QUANTIFICATION AND CHARACTERISATION OF CIRCULATING EXTRACELLULAR VESICLES IN CERVICAL CANCER PATIENTS BEFORE, DURING AND AFTER TREATMENT.

by

Noluthando Ncebakazi Gasa

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Supervisor: Prof M. Meiring, Department of Haematology and Cell Biology, Faculty of Health Sciences, University of the Free State

Co-supervisor: Prof MJC Coetzee, Department of Haematology and Cell Biology, Faculty of Health Sciences, University of the Free State

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'I can do all things through Christ who strengthens me.' *Philippians 4:13*

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
APC-CY7	Allophycocyanin: Cy-7 Tandem Conjugate
ApoBDs	Apoptotic bodies
BD	Becton Dickinson
CaCx	Cervix cancer
CD	Cluster of differentiation
CIN	Cervical intraepithelial neoplasm
COVID-19	Coronavirus disease of 2019
EBRT	External beam radiation therapy
EpCAM+	Epithelial cellular adhesion molecule
EVs	Extracellular vesicles
FBC	Full blood count
FIGO	International Federation of Gynaecology and Obstetrics
FITC	Fluorescein isothiocyanate
HER2	Human epidermal growth factor receptor 2
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
^{192}Ir	Iridium 192
ISTH	International Society for Thrombosis and Haemostasis
IZON	IZON Science
LMIC	Low and middle-income countries
LPS	Lipopolysaccharide
MicroRNA	Microribonucleic acid
MISEV 2018	The minimal information for studies of extracellular vesicles 2018
MWCO	Molecular weight cut off
MV	Microvesicles
MVB	Multivesicular bodies
NaCl	Sodium chloride
nt	Nucleotide(s)
NTA	Nanoparticle tracking and analysis
PacO	Pacific Orange
Pap	Papanicolaou
Pap smear	Papanicolaou smear
PE	Phycoerythrin
PercP 5.5	5 PerCP-Cyanine5.5
PMT	Photomultiplier tube
RT	Room temperature
RNA	Ribonucleic acid

SCC	Squamous cell carcinoma
SEC	Size exclusion chromatography
TF/PS	Tissue Factor/Phosphatidylserine
UF	Ultrafiltration
UC	Ultracentrifugation
WHO	World Health Organisation

LIST OF CD ANTIGENS

CD9	p24 (Involved in platelet activation and aggregation, cell adhesion and cell motility. One of the tetraspanins))
DC41	Integrin αIIb
CD63	Granulophysin (one of the tetraspanins)
CD81	One of the tetraspanins
CD133	Hematopoietic stem cell antigen
CD151	Raph blood group (one of the tetraspanins)

ABSTRACT

All cells actively release extracellular vesicles (EVs) upon activation and apoptosis. EVs mediate intercellular communication in normal physiology and pathology. Cancer cells, like normal cells, secrete cancer specific EVs into the blood stream. EVs can possibly be used to monitor disease progression and response to treatment. This research quantified and characterised circulating EVs in the plasma of ten patients with stage II cervical cancer before, during and up to six weeks after treatment. EVs were isolated from plasma by size exclusion chromatography. Flow cytometry was used to count and characterise the EVs. The total number of EVs was identified by using an anti-CD63 monoclonal antibody. Cancer derived EVs were identified using an anti-CD133, while anti-CD11b was used to identify platelet derived EVs, and anti-CD41 to identify neutrophil EVs.

After the start of radiotherapy (week 1) the number of CD63+ EVs, increased as large numbers of cancer cells were killed. The number of CD63 positive events significantly decreased in week six compared to baseline. There was also a significant decrease in CD133 positive and CD41 positive events in week six compared to baseline. This study demonstrated a significant increase at the start of treatment, followed by a decrease in circulating EVs after treatment compared to baseline. These findings suggest that EVs can possibly be used to monitor a patient's response to treatment.

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Keywords: Extracellular vesicles, EVs, Cervical cancer

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous group of small, membrane-bound structures. EVs are released by all cell types into the extracellular space and body fluids (Thery et al., 2018). The release of EVs occurs during cell activation and programmed cell death (also called apoptosis). EVs are formed through shedding or outward budding of the lipid bilayer plasma membrane (Figure 1) (Sheta et al., 2023). EVs play an essential role in intercellular communication by transferring biological molecules between cells (Figure 1) (van Niel et al., 2022). This cell-to-cell communication by EVs is crucial in maintaining normal physiological functions and homeostasis (Yates et al., 2022).

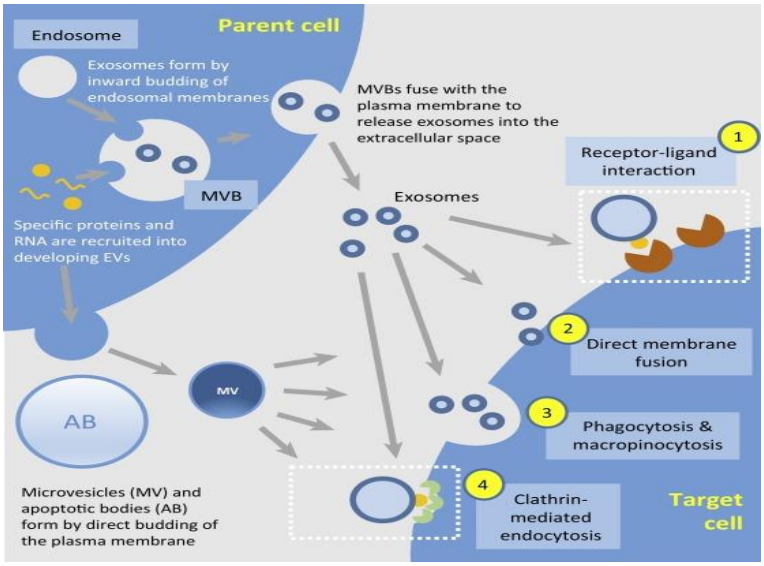

FIGURE 1: BIOGENESIS AND FORMATION OF EXTRACELLULAR VESICLES SUBTYPES FROM PARENT CELL TO THE RECIPIENT CELL, AS WELL AS CELL-TO-CELL COMMUNICATION MEDIATED BY EXTRACELLULAR VESICLES (HUANG-DORAN ET AL., 2017)**. MVB- multivesicular bodies**

Cells communicate with each other by secreting signaling molecules that include proteins, lipids, and nucleic acids. These molecules are then packaged, secreted, and transported in EVs (Jeppesen et al., 2023). Since EVs carry biological molecules such as nucleic acids, proteins, and lipids, we can trace their origin back to the parent cell (Jeppesen et

al., 2023). Because EVs can be traced back to their parent cells, an increasing number of publications have reported the use of extracellular vesicles as disease biomarkers. For instance, EVs derived from cancer cells carry cancer-specific biomolecules. Additionally, EVs report the current state of the cell during cancer development and during treatment. This is reflected by the number of EVs secreted into body fluids by that cell (Stevic et al., 2020). Signaling via EVs has also been identified in other disease states such as coagulopathies, cardiovascular diseases, and immunological disorders (Buzas et al., 2014; Sahoo et al., 2021).

EVs were first discovered in about 1940 when Chargaff and West were setting up centrifugation protocols. The researchers discovered that high speed centrifugation resulted in the sedimentation of “minute reddish brown translucent pellets” that shortened clotting time (Chargaff & West, 1946). In the 1960s EVs were assumed to be platelet dust. However, it is currently understood that EVs can be derived from many different cell types, not just from platelets. EVs derived from platelets are currently known as platelet derived-extracellular vesicles (Couch et al., 2021). Platelet-derived EVs are one of the most abundant extracellular vesicles in the blood. Platelet-derived EVs are covered with CD41 also called glycoprotein IIb/IIIa, P-Selectin and other platelet-specific proteins (Ying et al., 2018). It has been well established that cancer leads to platelet activation. Cancer-cell-induced platelet activation leads to the generation of platelet derived EVs (Vismara et al., 2022). EVs are found in biological fluids such as saliva, urine, and blood. EVs occur in three main subsets and can be classified based on their biogenesis and size (Cheng et al., 2014; Sheta et al., 2023; Yap et al., 2018).

EVs are commonly classified as exosomes, microvesicles, and apoptotic bodies. Exosomes range from 50 to 100 nm in size. Exosomes are formed by the fusion of the multivesicular bodies (MVBs) with the plasma membrane. Microvesicles are 100-1000 nm in size and are formed by the outward budding and blebbing from the plasma membrane (Figure 1) (Sheta et al., 2023). Apoptotic bodies are approximately the size of platelets with a diameter of 1000-5000 nm. Apoptotic bodies are formed during the final stages of apoptosis (Zhou et al., 2021). These apoptotic bodies are later phagocytosed

by macrophages (Kakarla et al., 2020; Santavanond et al., 2021). Interestingly, there is a substantial overlap of protein profile that is observed in the subsets of EVs despite their size. There are also no specific markers at present to distinguish between the different types of EVs (Doyle & Wang, 2019). However, all EVs are enriched with a subset of diverse lipids, proteins and non-coding microRNAs that are derived from the parent cells. Furthermore, EVs are enriched with tetraspanins, a superfamily of proteins with four transmembrane domains with characteristic structural features that organize the plasma membrane into microdomains. Tetraspanins are involved in biological processes which involve cell adhesion, invasion, signaling and protein trafficking. The tetraspanins CD9, CD81, and CD63 have frequently been identified in EVs (Chen et al., 2020; Doyle & Wang, 2019).

The minimal information for studies of extracellular vesicles 2018 (MISEV 2018) guidelines by Thery et at., (2018) recommends the use of physical characteristics of EVs such as small EVs <200 nm, medium and large EVs >200 nm. Moreover, the authors recommend the antibodies to the tetraspanins as markers for biochemical composition such as CD63+/CD81+/CD9+/Annexin A5 stained EVs (Thery et al., 2018). CD63 forms part of the tetraspanins CD9, CD81 and CD63 which are found in EVs. It is one of the classical markers for the majority of EVs (Chen et al., 2020).

CD133 is a widely known cancer stem cell marker that has been recently detected in EVs. CD133, a transmembrane glycoprotein, has been used as a cell surface marker of cancer stem cells (Yang et al., 2018). A study by Javed et al., (2018) concluded that CD133 positivity is a phenotypic marker of cancer stem cells in cervix carcinoma (Javed et al., 2018).

As mentioned previously, there is an increase in the number of platelets in patients with cancer. CD41 is a putative marker for platelet glycoprotein IIb. Platelet-derived EVs express CD 41 and P selectin (French et al., 2020). For this present study we chose to use the markers CD63 (EVs), CD41 (platelet glycoprotein IIb) and CD133 (cervical cancer).

Different methods have been developed to isolate and quantify EVs from different samples, such as blood, urine, serum, and cell culture medium. Some of these methods include, but are not limited to, precipitation, ultrafiltration, size exclusion chromatography and ultracentrifugation (Akbar et al., 2022). Flow cytometry has remained the most common method to visualise and characterise EVs. Nano and micro-flow cytometers have vastly improved the characterisation of EVs. The micro-flow cytometers, unlike conventional flow cytometers, can analyse small particles such as EVs, bacteria and viruses (Botha et al., 2021; Welsh et al., 2017). These techniques have allowed analysis of EVs for their prognostic potential in diseases such as cancer. They have also brought to light the role played by EVs in cell-to-cell communication, for instance in promoting cancer metastasis.

As previously mentioned, EVs are released by all cell types into the extracellular space and body fluids. Cancer cells, relative to normal cells, can constitutively secrete large numbers of EVs. Cancer cell-derived EVs carry cancer-specific biological molecules into the bloodstream and other biological fluids. Research into EVs in cancer is rapidly growing as cancer derived EVs may be involved in cancer metastasis, and can be used as potential biomarkers, targeted therapy, and could possibly be utilized to monitor disease progression and response to treatment (Zhou et al., 2021). EVs have been studied for their prognostic potential in different types of cancers such as lung cancer, breast cancer, prostate cancer, and glioblastoma (Skog et al., 2008; Zhou et al., 2021). For a better understanding of the diagnostic significance of cancer derived EVs, it is crucial to characterise EVs derived from cancer cells.

Minimal research studies have been done to assess the presence of EVs in the blood of patients with cervical cancer. Cervical cancer is a major gynaecological cancer in especially underdeveloped, but also in developed countries (Fan et al., 2021). According to the World Health Organisation (WHO) cervical cancer is the fourth most frequent cancer in females (Singh et al., 2023). An estimated 90% of deaths due to cervical cancer occur in low-and-middle-income countries. Over the past 30 years, the proportion of young women affected by cervical cancer has increased from ten percent to forty percent

(Zhang et al., 2020). Over ninety-nine percent of all cervical cancer cases are attributable to human papillomavirus (HPV). HPV is a sexually transmitted virus that occurs in different subtypes. The high-risk HPVs are types 16, 18, 31 and 45. Recent studies suggest that EVs derived from cancer cells can be detected in the systemic circulation (Choi et al., 2013; He et al., 2020; Mittal et al., 2020).

The aim of this present study is to quantify and characterise EVs in the systemic circulation of cervical cancer patients before, during and after treatment. The hypothesis of the project is that there are extracellular vesicles derived from the cervical cancer in systemic circulation of cervical cancer patients and they decrease with treatment.

CHAPTER 2: LITERATURE REVIEW

2.1 History Of Extracellular Vesicles

EVs were first discovered in normal plasma as procoagulant platelet-derived particles in 1946 by Chargaff and West (Chargaff & West, 1946) (Figure 2). Wolf referred to them as "platelet dust" in 1967 (Wolf, 1967). When spinning down blood to set up a centrifugation protocol to separate clotting factors from cells, Chargaff observed that the addition of the sediment obtained after high-speed centrifugation to the supernatant plasma considerably shortened the clotting time. Other early discoveries also included matrix vesicles that were discovered by Anderson in the year 1969 during bone calcification (Anderson, 2003) During the 1970s and 1980s, it was discovered that rectal adenoma microvillus cells released plasma membrane vesicles (De Broe et al., 1977). The first observations of membrane fragments from malignant tumours were made about the same time (Taylor & Doellgast, 1980). Researchers wondered if viruses caused diseases other than clinical infections, like cancer. While looking for virus-like particles in biological fluids researchers frequently came across a particulate matter but could not find anything that was of viral nature (Levine et al., 1967). These particles were observed in fluids of both cancer patients and healthy controls. About 10 years later Raposo et al. (1996) showed that particles (which at that time were called exosomes) that were extracted from Epstein-Barr virus B cell lines contained major histocompatibility proteins (Raposo et al., 1996). These exosomes presented antigens and triggered T-cell reactions (Raposo et al., 1996). When it was discovered in 2006–2007, that EVs contained RNA, including microRNA, interest in EVs as cell-to-cell communication mediators was awakened (Ratajczak et al., 2006; Valadi et al., 2007). Building on these innovative investigations, EVs have been isolated from most cell types and biological fluids, including breast milk, plasma, serum, amniotic fluid, nasal and bronchial lavage fluid, and saliva. Figure 2 shows the timeline of selected milestones in the extracellular vesical field.

