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Characterization of circulating extracellular vesicles and detection of the CD147 biomarker in a cohort of patients with colorectal cancer at the Hospital Universitario de San Agustín

MASTER IN BIOTECNOLOGY OF ENVIRONMENT AND HEALTH

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The research work entitled "Characterization of circulating extracellular vesicles and detection of the CD147 biomarker in a cohort of patients with colorectal cancer at the Hospital Universitario de San Agustín" has been made under our supervision by Hugo Pérez Botas, during his studies at the Master of Biotechnology of Environment and Health by Oviedo University. The work was carried out in the laboratory of the Nanoparticles, Membranes and Bioanalysis Research Group (NanoBioMem-Lablink) at the Physical and Analytical Chemistry Department in Oviedo University.

We hereby state that we have read and corrected the present Master Thesis document, and that it is suitable for its public defence by the student at the designed tribunal. Therefore, we authorize the submission of this Master Thesis to the University of Oviedo, MBEH academic commission.

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Abstract

Colorectal cancer places a huge burden on the healthcare systems around the world. With the aging of the population and without radical changes in lifestyle, cases are expected to increase in the coming decades. Current methods of diagnosing colon cancer are colonoscopy and Fecal-occult blood tests. Colonoscopy is invasive, requires specialized equipment and personnel, and waiting lists delay diagnosis. Fecal occult blood tests, on the other hand, are not capable of detecting all cases, their handling of the sample is laborious, and there is a risk of giving a false positive. For this reason, new diagnostic methods are being sought, preferably to be used in blood and based on point-of-care technology, that is, they can be used in the place and time in which the patient is attended. Extracellular vesicles, small lipid membranes that cells release to communicate, have the potential to be biomarkers for colon cancer. Abnormally high levels of the CD147 metalloprotease in extracellular blood borne extracellular vesicles have been associated with colorectal cancer patients, although it has not yet been investigated how this expression varies according to the development of the disease. One method for studying extracellular vesicles is lateral flow immunoassays, a simple and rapid technique that is very popular within point-of-care technologies. This work aims to use lateral flow immunoassays to study the variation of the CD147 protein in patients at different stages of colorectal cancer development, while evaluating the potential of these immunoassays as a diagnostic tool for colon cancer.

Resumen

El cáncer colorrectal supone una enorme carga a los sistemas de salud de todo el mundo. Con el envejecimiento de la población y sin unos cambios radicales en los hábitos de vida, se espera que los casos aumenten en las próximas décadas. Los métodos actuales de diagnóstico del cáncer de colon son la colonoscopia y las pruebas de sangre oculta en heces. La colonoscopia es invasiva, requiere equipamiento y personal especializado, y las listas de espera retardan el diagnostico. Las pruebas de sangre oculta en heces, por su parte, no son capaces de detectar todos los casos, su manipulación de muestra es laboriosa, y tiene riesgo de dar falso positivo. Por esta razón se están buscando nuevos métodos de diagnóstico, preferiblemente para usarse en sangre y basados en tecnología *point-of-care*, es decir, que puedan ser utilizados en el lugar y momento en que se atiende al paciente. Las vesículas extracelulares, pequeñas membranas lipídicas que las células liberan para comunicarse, tienen el potencial de ser biomarcadores para el cáncer de colon. Niveles anormalmente elevados de la metaloproteasa CD147 en vesículas extracelulares de plasma sanguíneo han sido asociados con pacientes de cáncer colorrectal, aunque aún no se ha investigado como esta expresión varía según el desarrollo de la enfermedad. Un método para estudiar las vesículas extracelulares son los inmunoensayos de flujo lateral, una técnica sencilla y rápida que es muy popular entre las tecnologías *point-of-care*. Con este trabajo se pretende utilizar inmunoensayos de flujo lateral para estudiar la variación de la proteína CD147 en pacientes en distintas fases de desarrollo de cáncer colorrectal, al tiempo que se evalúa el potencial de estos inmunoensayos como método de diagnóstico del cáncer de colon.

Abbreviations

AdH: High-risk Adenocarcinoma

AdL: Low-risk Adenocarcinoma

CM: chylomicrons

CRC: Colorectal cancer

CSPY: Colonoscopy

CT: Control

EBV: Epstein-Barr virus

ELISA: Enzyme Linked Immunosorbent Assay

EVs: Extracellular vesicles

FIT: Fecal Immunochemical Test

FOBT: Fecal Occult blood test

gFOBT: Guaiac Fecal Occult Blood Test

HDL: high-density lipoprotein

LDL: Low-density lipoprotein

LFIA: Lateral Flow Immunoassay

MVBs: Multivesicular bodies

NTA: Nanoparticle Tracking Analysis

POC: Point-of-care

VLDL: Very low-density lipoprotein

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1. INTRODUCTION

1.1. The problem of colorectal cancer

Cancer is considered one of the heaviest burdens for the healthcare systems in the world. According to WHO, it is the third greater cause of death worldwide and the second in developed countries, only behind cardiovascular diseases. GLOBOCAN (Global Cancer Observatory) is a project promoted by the OMS that seeks to provide national's level information of incidence, mortality, and prevalence for the main types of cancer in 184 countries (Bray et al., 2018). These estimations are made from the data gathered by the IARC (International Agency Research Centre) and is disposable to the public via Internet. According to GLOBOCAN, colorectal cancer was responsible of 1 931 590 cases worldwide in 2020, being the third most common type of cancer (Figure 1). Nevertheless, regarding mortality, colorectal cancer caused 935 173 deaths, becoming the most lethal behind lung cancer.

