonly used methods for extracellular vesicles’ enrichment: Implications in downstream analyses and use’, *European Journal of Cell Biology*, 101(3), p. 151227. Available at: https://doi.org/10.1016/j.ejcb.2022.151227.

Colombo, M., Raposo, G. and Théry, C. (2014) ‘Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles’, *Annual review of cell and developmental biology*, 30, pp. 255–289.

Çorbacıoğlu, Ş.K. and Aksel, G. (2023) ‘Receiver operating characteristic curve analysis in diagnostic accuracy studies: A guide to interpreting the area under the curve value’, *Turkish Journal of Emergency Medicine*, 23(4), pp. 195–198. Available at: https://doi.org/10.4103/tjem.tjem_182_23.

Coumans, F.A.W. *et al.* (2017) ‘Methodological Guidelines to Study Extracellular Vesicles’, *Circulation Research*, 120(10), pp. 1632–1648. Available at: https://doi.org/10.1161/CIRCRESAHA.117.309417.

Crowther, J.R. (2000) *The ELISA Guidebook*. New Jersey: Humana Press. Available at: https://doi.org/10.1385/1592590497.

Dai, J. *et al.* (2020) 'Exosomes: key players in cancer and potential therapeutic strategy', *Signal Transduction and Targeted Therapy*, 5(1), pp. 1–10. Available at: https://doi.org/10.1038/s41392-020-00261-0.

Dejima, H. *et al.* (2017) 'Exosomal microRNA in plasma as a non-invasive biomarker for the recurrence of non-small cell lung cancer', *Oncology letters*, 13(3), pp. 1256–1263.

Demirci, N.S. *et al.* (2023) 'Is the serum level of survivin, an antiapoptotic protein, a potential predictive and prognostic biomarker in metastatic pancreatic cancer?', *Medicine*, 102(25), p. e34014. Available at: https://doi.org/10.1097/MD.0000000000034014.

Derin, D. *et al.* (2008) 'Serum levels of apoptosis biomarkers, survivin and TNF-alpha in nonsmall cell lung cancer', *Lung cancer*, 59(2), pp. 240–245.

Dimitriadis, G.J. (1979) 'Effect of detergents on antibody-antigen interaction', *Analytical Biochemistry*, 98(2), pp. 445–451. Available at: https://doi.org/10.1016/0003-2697(79)90165-9.

Dohi, T. *et al.* (2004) 'Mitochondrial survivin inhibits apoptosis and promotes tumorigenesis', *Journal of Clinical Investigation*, 114(8), pp. 1117–1127. Available at: https://doi.org/10.1172/JCI200422222.

Dong, H. *et al.* (2015) 'Survivin expression and serum levels in pancreatic cancer', *World Journal of Surgical Oncology*, 13(1), p. 189. Available at: https://doi.org/10.1186/s12957-015-0605-7.

Du, C. *et al.* (2000) 'Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition', *Cell*, 102(1), pp. 33–42. Available at: https://doi.org/10.1016/s0092-8674(00)00008-8.

Dutta, H. and Jain, N. (2023) 'Post-translational modifications and their implications in cancer', *Frontiers in Oncology*, 13, p. 1240115. Available at: https://doi.org/10.3389/fonc.2023.1240115.

Ekka, D. (2023) *Understanding the Hook Effect*. Available at: https://www.linkedin.com/pulse/understanding-hook-effect-dr-dipti-ekka/ (Accessed: 7 May 2024).

Fawzy, A. *et al.* (2012) 'Importance of serum levels of angiopoietin-2 and survivin biomarkers in non-small cell lung cancer', *Journal of the Egyptian National Cancer Institute*, 24(1), pp. 41–45.

Figel, S. *et al.* (2021) 'Wild type, dEX3 and 2B survivin isoforms localize to the tumor cell plasma membrane, are secreted in exosomes, and interact with extracellular

tubulin', *Biochemistry and Biophysics Reports*, 28, p. 101174. Available at: https://doi.org/10.1016/j.bbrep.2021.101174.

Fortugno, P. *et al.* (2002) 'Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function', *Journal of Cell Science*, 115(3), pp. 575–585. Available at: https://doi.org/10.1242/jcs.115.3.575.

Frazzi, R. (2021) 'BIRC3 and BIRC5: multi-faceted inhibitors in cancer', *Cell and Bioscience*, 11(1), pp. 1–14. Available at: https://doi.org/10.1186/s13578-020-00521-0.

Fukuda, S. and Pelus, L.M. (2006) 'Survivin, a cancer target with an emerging role in normal adult tissues', *Molecular Cancer Therapeutics*, 5(5), pp. 1087–1098. Available at: https://doi.org/10.1158/1535-7163.MCT-05-0375.