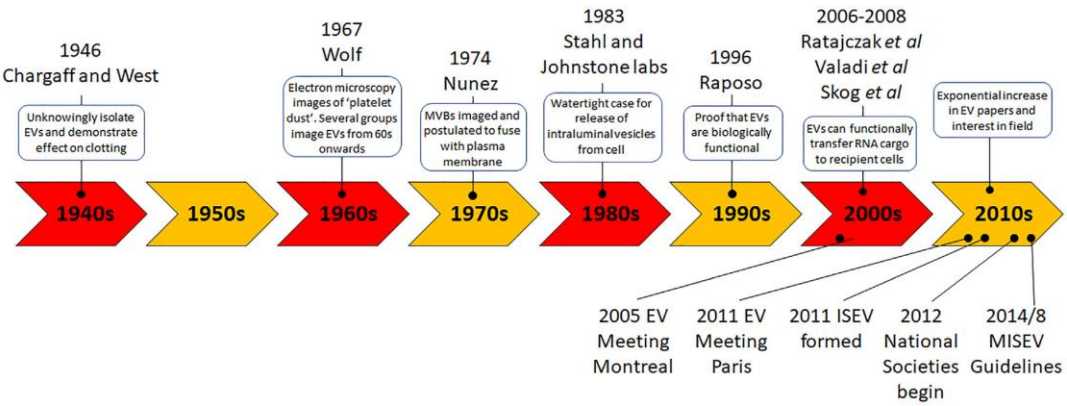

FIGURE 2: TIMELINE OF SELECTED MILESTONES IN THE EV FIELD (COUCH ET AL., 2021).

Once released into the extracellular space, EVs migrate to target cells where they deliver their contents and trigger functional responses. The migration into the target/recipient cell are mediated by tetraspanins, lipids and extracellular matrix components. This interaction leads to various downstream responses and processes. For instance, in dendritic cells, the protein cargo of EVs derived from intestinal epithelial cells is processed in the endocytic compartment in a similar way to which antigens are processed, and then used in antigen presentation (Mallegol et al., 2007).

As previously mentioned, the interest in EV expanded because of their cell-to-cell communication in healthy cells. Diseased cells such as cancer cells, package their biological molecules in EVs, then transport them to healthy cells, creating a pre-metastatic niche where cells can settle and grow, ultimately leading to cancer metastasis. The role of EVs in cancer metastasis has been widely studied (Ang et al., 2004; Becker et al., 2016; Voloshin et al., 2014). In the context of coagulation, platelet derived EVs carry tissue factor which contributes to thrombosis (Rosas et al., 2020). Thus, the concept that EVs could have physiological roles, that they could be used as biomarkers, and that they could have therapeutic applications, has led to the explosion of interest in EVs in the early 21st century.

2.2 EVs and their functions

EVs are plasma membrane fragments released by all cell types in response to chemical, physical activation, or apoptosis (Théry et al., 2018; Yáñez-Mó et al., 2015). They are small membrane-enclosed vesicles derived from the bi-lipid plasma membrane (Théry et al., 2018).

There are three main types of EV namely microvesicles, exosomes and apoptotic bodies (Couch et al., 2021). Their cargo consists of lipids, nucleic acids such as microRNAs, and proteins, and particularly proteins that are derived from the plasma membrane of the parent cell (Théry et al., 2018). Figure 3 shows the biogenesis and components of EVs. There are currently no specific protein markers to differentiate between the distinct types of EVs (Couch et al., 2021).

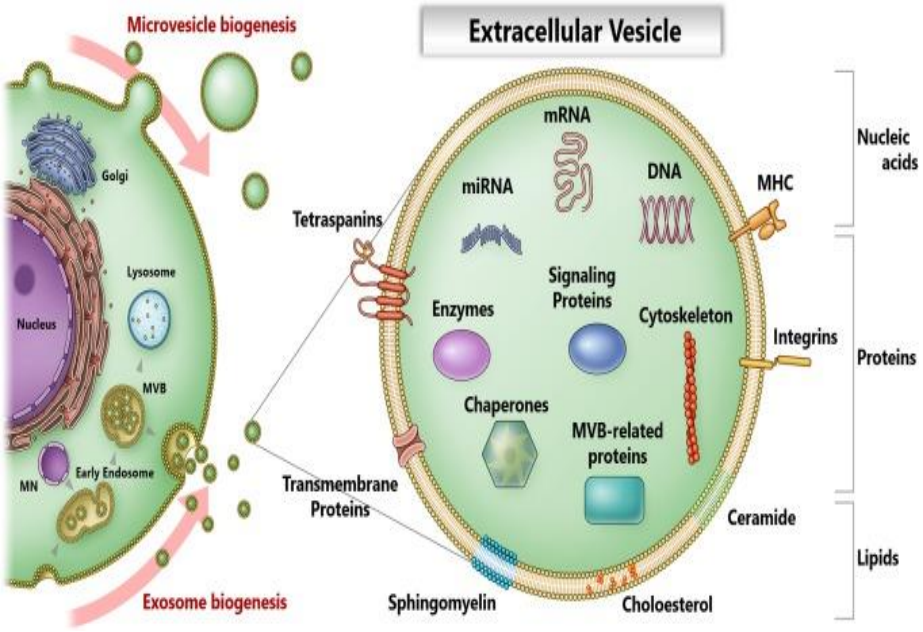

FIGURE 3: EXTRACELLULAR VESICLE BIOGENESIS AND COMPONENTS (YOKOI & OCHIYA, 2021)

2.2.1 EXOSOMES

Exosomes are the smallest types of EVs ranging from size 30-100 nm (Gouwens et al., 2018). They are formed through the early inward budding of the plasma membrane of the early endosomes (Doyle & Wang, 2019). Exosomes are particularly involved in protein sorting, recycling, storage, transport, and release (Donoso-Quezada et al., 2021). Initially, exosomes were believed to play a significant role in the way cells get rid of the unwanted material (Couch et al., 2021). However, it has subsequently been found that exosomes play a significant role in stimulating immune responses by acting as antigen presenting vesicles (Couch et al., 2021). In the nervous system, exosomes have been reported to play a key role in the formation of myelin, neurite growth and neuronal survival, therefore facilitating tissue repair and survival (Huo et al., 2021). Exosomes are also the subset of EVs that facilitates cell-to-cell communication. Because of their small size, they migrate between cells easier (Huang-Doran et al., 2017).

2.2.2 Microvesicles

Microvesicles (MV), also known as ectosomes, were initially believed to be part of cellular dumping (Couch et al., 2021). They are formed by the outward budding or pinching of the plasma membrane. MVs range between sizes 100 to 1000 nm (Doyle & Wang, 2019). MVs typically consist of cytosolic and plasma membrane proteins which form a cluster at the membrane surface called a tetraspanins (Couch et al., 2021). MVs are currently known to carry cargo that is like that of the parent cell. Thus, they have potential as clinical biomarkers of disease and can be used to monitor the response to treatment (Zhang et al., 2019).

2.2.3 APOPTOTIC BODIES

Apoptotic bodies are released by dying cells into extracellular space (Couch et al., 2021). They size range is 1000-5000 nm in diameter (Doyle & Wang, 2019). Unlike exosomes and MVs apoptotic bodies carry intact cell organelles, chromatin, and insignificant amounts of glycosylated proteins (Kakarla et al., 2020). Apoptotic bodies are secreted by cells undergoing programmed cell death. Initially apoptotic bodies are formed during plasma membrane blebbing which further develop into apoptotic bodies (Figure 4). Apoptotic bodies are then taken up by macrophages in the process called phagocytosis. The phagosome then fuses with lysosomes to form phagolysosome within the macrophage to prevent damage to the surrounding cell. Figure 4 shows the process of extracellular vesicles budding from normal and tumour cells. Table 1 classifies extracellular vesicles according to size, biogenesis and function.

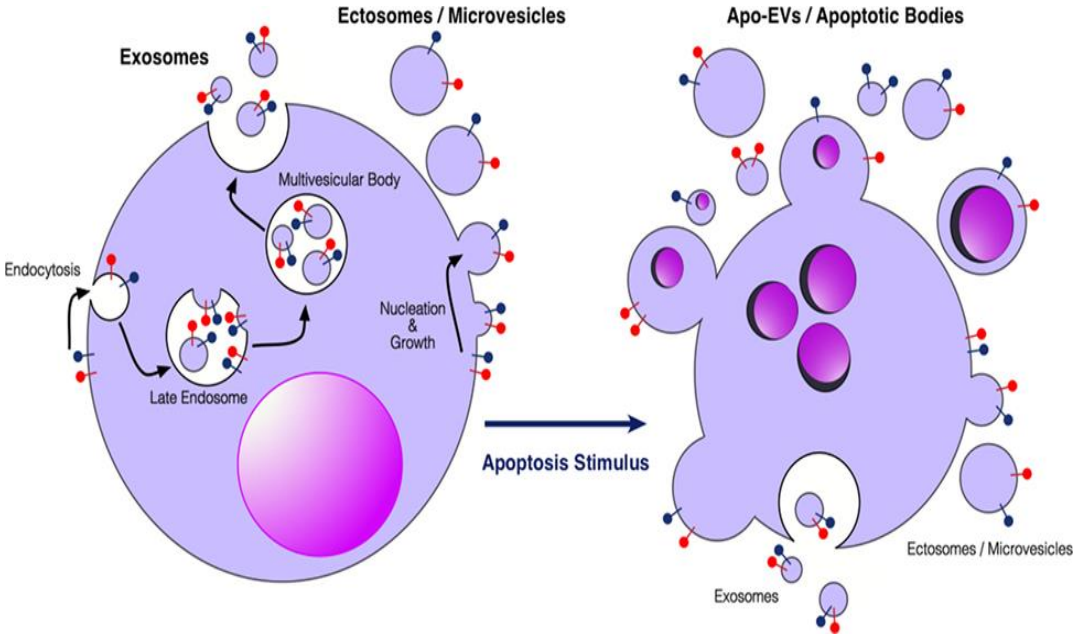

FIGURE 4: EXTRACELLULAR VESICLES BUDDING FROM NORMAL OR TUMOUR CELLS (LYNCH ET AL., 2017)**.**

TABLE 1: CLASSIFICATION OF EXTRACELLULAR VESICLES

Type	Size	Biogenesis	Function
Exosomes	30-100	Exocytosis of multivesicular bodies	Exosomes facilitate cell-to-cell communication
Microvesicles	100-1000	Budding of plasma membrane	Can be used as clinical biomarkers of diseases
Apoptotic bodies	1000-5000	Budding of plasma membrane during apoptosis	Carry information and substances from dying cells.

2.2.4 MICRORNAS

Some of the major components of the EV cargo include microRNAs (miRNAs) (Vaka et al., 2023). MiRNAs are small non-coding, single stranded RNAs 19-22 nucleotides (nt) in length (Xu et al., 2022). The miRNAs play a crucial role in post translational regulation of the gene expression. A small percentage of miRNAs are detected in the external environment, including bodily fluids in vivo, as well as in cell-culture medium in vitro (Albanese et al., 2021). Most miRNAs have an intracellular function. Theoretically, extracellular miRNAs can be potential circulating biomarkers for several cancers. miRNAs from EVs have been widely characterised. EVs released from different cell types contain miRNAs and are delivered to other target cells, where the miRNAs regulate their cognate target genes at the posttranscriptional level. At post transcriptional level, miRNAs regulate gene expression by modulating target messenger RNA resulting in altered levels of target protein (Albanese et al., 2021). These miRNAs are present in all types of body fluid, including serum, plasma, urine, and cerebrospinal fluid (CSF) (Gayosso-Gómez & Ortiz-Quintero, 2021). They are highly stable, relatively tissue-specific, associated with pathological states and readily detected, manipulated, and measured; therefore, they are considered a promising and sensitive biomarker for a wide spectrum of diseases. Importantly, evidence indicates that circulating miRNAs are actively packaged and transported by EVs (Fullerton et al., 2022). Figure 5 presents miRNA biogenesis, and the sorting of miRNA into extracellular vesicles and inter-cellular communication.

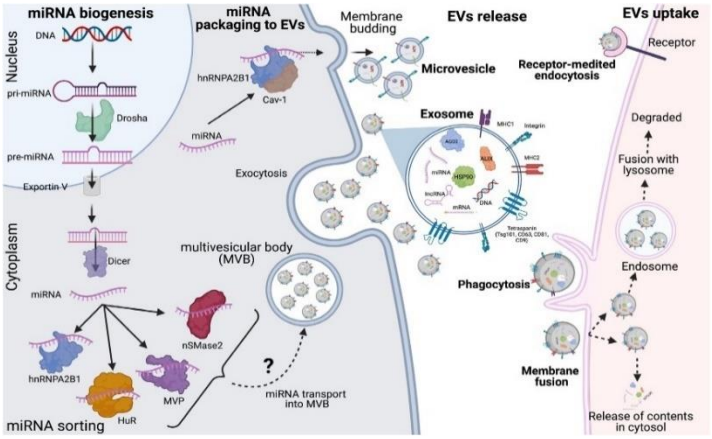

FIGURE 5: MIRNA BIOGENESIS, SORTING INTO EXTRACELLULAR VESICLES AND INTER-CELLULAR COMMUNICATION (FULLERTON ET AL., 2022).

2.3 EV ISOLATION TECHNIQUES

Several methods can be utilised to quantify and characterise EVs from cell-conditioned media and biofluids. The most popular techniques are ultracentrifugation (UC), ultrafiltration (UF) and size-exclusion chromatography (SEC). Historically the UC method has been the most popular. It was the most commonly available technique with simple protocols and cost effective (Brennan et al., 2020; Zhao et al., 2021)

2.3.1 ULTRACENTRIFUGATION

UC is the most classical method of EV isolation. It was the first method that was used for EV isolation and remains the gold standard to date. UC methods separates EVs based on size by multiple steps of centrifugation. Larger cells, platelets and apoptotic bodies are separated first at low-speed centrifugation) usually 10 000-20 000g for 1-2 hours. Smaller EVs are then pelleted at a higher speed 100 000-200 000g for 2 hours (Brennan et al., 2020; Zhao et al., 2021).

2.3.2 ULTRAFILTRATION

Ultrafiltration is the size-based EV isolation technique that is used most. The principle of UF is based on particle size and molecular weight cut off (MWCO) of the membrane filter. This means that particles larger than the MWCO filter will be retained while particles smaller will pass through the filter into the filtrate. To avoid clogging the pores it is

recommended to use multiple serial filters, starting with larger MWCO filters and gradually moving to smaller MWCO filters (Shu et al., 2021; Zhao et al., 2021).

2.3.3 Size exclusion chromatography

Size exclusion chromatography (SEC) is the most recent technique to isolate and purify EVs. It is a technique that separates particles based on size using gel resin. This technique uses simple membrane filters with specific size exclusion limits that separate particles based on their hydrodynamic volumes. SEC is considered to be the most efficient technique. The SEC setup consists of stationary and a mobile phase. The stationary phase contains a porous gel resin that is placed in a column. The mobile phase contains the sample that is injected into the gel resin. Molecules that are larger than the pore volume will be eluted first due to their inability to penetrate the pores, while the smaller particles will take longer to elute because they can penetrate the pores and are retained longer (Shu et al., 2021; Y. Yang et al., 2021; Zhao et al., 2021).

In a previous study conducted in 2022 we compared the three most common EVs isolation techniques (UC, UF and SEC). 20 Archived platelet-free plasma samples that had been collected from patients with infectious disease were used for this study. EVs were isolated from these samples using three different techniques. The results indicated that of the three isolation techniques SEC had the highest yield of EVs. We concluded that SEC was less time-consuming and more effective than to UC and UF (submitted for publication).

2.4 EV characterization techniques

2.4.1 Enzyme-linked Immunosorbent Assay (ELISA)

ELISA is one of the standard techniques used for the detection and quantification of EVs. The principle of ELISA is based on an antibody-antigen reaction in which an antibody is used to detect a target molecule. In this technique isolated EVs are added to the wells of a 96-well plate coated with the capture antibody against the target EV antigen. After washing steps, a detection antibody linked to an enzyme which will then produce a colour change is added. The colour change is proportionate to the amount of EVs present in the reaction. The ELISA assay is usually performed in 96 polystyrene plates, which enables not only the immobilization of antibodies and proteins, but also the analysis of multiple

samples per assay. This method offers a high flexibility concerning its design and procedure (Tan et al., 2021).