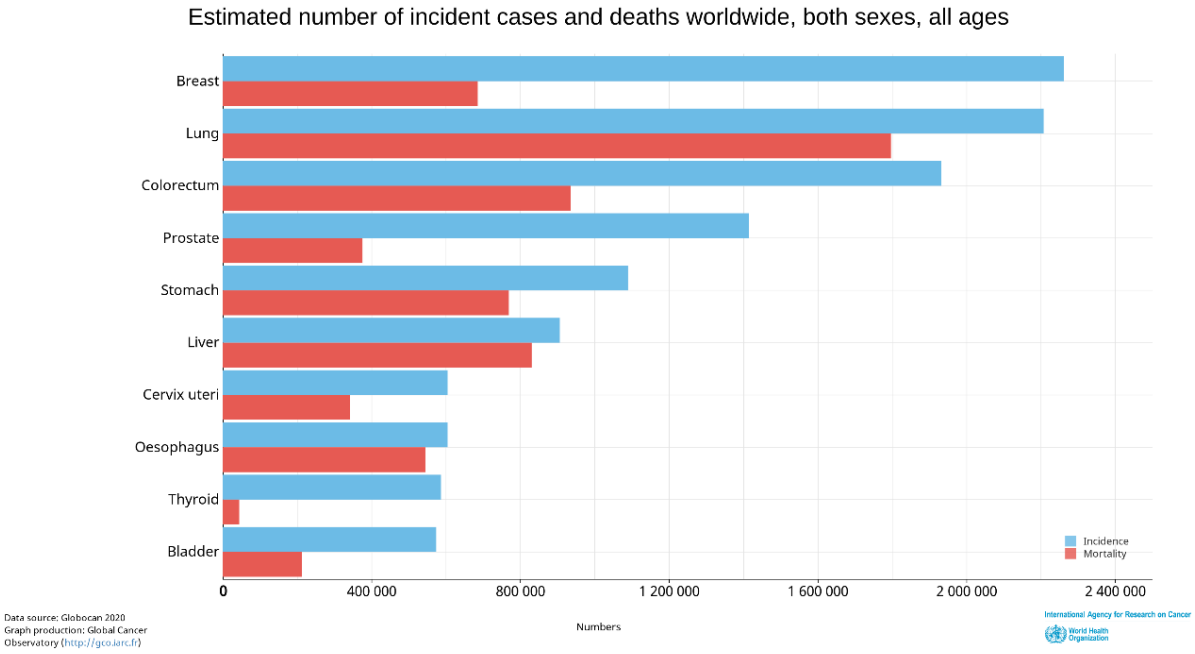


Figure 1 Bar chart representing the estimated incidence (blue bar) and mortality (red bar) worldwide of the ten most frequent types of cancer in raw numbers. (Source: GLOBOCAN)

In Spain, colorectal cancer represents the most common tumour type diagnosed in 2019 when both sexes are considered together (44.937 cases), only surpassed by prostate cancer on males and breast cancer on women (*INE. Instituto Nacional de Estadística*, n.d.). Regarding mortality, as INE (Instituto Nacional de Estadística) statistics shows, in 2018 colorectal cancer was responsible of the second highest number of deaths (11265 deaths), behind lung cancer. Separated by sex, colorectal cancer was the second most lethal type of cancer in men (after lung cancer) and the third in women (after breast and lung cancer).

Although cancer can occur in people of all ages, it is predominant in people over 50 years of age. For colon cancer, the average age at diagnosis is 68 years for men and 72 years for women. For rectal cancer, the age is 63 for both genders. Colorectal cancer affects men slightly more (*Colorectal Cancer - Risk Factors and Prevention*, 2012). The trend in recent years and estimations indicate an increase in the cases of this neoplasm incidence due to the increase in the population and its aging. However, the mortality and incidence of colorectal cancer seems to change more quickly than in other tumors. Mainly because it is more susceptible to changes in lifestyle and diet. Therefore, attending to risk factors is a key step in its prevention and treatment (Arnold et al., 2017).

1.2. Current techniques in colorectal cancer diagnosis

Nowadays, the techniques used for CRC diagnosis are grouped into two major categories: stool test, that includes hidden blood detection and exfoliated DNA, and structural exams, which includes several imaging-based techniques as flexible sigmoidoscopy, colonoscopy (CSPY), double-contrast barium enema; and computed tomographic colonography. Stool test are adequate for the detection of cancer, and in a few cases, for the detection of advanced adenomas (allowing polypectomy and prevention of CRC), although this is not the final goal of fecal tests. Meanwhile, structural exams are capable of detect both adenocarcinomas as well as adenomatous polyps (Levin et al., 2008).

1.2.1. Stool tests

The most common options among the stool tests are stool blood tests, also known as faecal occult blood tests (FOBT) since they are focused on finding hidden blood in the stool. Colorectal polyps and cancers are weak structures that can be easily damaged by the passage of stool, the damaged vessels release their blood into the colon or rectum, but in normal circumstances there is not enough blood to be seen by the naked eye. Blood in the stool is non-specific, it can be caused by CRC and large polyps (>1 to 2 cm), but also from injuries of the rectum that pose no risk of developing CRC. Also, small adenomatous polyps do not bleed, and bleeding from large polyps or cancer tend to be intermittent (Levin et al., 2008; Rex et al., 2006; Smith et al., 2001; Winawer et al., 2003). For this reasons, a proper use of the stool blood test demands annual testing consisting of 2 or 3 samples from consecutive bowel movements (Levin et al., 2008; Rex et al., 2006; Smith et al., 2001; Winawer et al., 2003). There are two formats of FOBT depending on the analyte: Guaiac Fecal Occult Blood Test (gFOBT) and Fecal Immunochemical Test

(FIT). gFOBT is the most common stool blood test used for CRC screening and the only one with confirmed efficacy from prospective randomized controlled trials. Guaiac-based is an enzymatic test that detects blood through pseudo-peroxidase activity of heme/haemoglobin (Hb), meanwhile FIT is an immunochemical method that detects directly human haemoglobin (Levin et al., 2008).

gFOBT protocols usually involve collecting 3 samples from 3 consecutive bowel movements at home. gFOBT detect Hb concentrations above 600µg/g of stool, although sensitivity and specificity of the gFOBT are highly variable (Levin et al., 2008; Schreuders et al., 2016; Tinmouth et al., 2015). Some studies show sensitivity values varies depending on test variant (Allison et al., 1996); test specimen collection technique (Collins et al., 2005); number of samples collected per test (Lieberman et al., 2001); rehydration of the specimen (i.e., adding a drop of water to the slide window before processing) (Simon, 1985); and variations in interpretation, screening interval, and other factors (Ransohoff & Lang, 1997). Diet must be carefully controlled during testing, gFOBT are not specific for human Hb, giving false positive results with foods that contain peroxidase activity (like red meat or uncooked vegetables); gFOBT tests detect Hb from the upper digestive tract, so gastrolesive drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) or aspirin can also cause false positive results. Finally, vitamin C inhibits the pseudo-peroxidase activity from Hb, so consumption of quantities of vitamin C higher than 250mg/ day can increase the number of false negative results (Schreuders et al., 2016).

FIT (Fecal Immunochemical Tests) is an immunochemical method of detection hidden blood on stool. FIT has multiple technological advantages in comparison with gFOBT. FIT detects human globin, a protein fundamental in the structure of haemoglobin along with heme (Levin et al., 2008; Schreuders et al., 2016). This means it is much more specific than guaiac-based tests. Since FIT test are independent from peroxidase activity, they are also compatible with high-vitamin C consumption. Cause globin is vulnerable to digestion on the upper gastrointestinal tract, FIT is also more specific to low-gastrointestinal bleeding, and thus, to CRC (Schreuders et al., 2016). Finally, FIT sampling is less demanding, requiring fewer samples and less handling of stool. Regarding FIT tests performance, there is no evidence of superior performance of FIT tests in comparison with high-sensitivity gFOBT tests, which detect from 40 to 300µg/g of blood in feces (Levin et al., 2008; Schreuders et al., 2016).

1.2.2. Structural Exams

Structural examinations are based on detecting abnormalities in the wall of the colon and rectum by direct observation. They are a much more invasive option, and they have more patient requirement, as previous bowel preparation, an office or hospital visit with the proper equipment and resources and have various levels of risk to patients. The most important options for structural examinations colonoscopy (CSPY) (Levin et al., 2008).