Fulda, S. (2009) 'Tumor resistance to apoptosis', *International Journal of Cancer*, 124(3), pp. 511–515. Available at: https://doi.org/10.1002/ijc.24064.

Galbo, P.M. *et al.* (2017) 'Circulating CD9+/GFAP+/survivin+ exosomes in malignant glioma patients following survivin vaccination', *Oncotarget*, 8(70), pp. 114722–114735. Available at: https://doi.org/10.18632/oncotarget.21773.

Gámez-Valero, A. *et al.* (2016) 'Size-Exclusion Chromatography-based isolation minimally alters Extracellular Vesicles' characteristics compared to precipitating agents', *Scientific Reports*, 6(1), p. 33641. Available at: https://doi.org/10.1038/srep33641.

Garg, H. *et al.* (2016) 'Survivin: A unique target for tumor therapy', *Cancer Cell International*, 16(1), pp. 1–14. Available at: https://doi.org/10.1186/s12935-016-0326-1.

Goksel, G. *et al.* (2007) 'Serum her-2/neu and survivin levels and their relationship to histological parameters in early-stage breast cancer', *Journal of international medical research*, 35(2), pp. 165–172.

Gonda, A. *et al.* (2018) 'Exosomal survivin facilitates vesicle internalization', *Oncotarget*, 9(79), p. 34919.

Goričar, K. *et al.* (2015) 'Serum Survivin Levels and Outcome of Chemotherapy in Patients with Malignant Mesothelioma', *Disease Markers*, 2015, p. 316739. Available at: https://doi.org/10.1155/2015/316739.

Gottfried-Blackmore, A. *et al.* (2020) 'Effects of processing conditions on stability of immune analytes in human blood', *Scientific Reports*, 10(1), p. 17328. Available at: https://doi.org/10.1038/s41598-020-74274-8.

Grant, B.D. and Donaldson, J.G. (2009) 'Pathways and mechanisms of endocytic recycling', *Nature reviews Molecular cell biology*, 10(9), pp. 597–608.

Gravina, G. *et al.* (2017) 'Survivin in autoimmune diseases', *Autoimmunity Reviews*, 16(8), pp. 845–855. Available at: https://doi.org/10.1016/j.autrev.2017.05.016.

Gunaldi, M. *et al.* (2018) ‘The value of serum survivin level in early diagnosis of cancer’, *Journal of Cancer Research and Therapeutics*, 14(3), p. 570. Available at: https://doi.org/10.4103/0973-1482.171369.

Guney, N. *et al.* (2006) ‘Serum and urine survivin levels in breast cancer’, *Medical Oncology*, 23, pp. 335–339.

Gurung, S. *et al.* (2021) ‘The exosome journey: from biogenesis to uptake and intracellular signalling’, *Cell Communication and Signaling*, 19(1), p. 47. Available at: https://doi.org/10.1186/s12964-021-00730-1.

Hanahan, D. and Weinberg, R.A. (2011) ‘Hallmarks of cancer: The next generation’, *Cell*, 144(5), pp. 646–674. Available at: https://doi.org/10.1016/j.cell.2011.02.013.

Hjelmeland, L.M. (1980) ‘A nondenaturing zwitterionic detergent for membrane biochemistry: design and synthesis’, *Proceedings of the National Academy of Sciences of the United States of America*, 77(11), pp. 6368–6370. Available at: https://doi.org/10.1073/pnas.77.11.6368.

Hong, B.S. *et al.* (2009) ‘Colorectal cancer cell-derived microvesicles are enriched in cell cycle-related mRNAs that promote proliferation of endothelial cells’, *BMC Genomics*, 10, p. 556. Available at: https://doi.org/10.1186/1471-2164-10-556.

Hu, W. *et al.* (2020) ‘Therapeutic Potentials of Extracellular Vesicles for the Treatment of Diabetes and Diabetic Complications’, *International Journal of Molecular Sciences*, 21(14), p. 5163. Available at: https://doi.org/10.3390/ijms21145163.

Huang, J. *et al.* (2015) ‘Influence of survivin-targeted therapy on chemosensitivity in the treatment of acute myeloid leukemia’, *Cancer Letters*, 366(2), pp. 160–172. Available at: https://doi.org/10.1016/j.canlet.2015.05.033.

Huang, J. *et al.* (2021) ‘Assessing the Preanalytical Variability of Plasma and Cerebrospinal Fluid Processing and Its Effects on Inflammation-Related Protein Biomarkers’, *Molecular & Cellular Proteomics*, 20, p. 100157. Available at: https://doi.org/10.1016/j.mcpro.2021.100157.