2.4.2 NANOPARTICLE TRACKING ANALYSIS (NTA)

Nanoparticle tracking and analysis is another frequently used technique for the characterisation of EVs. NTA can determine the size distribution and concentration of EVs in a sample. This technique displays the Brownian movement of particles in liquid suspension in real time and can estimate EV sizes ranging from 60 to 1000 nm. Brownian motion of the particles is measured using laser light scattering microscopy, with a camera as the detector, and their hydrodynamic diameter is calculated using the Stokes–Einstein equation (Auger et al., 2022).

2.4.3 FLOW CYTOMETRY

Flow cytometry is a technique used to detect the expression of cell surface and intracellular molecules. The molecule targets being studied are also called cluster of differentiation (CD) markers. Flow cytometry is used to characterise and define different cell types in a heterogeneous cell population, assessing the purity of isolated subpopulations, and analysing cell size and volume. Flow cytometry uses hydrodynamic focusing to focus cells into a single file as they pass through a laser beam aimed at fluorescent light detectors. As the cells pass through the laser beam, they are excited to a higher energy state, and emit light. This is measured as fluorescence intensity. Conventional flow cytometers cannot be used to study EVs as the limit of detection is more than 300 nm. The recently developed micro and nano flow cytometers allow rapid analysis of smaller particles in a single sample (Botha et al., 2021). Micro-flow cytometers can measure light scatter signals 1000 times weaker than that which can be detected by conventional flow cytometers.

2.4.3.1 MICRO FLOW CYTOMETER

Micro flow cytometers are specifically designed to detect small particles such as viruses, bacteria, and extracellular vesicles. They use the principle of light scattering and emission of fluorescence to generate data from small particles. These instruments use both silica and polystyrene beads with sizes ranging from 80 to 1300 nm as quality control for comparison. Parida et al. (2015) concluded that silica beads are the preferred standard

to define extracellular vesicle populations without platelet contamination. Silica beads are also to determine extracellular vesicle sizes using a micro flow cytometer (Parida et al. 2015). The International Society for Thrombosis and Haemostasis (ISTH) established working groups of experts on extracellular vesicles research that set up guidelines for using CD markers and controls for extracellular vesicle research (van der Pol et al., 2018). The markers and controls used by Van der Pol et al. (2018) complied with the latest guidelines. Furthermore, this research study also adhered to the 2014 MISEV guidelines for isolation of EVs from plasma samples (Thery et al., 2018).

2.5 EXTRACELLULAR VESICLES IN CANCER

Tumour cells can constitutively secrete large numbers of EVs carrying tumour-specific antigens into the bloodstream and other biological fluids (Palazzolo et al., 2020). The quantity and molecular characteristics of EVs in the circulation do not only reflect the origin of the parent cells but also reflect the stimulus which caused their release (Rousseau et al., 2015). It has been suggested that cancer cell-derived extracellular vesicles play diverse roles in cancer prognosis (Becker et al., 2016). EVs have been observed to be significantly elevated in circulation of cancer patients when compared to healthy controls. Recent high-throughput proteomic and transcriptomic studies of these complex extracellular organelles have accelerated the discovery of cancer-specific biomarkers and the development of novel diagnostic tools based on extracellular vesicles. Circulating extracellular vesicles have been observed in patients with colorectal, prostate, breast, gastric and lung cancers (Eguchi et al., 2020; Hosseini-Beheshti et al., 2012; Nanou et al., 2020; Rodríguez-Martínez et al., 2019; Toth et al., 2008; Tseng et al., 2013). Tseng et al., (2013) assessed levels of circulating extracellular vesicles in patients with lung cancer using flow cytometry. They reported that circulating levels of extracellular vesicles derived from platelets and endothelial cells were significantly increased in patients with lung cancer compared to normal patients (Tseng et al., 2013). Toth et al. (2008) showed increased levels of EVs derived from breast cancer correlated with the tumour size, thus reflecting the stage of the disease (Toth et al., 2008). EVs are obtained by potential liquid biopsies that could allow non-invasive blood-based tests and real time monitoring of tumour progress and treatment effectiveness. Li et al. (2018) established a

molecular phenotyping technique of breast cancer derived EVs using micro flow cytometry (Li et al., 2018). These authors were able to establish EpCAM+ (epithelial cell adhesion molecule) and HER2+ (human epidermal growth factor receptor 2) EVs as diagnostic indicators of breast cancer in a sample of 31 patients with metastatic breast cancer and seven healthy controls. They could distinguish between HER2+ and HER2- tumours (Li et al., 2018).

Wang et al, (2022) assessed that cervical cancer cells derived extracellular vesicles containing microRNA-146a-5p. They further characterised microRNA-146a-5p expression in clinical cervical cancer tissue samples and reported that microRNA-146a-5p in EVs activates pathways that promoted cervical cancer metastasis (Wang et al., 2022).

Melo et al. (2015), reported that glypican-1 is a highly specific cancer-derived EV marker that can be used to differentiate between patients with pancreatic ductal adenocarcinoma from healthy controls or patients with benign pancreas disease (Melo et al., 2015). EVs derived from prostate cancer contain CD151 which distinguishes them from extracellular vesicles derived from normal prostate cells. Hosseini-Beheshti et al., (2012) characterised cholesterol, lipids, and proteins from EVs derived from six prostate cell lines (Hosseini-Beheshti et al., 2012). They reported that EVs derived from these cell lines consisted of at least four markers that were CD9, CD63, actin and LAMP2. They also confirmed the presence of tetraspanins CD151 in prostate cancer derived EVs. Interestingly, this protein has been shown to be overexpressed in multiple cancers and it predicts low grade primary prostate cancer (Ang et al., 2004; Hosseini-Beheshti et al., 2012). Increasing evidence shows that EVs released from cancer cells promote cancer progression by transferring their cargo into normal cells. Furthermore, the role of EVs response to cancer treatment is increasingly studied (García Garre et al., 2018; Hong et al., 2020; König et al., 2018). For instance, Konig et al., (2018) observed that the concentration of EVs increased during therapy, and they associated the elevated EV concentration with lymph node infiltration in breast cancer patients. Circulating EV counts were used as additional markers for disease monitoring and prediction of prognosis in breast cancer patients (König et al.,

2018). Although the role of EVs in a clinical setting is still debatable, analysis of circulating EVs may allow rapid and repeated evaluation of cancer.

2.6 CERVICAL CANCER

Cervical cancer starts at the cervix which is a narrow opening between the vagina and the uterus connected to the vagina through the endocervical canal as shown in figure 6. The cervix is divided into two areas, the ectocervix and endocervix. Ectocervix is covered in stratified squamous epithelial cells while the endocervix comprises columnar epithelial cells. The stratified squamous and the columnar epithelial cells form the squamocolumnar junction in the endocervix. These cells meet at an area called the transformation zone. The transformation zone comprises metaplastic epithelium, which replaces the columnar lined epithelium of the endocervix. This zone is prone to the development of cervical cancer because it is a significant site for premalignant transformation through persistent HPV infection. Cervical cancer is divided into two major histological subtypes: squamous cell carcinoma (SCC) and adenocarcinoma. SCC develops from the squamous cells in the ectocervix and accounts for about 75% of all cervical cancer cases. Adenocarcinoma on the other hand develops from the glandular cells which are mucus producing cells in the endocervix (Bhatla et al., 2021; Burmeister et al., 2022)

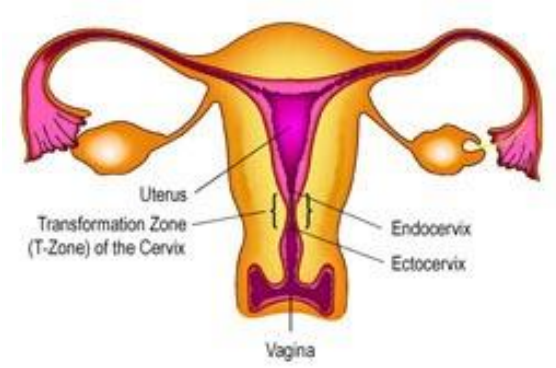

FIGURE 6: ANATOMICAL STRUCTURE OF THE UTERUS,

FROM NATIONAL CERVICAL CANCER COALITION (HTTPS://WWW.NCCC-ONLINE.ORG/HPVCERVICAL-CANCER/CERVICAL-CANCER-OVERVIEW/)

2.6.1 EPIDEMIOLOGY OF CERVICAL CANCER

Cervical cancer is the most common gynaecological cancer among females (Fan et al., 2021). Over 99% of all cervical cancer cases are caused by sexually transmitted persistent human papillomavirus (HPV) infections virus (Johnson et al., 2019). The high-risk HPVs are types 16 and 18 and they are responsible for 71% of cervical cancer cases globally (Lin et al., 2021). Other risk factors are the human immunodeficiency virus (HIV), the use of oral contraceptives, and high-risk sexual behaviours (Lin et al., 2021). Currently, there is no effective HPV treatment (Ault, 2006), however, vaccines are available in most countries (Randall et al., 2021). The first-generation HPV vaccines offer protection against the two oncogenic HPV types (16 and 18) (Davies-Oliveira et al., 2021). The second generation then protects against the five additional oncogenic types which include HPV 31, 33, 45, 52, and 58 (Davies-Oliveira et al., 2021). These vaccines have been shown to be safe and effective and are projected to prevent 70-90% of cervical cancer. The current HPV vaccines do not however treat pre-existing HPV infections and associated precancerous lesions (Zhu et al., 2018). Thus, there are several generations of females who are still at risk.

Gynaecological cancers inflict a major burden worldwide (Ginsburg et al., 2017). The World Health Organization reported cervical cancer as the fourth most frequent cancer in females with an estimated 570 000 new cases in 2018, causing 7.5 percent of all cancer deaths in females (Arbyn et al., 2020; Bray et al., 2018). In 2020 the Globocan global cancer estimates showed an estimated 604 000 new cases and 342 000 deaths in women worldwide (Sung et al., 2021). The distribution of cervical cancer differs widely, with 90% of all deaths occurring in low- and middle-income countries (Kombe Kombe et al., 2021; Sung et al., 2021). The peak regional incidence and mortality are reported in sub-Saharan Africa, with the rates most elevated in Eastern Africa and Southern Africa (Sung et al., 2021). Despite the vaccine for cervical cancer being available, less than 30% of low- and middle-income countries (LMIC) had implemented national HPV vaccine programs compared to more than 80% of high-income countries by May 2020 (Sung et al., 2021). Moreover, only about 44% females in LMIC have ever been screened for cervical cancer (Sung et al., 2021). In South Africa, 21% of the female population is estimated to harbour cervical HPV infection. In 2006, the South African Cancer Association reported that the

age-standardized incidence rate for cervical cancer was 24.71 per 100,000 population (Akokuwebe et al., 2021). The WHO has produced the Global strategy to accelerate the elimination of cervical as a public health problem. In this global strategy, 70% of women aged 35-45 are targeted to be screened at least twice with a high-performance test and 90% of females identified with lesions will get treatment (Serrano et al., 2022).

Cervical cancer is severe in women living with HIV. It is classified as an AIDS (acquired immunodeficiency syndrome) defining illness (Stelzle et al., 2021; Tisler et al., 2021). Females living with HIV have a six-fold risk of developing cervical cancer (Stelzle et al., 2021). The antiretroviral therapy has significantly decreased the mortality of HIV/AIDS. Thus, the number of adult females living with HIV has increased from an estimated 3.3 million in 1990 to 18.8 million in 2018, with about 60% of them living in Eastern and Southern Africa (Stelzle et al., 2021). Thus, there is still a high burden of HIV and cervical cancer despite both the diseases being preventable. In 2018, there was an estimated 6% of new cervical cancer cases in females living with HIV (Stelzle et al., 2021). Figure 7 shows the Age standardized incidence and mortality rates of cervical cancer by country in 2020.

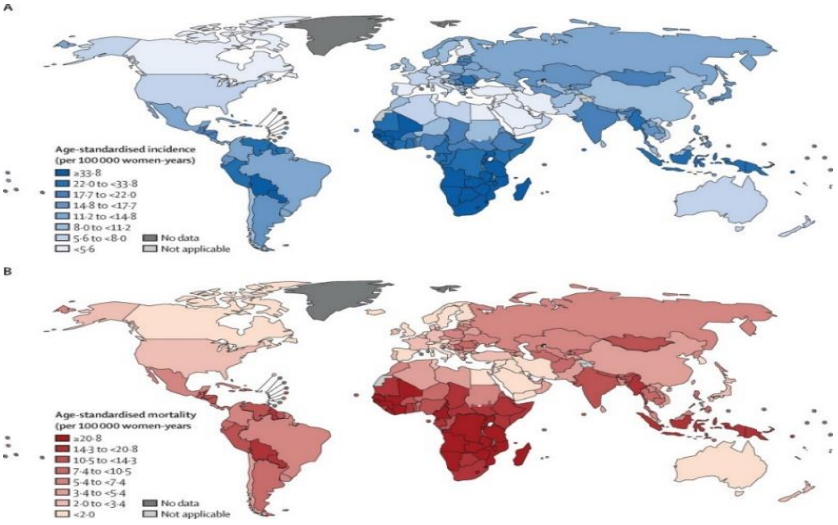

FIGURE 7: AGE STANDARDIZED INCIDENCE AND MORTALITY RATES OF CERVICAL CANCER BY COUNTRY IN 2020 (SINGH ET AL., 2023)

2.6.2 RISK FACTORS OF CERVICAL CANCER

2.6.2.1 HIGH RISK SEXUAL BEHAVIOR

Behavioral and infectious factors can increase the risk of developing cervical cancer. Behavioral factors include sexual activity and lifestyle factors. Cervical cancer is caused by HPV which is transmitted sexually. Participating in sexual activities at a younger age increases the risk of developing cervical cancer. The reason for this is assumed to be due to the cervical tissue still undergoing physiological changes where the transformation zone in the ectocervix is enlarged. Exposure to HPV at that period may enable infection making this area more susceptible to development of dysplastic cervical squamous cancer. Furthermore, having multiple sexual partners also significantly increases the risk of developing cervical cancer (Pedroza-Gonzalez et al., 2022; Sierra et al., 2021). Bruni et al. (2023) have shown that 21% of adult men carry high-risk HPV. Almost one in three men worldwide is infected with at least one genital HPV type. Moreover, the prevalence is high in men over the age of 15 years (Bruni et al., 2023)

2.6.2.2 ORAL CONTRACEPTIVES

Oral contraceptives (OC) are also associated with cervical cancer. The world health organization has regarded combined contraceptives as Group 1 carcinogens for their possible relationship to liver cancer, invasive cancer of the uterine cervix, and breast cancer. The long-term use of OC, which is greater than 5 years is associated with an elevated risk of cervical cancer (Trivedi et al., 2017). This was confirmed by Iversen et al. (2021) who studied about two million women of reproductive age. They reported an increased risk of cervical cancer among females who were recent and current users of OC. The risk was similar for both adenocarcinoma and squamous cancer. However, the risk declined after discontinuation of the OC (Iversen et al., 2021)

2.6.2.3 HUMAN PAPILLOMAVIRUSES

Human papillomaviruses (HPV) belong to the papillomaviridae family, a small group of nonenveloped viruses with a systemic classification of five genera. There are over 200 HPV subtypes with a genome in the form of circular double-stranded DNA (Hu & Ma, 2018). HPV is the most common type of sexually transmitted virus. Almost all cervical cancer cases are attributed to persistent HPV infections (Zhu et al., 2022). HPV has been

identified as the major factor leading to cervical cancer. The high-risk HPV are 16, 18, 31, 33, and 3 the low-risk types are 6, 11, 42, and 44 (Xing et al., 2021; Zhu et al., 2022). Among the high-risk types, 16 is the most carcinogenic accounting for more than half of cervical cancer cases in the world while 18 makes up 16.5% as the second most carcinogenic type (Hu & Ma, 2018). HPV infections are also associated with penile, anal, and oropharyngeal cancers. More than 90% of HPV infections clear up by themselves, however, there is a risk for all women that HPV infection may become chronic and cause pre-cancerous lesions which may progress to invasive cervical cancer.