CSPY is one of the most performed medical procedures, 14 million were performed in 2003 only in the USA (Seeff et al., 2004). CSPY allows direct mucosal inspection of the entire colon from the appendiceal orifice to the dentate line and same-session biopsy sampling or definitive treatment by polypectomy, in the case of precancerous polyps and some early-stage cancers. The colonoscope can examine the entire bowel, with the examination terminating at the cecum (Levin et al., 2008). CSPY is used as comparison to other positive screening tests, that CSPY and polypectomy have shown incidence reductions in randomized controlled trials of other screening tests (Levin et al., 2008; Mandel et al., 2000). It is strictly obligatory to follow a liquid diet one or more days before the examination, followed by either ingestion of oral lavage solutions or saline laxatives to stimulate bowel movements until the bowel is clean. Proper bowel preparation is a critical element in the accuracy and cost-effectiveness of screening with CSPY (Levin et al., 2008). It is possible to apply a mild sedative prior to the procedure, but its optional, the patient can decide if endure a mild discomfort during the procedure. Despite this, CSPY cannot be considered a "gold standard". Controlled studies have shown the CSPY miss rate for large adenomas (> 10 mm) to be 6% to 12% (Rex et al., 1997), and the reported CSPY miss rate for cancer is about 5% (Bressler et al., 2004). Also, CSPY can result in significant harm, most often associated with post-polypectomy bleeding. Post-polypectomy bleeding is favoured by large polyp size and proximal colon location; however, small polyp bleeds are more numerous than large polyp bleeds because small polyps are so numerous. Other potential harms are perforation or cardiopulmonary complications due to anaesthesia (Levin et al., 2008).

1.2.3. The need of new biomarkers

The diagnosis of CRC has several problems, the methods described above are based on the presence of cancer symptoms, thus limiting their precision. In addition, in stool tests determining the stage of cancer is not always possible and the handling of feces is laborious. Observation by CSPY, on the other hand, consumes a large amount of resources,

is laborious and requires much more time, it pose a risk of complications like bleeding, bowel tears or the utilization of anaesthesia, although it is much more effective in determining the stage of cancer at the time of diagnosis. Moreover, physical observation methods require advanced instrumentation and well-trained experts for diagnosis. Thus, there is an urgent need to develop alternative methods that are user-friendly, have high throughput screening and are relatively inexpensive for CRC diagnosis (Kekelidze et al., 2013; Quinchia et al., 2020).

1.2. Extracellular vesicles

1.2.1. Types and definitions

As a result of this intense search for new biomarkers, multiple candidates have emerged, among the most promising and which has led to an intense generation of scientific literature for the last years are extracellular vesicles. Extracellular vesicles (EV) are lipid-bilayer particles that are released from cells to its surroundings, and unlike them, are unable to replicate (Battistelli & Falcieri, 2020; Borges et al., 2013; Doyle & Wang, 2019; Raposo & Stoorvogel, 2013; van der Pol et al., 2012). They contain a variety of molecules, such as lipids, metabolites, proteins, nucleic acids, and even organelles from the cell that produced them. Their release has been evolutionally preserved by cells, from prokaryotes to eukaryotes, (some bacteria, fungi, and plant cells) (van der Pol et al., 2012; Yáñez-Mó et al., 2015).

EV population is heterogeneous in terms of size, ranging from 20-30 nm to 1 µm (Figure 2), content and membrane composition, and they may be produced via different biogenesis pathways. They can be found in several body fluids, such as blood, saliva, urine, milk, and in cell culture supernatants. According to their origin, EV are broadly classified into:

- Exosomes: they are formed by a phospholipid bilayer around 50–100 nm in diameter, their size range roughly overlaps that of the viruses (Borges et al., 2013; György et al., 2011; van der Pol et al., 2012). Their release into the environment can be either constitutive or induced and it is carried out by exocytosis of multivesicular bodies (MVBs). MVBs are structures formed by multiple brane (György et al., 2011; Raposo & Stahl, 2019; van der Pol et al., 2012). In turn, MVBs are formed from early endosomes, prelysosomal structures that make part of the degradative endosomal pathway of internalized proteins. Early endosomes take part of key cellular processes such as protein sorting, recycling, transport, storage, and release. Early endosomes come

from the endocytosis of the cell membrane induced by environmental changes. They usually interact with the Golgi apparatus and the endoplasmic reticulum by bidirectional vesicle exchange. Exosomes, still in form of ILV, originate from endocytosis of the early endosome membrane (Borges et al., 2013; György et al., 2011; van der Pol et al., 2012). This type of EVs have been study deeply in the case of immune cells (dendritic cells, T cell, B cells, macrophages) and tumors (György et al., 2011). Regarding its composition, they exhibit phosphatidylserine on the outer membrane leaflet and some markers that include CD63, CD81, CD9 (tetraspanins), Alix, LAMP1, TSG10, and other proteins that reflect their cellular source (György et al., 2011; Yáñez-Mó et al., 2015). They accomplish their biological function over cells through multiple biological mechanism such as direct contact between surface molecules of vesicles and cells, endocytosis of vesicles, and vesicle-cell membrane fusion (György et al., 2011; van der Pol et al., 2012). Exosomes have also been shown to intervene in horizontally transference of genetic material (mRNA and miRNA) (Valadi et al., 2007), intercellular transfer of oncogenic receptor (Al-Nedawi et al., 2008) and also transfer of HIV particles (N et al., 2008).

- Microvesicles: like exosomes, they are formed by a phospholipid bilayer although with greater diameters, between 100-1000 nm, when they are isolated in blood plasma, they tend to have a narrower range of sizes (100-400 nm) (György et al., 2011; van der Pol et al., 2012). They are formed through regulated release by budding/blebbing of the plasma membrane. Constitutive release of microvesicles is low in most cell types, one exception is for example tumoral cells. Instead, it is activated via surface receptors and increasing of intracellular Ca^{2+} in response to stress signals or damage (György et al., 2011). Cell types used for studying microvesicles are platelets, red blood cells and endothelial cells. Regarding their composition, phosphatidylserine is present in certain types of microvesicles, they also show in their surface proteins like Annexin V, Flotillin-2, selectins, integrins, CD40 metalloproteinase, which are often used as biomarkers (György et al., 2011). Some examples of the functions of microvesicles are procoagulant activity (Leroyer et al., 2008), secretion of IL1b (MacKenzie et al., 2001), enhancement of the proinvasive character of some tumors (Giusti et al., 2008), oncogenic cellular transformation induction (Antonyak et al., 2011) and fetomaternal communication (Pap et al., 2008).

- Apoptotic bodies: They are 1–5 µm in diameter (the largest type of EVs) and produced by cells as bubbles when undergoing apoptosis processes, so their composition imitates the cell's membrane (György et al., 2011; van der Pol et al., 2012). Common proteins of their composition are Annexin V and histones (Battistelli & Falcieri, 2020). The have phosphatidylserine in the surface. Nevertheless, there is a certain degree of heterogenicity in their composition, since they usually contain cellular debris produced during apoptosis such as organelles or DNA fragments, that depends on the cell type and its composition. Apoptotic bodies have multiple functions, vector for horizontal transfer of DNA (in example, oncogenes) (Bergsmedh et al., 2001), uptake by phagocytic cells to generate T cell epitopes (Bellone et al., 1997) and presentation of B cell autoantigens (Cocca et al., 2002). Uptake of apoptotic bodies has been associated with immunosuppression (Savill et al., 2002).