Hurley, J.H. (2015) ‘ESCRTs are everywhere’, *The EMBO Journal*, 34(19), pp. 2398–2407. Available at: https://doi.org/10.15252/embj.201592484.

Hurwitz, S.N. *et al.* (2016) ‘Proteomic profiling of NCI-60 extracellular vesicles uncovers common protein cargo and cancer type-specific biomarkers’, *Oncotarget*, 7(52), pp. 86999–87015. Available at: https://doi.org/10.18632/oncotarget.13569.

Jaiswal, P.K., Goel, A. and Mittal, R.D. (2015) ‘Survivin: A molecular biomarker in cancer’, *Indian Journal of Medical Research*, 142(April), pp. 389–397.

Jakubowska, K. *et al.* (2016) ‘Immunohistochemical expression and serum level of survivin protein in colorectal cancer patients’, *Oncology Letters*, 12(5), pp. 3591–3597. Available at: https://doi.org/10.3892/ol.2016.5075.

Jeyaprakash, A.A. *et al.* (2007) ‘Structure of a Survivin-Borealin-INCENP Core Complex Reveals How Chromosomal Passengers Travel Together’, *Cell*, 131(2), pp. 271–285. Available at: https://doi.org/10.1016/j.cell.2007.07.045.

Jiang, X. *et al.* (2018) ‘Effects of poly(I:C) and MF59 co-adjuvants on immunogenicity and efficacy of survivin polypeptide immunogen against melanoma’, *Journal of Cellular Physiology*, 233(6), pp. 4926–4934. Available at: https://doi.org/10.1002/jcp.26317.

Johnson, M. (2023) ‘Detergents: Triton X-100, Tween-20, and More’, *Materials and Methods* [Preprint]. Available at: https://www.labome.com/method/Detergents-Triton-X-100-Tween-20-and-More.html (Accessed: 8 May 2024).

Kajimoto, T. *et al.* (2013) ‘Ongoing activation of sphingosine 1-phosphate receptors mediates maturation of exosomal multivesicular endosomes’, *Nature Communications*, 4, p. 2712. Available at: https://doi.org/10.1038/ncomms3712.

Kalluri, R. and LeBleu, V.S. (2020) ‘The biology, function, and biomedical applications of exosomes’, *Science*, 367(6478), p. eaau6977. Available at: https://doi.org/10.1126/science.aau6977.

Karnezis, T. *et al.* (2012) ‘VEGF-D Promotes Tumor Metastasis by Regulating Prostaglandins Produced by the Collecting Lymphatic Endothelium’, *Cancer Cell*, 21(2), pp. 181–195. Available at: https://doi.org/10.1016/j.ccr.2011.12.026.

Keklikoglou, I. *et al.* (2019) ‘Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models’, *Nature cell biology*, 21(2), pp. 190–202.

Kenny, G.E. and Dunsmoor, C.L. (1987) ‘Effectiveness of detergents in blocking nonspecific binding of IgG in the enzyme-linked immunosorbent assay (ELISA) depends upon the type of polystyrene used’, *Israel Journal of Medical Sciences*, 23(6), pp. 732–734.

Khan, S. *et al.* (2009) ‘Extracellular, cell-permeable survivin inhibits apoptosis while promoting proliferative and metastatic potential’, *British journal of cancer*, 100(7), pp. 1073–1086.

Khan, S. *et al.* (2011) ‘Survivin is released from cancer cells via exosomes’, *Apoptosis*, 16, pp. 1–12.

Khan, S. *et al.* (2012) ‘Plasma-Derived Exosomal Survivin, a Plausible Biomarker for Early Detection of Prostate Cancer’, *PLoS ONE*. Edited by J. Li, 7(10), p. e46737. Available at: https://doi.org/10.1371/journal.pone.0046737.

Khan, S. *et al.* (2014) ‘Early diagnostic value of survivin and its alternative splice variants in breast cancer’, *BMC Cancer*, 14(1), p. 176. Available at: https://doi.org/10.1186/1471-2407-14-176.

Khan, S. *et al.* (2015) ‘Localization and upregulation of survivin in cancer health disparities: a clinical perspective’, *Biologics: Targets and Therapy*, 9, pp. 57–67. Available at: https://doi.org/10.2147/BTT.S83864.

Khan, S. *et al.* (2017) ‘Racial differences in the expression of inhibitors of apoptosis (IAP) proteins in extracellular vesicles (EV) from prostate cancer patients’, *PLOS ONE*, 12(10), p. e0183122. Available at: https://doi.org/10.1371/journal.pone.0183122.