2.6.3 SCREENING AND MANAGEMENT OF CERVICAL CANCER.

The most crucial strategy in reducing cervical cancer globally is screening (Agboola & Bello, 2021). Cervical cancer screening allows the early detection and effective treatment of prevalent cervical cancer precancerous lesions. These precancerous lesions include high-grade cervical intraepithelial neoplasm (CIN) and adenocarcinoma in situ. When detected early, precancerous lesions are treated effectively, therefore, preventing invasive cervical cancer, and subsequently reducing mortality (Mishra et al., 2011). Currently, several tests are being used to screen for cervical cancer. The Papanicolaou (Pap) smear is the current screening method (Yoo & Lim, 2019). Preventive education is also highly recommended in providing awareness of HPV risk factors and ways to reduce the risk (Ghosh et al., 2021). It is essential to understand the clinical presentation, physical assessment, diagnosis, staging, and treatment of cervical cancer. The earliest and most common clinical presentation of cervical cancer is irregular heavy bleeding. A physical exam is recommended in cases where cervical cancer is suspected.

2.6.4 FIGO STAGING OF THE CERVIX UTERI CARCINOMA.

Cervical cancer is staged by the International Federation of Gynaecology and Obstetrics (FIGO) staging. The FIGO staging of cervical cancer ranges from stage I to IV (Bhatla et al., 2021). The treatment of cervical cancer depends on the stage. Radical/curative treatment involves surgery or radiation therapy with concurrent chemotherapy. The patients at Universitas Academic Hospital that will be enrolled in the study will be patients with stage IB to IIIB disease (see FIGO staging in Table 2 below) who did not qualify for surgery, but who qualify for radical/curative treatment, taking into consideration

comorbidities. They received external beam radiation therapy, high dose rate brachytherapy with an iridium-192 source, and cisplatin chemotherapy concurrently. Table 2 outlines the FIGO staging of the cervical cancer.

TABLE 2: FIGO STAGING ACCORDING TO BHATLA ET AL. (2021)

Stage	Description
1	The carcinoma is strictly confined to the cervix (extension to the corpus should be disregarded).
IA	Invasive carcinoma that can be diagnosed only by microscopy with maximum depth of invasion ≤5 mm[a]
IA1	Measured stromal invasion ≤3 mm in depth
IA2	Measured stromal invasion >3 mm and ≤5 mm in depth
IB	Invasive carcinoma with measured deepest invasion >5 mm (greater than stage IA); lesion limited to the cervix uteri with size measured by maximum tumor diameter[b]
IB1	Invasive carcinoma >5 mm depth of stromal invasion and ≤2 cm in greatest dimension
IB2	Invasive carcinoma >2 cm and ≤4 cm in greatest dimension
IB3	Invasive carcinoma >4 cm in greatest dimension
II	The cervical carcinoma invades beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall
IIA	Involvement limited to the upper two-thirds of the vagina without parametrial invasion
IIA1	Invasive carcinoma ≤4 cm in greatest dimension
IIA2	Invasive carcinoma >4 cm in greatest dimension
IIB	With parametrial invasion but not up to the pelvic wall
III	The carcinoma involves the lower third of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or non-functioning kidney and/or involves pelvic and/or para-aortic lymph nodes
IIIA	Carcinoma involves lower third of the vagina, with no extension to the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or non-functioning kidney (unless known to be due to another cause)
IIIC	Involvement of pelvic and/or paraaortic lymph nodes (including micrometastases)[c] irrespective of tumor size and extent (with r and p notations).[d]
IIIC1	Pelvic lymph node metastasis only
IIIC2	Paraaortic lymph node metastasis
IV	The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum. A bullous edema, as such, does not permit a case to be allotted to stage IV

IVA	Spread of the growth to adjacent organs
IVB	Spread to distant organs

[a]Imaging and pathology can be used, where available, to supplement clinical findings with respect to tumour size and extent, in all stages. Pathological findings supersede imaging and clinical findings.
[b]The involvement of vascular/lymphatic spaces should not change the staging. The lateral extent of the lesion is no longer considered.
[c]Isolated tumour cells do not change the stage but their presence should be recorded.
[d]Adding notation of r (imaging) and p (pathology) to indicate the findings that are used to allocate the case to Stage IIIC. For example, if imaging indicates pelvic lymph node metastasis, the stage allocation would be Stage IIIC1r; if confirmed by pathological findings, it would be Stage IIIC1p. The type of imaging modality or pathology technique used should always be documented. When in doubt, the lower staging should be assigned.

2.6.5 RADICAL TREATMENT

Cisplatin is a common chemotherapeutic drug that is administered intravenously (Trummer et al., 2018). It is used widely to treat cancers such as ovarian, lung, breast, and cervical cancer (Helm & States, 2009; Ma et al., 2019; Michalska et al., 2018). It has clinically proven effectiveness against carcinomas, germ cell tumours, lymphomas, and sarcomas. The cisplatin crosslinks with the N7 reactive centre of the purine bases of the deoxyribonucleic acid (DNA) interfering with the repair of DNA (Kiss et al., 2021). This mechanism thus leads to DNA damage and subsequently lead to cell apoptosis (Kiss et al., 2021).

Radiation therapy uses a controlled dose of radiation to destroy cancer cells (Chino et al., 2020). X-ray beams are targeted to the areas of the body where cancer cells are located or might have spread (Williamson et al., 2021). The external beam radiation therapy (EBRT) machine precisely directs radiation from outside the body to the areas like the cervix (Kisling et al., 2019). Brachytherapy also known as internal radiation therapy delivers radiation therapy directly to the tumour inside the body (Holschneider et al., 2019). It is administered as a boost after the EBRT. The high dose brachytherapy is imbedded to the HDR-miniaturized radionuclide sources, mostly iridium-192 and cobalt-60 (Dayyani et al., 2021). Patients at the Universitas Academic Hospital Complex mostly

receive iridium-192. Iridium-192 emits gamma rays which deliver a cytotoxic dose to the tumour site (Dayyani et al., 2021).

2.6.6 Effect of radiation and chemotherapy on EVs.

Treatment of cancer cells with cytotoxic compounds such as cisplatin may lead to increased secretion of extracellular vesicles. The elevated release of EVs after treatment is probably because of cellular stress induced by the treatment (Samuel et al., 2018). Sukati et al., (2022) showed that cranial radiation releases extracellular vesicles in the context of brain injury (Sukati et al., 2022).

Circulating EVs have been studied in cancers, such as lung and breast cancer, and have been reported as potential biomarkers of the disease (Toth et al., 2008; Tseng et al., 2013). There is however no available information on circulating extracellular vesicles in cervical cancer.

2.7 Aim

This study aimed to quantify and characterise extracellular vesicles in the systemic circulation before, during, and after the treatment of cervical cancer patients.

2.8 Objectives

- To quantify and characterize extracellular vesicles in patients with cervical cancer before, during and after treatment.

CHAPTER 3: METHODOLOGY

3.1 ETHICS

Ethics approval for this study was obtained from the Health Sciences Research Ethics committee of the University of the Free State (UFS-HSD2022/1282/3101), with permission to conduct the research from the Free State Department of Health Provincial Research Committee (FS_202211_010)

3.2 STUDY LOCATION

This study was conducted at the Special Haemostasis Laboratory of the National Heath Laboratory Service (NHLS), Universitas Academic Laboratories, and the University of the Free State, Bloemfontein, South Africa. Patient samples were obtained from patients with cervical cancer treated at the Department of Oncology, Universitas Academic Hospital Complex, Bloemfontein.

3.3 STUDY DESIGN

This was a short cohort longitudinal study.

3.4 STUDY PARTICIPANTS

All patients aged 18 years and older diagnosed with cervical cancer and for whom radical/curative, treatment was planned, were eligible for this study (a maximum of 20 patients). Informed consent was obtained prior to participants being enrolled to the study. This study was conducted in accordance with the Declaration of Helsinki. The radical treatment entailed the following: external beam radiation (EBRT) (50 Gy in 25 fractions), high dose rate brachytherapy of five implants to a total equivalent dose of more than 35 Gy, and weekly cisplatin 30 mg/m^2 infusions.

3.5 EXCLUSION CRITERIA

Patients who had early stage (stage I) or late stage (stage IV) cancer were excluded from the study. This was because patients with stage I disease were more likely to be treated with surgery. Patients with stage IV cervical cancer were at an advanced stage of the

disease, and often presented with hydronephrosis and kidney failure and therefore could not receive the cisplatin chemotherapy. The kidney function (an assessment of renal failure) was recorded as the glomerular filtration rate (GFR). The staging was be done according to the FIGO system. Patients who had already started treatment were excluded from this research study. Patients for whom palliative treatment is planned were also excluded from the study, as the aim of the study is to observe extracellular vesicles throughout the diagnosis and treatment with radiation and concurrent chemotherapy.

Ten patients, who were histologically confirmed to have cervical cancer were enrolled in this study. Clinical data was collected from the patient's hospital file. Baseline clinical parameters that were collected included the cancer stage, HIV status, Tumour histology, glomerular filtration rate, platelet count and neutrophil count (see table 5). These parameters were then collected weekly as citrate blood was collected each week for six weeks from the study participants (see figure 8 and table 3) (appendix 1 for supplementary data). All patients received 2 Gy EBRT daily for the first four weeks of treatment with concurrent chemotherapy. Thereafter, they received high dose iridium^{192} brachytherapy twice a week at week five and three times a week respectively at week two. (see Table 3)

3.6 SPECIMEN COLLECTION, PROCESSING, AND STORAGE

Blood was collected into 4.5 mL Vacutainer® tubes (BD-Plymouth, UK) containing 3.2% trisodium citrate by experienced medical personnel at the Department of Oncology. One blood sample was collected on seven occasions for each participant: before treatment, when a patient arrived to start with the treatment course, and once a week in the morning during treatment (six times as the treatment lasts six weeks). During the six-week treatment period, blood for this research project was collected during the phlebotomy for the routine blood samples before the weekly chemotherapy. See Table 3 for the weekly blood collection. The researcher remained in daily contact with the oncology personnel who collected patient samples. Figure 8 outlined the blood collection process during the study.

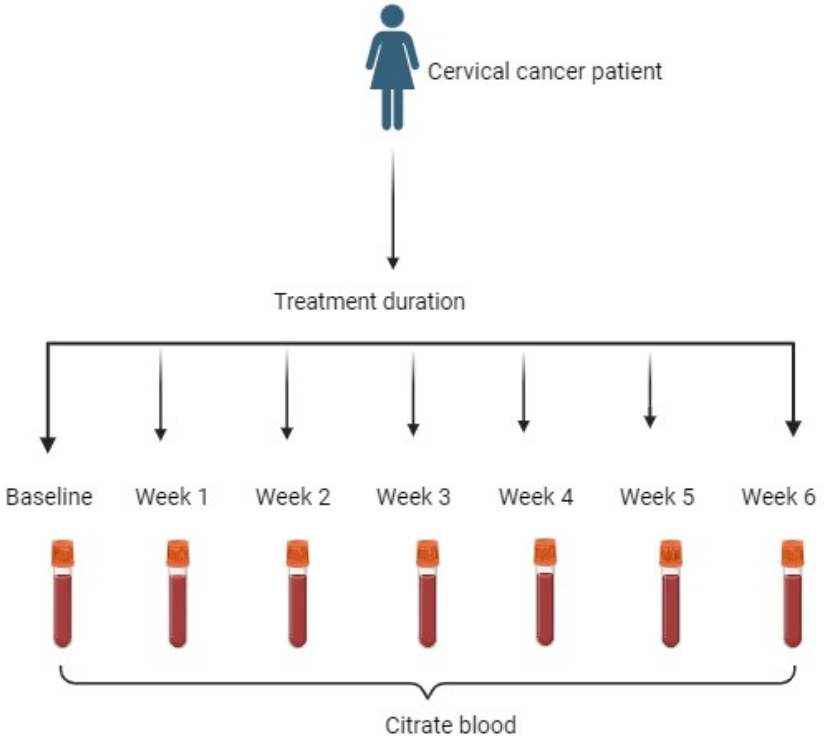

FIGURE 8: BLOOD COLLECTION OUTLINE

Because the period of data collection each patient included six weeks of treatment and three months of follow-up, a minimum of ten patients was acceptable. Samples were collected in the morning prior to chemotherapy infusion to avoid apoptotic bodies contamination and transported to the Special Haemostasis Laboratory within four hours at room temperature for processing. Table 3 shows an outline of the study from week 0 (arrival at the clinic) to week 6 (discharge of patient). Unfortunately, all patients were lost to follow up, therefore we could not report the after-treatment data because majority of the participants were from Lesotho, and they did their follow up in their country.

TABLE 3: CERVICAL CANCER STUDY OUTLINE FROM WEEK 0 WHEN PATIENTS FIRST ARRIVE AT THE UNIVERSITAS ACADEMIC HOSPITAL ONCOLOGY ANNEX, ONCOLOGY TREATMENT CENTER.

Weeks						
Week 0 (Arrival at Oncology treatment centre)	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
	Weekly Bloods					
1 EDTA tube & 1 ACD tube (Done as routine at Oncology) + citrate tube for the research study.	✓	✓	✓	✓	✓	✓
	Treatment					
Recruitment Consent forms	Daily EBRT (2 Gy)	Daily EBRT (2 Gy)	Daily EBRT (2 Gy)	Daily EBRT (2 Gy)	Daily EBRT (2 Gy) + High dose brachytherapy (1 x ^{192}Ir implant daily for 3 days)	High dose brachytherapy (1 x ^{192}Ir implant daily for 2 days)
Before (baseline) blood tube (citrate) Collection of baseline clinical information	Cisplatin chemotherapy (30 mg/m^2) (once a week)	Cisplatin chemotherapy (30 mg/m^2) (once a week)	Cisplatin chemotherapy (30 mg/m^2) (once a week)	Cisplatin chemotherapy (30 mg/m^2) (once a week)	Cisplatin chemotherapy (30 mg/m^2) (once a week)	Cisplatin chemotherapy (30mg/m^2) (once a week)
NB. EBRT was administered every day during the week for five days in the first five weeks, there was no treatment on weekends. Chemo infusions were administered once a week. On the last two weeks of treatment, high dose brachytherapy implants were delivered three times in the 5th week, and two times in the 6th week. Please note that the before blood was collected on week 0 after the patient had signed consent.						

3.7 PREPARATION OF PLASMA

Upon arrival at the Special Haemostasis laboratory, samples were centrifuged (Sigma, Burlington, Massachusetts, United States) at 200 x g for 20 minutes at room temperature. Plasma was collected above the buffy coat using a plastic Pasteur pipette and transferred into a clean (5 mL) plastic tube. Subsequently, the plasma was centrifuged at 1550 x g for 20 minutes at room temperature to minimise platelets. Afterwards, plasma was collected into 5 mL plastic tubes using Pasteur pipette leaving approximately 100 µL of plasma at the bottom of the tube to avoid platelet contamination. Plasma samples was stored at -80°C.