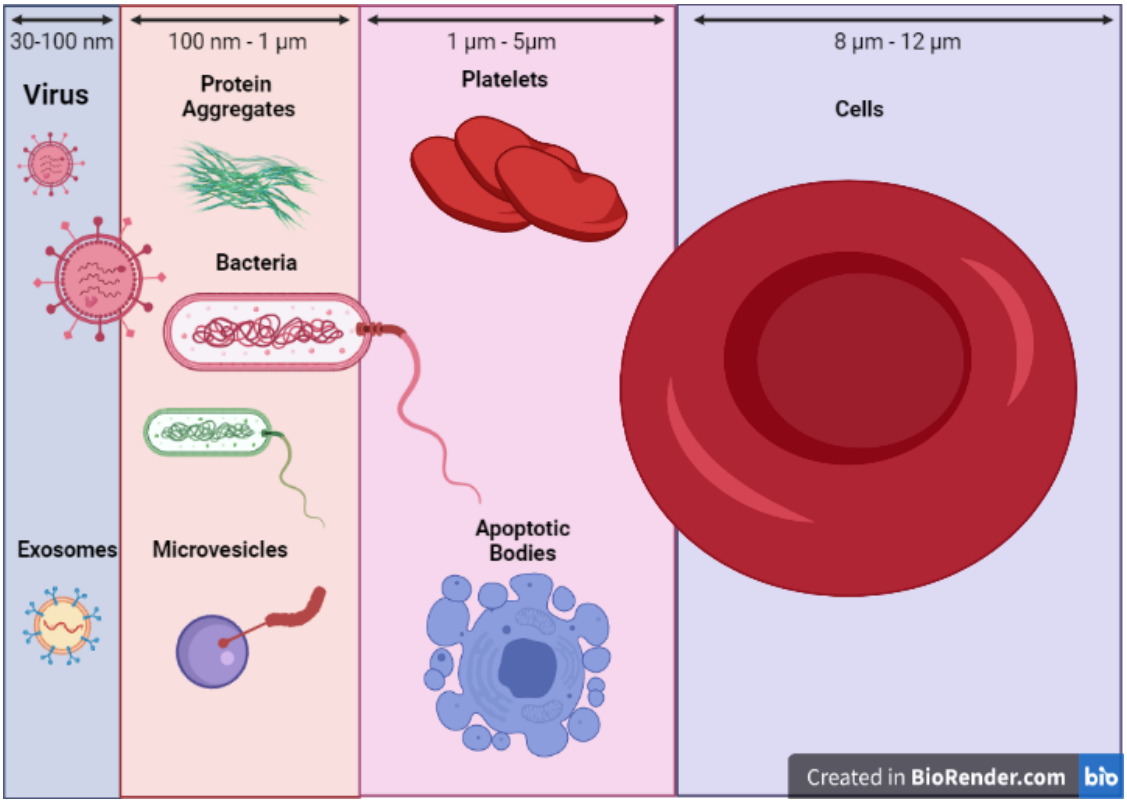


Figure 2 Graph presenting the three main types of extracellular vesicles (exosomes, microvesicles and apoptotic bodies) and their size range along with other biological structures of similar dimensions for comparison.

1.2.2. The role of extracellular vesicles in cell communication

EV have recently emerged as means of communication between cells, being released in response to intracellular or extracellular stimuli (refs). This communication has demostrated to be highly specific, probably caused by the presence of membrane proteins in the EVs surface with high affinity with ligands in the receptor cell. The fact that EVs are ubiquitous in body fluids (blood, urine, milk) implies also that this mechanism is not limited to neighbouring cells (paracrine mechanism) but also systemic communication (endocrine mechanism) (Borges et al., 2013). Their cargo, mainly proteins and RNA, may interact with recipient cells and regulate a variety of signalling pathways, not only in physiological processes, but also under pathological conditions. For example, EVs have an important role in the modulation of the immune system. In pregnancy, the trophoblast evades maternal immunity releasing exosomes exposing FasL, a programmed cell death inducer, to kill activated T cells that were sensitized to paternal alloantigens (Abrahams et al., 2004). This same systems is also used by certain tumour types to bypass the homing and antitumoral activity of the immunocompetent cells (Martínez-Lorenzo et al., 2004). Viruses and parasites, in a similar fashion, exploit this ability of EVs to expose ligands and kill immune cells at a distance to bypass the immune system. The latent membrane protein-1 (LMP-1) of Epstein-Barr virus (EBV) is exposed on exosomes and microvesicles from EBV-infected cells and inhibits the proliferation of peripheral blood mononuclear cells (Flanagan et al., 2003). Other example are the kidney and the renal epithelium, that are continuously releasing EVs into urine. The released EVs work as protein transporters carrying the vasopressin-regulated water channel aquaporin-2 (AQP2), an apical Na+ transporter protein, whose presence modulates the water permeability across the nephron (SINLAPADEELERDKUL et al., 2019). In neurodegenerative diseases, EVs work as protein transporters of misfolded proteins (β-amyloid and tau in Alzheimer disease, α-synuclein in Parkinson disease, TDP-43 in ALS, mutant huntingtin in Huntington's disease) spreading the protein aggregates to naïve recipient cells (Thompson et al., 2016). Exosomes from human bone marrow mesenchymal stem cells can stimulate proximal tubular cells to proliferate through the transfer of hepatocyte growth factor-1 receptor mRNA (Tomasoni et al., 2013). Similarly, exosomes from nasopharyngeal carcinoma cells harbouring latent EBV, thus helping on the spreading of the virus (Gourzones et al., 2010).

1.2.3. Extracellular vesicles in colorectal cancer

Studies show that the exosomes isolated from CRC (and all kinds of cancer) patients have a completely different miRNA, lncRNA, and protein profiles compared to normal controls (ND) (Mathew et al., 2020). The aspects could make exosomes a suitable early detection and prognosis of either primary tumors or metastatic disease, including CRC (Moyano et al., 2021).

Some miRNAs and lncRNA (long non-coding RNAs, non-coding RNA sequences with a length greater than 200 nt) that have been observed being expressed in blood exosomes of CRC patients, and after surgical resection, being reduce drastically. This suggest they have potential as biomarkers for CRC (Mathew et al., 2020; Vella et al., 2016). The differential expression of proteins on exosomes from serum of CRC patient has also been proposed as a potential diagnosis tool. One study discovered 36 proteins upregulated and 22 downregulated in the serum exosomes of CRC patients. Upregulated proteins tend to participate in modulating the metastasis microenvironment meanwhile downregulated proteins intervene in tumour cell growth and survival (Mathew et al., 2020).