King, H.W., Michael, M.Z. and Gleadle, J.M. (2012) ‘Hypoxic enhancement of exosome release by breast cancer cells’, *BMC Cancer*, 12(1), p. 421. Available at: https://doi.org/10.1186/1471-2407-12-421.

Konoshenko, M.Yu. *et al.* (2018) ‘Isolation of Extracellular Vesicles: General Methodologies and Latest Trends’, *BioMed Research International*, 2018(1), p. 8545347. Available at: https://doi.org/10.1155/2018/8545347.

Kreger, B.T. *et al.* (2016) ‘The Enrichment of Survivin in Exosomes from Breast Cancer Cells Treated with Paclitaxel Promotes Cell Survival and Chemoresistance’, *Cancers*, 8(12), p. 111. Available at: https://doi.org/10.3390/cancers8120111.

Kumar, V., Abbas, A.K. and Aster, J.C. (2017) *Robbins Basic Pathology*. Elsevier Health Sciences.

Kut, C., Mac Gabhann, F. and Popel, A.S. (2007) ‘Where is VEGF in the body? A meta-analysis of VEGF distribution in cancer’, *British Journal of Cancer*, 97(7), pp. 978–985. Available at: https://doi.org/10.1038/sj.bjc.6603923.

Lamparski, H.G. *et al.* (2002) ‘Production and characterization of clinical grade exosomes derived from dendritic cells’, *Journal of Immunological Methods*, 270(2), pp. 211–226. Available at: https://doi.org/10.1016/s0022-1759(02)00330-7.

Lechler, P. *et al.* (2011) ‘The oncofetal gene survivin is re-expressed in osteoarthritis and is required for chondrocyte proliferation in vitro’, *BMC Musculoskeletal Disorders*, 12(1), p. 150. Available at: https://doi.org/10.1186/1471-2474-12-150.

Lee, J.-E., Kim, S.Y. and Shin, S.-Y. (2015) ‘Effect of Repeated Freezing and Thawing on Biomarker Stability in Plasma and Serum Samples’, *Osong Public Health and Research Perspectives*, 6(6), pp. 357–362. Available at: https://doi.org/10.1016/j.phrp.2015.11.005.

Lehmann, B.D. *et al.* (2008) ‘Senescence-associated exosome release from human prostate cancer cells’, *Cancer Research*, 68(19), pp. 7864–7871. Available at: https://doi.org/10.1158/0008-5472.CAN-07-6538.

Lequin, R.M. (2005) ‘Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA)’, *Clinical Chemistry*, 51(12), pp. 2415–2418. Available at: https://doi.org/10.1373/clinchem.2005.051532.

Li, D., Hu, C. and Li, H. (2018) ‘Survivin as a novel target protein for reducing the proliferation of cancer cells’, *Biomedical reports*, 8(5), pp. 399–406.

Li, F. (2005) ‘Role of survivin and its splice variants in tumorigenesis’, *British Journal of Cancer*, 92(2), pp. 212–216. Available at: https://doi.org/10.1038/sj.bjc.6602340.

Li, F., Aljahdali, I. and Ling, X. (2019) ‘Cancer therapeutics using survivin BIRC5 as a target: What can we do after over two decades of study?’, *Journal of Experimental and Clinical Cancer Research*, 38(1), pp. 1–22. Available at: https://doi.org/10.1186/s13046-019-1362-1.

Li, K. *et al.* (2020) ‘Survivin in breast cancer–derived exosomes activates fibroblasts by up-regulating SOD1, whose feedback promotes cancer proliferation and metastasis’, *Journal of Biological Chemistry*, 295(40), pp. 13737–13752. Available at: https://doi.org/10.1074/jbc.RA120.013805.

Li, P. *et al.* (2017) ‘Progress in Exosome Isolation Techniques’, *Theranostics*, 7(3), pp. 789–804. Available at: https://doi.org/10.7150/thno.18133.

Li, Y. *et al.* (2021) ‘Survivin as a biological biomarker for diagnosis and therapy’, *Expert Opinion on Biological Therapy*, 21(11), pp. 1429–1441. Available at: https://doi.org/10.1080/14712598.2021.1918672.

Liu, N. *et al.* (2015) ‘Survivin contributes to the progression of diabetic retinopathy through HIF-1α pathway’, *International Journal of Clinical and Experimental Pathology*, 8(8), pp. 9161–9167.

Liu, W., Ma, Z. and Kang, X. (2022) ‘Current status and outlook of advances in exosome isolation’, *Analytical and Bioanalytical Chemistry*, 414(24), pp. 7123–7141. Available at: https://doi.org/10.1007/s00216-022-04253-7.