3.8 ISOLATION OF EVS USING IZON SIZE EXCLUSION CHROMATOGRAPHY (SEC)

3.8.1 PREPARATION OF PHOSPHATE BUFFERED SALINE (PBS)

- Phosphate Buffered Saline (PBS) was prepared by adding 11.68 g of Sodium Chloride, 9.55 g of Disodium phosphate,5.28 g of Sodium phosphate monobasic dihydrate to 1 liter of deionized water (pH 7.2)

The PBS was filtered using a 0.22 µm sterile syringe filter (Fisher Scientific, Ireland)

3.8.2 SEC COLUMNS

Plasma EVs were isolated by size exclusion chromatography (SEC) using qEV1 columns (qEV1/70 nm/IC1-70, Izon Science, Christchurch, New Zealand). The qEV1 columns were equilibrated at room temperature for 15 minutes prior to use. The qEV1 columns were then mounted on the Izon Automatic Fraction Collector (AFC) (Izon Science, Christchurch, New Zealand). Subsequently the columns were flushed twice using filtered PBS (pH 7.2, 0.22 µm filtered). Plasma was thawed at room temperature for an hour. Then 1 mL of thawed plasma was loaded into the column, followed by elution with PBS (pH 7.2, 0.22 µm filtered). The eluate was collected in four fractions of 0.70 mL using the Izon AFC. The fractions were then pooled together for subsequent analysis.

3.8.3 Flow cytometry using an Apogee A60 Micro-PLUS® flow cytometer

High-resolution flow cytometry was performed on an A60 Micro-PLUS flow cytometer (Apogee Flow Systems, Hemel Hempstead, UK) equipped with 405 nm (300 mW), 638 nm (180 mW) and 488 nm (200 mW) diode lasers. FITC signals were collected from the 488 nm laser into a photomultiplier tube (PMT) fitted with a 530/40 bandpass filter. PE signals were collected from the 488 nm laser into a PMT with a 575/30 bandpass filter, and APC signals were collected from the 638 nm laser into a PMT with a 680/35 bandpass filter. Data were acquired for 120 seconds at a sample flow rate of 1.50 µL/min and the sheath fluid pressure of 150 mBar. This was done to keep the sample core as tight as possible and allow for adequate exposure of EVs by the lasers. Data was recorded as events/µL for each sample. The data were recorded in the Apogee Histogram v.1.21 software utility (Apogee Flow Systems, Hemel Hempstead, Hertfordshire, UK). All data from the A60 Micro-PLUS were acquired in the FCS v. 3.0 format and analysed using Apogee Histogram v.1.21 and FlowJo v.10.8.1 (www.flowjo.com) software.

3.8.4 Calibration beads

Before sample analysis, calibration of the flow cytometer was performed using a reference bead mix (ApogeeMix, #1527 Apogee Flow Systems, UK). The bead mix is composed of a mixture of silica nanoparticles with diameters of 80, 180, 240, 300, 590, 880 and 1 300 nm. The beads have a refractive index that is comparable to that of extracellular vesicles. The reference bead populations were used to provide points of reference in the light scatter data, for instance the 300 nm EV population scatters roughly the same as the 300 nm silica test bead. The ApogeeMix bead mix populations were also used to measure the micro-Flow cytometer's light scatter and fluorescence performance.

Before any sample analysis, a fresh filtered water sample was run as procedural control. The average events per second were less than 50 events/µL. Filtered PBS (pH 7.2, 0.22 µm filtered) was also run with the average events per second being less than 400 events/µL. To determine the background caused by each antibody, the antibodies were each incubated in filtered PBS for an hour, after which the events per microlitre were measured.

3.9 EV STAINING AND FLOW CYTOMETRIC ANALYSIS

3.9.1 EV STAINING

To characterise EVs using flow cytometry, the following primary monoclonal fluorescently labelled antibodies were used: (All EVs) PE-conjugated anti-CD63 (H5C6, #353004), (Platelet derived EVs) Pac-Blue conjugated anti-CD41 (HIP8, #303714), (Cancer derived EVs) APC-conjugated anti-CD133 (W6B3C1, #397906), and (Neutrophil derived EVs) Alexa Flour®488 conjugated anti-CD11b (LM2, #393108). All antibodies were obtained from BioLegend, London, UK). Table 4 list the antibody concentrations used in this study. The total amount of EV's, platelet-derived EVs, cervical cancer derived EVs and neutrophil-derived EVs were measured in all study samples.

TABLE 4: LIST OF ANTIBODIES THAT WERE USED IN THIS RESEARCH STUDY.

Fluorochrome	Clone	Concentration (µg/mL)	Catalogue number
PE-CD63	H5C6-Mouse IgG1 $_k$	100	353004
PacificBlue-CD41	HIP8-Mouse IgG1 $_k$	80	303714
APC-CD133	W6B3C1-Mouse IgG1 $_k$	200	397906
Alexa flour 488-CD11b	LM2-Mouse IgG1 $_k$	100	393108

Please note that no antibody titrations were performed in this research and the volume of used for each antibody was according to the manufacturer's instruction.

3.9.1.1 ALL EVS

Fifty µl of EVs sample were labelled with 5 µL of PE-labelled anti-CD63 (H5C6 #353004, Mouse IgG1-kappa, concentration: 100µ g/mL) and incubated in the dark at RT for an

hour. Subsequently the sample was diluted with 445 µl 0.22 µm filtered PBS and analysed on the Apogee Microflow cytometer.

3.9.1.2 Platelet-derived EVs

Fifty µl of EVs sample were stained with 5 µL Pac-Blue labelled anti-CD41 (HIP8, #303714 80ug/mL) and incubated in the dark for an hour at RT. Subsequently the sample was diluted with 445 µl 0.22 µm filtered PBS and analysed on the Apogee Microflow cytometer.

3.9.1.3 Cervical cancer derived EVs

Again, fifty µl of isolated purified EVs sample were stained with 5 µL APC-labelled anti-CD133 (W6B3C1, #397906 200ug/mL mouse igG1-kappa). Subsequently the sample was diluted with 445 µl 0.22 µm filtered PBS and analysed on the Apogee Microflow cytometer.

3.9.1.4 Neutrophil derived EVs

Neutrophil derived EVs were characterised by adding 5 µL Alexa Flour ®488 labelled anti-CD11b (LM2, #393108 100ug/mL mouse igG1-kappa) to 50 µL EV sample. Subsequently the sample was diluted with 445 µL 0.22 µm filtered PBS and analysed on the Apogee Microflow cytometer.

3.9.1.5 Measurement of correlation

Spearman rank correlation was used to measure the correlation between the clinical platelet count and the platelet derived EVs. Moreover, correlation between the clinical neutrophil count and the neutrophil derived EVs was also measured.

3.9.1.6 Statistical analysis

Results were exported to Excel, and descriptive statistics were applied. Data was summarized by frequencies, percentages and means, standard deviations or percentiles (numerical variables). Data from the flow cytometry was analysed using the Apogee Histogram software v.1.21 and FloJo v10.8.1 (www.flowjo.com).

CHAPTER 4: RESULTS

TABLE 5: PATIENT DEMOGRAPHIC DISTRIBUTION

Please refer to Table 5 for a summary of the ten patients included in the study. Most patients were older than 50 years, and half were HIV positive. All had squamous cell cancer with 90% being stage IIB.

	n=10	**%**
Age *(years)*		
<50	3	30
50-65	7	70
Sex		
Female	10	100
Stage *(IIB-IIIB)*		
IIB	9	90
III	1	10
IIIA	0	0
IIIB	0	0
Histology		
SSC	10	100
HIV		
Negative	5	50
Positive	5	50

TABLE 6: CLINICAL AND LABORATORY DATA COLLECTED FROM EACH PARTICIPANT BEFORE TREATMENT (BASELINE).

Study				**CA-…**			
Clinical data							
Patient details	**Baseline**	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**
Date admitted	2/1/2023						
Date of birth (dd/mm/yyyy)	10/12/1977						
Stage (IIB-IIIB) (FIGO)	IIB						
Tumour histology	SCC						
Tumour volume (cm^3)							
Glomerular filtration rate (eGFR) (mL/min/1.73 m^2)	88	83	91	90	84	76	
HIV status (+/-)	Negative						
Platelet count x10^9/L	273	253	226	293	246	178	
Neutrophil count x10^9/L	1.78	1.8	2.08	2.74	0.85	1.82	
Weekly laboratory data							
PE-CD63 (events/µL)	77.866	163.934	55.000	52.466	50.121	35.914	19.590
Pac-Blue-CD41 (events/µL)	72.183	31.950	12.706	26.780	16.212	13.620	10.762
APC-CD 133 (events/µL)	28.963	77.926	16.261	14.861	14.041	12.000	8.244
Alexa flour 488-CD11b (events/µL)	142.720	23.529	17.738	9.523	37.957	46.059	13.977

SCC, *squamous cell carcinoma.* CA, *the study numbers all began with the prefix CA.*
Participants were discharged at week 6, however, bloods were still drawn on the 6th week for the research study.
No clinical parameters were measured for some participants because they were being discharged.

Clinical and Laboratory baseline parameters collected are outlined in Table 6, Glomerular filtration rate, Platelet count, and Neutrophil count was collected weekly from each participant for six weeks. (See Appendix 5 for all participants data)

Laboratory parameters measured weekly included the quantification of EVs using PE conjugated anti-CD63, Pacific blue conjugated anti-CD41, APC conjugated anti-CD133, Alexa flour 488® conjugated anti-CD11b and it was measured as events/µL (see Table 6). To our understanding this is the first study to investigate the presence of circulating extracellular vesicles in the plasma of cervical cancer patients before and during treatment and to study the effect of treatment on the presence of EVs in these patients. There are no other studies that have investigated extracellular vesicles in cervical cancer.

EVs quantifications were measured as events/µL from each participant at each interval. This included baseline and week one to week six. Data was reported as percentage of the baseline of each patient. The mean number of events/µL at baseline was treated as 100%. EVs measured weekly were then compared to baseline percentage. The mean and standard deviation of all participants at each interval were determined. Figure 9 shows the gating strategy that was used to define positive EV populations.

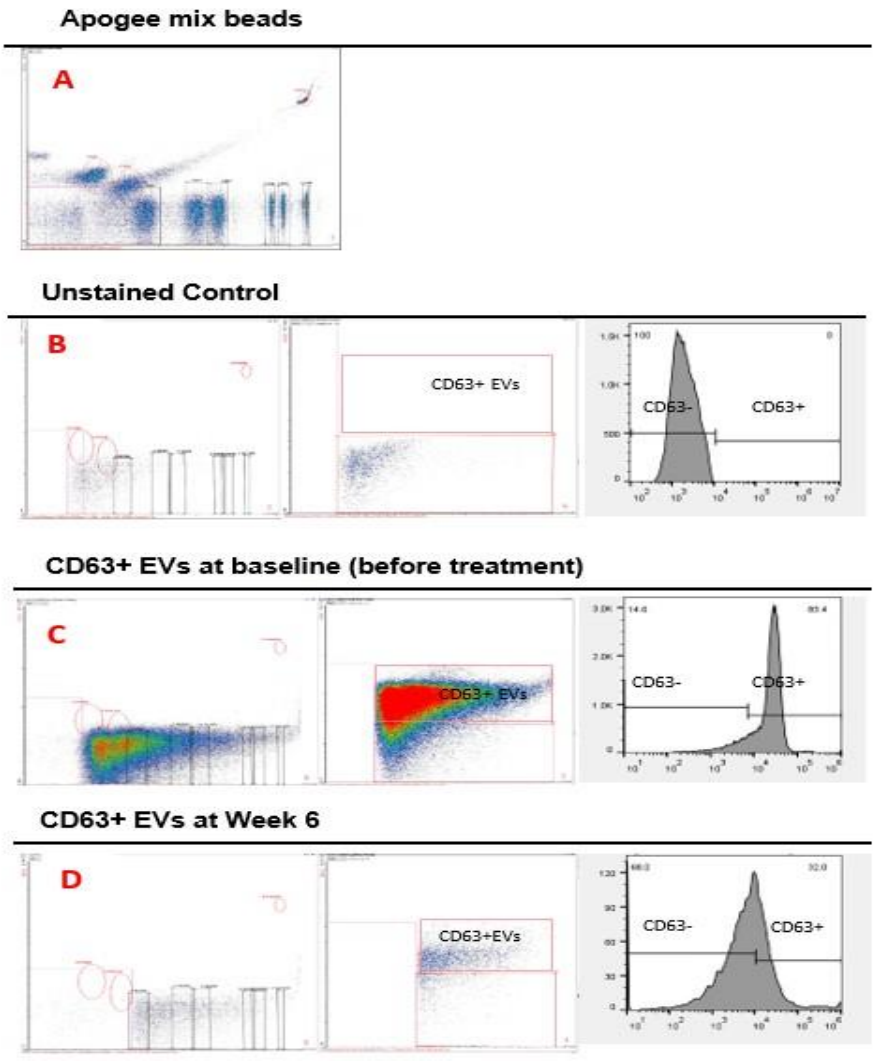

FIGURE 9: GATING STRATEGY TO DEFINE POSITIVE EV POPULATIONS. (A) APOGEE MIX BEADS CATALOG #1527 WAS USED TO PREPARE THE MICRO-FLOW CYTOMETER FOR THE ANALYSIS OF EVS. APOGEE MIX BEADS WERE USED TO ASSESS THE CYTOMETERS PERFORMANCE, SENSITIVITY AND ALSO TO DEFINE THE SIZES OF EVS. EACH GATE ON THE APOGEE MIX BEAD WINDOW REPRESENTS SILICA BEADS AND POLYSTYRENE BEADS ACCORDING TO THEIR SIZES. (B) UNSTAINED NORMAL CONTROL. (C) EVS WERE STAINED USING PE CONJUGATED ANTI-CD63 TO CONFIRM AND DEFINE POSITIVE EV POPULATIONS ON THE APOGEE MICROFLOW CYTOMETER AT BASELINE (BEFORE TREATMENT). THE EV POPULATION RANGED FROM 80–300 NM. (D) CD63 POSITIVE EVS AT WEEK AT WEEK 6.

Figures 10 to 13 show the changes in the EV compositions during the treatment period. The total number of EVs, platelet-derived EVs and cervical cancer-derived EVs increased during the first week of treatment but decreased gradually over the remaining treatment period (Figures 10-12). The neutrophil derived EVs (Fig 13) increased during the treatment period, with a maximum at week 4.