Examples of proteins suitable for CRC diagnosis are the heat shock protein 60 (Hsp60), a chaperonin involved in tumorigenesis, or glypican-1 (GPC1), both were highly expressed in plasma exosomes of patients until surgical removal, when protein levels quickly decreased (Mathew et al., 2020). One of these candidates as biomarkers that is of particular interest in this work is CD147/EMMPRIN, also known as basigin or M6 antigen, a member of the immunoglobulin family which is widely expressed in a variety of human tissues and cells. CD147 is found embedded in EVs associated with cancer and among its functions can be found: facilitating the secretion of extracellular matrix metalloproteases (MMPs) from cancer cells, fibroblasts and endometrial cells, and thus promoting tumour proliferation, invasion and metastasis; boost tumour angiogenesis; regulate expression and activity of monocarboxylate transporters and form complexes on the membrane to evacuate the lactic acid produced by anaerobic glycolysis; develop chemoresistance; and interact some integrins to regulate adhesion and also enhance the expression of growth promoters (Landras et al., 2019, p. 147; Lucchetti et al., 2020, p. 147; Xin et al., 2016, p. 147).

1.3. Point-of-care Devices

1.3.1. The utility of point-of-care testing

Point-of-care testing (POC or bedside testing) is defined as medical diagnostic testing close to the point of care. It provides clinicians access to rapid and actionable diagnostic results. It reflects a paradigm shift within modern medicine, from a reactive and episodic model to one more based on prevention and continuous observation. It also arises as a response to the interest of patients to be involved in managing their own health status. Point-of-care technologies tend to meet a series of very specific characteristics that connect with this change in medicine strategy: they must be single-use, compatible with being mass-produced, low cost, quick results, easy to transport or even wearable, minimally invasive, user-friendly, analytically, and clinically valid. Examples of POC technologies would be glucometers, pregnancy tests and rapid tests for infectious diseases such as HIV or COVID-19 (Moyano et al., 2021; Oliveira-Rodríguez et al., 2017).

1.3.2. Lateral Flow Immunoassays

Lateral flow immunoassay (LFIA) is an immunochromatographic technique. When the sample is added to one of the ends of a nitrocellulose strip, the analyte and label are subjected to chromatography-like migration through a membrane, and a result is coloured line at the site of an immobilized capture reagent (Figure 3). Traditionally, it has been considered a qualitative technique that allowed detecting the presence of the analyte in the sample above certain thresholds. Nowadays, the development of multiple optical, conductance, and magnetic devices have allowed obtaining qualitative results from LFIAs, thus improving their sensitivity, reproducibility and turning this technology in one of the most important POC techniques for clinical research (Moyano et al., 2021; Oliveira-Rodríguez et al., 2016; Sajid et al., 2015; Urusov et al., 2019).

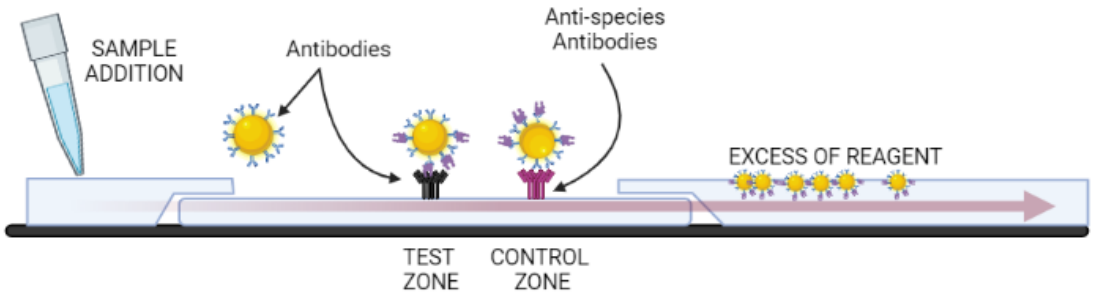


Figure 3 LFIA mechanism for sandwich format: the sample is deposited at one end of the strip, and it travels to the other end by capillarity. At the sandwich format, the analyte is captured by bioreagents at the test line. The detection reagent is usually labeled with nanoparticles. The antibodies of the test line are specific for the analyte, the antibodies of the control line are specific against the capture antibodies. The excess of reagent is deposited at the end of the strip.

Lateral flow immunoassays (LFIA) represent a powerful POC in vitro test (Moyano et al., 2021). LFIA has many advantages, among these are: great ease of manufacture with a low associated cost; simplicity; small sample volumes; no need for pre-treatment; format versatility, which also gives it many potential applications; high sensitivity and specificity, that can be improved by the coupling of electronic devices; and that these tests can be kept for long periods of time without losing their viability (robustness). On the other side, it also has some major drawbacks: reproducibility is still a big issue regarding the strips; certain samples, due to its nature (viscosity, surface tension…), are incompatible with this test type or may require a pre-treatment; and there is a lack of control in the velocity of the sample flow through the strip (Sajid et al., 2015).

1.3.3. Point-of-care devices in Colorectal cancer diagnosis

Multiple POC devices have already been developed for the diagnosis of colorectal cancer: electrochemical biosensors (Quinchia et al., 2020), aptamer biosensors (Raji et al., 2015), surface plasmon resonance biosensors (Narayan et al., 2019), and LFIA / magnetic LFIA biosensors (Moyano et al., 2021; Oliveira-Rodríguez et al., 2016). In all cases, these biosensors are capable of providing quantitative information about the sample. The results were generally very positive, obtaining devices with similar or even superior sensitivities to other methods such as ELISA.

2. OBJECTIVES

Extracellular vesicles participate in a variety of biological processes. Their number and composition may be altered under different conditions. In addition, since they can be isolated from different body fluids, EVs are emerging as potential biomarkers in several diseases, including colorectal cancer. The protein CD147 has been recently proposed as a promising CRC biomarker, as elevated levels of CD147-positive EVs were found in comparison with healthy controls. However, whether this increase in EVs levels and CD147 expression in CRC is specific to a late stage of CRC or a progression according to the development of the disease has not been assessed so far. On the other hand, there are difficulties in carrying out population screening strategies for diseases such as colorectal cancer, due to the lack of low invasive test or expensive procedures such colonoscopy. Alternative diagnostic methods more reliable than other lab tests such as fecal occult blood test are required. LFIAs, as powerful point-of-care platforms, appears to be a good candidate to meet the requirements sought in new colorectal cancer screening methods.

The general objective of this work was to evaluate CD147-positive EVs as potential biomarkers in CRC in a cohort of patients at the Hospital Universitario de San Agustín. This work was carried out under the framework of the project "*Biosensor based on superparamagnetic nanoparticles for early diagnosis of colorectal cancer*" (MINECO18-MAT2017-84959-C2-1-R). The specific objectives of this study were:

- To characterise plasma-derived EVs from a cohort of CRC at different stages in terms of size and concentration, and to determine their protein content.
- To design and develop a LFIA for the detection of isolated plasma-derived EVs and for detection of CD147-positive EVs.
- To compare the results obtained in the parameters studied among the patients at different stages of colorectal cancer.
- To evaluate the potential of LFIA as a suitable method for diagnosis of colorectal cancer.

3. MATERIALS AND METHODS

3.1. Cohort

Patients who participated in the Colorectal Cancer Screening in Asturias and were about to undergo a colonoscopy because of a positive Occult Blood Test in Feces, were recruited. This project was approved by the Ethics Committee of Hospital Universitario San Agustín (HUSA). The following exclusion criteria were applied: patients under 18 years; patients who did not give their permission to obtain the sample; patients unable to read, understand explanations or give informed consent; patients affected by any chronic or acute systemic inflammatory process at the time of the examination; patients with active inflammation (ischemic, infectious, chronic inflammatory bowel) in the colon or ileum at the colonoscopy performed; patients with a previous history of neoplasia or affected by extracolonic tumors at the time of the test.