Lohmann, L.J. and Strube, J. (2020) ‘Accelerating Biologics Manufacturing by Modeling: Process Integration of Precipitation in mAb Downstream Processing’, *Processes*, 8(1), p. 58. Available at: https://doi.org/10.3390/pr8010058.

Ludwig, A.-K. *et al.* (2018) ‘Precipitation with polyethylene glycol followed by washing and pelleting by ultracentrifugation enriches extracellular vesicles from tissue culture supernatants in small and large scales’, *Journal of Extracellular Vesicles*, 7(1), p. 1528109. Available at: https://doi.org/10.1080/20013078.2018.1528109.

Mace, P.D., Shirley, S. and Day, C.L. (2010) ‘Assembling the building blocks: structure and function of inhibitor of apoptosis proteins’, *Cell Death & Differentiation*, 17(1), pp. 46–53. Available at: https://doi.org/10.1038/cdd.2009.45.

Mahmoudzadeh-Sagheb, A. *et al.* (2024) ‘Survivin as a potential biomarker for early diagnosis of the progression of precancerous lesions to gastric cancer’, *The International Journal of Biological Markers*, 39(1), pp. 52–58. Available at: https://doi.org/10.1177/03936155231217268.

Marenduzzo, D., Finan, K. and Cook, P.R. (2006) ‘The depletion attraction: an underappreciated force driving cellular organization’, *The Journal of Cell Biology*, 175(5), pp. 681–686. Available at: https://doi.org/10.1083/jcb.200609066.

Marivin, A. *et al.* (2012) ‘The Inhibitor of Apoptosis (IAPs) in Adaptive Response to Cellular Stress’, *Cells*, 1(4), pp. 711–737. Available at: https://doi.org/10.3390/cells1040711.

Martini, E. *et al.* (2016) ‘Survivin is a guardian of the intestinal stem cell niche and its expression is regulated by TGF-β’, *Cell Cycle (Georgetown, Tex.)*, 15(21), pp. 2875–2881. Available at: https://doi.org/10.1080/15384101.2016.1231286.

McCabe, J.P., Fletcher, S.M. and Jones, M.N. (1988) ‘The effects of detergent on the enzyme-linked immunosorbent assay (ELISA) of blood group substances’, *Journal of Immunological Methods*, 108(1–2), pp. 129–135. Available at: https://doi.org/10.1016/0022-1759(88)90411-5.

McIlwain, D.R., Berger, T. and Mak, T.W. (2013) ‘Caspase functions in cell death and disease’, *Cold Spring Harbor perspectives in biology*, 5(4), p. a008656.

Melo, S.A. *et al.* (2014) ‘Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis’, *Cancer Cell*, 26(5), pp. 707–721. Available at: https://doi.org/10.1016/j.ccell.2014.09.005.

Merck (no date) *Amicon® Ultra-15 Centrifugal Filter Units - Sample Prep Centrifugal Filter Units*. Available at: https://www.merckmillipore.com/GB/en/product/Amicon-Ultra-15-Centrifugal-Filter-Units,MM_NF-C7715 (Accessed: 12 January 2025).

Mulcahy, L.A., Pink, R.C. and Carter, D.R.F. (2014) ‘Routes and mechanisms of extracellular vesicle uptake’, *Journal of Extracellular Vesicles*, 3, p. 10.3402/jev.v3.24641. Available at: https://doi.org/10.3402/jev.v3.24641.

Musi, A. and Bongiovanni, L. (2023) ‘Extracellular Vesicles in Cancer Drug Resistance: Implications on Melanoma Therapy’, *Cancers*, 15(4), p. 1074. Available at: https://doi.org/10.3390/cancers15041074.

Naumnik, W. *et al.* (2009) ‘Serum levels of HMGB1, survivin, and VEGF in patients with advanced non-small cell lung cancer during chemotherapy.’, *Folia Histochemica et Cytobiologica*, 47(4), pp. 703–709.

Nogueira-Ferreira, R. *et al.* (2013) ‘Exploring the role of post-translational modifications on protein–protein interactions with survivin’, *Archives of Biochemistry and Biophysics*, 538(2), pp. 64–70. Available at: https://doi.org/10.1016/j.abb.2013.07.027.

Novais, A. *et al.* (2023) ‘Small Extracellular Vesicles and Survivin as Diagnostic and Prognostic Marker for Breast Cancer: A Pilot Study’, *Clinics of Oncology*, 06(22), pp. 01–07. Available at: https://doi.org/10.47829/COO.2023.62202.

Ostrowski, M. *et al.* (2010) ‘Rab27a and Rab27b control different steps of the exosome secretion pathway’, *Nature Cell Biology*, 12(1), pp. 19–30. Available at: https://doi.org/10.1038/ncb2000.