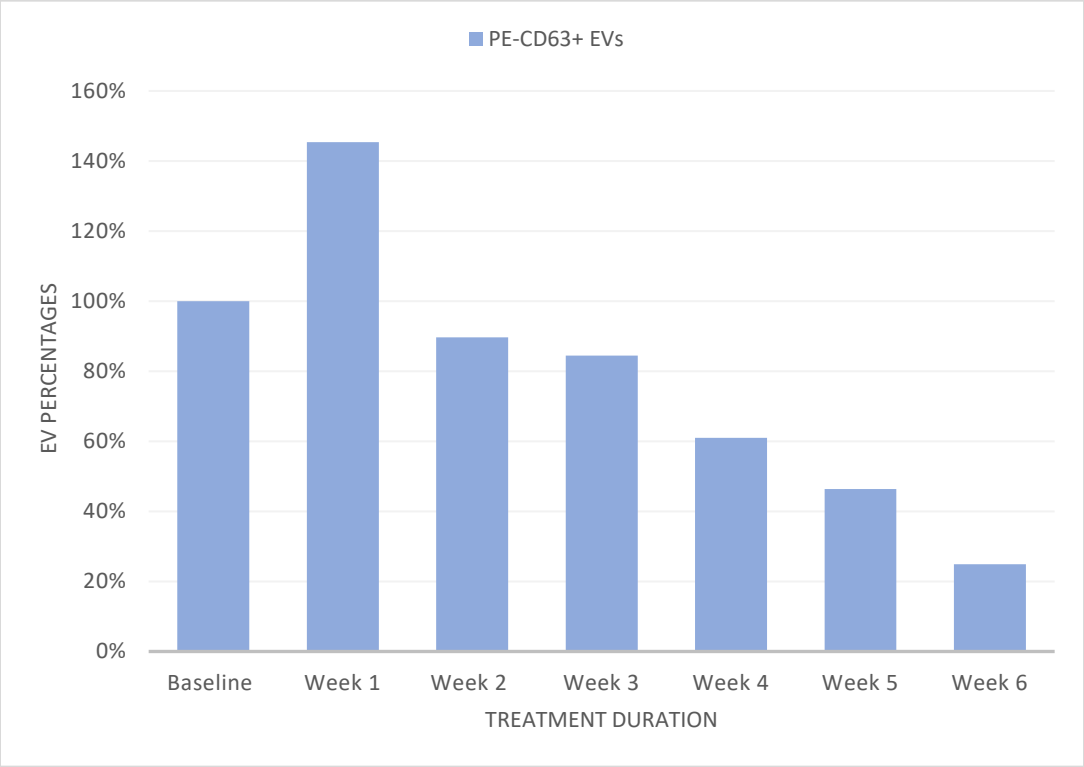

FIGURE 10: PERCENTAGES OF CD63 POSITIVE EXTRACELLULAR VESICLES FROM PLASMA OF PATIENTS WITH CERVICAL CANCER FROM BASELINE FOLLOWED THROUGHOUT THE TREATMENT PERIOD.

The main aim of this research study was to identify the presence of CD63+ EVs in plasma of patients with cervical cancer and measure their concentration throughout the treatment duration (see Figure 10). We measured the number of EVs at baseline i.e., before treatment. Subsequently, the number of EVs (events/µl) were measured weekly as the patients received chemoradiation weekly and compared each week to baseline as percentages. At week 1 we noted an elevated number of circulating CD63 positive EVs in plasma compared to baseline. This was followed by a significant decrease from week two and the following weeks respectively.

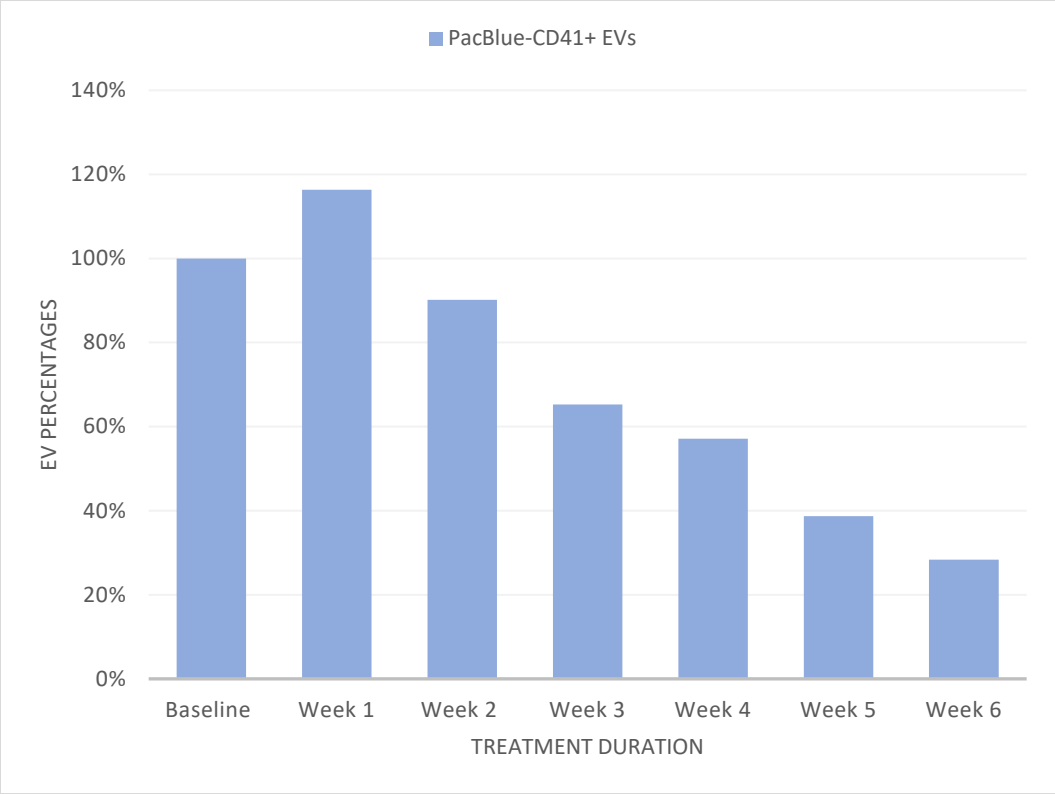

FIGURE 11: PERCENTAGES OF CD41 POSITIVE EVS

We measured the number of EVs at baseline i.e., before treatment. Subsequently, the number of EVs (events/µL) were measured weekly as the patients received chemoradiation weekly and compared each week to baseline as percentages. As can be seen in Figure 11, at week 1 we noted an elevated number of circulating CD41 positive EVs in plasma compared to baseline. This trend in platelet-derived EVs followed a similar trend as the total number of CD63+ EVs. This was followed by a significant decrease from week two and the following weeks respectively.

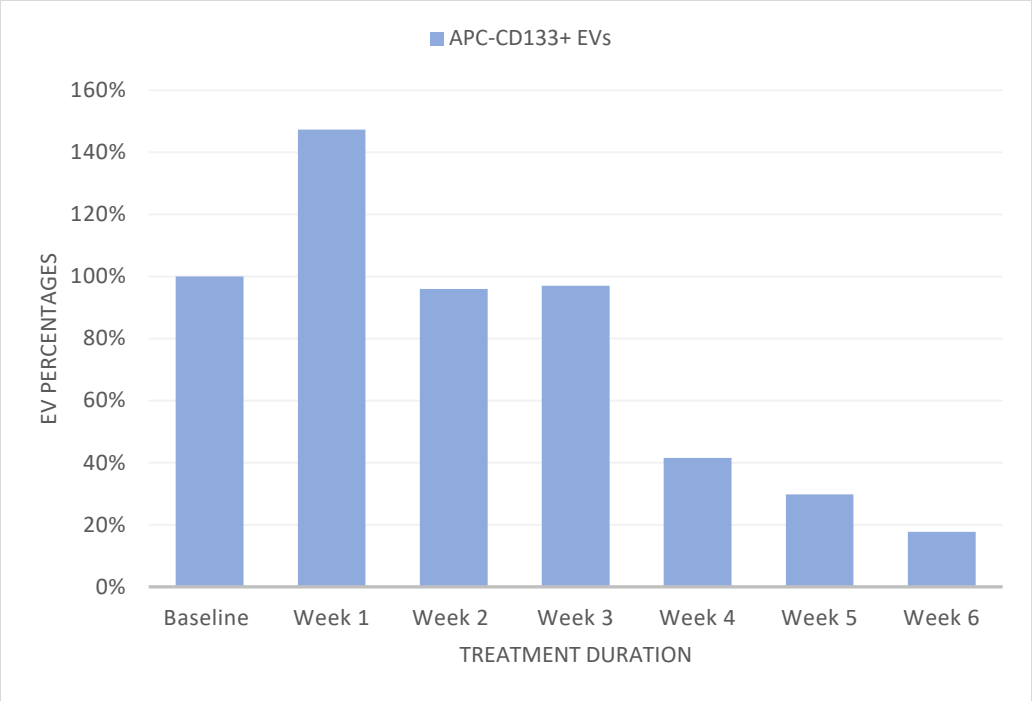

FIGURE 12: PERCENTAGES OF CD133+ EVS THROUGH THE TREATMENT DURATION.

We measured the number of CD133+ EVs at baseline i.e., before treatment as a measure of cancer derived EVs. Subsequently, the number of EVs (events/µL) were measured weekly. Mainly because patients received chemoradiation weekly and compared each week to baseline as percentages. At week 1 (Figure 12) we noted an elevated number of circulating CD133 positive EVs in plasma compared to baseline. This was followed by a significant decrease from week two and the following weeks respectively.

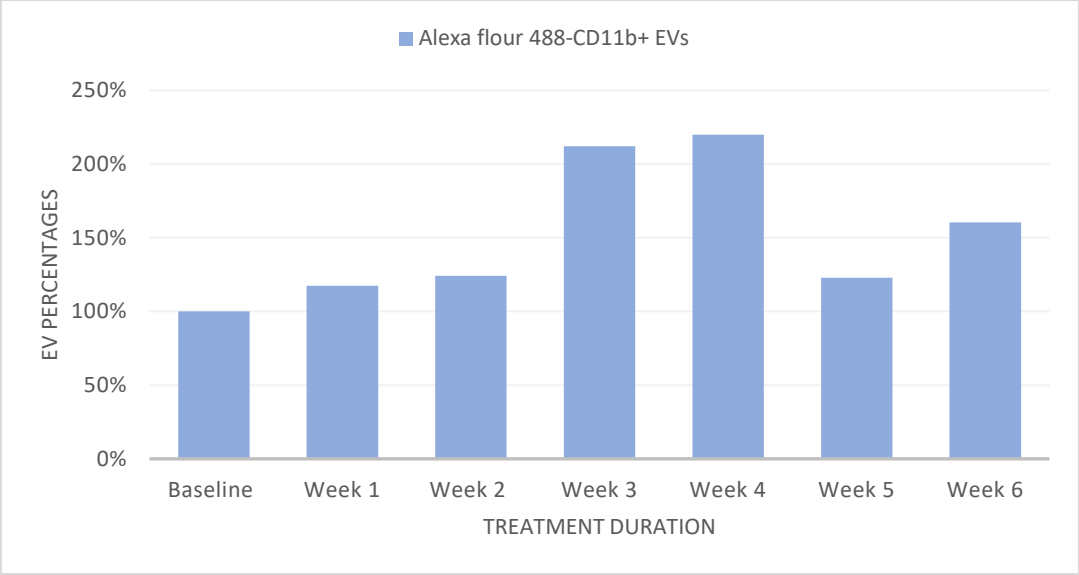

FIGURE 13: PERCENTAGES OF ANTI-CD11B POSITIVE EXTRACELLULAR VESICLES DERIVED NEUTROPHILS IN PLASMA OF PATIENTS WITH CERVICAL CANCER FROM BASELINE THROUGHOUT THE TREATMENT PERIOD.

There was a gradual increase in neutrophil derived EVs from week 1 to week 2 (see Figure 13). With the highest EV percentages observed at week 3 and week 4. There was a decrease at week 5 and week 6.

TABLE 7: SUMMARY OF ALL PERCENTAGES OF EV POPULATIONS FROM BASELINE THROUGHOUT THE TREATMENT PERIOD.

EV characteristic	Baseline (%)	Week 1 (%)	Week 2 (%)	Week 3 (%)	Week 4 (%)	Week 5(%)	Week 6 (%)
CD63+	100	145	90	85	61	46	25
CD41+	100	116	90	65	57	39	28
CD133+	100	147	96	97	42	30	18
CD11+	100	117	124	212	220	123	160

Table 7 shows the percentage of the various EV populations measured over course of the study.

The mean number of EVs collected before treatment (baseline) was measured as 100% and each week was compared to baseline throughout the treatment duration for each EV population. The highest number of CD133+ (cancer derived) EVs was observed at week 1. At week 6 CD63+, CD41+ and CD133+ significantly decreased.

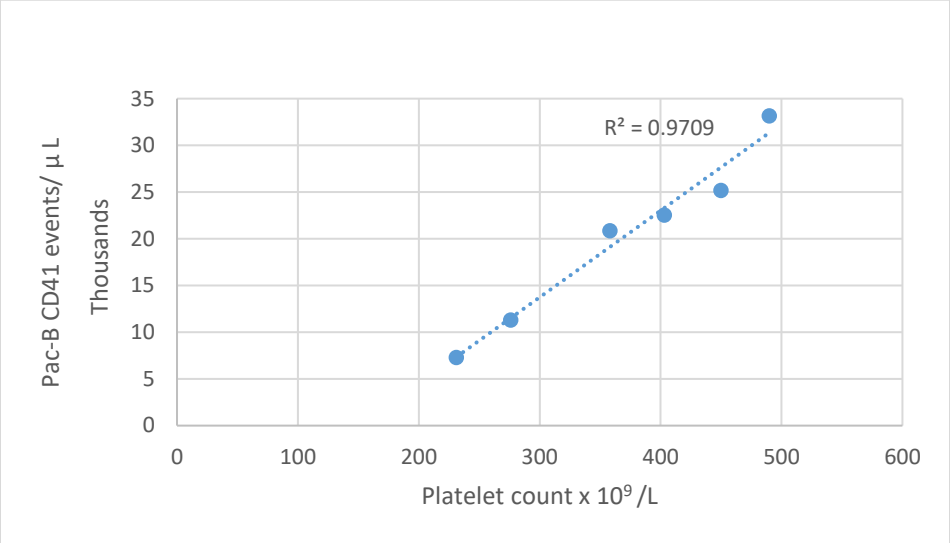

FIGURE 14: THE CORRELATION BETWEEN PLATELET DERIVED EVS AND PLATELETS WAS ASSESSED USING SPEARMAN RANK CORRELATION. WHEN THE PLATELET COUNTS INCREASED, THE NUMBER OF CIRCULATING CD41+ EVS ALSO INCREASED.

We were interested in the relationship between the platelet count (measured as part of the full blood count) and the platelet derived EVs (measured by flow cytometry). Figure 15 shows that there was a linear positive correlation between the two.

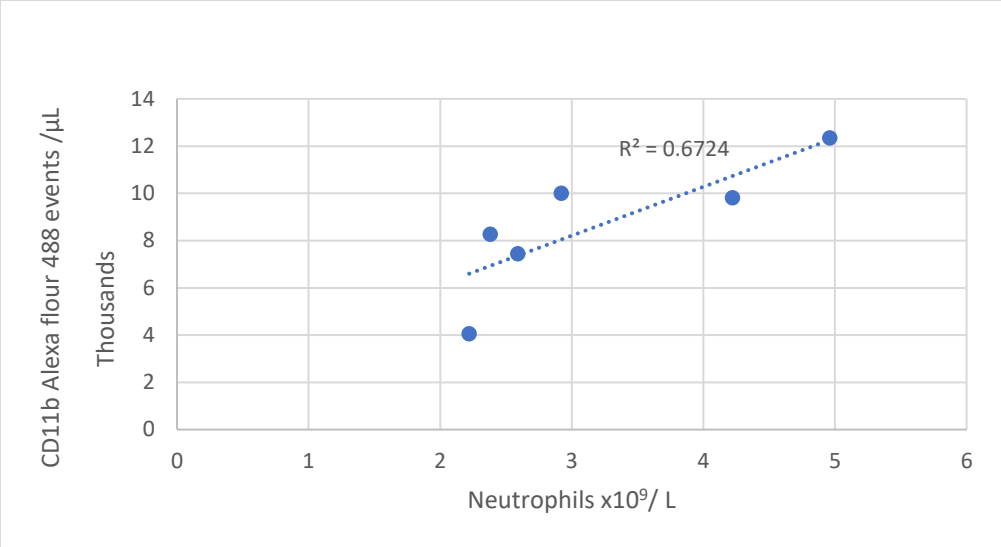

FIGURE 15: NEUTROPHIL COUNT AGAINST NEUTROPHIL DERIVED EXTRACELLULAR VESICLES IN PLASMA, THERE WAS A POSITIVE CORRELATION BETWEEN THE NEUTROPHIL COUNT AND THE NEUTROPHIL DERIVED EVS.