Patients were classified in four groups:

- Group I: patients with no pathological findings during colonoscopy (CT).
- Group II: patients with low-grade adenomas (AdL).
- Group III: patients with high-grade adenomas (AdH).
- Group IV: patients with adenocarcinomas (CRC).

The profile of the population that participated in this study is showed below (Table 1).

Table 1 Cohort description

	Group of Study			
	CT	**AdL**	**AdH**	**CRC**
Overall (%)	**14**	**13**	**8**	**8**
Sex (%)				
Male (53,49%)	4	8	4	7
Female (46,51%)	10	5	4	1
Age				
Mean (SD)	61,64 (5,23)	66,31 (5,56)	58,5 (5,08)	62,65 (11,97)
Median (Q1;Q3)	61,5 (59,25;66,25)	61 (57;66)	61,5 (60,5;65)	67 (60,5;78,25)
Min; Max	51; 70	51; 67	53; 69	49; 83
Factors (%)				
Obesity [Yes] (23,36%)	3	3	3	1
Dyslipidaemia [Yes] (53,49%)	9	7	4	3
Smoker [Yes] (23,26%)	1	6	2	1
Alcohol [Yes] (23,26%)	3	2	2	3

3.2. Sample collection

Peripheral venous blood was collected in 10 mL Vacutainer (Becton Dickinson) tubes with EDTA as an anticoagulant after discarding the first millilitre and processed within 30 min of collection. Blood was first centrifuged for 30 min at 1550 g to remove cells. Aliquots of plasma were stored at −80 °C until use or further centrifuged to isolate extracellular vesicles.

3.3. Enrichment of plasma-derived extracellular vesicles

For the isolation of EV, the ExoQuick™ precipitation solution (System Biosciences, Palo Alto, CA) was used, following the manufacturer's instructions (Figure 4).

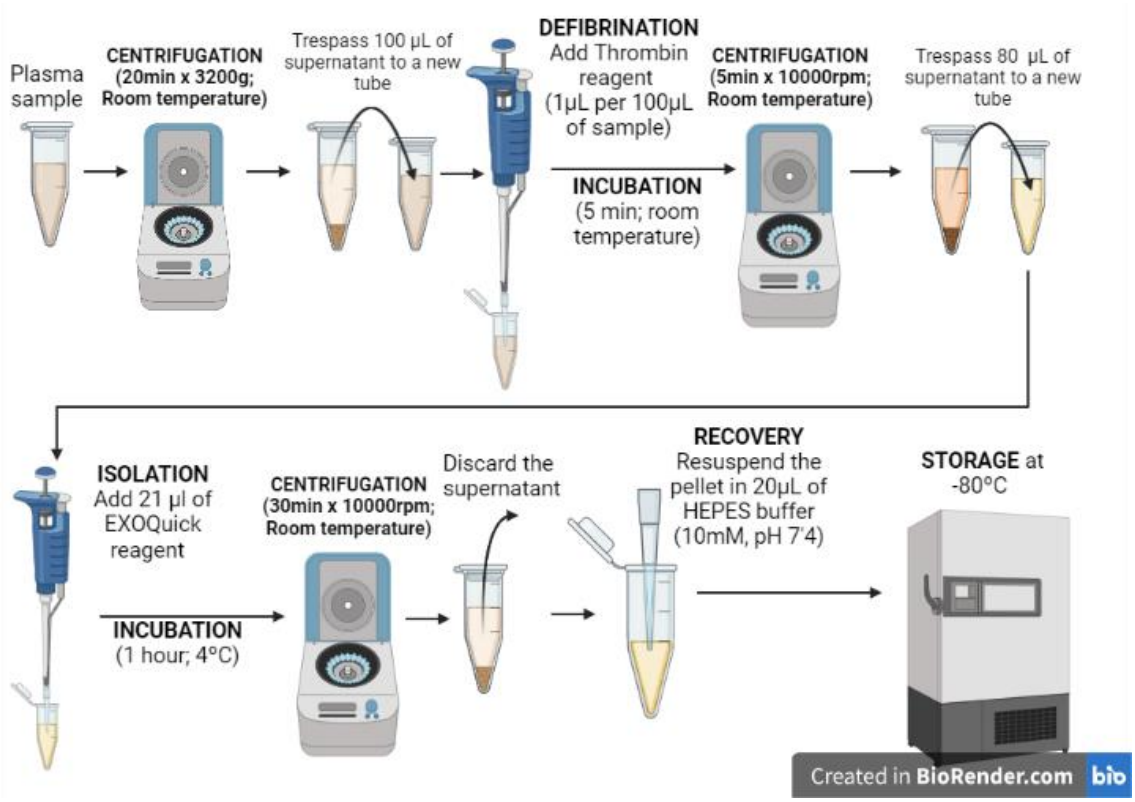


Figure 4 Protocol for the isolation of EVs from plasma samples.

Plasma samples were first centrifuged at 3200 x g to remove platelets and cell debris. Next, samples were pre-treated with thrombin for defibrinating at room temperature for 5 minutes and centrifuged for 5 min at 10000rpm for clearing. Then, the plasma samples were mixed with the ExoQuick reagent and incubated for 1 hour at 4ºC. After incubation, EV were precipitated by low-speed centrifugation (1700 x g) and the supernatant was discarded. After additional centrifugation, all traces of the ExoQuick solution were removed and the remaining pellet was resuspended in HEPES buffer (10mM, pH 7.4) and stored for further processing.

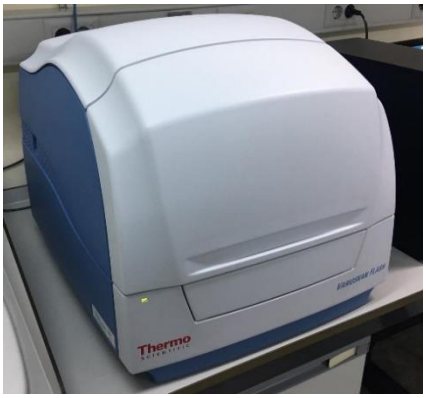
3.4. Characterization of extracellular vesicles by nanoparticle tracking analysis (NTA)

Freshly isolated EV fractions were characterized in terms of size distribution and concentration using a NanoSight LM10 instrument (Malvern, UK) at Nanovex Biotechnologies S.L (Asturias, Spain). The samples were diluted to achieve a concentration of 10^6-10^7 EV/mL to perform the analysis.

3.5. Protein concentration of the extracellular vesicles' fractions

Quantification of the protein content in the EV fractions was carried out using the Pierce BCA Protein Assay kit (Thermo Scientific). This colorimetric assay is based on the reduction in an alkaline medium of Cu^{2+} to Cu^{+} by protein combined with the reaction between cuprous cation (Cu^{+}) and bicinchoninic acid (BCA), that yields a product that allows a highly sensitive and selective colorimetric detection.