Parolini, I. *et al.* (2009) ‘Microenvironmental pH is a key factor for exosome traffic in tumor cells’, *The Journal of Biological Chemistry*, 284(49), pp. 34211–34222. Available at: https://doi.org/10.1074/jbc.M109.041152.

Pavlidou, A., Kroupis, C. and Dimas, K. (2014) ‘Association of survivin splice variants with prognosis and treatment of breast cancer’, *World Journal of Clinical Oncology*, 5(5), pp. 883–894. Available at: https://doi.org/10.5306/wjco.v5.i5.883.

Pierce™ BCA Protein Assay Kits (no date) *Pierce™ BCA Protein Assay Kits*. Available at: https://www.thermofisher.com/order/catalog/product/23225 (Accessed: 19 March 2024).

Poggio, M. *et al.* (2019) ‘Suppression of Exosomal PD-L1 Induces Systemic Anti-tumor Immunity and Memory’, *Cell*, 177(2), pp. 414-427.e13. Available at: https://doi.org/10.1016/j.cell.2019.02.016.

Qualtiere, L.F., Anderson, A.G. and Meyers, P. (1977) ‘Effects of ionic and nonionic detergents on antigen-antibody reactions’, *Journal of Immunology (Baltimore, Md.: 1950)*, 119(5), pp. 1645–1651.

Raiborg, C. and Stenmark, H. (2009) ‘The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins’, *Nature*, 458(7237), pp. 445–452. Available at: https://doi.org/10.1038/nature07961.

R&D Systems (no date) *Human Survivin Quantikine ELISA Kit*, *www.rndsystems.com*. Available at: https://www.rndsystems.com/products/human-survivin-quantikine-elisa-kit_dsv00 (Accessed: 17 September 2024).

Rebucci, M. and Michiels, C. (2013) ‘Molecular aspects of cancer cell resistance to chemotherapy’, *Biochemical pharmacology*, 85(9), pp. 1219–1226.

Rider, M.A., Hurwitz, S.N. and Meckes, D.G. (2016) ‘ExtraPEG: A Polyethylene Glycol-Based Method for Enrichment of Extracellular Vesicles’, *Scientific Reports*, 6(1), p. 23978. Available at: https://doi.org/10.1038/srep23978.

Sah, N.K. and Seniya, C. (2015) ‘Survivin splice variants and their diagnostic significance’, *Tumor Biology*, 36(9), pp. 6623–6631. Available at: https://doi.org/10.1007/s13277-015-3865-5.

Salvesen, G.S. and Duckett, C.S. (2002) ‘IAP proteins: Blocking the road to death’s door’, *Nature Reviews Molecular Cell Biology*, 3(6), pp. 401–410. Available at: https://doi.org/10.1038/nrm830.

Saman, Sudad *et al.* (2012) ‘Exosome-associated Tau Is Secreted in Tauopathy Models and Is Selectively Phosphorylated in Cerebrospinal Fluid in Early Alzheimer Disease*’, *Journal of Biological Chemistry*, 287(6), pp. 3842–3849. Available at: https://doi.org/10.1074/jbc.M111.277061.

Samarkos, M. *et al.* (2018) ‘Significance of survivin mRNA blood levels in patients with melanoma’, *Journal of B.U.ON.*, 23(Suppl 1), pp. 96–103.

Sartorius (2025) *Centrifugal Concentrators*, *Sartorius*. Available at: https://www.sartorius.com/en/products/lab-filtration-purification/ultrafiltration-devices/centrifugal (Accessed: 13 January 2025).

Schwickart, M. *et al.* (2014) ‘Interference in Immunoassays to Support Therapeutic Antibody Development in Preclinical and Clinical Studies’, *Bioanalysis*, 6(14), pp. 1939–1951. Available at: https://doi.org/10.4155/bio.14.127.

Shakeel, E. *et al.* (2017) ‘Molecular docking analysis of aplysin analogs targeting survivin protein’, *Bioinformation*, 13(9), p. 293.

Shao, Q. *et al.* (2018) ‘The expressions of YAP1, β-catenin and survivin in colon cancer tissues and their clinical significance’, *Int J Clin Exp Pathol*, 11(12), pp. 6032–6038.

Sheta, M. *et al.* (2023) ‘Extracellular Vesicles: New Classification and Tumor Immunosuppression’, *Biology*, 12(1), p. 110. Available at: https://doi.org/10.3390/biology12010110.