We were interested in the relationship between the neutrophil count (measured as part of the full blood count) and the neutrophil derived EVs (measured by flow cytometry). Figure 16 shows that there was a linear positive correlation between the two.

CHAPTER 5: DISCUSSION

All the study participants were in stage II of the disease and had squamous cell carcinoma. Five (50%) participants were confirmed HIV positive. Cervical cancer is primarily diagnosed in older females. Seven out of ten patients (70%) in our research study were older than 50 years of age (see Table 5). A similar pattern was observed in a study by Matovelo et al., (2012) (Matovelo et al., 2012). Cervical cancer is mostly diagnosed in older females (Quick et al., 2020). Seven (70%) out of ten patients in our research study were older than 50 years of age. Five (50%) participants were confirmed HIV positive (see Table 5). This is similar to the prevalence of 63.8% of new cases being HIV positive in southern Africa that was reported by Stelzle et al., (2021) (Stelzle et al., 2021)

Isolation of EVs was achieved by IZON SEC. SEC, a technique that separates particles based on size, has been shown to be high-throughput and adaptable method for the isolation of EVs from biofluids such as plasma and urine. EVs obtained from SEC demonstrates better purity and are more useful therefore the use of SEC is recommended in EV isolation (Yang et al., 2021). A similar study was conducted in our laboratory where three EV isolation techniques were compared (ultrafiltration, ultracentrifugation, and SEC). (personal communication) SEC had the highest number of CD63+ EVs compared to ultracentrifugation and ultrafiltration. The consistency of these methods was assessed over a period of three days, and SEC still had the highest number of CD63+ EVs over the period of three days. The EVs were visualised using Apogee A60 Micro-PLUS Flow cytometry. Flow cytometry has remained the gold standard in characterisation of multiple parameters. A study by Botha et al. (2021) compared conventional FACS Aria III, Apogee A60 Micro-PLUS and ImageStream X II imaging flow cytometers (Botha et al., 2021). The authors evaluated the ability of the three platforms to detect different types of EV phenotypes in blood plasma using fluorescently labelled antibodies. Their findings suggest that Apogee A60 Micro-PLUS and ImageStream X Mk II were able to detect all nanospheres. The high sensitivity of the Apogee A60 Micro-PLUS is attributed to the slow

sheath and sample flow rate, and the use of powerful lasers for illumination of particles (Botha et al., 2021).

The main findings of this research study demonstrate a significant increase in the mean number of circulating EVs at week 1 in all participants as treatment started. This was reflected in counts of CD63+ (total EVs), CD41+ (platelet-derived EVs), CD133+ (cancer-derived EVs) (see Figures 10 to 12). Week 1 is when radiation therapy is started (Table 3). Tortolici et al. (2021) reported that initial exposure to radiation therapy increases the release of EVs in patients with cancer (Tortolici et al., 2021) and non-human primates. This increase in circulating EVs is mostly a manifestation of the effect of radiation damage on the cancer cells (Cheema et al., 2018, Tortolici et al., 2021).

The rise in the EV concentrations (Figures 10-12) in the plasma of cervical cancer patients was a novel finding because no other study has investigated EVs in plasma of these patients. CD63 one of the tetraspanin antigens found on the surface of EVs (see Figure 10). Published studies have used cancer cell culture cells lines as a source of EVs (Hu et al., 2021; Stridfeldt et al., 2023).

The second novel finding was the observation of CD133+ EVs in plasma of patients with cervical cancer (see Figure 12). CD133 is pentaspan transmembrane protein that is used as a surface marker for cancer stem cells (Barzegar Behrooz et al., 2019). A series of studies have shown that CD133 promotes tumorigenesis, angiogenesis, self-renewal, and cell migration (Garzia et al., 2009; Y. Li et al., 2019; Liou, 2019). CD133 is released into various body fluids and has been reported in various types of cancers such colon, liver, and cervical cancer (Chai et al., 2016; Garzia et al., 2009; Wei et al., 2013; Yuan et al., 2022).

Platelet derived EVs in plasma have been reported in most cancer patients (Janowska-Wieczorek et al., 2005; Mezouar et al., 2014; Vismara et al., 2022). Platelet derived EVs are naturally occurring in plasma during normal physiological conditions. However, an increase in platelet derived EVs has been observed in clinical conditions associated with platelet activation such as cancer. CD41 positive EVs are released from activated platelets during the cytoskeleton membrane adhesion loss (Contursi et al., 2023).

Therefore, Platelet derived EVs are used in disease prognosis. Wang et al., (2017) showed that that circulating platelet derived EVs were higher in patients with non-small lung cancer patients compared to normal controls, but after three months of treatment, the circulating platelet and endothelial EVs were significantly lower, and were comparable to those of the normal controls (Wang et al., 2017). Our study did not compare cancer patients to healthy individuals.

From week 2 to week 6 of treatment, there was a significant decrease in the mean number of circulating EVs in plasma from all the participants. Once again, this was observed in CD63 + (total EVs), CD41+ (platelet-derived EVs), CD133+ (cancer-derived) EVs (Figures 10 to 12). This demonstrates that the effects of chemoradiation therapy causes an initial release of EVs, but as the number of cancer cells decreases during the therapy, the number of circulating EVs decreases. Similar to our findings, a study conducted in breast cancer patients reported that endothelial cell derived EVs in plasma decreased after chemotherapy and this was associated with better prognoses of breast cancer patients (García Garre et al., 2018). In contrast, Hong et al., (2020) Reported that in acute myeloid patients, the levels of extracellular vesicles remained high after chemotherapy, this was associated with therapy resistance. None of the patients in our study demonstrated resistance to chemotherapy (Hong et al., 2020). At week 6 the lowest mean percentage of EVs was CD133+ compared to CD63+ and CD41+ (see Table 7). This was expected because platelet derived EVs are naturally occurring in plasma of all individuals and are always present.

Interestingly, neutrophil derived EVs did not demonstrate a steady trend (see Figure 13). From baseline to week 2, there was an increase in the mean number of neutrophil derived EVs in plasma of all the participants. Neutrophils indicate the state of the acute host inflammation, which is induced by the radiation on top of the cancer. Additionally, neutrophils infiltrate tumour microenvironment causing tumour proliferation by weakening the immune system (Xiong et al., 2021). However, this was not observed in plasma circulating CD11b+ EVs which was used as a marker for EVs. However, at week 3 and 4 of treatment, we observed a higher percentage of CD11b+ EVs compared to baseline (as shown in Figure 13). This finding can be attributed to inflammation caused by

chemotherapy. Similar to the clinical neutrophil count, neutrophil derived EVs were elevated in the plasma of the patients with cervical cancer with the highest percentages at week three and week four.

A decrease in the mean number of plasma neutrophil derived EVs (CD11b+) was observed at week 5 and week 6 (as shown in Figure 13). Based on this finding, we conclude that neutrophil derived EVs can be used in monitoring the effect of chemotherapy in cervical patients. Unfortunately, no other studies have explored the presence of neutrophil derived EVs in plasma of cervical cancer patients. Neutrophil derived EVs were first discovered by Stein and Luzio. They are produced in response to immunological stimuli during inflammatory processes (Stein & Luzio, 1991). Cisplatin has been shown to induce pro-inflammatory signaling in cervical intraepithelial neoplasia (Domingo et al., 2022). Based on our findings we can suggest that inflammation caused by chemotherapy can be observed by the number of neutrophil derived EVs in plasma.

Lastly, it has been demonstrated that circulating EVs report the current state of a cell or reflect the stimulus at which they produced. To prove this, we measured correlation between platelet derived EVs and the clinical platelet count (Figure 14). We further measured the correlation between neutrophil derived EVs and the clinical neutrophil count (Figure 15). A positive correlation was observed in both parameters respectively. Based on these findings we confirmed that circulating EVs do reflect on the parent of origin and stimulus at which they are produced because.

A study conducted by Javed et al., (2018) reported that cervical cancer stem cells display a CD133 positive phenotype. A study by Brocco et at., (2020) looked at circulating CD133-derived EVs as a novel biomarker for clinical outcome evaluation in cancer (Brocco et al., 2020). In their research, they demonstrated that the total blood concentration of EVs was significantly elevated in patients with cancer compared to healthy volunteers (Brocco et al., 2020). Our research study did not compare cervical cancer patients to healthy volunteers, however looked at the number of circulating EVs in cervical cancer patients before treatment initiation and during treatment for the six-week treatment duration. Our findings demonstrated an overall significant decrease in the number of circulating CD63+ (all EVs), CD41+ (platelet-derived), CD133+ (cancer-derived) and CD11b+ (neutrophil-

derived) EVs from before treatment initiation compared to the week six which was the last week of treatment with CD133+ being the lowest.

Excessive concentration of CD31+ (platelet derived EVs) EVs have been detected in breast and colorectal cancer patients, while an excessive concentration of CD133+ EVs have been identified in lung cancer patients (Brocco et al., 2020). Our findings demonstrated a higher amount of CD133+ and CD41+ EVs at week one compared to week 6 (Figure 11 and 12) (Table 7).

As can be expected, chemoradiation therapy causes an initial increase in circulating EVs, followed by a significant decrease in the number of circulating EVs over treatment duration (Figures 10-12). This has been demonstrated in other research studies. However, these studies used cell culture cells as a source of EVs. Our study is the first to measure EVs in plasma. A study by Ehteram Hassan et al., 2022, reported that CD133 as a colorectal cancer predictive prognostic biomarker in clinical assessment. Moreover, CD133 could serve as a therapeutic target for colorectal cancer. In their research they noted a higher expression of CD133 in advanced tumour stage (Ehteram et al., 2022). Similarly, a study by Stridfeldt., 2023 demonstrated that CD9+ and CD81+ EVs decreased after H1975 cells were treated with Osimertinib and erlotinib. This was when they isolated EVs from the H1975 cell culture media. They then treated the cells and measured the amount EVs after treatment (Stridfeldt et al., 2023).

CHAPTER 6: CONCLUSIONS

This study has provided a novel insight into the presence of EVs in plasma of cervical cancer patients. Cervical cancer ranks the fourth most common cancer in females. Despite the availability of vaccines cervical cancer still poses a major burden in low- and middle-income countries. Recent technologies have allowed the rapid analysis of EVs. Biological content and physical durability of EVs makes them a highly suitable and promising material in cancer monitoring. A population of CD63+ EVs was observed in plasma of cervical cancer patients. Furthermore, there were CD41+, CD133+, and CD11b+ EVs indicative of their parent cells (Figure 11 to 12) (Figure 14 and 15). Therefore, the present research study confirms that there are circulating EVs in plasma Which can be attributed to cancer. Treatment influences the number of circulating EVs, causing a significant gradual decrease in the number of circulating EVs over time. This can be directly attributed to the decrease in the cancer. Therefore, EVs present as a good treatment monitoring tool in real time. Our study has laid a foundation for future studies that aim to investigate EVs in plasma of cervical cancer patients. Taking into consideration the findings of this research, Cervical cancer metastasis can be explored by further investigating the cargo that the cervical cancer derived EVs carried.

LIMITATIONS

One of the limitations of this research study was that most of the study participants were from Lesotho and they did not come back for a follow-up in South Africa. Moreover, all the study participants were on stage II of the disease, therefore we could explore the differences in the number of circulating EVs at different stages of cervical cancer. The sample size was relatively small mainly because of the inclusion and exclusion of criteria of the research study. Due to late and delayed presentation and diagnosis, most cervical cancer patients seen at Universitas Academic Hospital Annex have more advanced stages where the cancer has infiltrated outside of the cervix into the surrounding tissue. Another limitation was that this research study did not compare the cervical cancer

patients to normal controls as it is mostly done in literature. The study of extracellular vesicles is relatively new and still requires extensive research to be fully understood.

FUTURE STUDIES/ RECOMMENDATIONS.

Future studies protein-based assays such as Western Blotting to characterise oncoproteins such as E6 and E7 from extracellular vesicles using Western Blotting is recommended. We further recommend a repeat of this study on a larger sample size and on participants that are from South Africa to avoid patients being lost to follow up. Studying the presence of EVs in patients with different stages of the cervical cancer is also recommended to investigate if the number of EVs reflect on the stage of the cancer. Lastly, we recommend studying the presence of EVs in patients on early stage such a stage I before and after surgery as it would be interesting to determine the effect of surgical treatment on circulating EVs.

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APPENDICES

APPENDIX 1: PE-CD63 EVENTS PER MICROLITER OF THE ALL THE PARTICIPANTS

Study ID	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
CA-01	5,157	4,220	3,477	3,853	4,011		
CA-02	77,866	163,934	55,000	52,466	50,121	35,914	19,590
CA-03	143,022	156,055	83,241	66,971	7,995	6,479	5,908
CA-04	83,612	159,077	126,405	114,990	111,369	97,335	50,085
CA-05	8,740	14,556	12,366	8,017	7,980	4,893	4,700
CA-06	36,935	43,871	27,870	16,811	12,293	10,319	7,319
CA-07	31,062	60,354	26,202	23,430	20,102	11,908	4,108
CA-08	30,785	78,165	38,162	102,985	68,633	17,340	11,698
CA-09	33,715	37,460	55,273	33,429	22,636	20,322	7,929
CA-10	75,366	47,262	43,829	21,741	15,826	14,916	6,437
Mean	**52,626**	**76,495**	**47,183**	**44,469**	**32,097**	**24,381**	**13,086**
STDEV	**42,178**	**61,066**	**36,103**	**39,147**	**34,618**	**28,863**	**14,661**
CV	**0.80**	**0.80**	**0.77**	**0.88**	**1.08**	**1.18**	**1.12**
% of Baseline	**100%**	**145%**	**90%**	**85%**	**61%**	**46%**	**25%**

APPENDIX 2: PACIFIC BLUE-CD41 EVENTS PER MICROLITER OF THE ALL THE PARTICIPANTS

Study ID	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
CA-01	6,308	3,320	2,888	2,869	2,335		
CA-02	72,183	31,950	12,706	26,780	16,212	13,620	10,762
CA-03	138,113	117,836	107,690	54,615	35,996	23,331	10,228
CA-04	50,606	104,106	85,674	83,020	76,970	44,514	48,400
CA-05	10,698	9,812	6,468	6,553	7,294	8,713	3,889
CA-06	25,180	33,146	20,852	22,528	11,286	7,281	6,191
CA-07	23,624	23,489	50,950	22,582	18,781	14,982	4,649
CA-08	46,192	107,660	47,227	23,361	22,830	12,290	8,218
CA-09	29,933	27,836	27,737	20,135	40,151	18,634	8,769
CA-10	40,909	57,245	37,912	27,165	21,734	11,306	12,224
Mean	**44,375**	**51,640**	**40,010**	**28,961**	**25,359**	**17,186**	**12,592**
STDEV	**38,304**	**42,788**	**34,332**	**23,482**	**21,594**	**11,362**	**13,715**
CV	**0.86**	**0.83**	**0.86**	**0.81**	**0.85**	**0.66**	**1.09**
% of Baseline	**100%**	**116%**	**90%**	**65%**	**57%**	**39%**	**28%**

APPENDIX 3: APC-CD133 EVENTS PER MICROLITER OF THE ALL THE PARTICIPANTS

Study ID	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
CA-01	2,711	3,832	3,313	2,314	2,299		
CA-02	28,963	77,926	16,261	14,861	14,041	12,000	8,244
CA-03	118,816	149,697	118,477	68,040	8,327	5,670	5,573
CA-04	41,873	49,316	48,492	48,256	27,663	22,730	16,343
CA-05	5,509	8,640	7,901	4,576	4,266	2,706	2,621
CA-06	21,406	28,434	19,140	16,218	10,458	7,051	5,318
CA-07	36,246	39,685	20,944	17,726	13,233	6,814	2,840
CA-08	28,700	79,127	45,359	130,807	36,996	15,048	6,968
CA-09	26,406	30,576	21,051	17,249	14,972	12,040	4,096
CA-10	33,925	40,428	29,968	14,213	10,769	8,392	2,998
Mean	**34,456**	**50,766**	**33,091**	**33,426**	**14,302**	**10,272**	**6,111**
STDEV	**32,179**	**42,746**	**33,300**	**39,749**	**10,556**	**6,004**	**4,294**
CV	**0.93**	**0.84**	**1.01**	**1.19**	**0.74**	**0.58**	**0.70**
% of Baseline	**100%**	**147%**	**96%**	**97%**	**42%**	**30%**	**18%**

APPENDIX 4: ALEXA FLOUR 488 ®-CD11B EVENTS PER MICROLITER OF THE ALL THE PARTICIPANTS

Study ID	Baseline	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
CA-01	15,604	47,160	42,168	11,606	16,258		
CA-02	142,720	23,529	17,738	9,523	37,957	46,059	13,977
CA-03	60,160	17,328	125,610	39,709	57,034	48,980	232,558
CA-04	72,873	206,506	130,937	299,813	318,122	35,489	161,756
CA-05	7,446	9,998	4,058	8,275	9,810	12,347	7,821
CA-06	22,579	13,725	35,962	9,614	19,158	73,809	27,523
CA-07	36,800	87,505	65,671	7,077	20,440	51,611	9,982
CA-08	6,441	15,595	16,387	15,120	15,030	11,602	10,051
CA-09	24,792	19,965	107,752	351,421	227,125	217,558	203,148
CA-10	77,321	106,724	32,687	237,171	305,491	18,902	6,960
Mean	**46,674**	**54,804**	**57,897**	**98,933**	**102,643**	**57,373**	**74,864**
STDEV	**42,573**	**62,951**	**47,216**	**139,044**	**127,725**	**63,485**	**95,091**
CV	**0.91**	**1.15**	**0.82**	**1.41**	**1.24**	**1.11**	**1.27**
% of Baseline	**100%**	**117%**	**124%**	**212%**	**220%**	**123%**	**160%**

Appendix 1- 4: Events/µL were measured on each participant weekly for six weeks, data was summarized as a mean and standard deviation.