The analysis was performed in a 96-well microplate. The standard curve (working range: 0–2000 µg/mL) was made with nine points of serial dilution with bovine serum albumin (BSA). The EV fractions were homogenised with 1x RIPA buffer (Fisher Scientific). The samples and the standard points were mixed with the working reagent according to the manufacturer's instructions. The plate was placed in a shaker for 30 seconds and then incubated for 30 minutes at 37ºC. After cooling the plate at room temperature, the absorbance at 562 nm was measured using a microplate reader (ThermoScientific, Varioskan Flash, Figure 5). All samples and standard points were replicated twice.



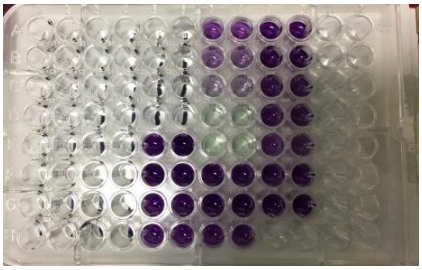


Figure 5 [Top] Image of the ThermoScientific Varioskan Flash [Down] 96-well microplate with BCA reaction

3.6. Conjugation of antibody to gold nanoparticles (AuNPs)

For the formation of the AuNP-Ab complex (Figure 6), gold nanoparticles (*40 nm; BBI Solutions*) were incubated with 0.15 mg/ml of the detection antibody (anti-human CD63; clone Tea 3/18, Immunostep) for one hour in rotation. Next, the nanoparticles were incubated for 45 minutes with 1mg/ml of BSA to block them from any unspecific interaction . After this, the nanoparticle solution was divided in two microcentrifuge tubes, and both were centrifuged 20 minutes at 10000 rpm. Most of the supernatant was discarded and the pellet was resuspended in a stabilizing solution (10% Sucrose, 1% BSA, PBS 2mM pH 7.4). To monitor the successful formation of the AuNP-Ab conjugate, the size distribution of the conjugates was analysed by Dynamic Light Scattering (DLS), to confirm the increase in the hydrodynamic diameter of the particles (Figure 7). Last, the AuNPs-antiCD63 were stored at -20ºC until further use was needed.

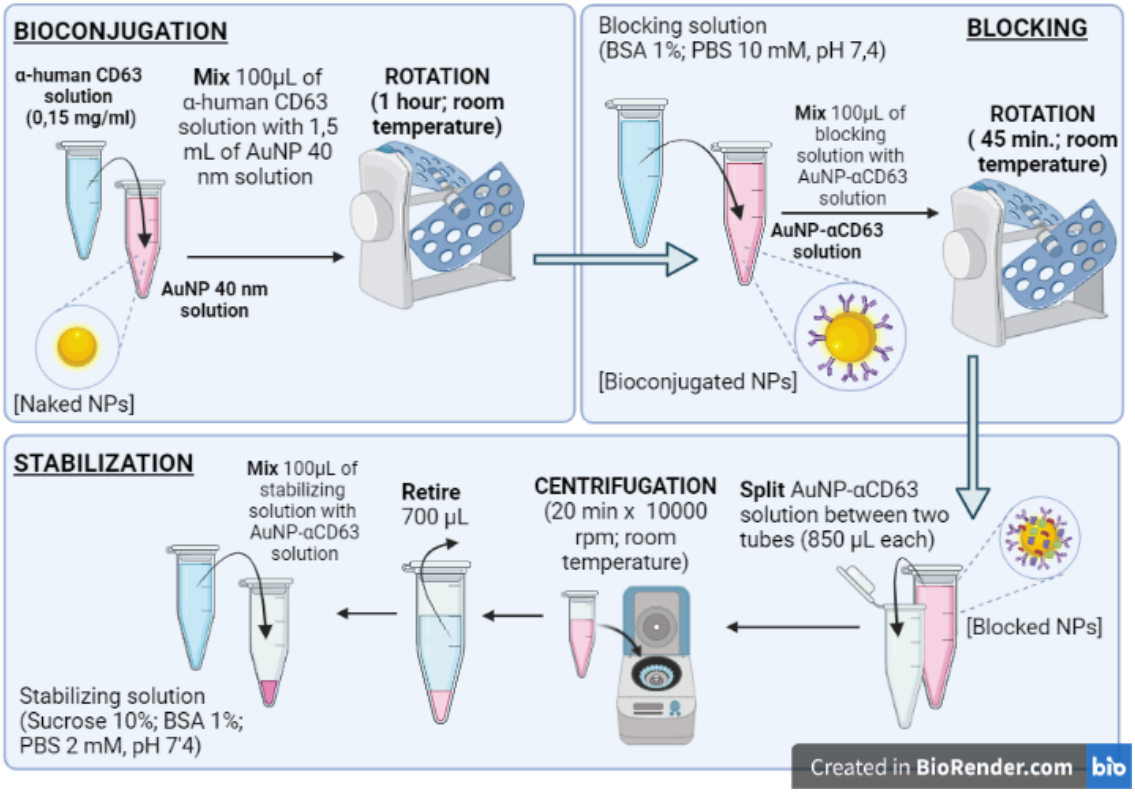


Figure 6 Protocol for the bioconjugation of AuNP (40 nm) with α-human CD63 antibodies.

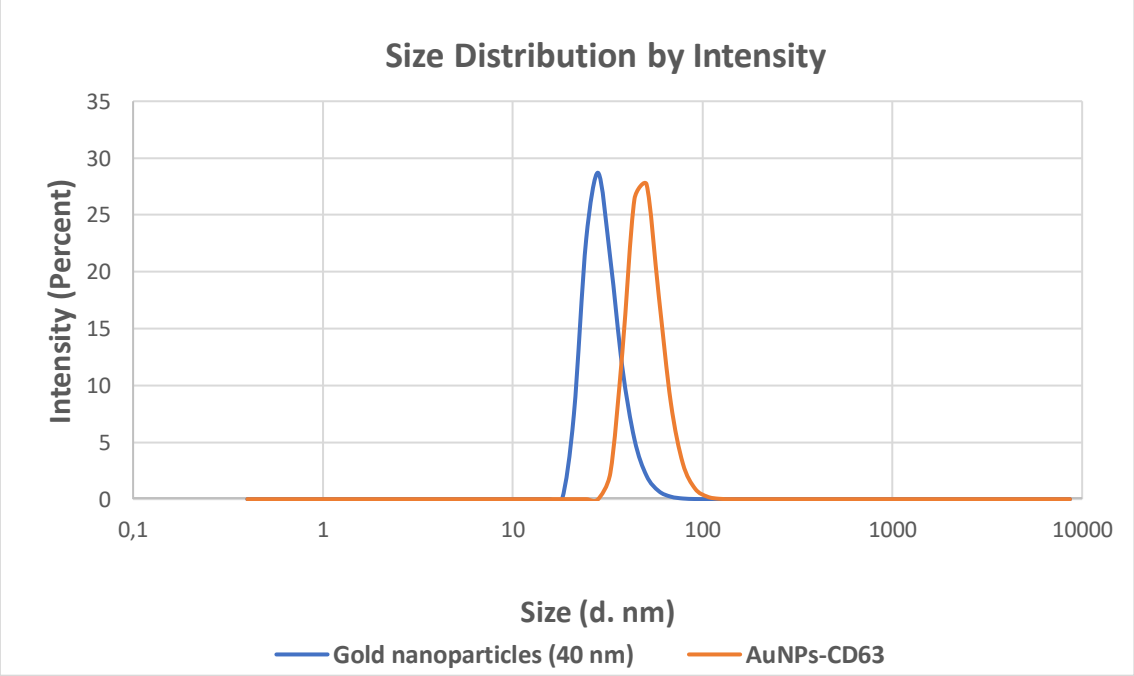


Figure 7 Successful conjugation of antibodies to gold nanoparticles was confirmed by DLS (Dynamic Light Scattering). Comparing the average hydrodynamic radius of a sample of bare nanoparticles (blue peak) with another subjected to the conjugation process (orange peak), it was observed that the latter had a larger particle size distribution. This allowed us to conclude that the conjugation has been successful.