Shojaei-Ghahrizjani, F. *et al.* (2020) ‘Does survivin overexpression enhance the efficiency of fibroblast cell-based wound therapy?’, *Molecular Biology Reports*, 47(8), pp. 5851–5864. Available at: https://doi.org/10.1007/s11033-020-05656-4.

Silke, J. and Vaux, D.L. (2001) ‘Two kinds of BIR-containing protein-inhibitors of apoptosis, or required for mitosis’, *Journal of cell science*, 114(10), pp. 1821–1827.

Singh, A.D. *et al.* (2023) ‘Polyethylene glycol-based isolation of urinary extracellular vesicles, an easily adoptable protocol’, *MethodsX*, 11, p. 102310. Available at: https://doi.org/10.1016/j.mex.2023.102310.

Skog, J. *et al.* (2008) ‘Glioblastoma microvesicles transport RNA and protein that promote tumor growth and provide diagnostic biomarkers’, *Nature cell biology*, 10(12), pp. 1470–1476. Available at: https://doi.org/10.1038/ncb1800.

Small, S. *et al.* (2010) ‘Overexpression of Survivin Initiates Hematologic Malignancies In Vivo’, *Leukemia*, 24(11), pp. 1920–1926. Available at: https://doi.org/10.1038/leu.2010.198.

Song, Z., Yao, X. and Wu, M. (2003) ‘Direct interaction between Survivin and Smac/DIABLO is essential for the anti-apoptotic activity of Survivin during Taxol-induced apoptosis’, *Journal of Biological Chemistry*, 278(25), pp. 23130–23140. Available at: https://doi.org/10.1074/jbc.M300957200.

Stoorvogel, W. *et al.* (1991) ‘Late endosomes derive from early endosomes by maturation’, *Cell*, 65(3), pp. 417–427.

Su, Z. *et al.* (2015) ‘Apoptosis, autophagy, necroptosis, and cancer metastasis’, *Molecular Cancer*, 14(1), pp. 1–14. Available at: https://doi.org/10.1186/s12943-015-0321-5.

Tas, F. *et al.* (2004) ‘Serum bcl-2 and survivin levels in melanoma’, *Melanoma Research*, 14(6), p. 543.

Tate, J. and Ward, G. (2004) ‘Interferences in Immunoassay’, *The Clinical Biochemist Reviews*, 25(2), pp. 105–120.

ThermoFisher Scientific (2024a) *Detergents for Protein Solubilization - UK*. Available at: https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-purification-isolation/cell-lysis-fractionation/detergents-protein-solubilization.html (Accessed: 10 May 2024).

ThermoFisher Scientific (2024b) *Overview of ELISA*. Available at: https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html (Accessed: 16 December 2024).

Trajkovic, K. *et al.* (2008) ‘Ceramide triggers budding of exosome vesicles into multivesicular endosomes’, *Science (New York, N.Y.)*, 319(5867), pp. 1244–1247. Available at: https://doi.org/10.1126/science.1153124.

Troy, C.M. and Jean, Y.Y. (2015) ‘Caspases: Therapeutic Targets in Neurologic Disease’, *Neurotherapeutics*, 12(1), pp. 42–48. Available at: https://doi.org/10.1007/s13311-014-0307-9.

Vergauwen, G. *et al.* (2017) ‘Confounding factors of ultrafiltration and protein analysis in extracellular vesicle research’, *Scientific Reports*, 7(1), p. 2704. Available at: https://doi.org/10.1038/s41598-017-02599-y.

Vesiclepedia (2024) *Vesiclepedia: BIRC5 Gene summary*, *Vesiclepedia*. Available at: http://microvesicles.org/gene_summary?gene_id=332 (Accessed: 29 February 2024).

Waligórska-Stachura, J. *et al.* (2014) ‘Survivin Delta Ex3 Overexpression in Thyroid Malignancies’, *PLoS ONE*, 9(6), p. e100534. Available at: https://doi.org/10.1371/journal.pone.0100534.

Wang, P. *et al.* (2023) ‘Polyethylene glycol precipitation is an efficient method to obtain extracellular vesicle-depleted fetal bovine serum’, *PLOS ONE*, 18(12), p. e0295076. Available at: https://doi.org/10.1371/journal.pone.0295076.

Warnecke-Eberz, U. *et al.* (2008) ‘Up-regulation of survivin mRNA might be a marker for non-invasive detection of non-small cell lung cancer rather than for prognosis’, *Anticancer Research*, 28(3A), pp. 1525–1529.

Weng, Y. *et al.* (2016) ‘Effective isolation of exosomes with polyethylene glycol from cell culture supernatant for in-depth proteome profiling’, *Analyst*, 141(15), pp. 4640–4646. Available at: https://doi.org/10.1039/C6AN00892E.