APPENDIX 5 (A-G): CLINICAL AND LABORATORY DATA OF ALL THE PARTICIPANTS WEEKLY.

A

Study ID	D.O.B	CaCx Histolo	Stage of CaC	Dose of cisplatin	Dose of EBRT	eGFR (mL/min/1.73m^2	Neutr	Platelet count x 10^9 /L	PE-CD 63 e	Pac-B CD41 ev	CD133 events / uL
CA-01	10/12/1977	SCC	IIB	30 mg/m^2	2 Gy	113	7.92	542	5.157	6.308	2.711
CA-02	9/16/1974	SCC	IIB	30 mg/m^2	2 Gy	88	3.68	273	77.866	72.183	28.963
CA-03	7/10/1959	SCC	IIB	30 mg/m^2	2 Gy	93	5.27	377	143.022	138.113	118.816
CA-04	4/3/1968	SCC	IIB	30 mg/m^2	2 Gy	75	6.59	308	83.612	50.606	41.873
CA-05	11/22/1979	SCC	IIB	30 mg/m^2	2 Gy	68	5.56	227	8.74	10.698	5.509
CA-06	10/28/2023	SCC	IIB	30 mg/m^2	2 Gy	96	9.38	450	36.935	25.18	21.406
CA-07	2/10/1959	SCC	IIB	30 mg/m^2	2 Gy	80	12.95	385	31.062	23.624	36.246
CA-08	9/3/1970	SCC	IIB	30 mg/m^2	2 Gy	104	6.37	264	30.785	46.192	28.7
CA-09	3/20/1968	SCC	IIB	30 mg/m^2	2 Gy	101	22.16	471	33.715	29.933	26.406
CA-10	7/12/1968	SCC	IIB	30 mg/m^2	2 Gy	63	9.08	391	75.366	40.909	33.925

Clinical and laboratory data at baseline for all the research participants. Clinical data (eGFR, neutrophil count, and platelet count) were collected from the participants hospital file

B

Study ID	Dose of cisplatin	Dose of EBRT	eGFR (mL/min/1.73m^2	Neutr	Platelet count x 10^9 /L	PE-CD 63 events /µ L	Pac-B CD41 events / µ L	APC-CD133 events / µ L
CA-01	30 mg/m^2	2 Gy	119	7.52	530	4.22	3.32	3.832
CA-02	30 mg/m^2	2 Gy	83	2.98	253	163.934	31.95	77.926
CA-03	30 mg/m^2	2 Gy	95	4.03	345	156.055	117.836	149.697
CA-04	30 mg/m^2	2 Gy	78	6.23	366	159.077	104.106	49.316
CA-05	30 mg/m^2	2 Gy	70	5.76	213	14.556	9.812	8.64
CA-06	30 mg/m^2	2 Gy	78	8.43	490	43.871	33.146	28.434
CA-07	30 mg/m^2	2 Gy	75	11.17	342	60.354	23.489	39.685
CA-08	30 mg/m^2	2 Gy	99	3.82	306	78.165	107.66	79.127
CA-09	30 mg/m^2	2 Gy	110	14.7	275	37.46	27.836	30.576
CA-10	30 mg/m^2	2 Gy	69	6.14	427	47.262	57.245	40.428

Clinical and laboratory data at week 1 for all the research participants. Clinical data (eGFR, neutrophil count, and platelet count) were collected from the participants hospital file.

C

Study ID	Dose of cisplatin	Dose of EBRT	eGFR (mL/min/1.73m^2	Neutr	Platelet count x 10^9 /L	PE-CD 63 events/µ L	Pac-B CD41 events/µ L	APC-CD133 events/µ L
CA-01	30 mg/m^2	2 Gy	118	6.51	414	3.477	2.888	3.313
CA-02	30 mg/m^2	2 Gy	91	2.95	226	55	12.706	16.261
CA-03	30 mg/m^2	2 Gy	72	4.54	267	83.241	107.69	118.477
CA-04	30 mg/m^2	2 Gy	63	7.11	275	126.405	85.674	48.492
CA-05	30 mg/m^2	2 Gy	80	3.62	172	12.366	6.468	7.901
CA-06	30 mg/m^2	2 Gy	85	5.78	358	27.87	20.852	19.14
CA-07	30 mg/m^2	2 Gy	82	11.65	469	26.202	50.95	20.944
CA-08	30 mg/m^2	2 Gy	108	2.67	292	38.162	47.227	45.359
CA-09	30 mg/m^2	2 Gy	110	13.56	266	55.273	27.737	21.051
CA-10	30 mg/m^2	2 Gy	74	4.85	387	43.829	37.912	29.968

Clinical and laboratory data at week 2 for all the research participants. Clinical data (eGFR, neutrophil count, and platelet count) were collected from the participants hospital file.

D

Study ID	Dose of cisplatin	Dose of EBRT	eGFR (mL/min/1.73m^2	Neutr	Platelet count x 10^9 /L	PE-CD 63 events / µ L	Pac-B CD41 events / µ L	APC-CD133 events / µ L
CA-01	30 mg/m^2	2 Gy	113	5.26	435	3.853	2.869	2.314
CA-02	30 mg/m^2	2 Gy	90	3.73	293	52.466	26.78	14.861
CA-03	30 mg/m^2	2 Gy	72	5.79	250	66.971	54.615	68.04
CA-04	30 mg/m^2	2 Gy	97	2.22	269	114.99	83.02	48.256
CA-05	30 mg/m^2	2 Gy	66	3.77	199	8.017	6.553	4.576
CA-06	30 mg/m^2	2 Gy	86	6.22	403	16.811	22.528	16.218
CA-07	30 mg/m^2	2 Gy	93	7.03	313	23.43	22.582	17.726
CA-08	30 mg/m^2	2 Gy	108	2.06	265	102.985	23.361	130.807
CA-09	30 mg/m^2	2 Gy	111	15.58	255	33.429	20.135	17.249
CA-10	30 mg/m^2	2 Gy	86	3.38	257	21.741	27.165	14.213

Clinical and laboratory data at week 3 for all the research participants. Clinical data (eGFR, neutrophil count, and platelet count) were collected from the participants hospital file.

E

Study ID	Dose of cisplatin	Dose of EBRT	eGFR (mL/min/1.73m^2	Neutr	Platelet count x 10^9 /L	PE-CD 63 events / µ L	Pac-B CD41 events / µ L	APC-CD133 events / µ L
CA-01	30 mg/m^2	2 Gy	115	3.89	405	4.011	2.335	2.299
CA-02	30 mg/m^2	2 Gy	84	1.44	208	50.121	16.212	14.041
CA-03	30 mg/m^2	2 Gy	85	2.74	220	7.995	35.996	8.327
CA-04	30 mg/m^2	2 Gy	99	2.62	275	111.369	76.97	27.663
CA-05	30 mg/m^2	2 Gy	76	5.49	292	7.98	6.294	4.266
CA-06	30 mg/m^2	2 Gy	100	3.97	276	12.293	11.286	10.458
CA-07	30 mg/m^2	2 Gy	82	6.68	269	20.102	18.781	13.233
CA-08	30 mg/m^2	2 Gy	115	1	262	68.633	22.83	36.996
CA-09	30 mg/m^2	2 Gy	111	6.91	315	22.636	40.151	14.972
CA-10	30 mg/m^2	2 Gy	83	2.37	237	15.826	21.734	10.769

Clinical and laboratory data at week 4 for all the research participants. Clinical data (eGFR, neutrophil count, and platelet count) were collected from the participants hospital file.

F

Study ID	Dose of cisplatin	Dose of EBRT	eGFR (mL/min/1.73m	Neutr	Platelet count x 10^9 /L	PE-CD 63 events/ µ L	Pac-B CD41 events/ µ l	APC-CD133 events/ µ L
CA-01								
CA-02	30 mg/m^2	2 Gy	76	1.82	178	35.914	13.762	12,000
CA-03	30 mg/m^2	2 Gy	72	2.1	130	6.479	23.331	5,670
CA-04	30 mg/m^2	2 Gy	101	1.7	131	97.335	44.514	22.73
CA-05	30 mg/m^2	2 Gy	80	6.2	435	4.893	8.713	2.706
CA-06	30 mg/m^2	2 Gy	79	3.91	231	10.319	7.281	7.051
CA-07	30 mg/m^2	2 Gy	79	5.67	258	11.908	14.982	6.814
CA-08	30 mg/m^2	2 Gy	117	2.99	227	17.34	12.29	15.048
CA-09	30 mg/m^2	2 Gy	111	3.44	333	20.322	18.634	12.04
CA-10	30 mg/m^2	2 Gy	94	2.68	161	14.916	11.306	8.392

Clinical and laboratory data at week 5 for all the research participants. Clinical data (eGFR, neutrophil count, and platelet count) were collected from the participants hospital file.

G

Study ID	Dose of cisplati	eGFR (mL/min/1.73m^2	Neutro	Platelet count x 10^9 /L	PE-CD 63 events / µ L	Pac-B CD41 events/ µ l	APC-CD133 events / µ L
CA-01							
CA-02	30 mg/m^2						
CA-03	30 mg/m^2						
CA-04	30 mg/m^2	92	1.47	163	50.085	48,400	16.343
CA-05	30 mg/m^2						
CA-06	30 mg/m^2						
CA-07	30 mg/m^2	79	4.54	213	4.108	4.649	2.84
CA-08	30 mg/m^2	124	3.88	127	11.698	8.218	6.968
CA-09	30 mg/m^2	103	2.45	245	7.929	8.769	4.096
CA-10	30 mg/m^2	89	1.93	177	6.437	3.224	2.998

Clinical and laboratory data at week 6 for all the research participants. Clinical data (eGFR, neutrophil count, and platelet count) were collected from the participants hospital file.

APPENDIX 6: HSREC ETHICS APPROVAL LETTER

Health Sciences Research Ethics Committee

05-Dec-2022

Dear **Ms Noluthando Gasa**

Ethics Clearance: **Qauntification and Characterisation of extracellular vesicles before during and after treatment**

Principal Investigator: **Ms Noluthando Gasa**

Department: **Haematology and Cell Biology Department (Bloemfontein Campus)**

Submission Page

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is:**UFS-HSD2022/1282/3101**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

Research conducted in any Department of Health facility: Researchers are required to sign and return the HSREC approval letters to the provincial Department of Health where they applied. It is also a requirement for researchers to submit electronic copies of their final research findings, and/or make a presentation of their findings and recommendations at departmental research days when and where indicated.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2020); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; International Council for Harmonisation (ICH) Harmonised Guideline, Integrated Addendum to ICH E6(R1), Guideline for Good Clinical Practice (GCP) E6(R2), 2016, SAHPRA Guidelines as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

The Principal Investigator (PI) bears final responsibility for the RIMS application. In the event of any misconduct or improper activities perpetuated by a third party, the PI will be held vicariously liable. The HSREC will bear no responsibility or liability for any actions of a PI and/or third party or breach of

APPENDIX 7: FREE STATE DEPARTMENT OF HEALTH APPROVAL LETTER

health
Department of Health
FREE STATE PROVINCE

29 November 2022

Ms N Gasa
Haematology and Cell Biology Department
University of the Free State

Dear Ms N Gasa

Subject: Quantification and Characterisation of extracellular vesicles before during and after treatment

- Please ensure that you read the whole document, Permission is hereby granted for the above–mentioned research on the following conditions:
- Participation in the study must be voluntary and written consent by each participant must be obtained.
- Serious adverse events to be reported to the Free State department of health and/ or termination of the study.
- Ascertain that your data collection exercise neither interferes with the day-to-day running of **Universitas Hospital** nor the performance of duties by the respondents or health care workers.
- The Department of Health expects that the researcher will be the responsible data manager according to the POPI Act. The responsibility thus lies with the researcher to ensure that the processing of all participants' personal information and research data is lawful according to the stipulations of the POPI Act (Protection of Personal Information Act 4 of 2013).
- Confidentiality of information will be ensured and please do not obtain information regarding the identity of the participants.
- Department of Health to be fully indemnified from any contravention of the POPI Act as you conduct this study.
- **Research results and a complete report should be made available to the Free State Department of Health upon completion of the study (a hard copy plus a soft copy).**
-
- Progress report must be presented not later than one year after approval of the project to the Ethics Committee of the University of Free State and to the Free State Department of Health.
- Any amendments, extensions, or other modifications to the protocol or investigators must be submitted to the Ethics Committee of the University of Free State and to the Free State Department of Health.
- **Conditions stated in your Ethical Approval letter should be adhered to and a final copy of the Ethics Clearance Certificate should be submitted to Sebeelats@fshealth.gov.za/Gwantshuws@fshealth.gov.za before you commence with the study**
- No financial liability will be placed on the Free State Department of Health.
- **Please discuss your study with the Institution Manager on commencement for logistical arrangements.**
- Department of Health is to be fully indemnified from any harm that participants and staff experience in the study.
- As part of the feedback, you will be required to present your study findings/results at the Free State Provincial Health Research Day.

Trust you find the above in order.

Kind Regards

MR. MNG MAHLATSI
HEAD: FREE STATE DEPARTMENT OF HEALTH

Date: 30/11/2022

Head: Health
PO Box 827, Bloemfontein, 9300
4th Floor, Executive Suite, Bophelo House, cnr Maitland and, Harvey Road, Bloemfontein
Tel: (051) 408 1646 Fax: (051) 408 1555 e-mail:khusem@fshealth.gov.za / chikobwupe@fshealth.gov.za
www.fs.gov.za