Weston, C.L., Glantz, M.J. and Connor, J.R. (2011) ‘Detection of cancer cells in the cerebrospinal fluid: current methods and future directions’, *Fluids and Barriers of the CNS*, 8(1), p. 14. Available at: https://doi.org/10.1186/2045-8118-8-14.

Wheatley, S.P. and Altieri, D.C. (2019) ‘Survivin at a glance’, *Journal of Cell Science*, 132(7), p. jcs223826. Available at: https://doi.org/10.1242/jcs.223826.

Wollert, T. and Hurley, J.H. (2010) ‘Molecular mechanism of multivesicular body biogenesis by ESCRT complexes’, *Nature*, 464(7290), pp. 864–869. Available at: https://doi.org/10.1038/nature08849.

Wong, L. *et al.* (1985) ‘Monoclonal antibodies to rat C apolipoproteins: production and characterization of a unique antibody whose binding to apoC-I is inhibited by nonionic detergents.’, *Journal of Lipid Research*, 26(5), pp. 528–539. Available at: https://doi.org/10.1016/S0022-2275(20)34339-X.

Wuttke, M. *et al.* (2009) ‘Identifying tumor antigens in endocrine malignancies’, *Trends in Endocrinology & Metabolism*, 20(3), pp. 122–129. Available at: https://doi.org/10.1016/j.tem.2008.12.003.

Xu, Y.-X. *et al.* (2018) ‘Survivin regulated by autophagy mediates hyperglycemia-induced vascular endothelial cell dysfunction’, *Experimental Cell Research*, 364(2), pp. 152–159. Available at: https://doi.org/10.1016/j.yexcr.2018.01.037.

Yahya, R.S. *et al.* (2012) ‘Serum survivin and tp53 gene expression in children with acute lymphoblastic leukemia’, *Iranian journal of public health*, 41(1), p. 37.

Yakubovich, E.I., Polischouk, A.G. and Evtushenko, V.I. (2022) ‘Principles and Problems of Exosome Isolation from Biological Fluids’, *Biochemistry (Moscow), Supplement Series A: Membrane and Cell Biology*, 16(2), pp. 115–126. Available at: https://doi.org/10.1134/S1990747822030096.

Yáñez-Mó, M. *et al.* (2015) ‘Biological properties of extracellular vesicles and their physiological functions’, *Journal of Extracellular Vesicles*, 4, p. 10.3402/jev.v4.27066. Available at: https://doi.org/10.3402/jev.v4.27066.

Ye, T. *et al.* (2019) ‘Role of Smac, survivin, XIAP, and Omi/HtrA2 proteins in determining the chemotherapeutic response of patients with cervical cancer treated with neoadjuvant chemotherapy’, *Cancer Biomarkers*, 26, pp. 249–259. Available at: https://doi.org/10.3233/CBM-182165.

Yıldırım, M. *et al.* (2022) ‘The role of exosomal survivin in the diagnosis of breast cancer’, *International Journal of Clinical Biochemistry and Research*, 9(3), pp. 254–259. Available at: https://doi.org/10.18231/j.ijcbr.2022.049.

Yu, J. *et al.* (2024) ‘Optimizing Conditions of Polyethylene Glycol Precipitation for Exosomes Isolation From MSCs Culture Media for Regenerative Treatment’, *Biotechnology Journal*, 19(9), p. e202400374. Available at: https://doi.org/10.1002/biot.202400374.

Zeng, X. *et al.* (2024) ‘Alzheimer blood biomarkers: practical guidelines for study design, sample collection, processing, biobanking, measurement and result reporting’, *Molecular Neurodegeneration*, 19(1), p. 40. Available at: https://doi.org/10.1186/s13024-024-00711-1.

Zhang, Y. *et al.* (2015) ‘Prognostic and clinicopathological value of survivin in diffuse large B-cell lymphoma: A meta-analysis’, *Medicine (United States)*, 94(36), pp. 1–10. Available at: https://doi.org/10.1097/MD.0000000000001432.

Zhang, Y. *et al.* (2023) ‘Survivin-Based Recombinant Overlapping Peptides Induce T Lymphocyte Cytotoxicity and Prolong the Survival in In Vivo Melanoma Model’, *Advanced Therapeutics*, 6(11), p. 2300253. Available at: https://doi.org/10.1002/adtp.202300253.

Zhou, L., Hu, Y. and Xiao, H. (2021) ‘The prognostic significance of survivin expression in patients with HNSCC: a systematic review and meta-analysis’, *BMC Cancer*, 21(1), p. 424. Available at: https://doi.org/10.1186/s12885-021-08170-3.