3.7. Preparation of the lateral flow strips

The following components were used to prepare the LFIA (Figure 9):

- Backing card, it is made of plastic and confers robustness to the strips, working as a structural support for the rest of the device.
- Conjugate pad (*Millipore, Glass Fibre, GFCP001000*), responsible of capturing the sample and moving it along the strip through capillarity.
- Absorbent pad (*Whatman, ref. 81162250*), that stores the excess of sample that runs in the strip.
- Nitrocellulose membrane (*Millipore HI-Flow Plus, HF07504XSS*), which acts as the base on which the flow is established and where the reagents are immobilized in either the test or the control line.

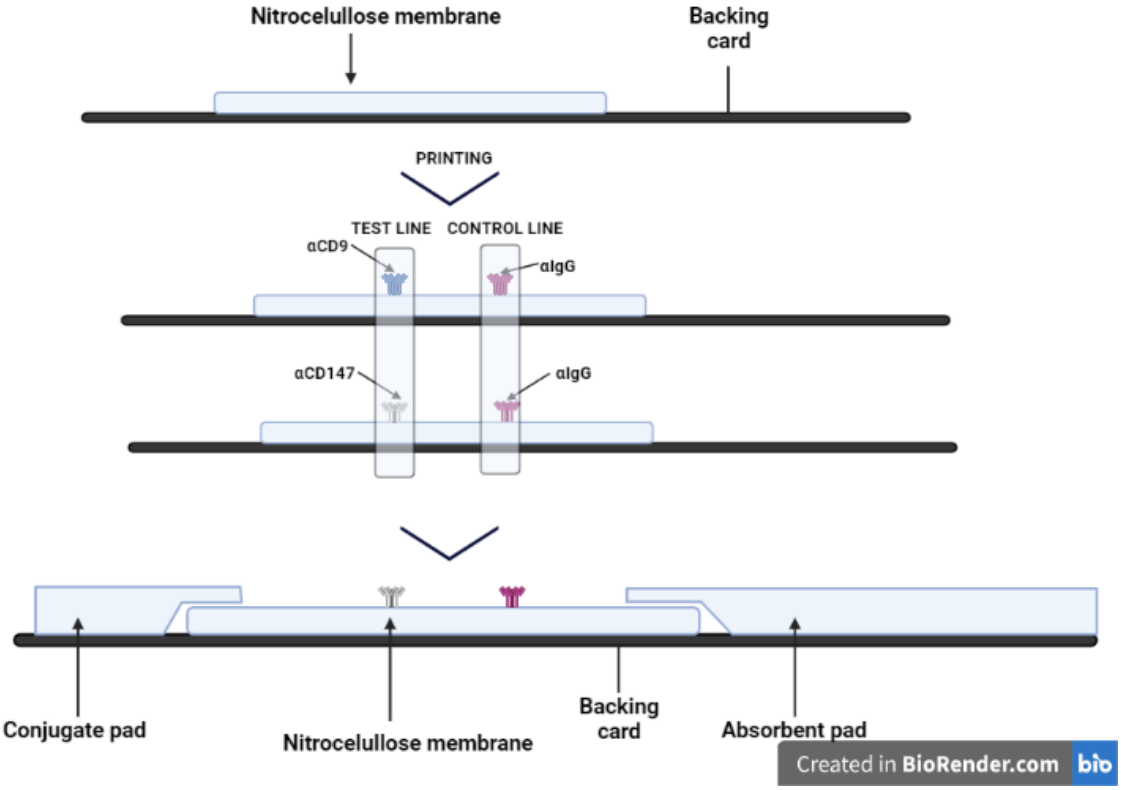


Figure 8 Steps followed for manufacturing LFIA strips: first, the nitrocellulose membrane and backing card are coupled; then, the test and control line are printed; finally, the conjugate and absorbent pad are added.

First, the nitrocellulose membrane was placed over the backing card. Then, the antibodies used in this work were deposited using an IsoFlow dispenser (Imagene Technology, Hanover, USA; Figure 9) at a rate of 0.100 µL/mm. Two types of membranes were prepared: one with 1mg/ml anti-CD9 (clone VJ1/20; Immunostep, Spain) as capture antibody at the

test line, and the other with 1 mg/ml anti-CD147 (clone VJ1/9) at the test line. In both cases, an anti-mouse IgG (1mg/ml; Sigma-Aldrich) was dispensed at the control line.

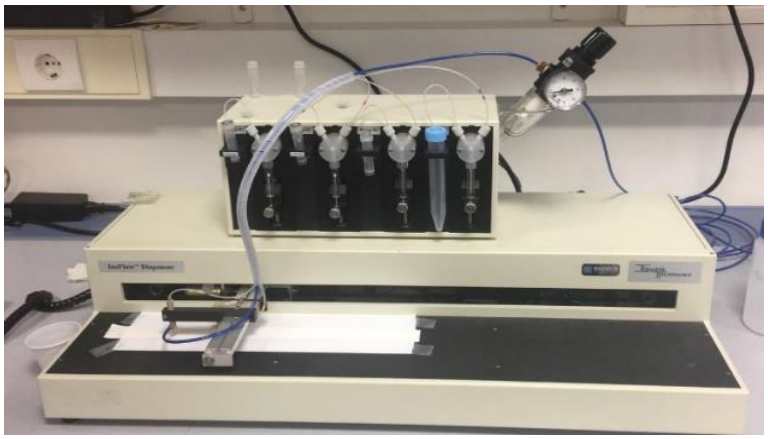


Figure 9 Image of the IsoFlow dispenser (Imagene Technology, Hanover, USA) used to draw the test and control line.

3.8. Lateral flow immunoassay for detection of extracellular vesicles

The tests were carried out in centrifuge microtubes (2 ml). Same volumes of isolated EVs were mixed with 10 µL of AuNPs-antiCD63 and brought to a total volume of 100µL with running buffer (1% BSA, 0.05 % Tween-20, 150 mM NaCl, HEPES 10 mM pH 7.4). The strips were added, and the samples were left to run for 10 minutes. After this time, a portable strip reader ESE-Quant LR3 lateral flow system (Qiagen Inc., Germany) was used to quantify the optical density of the lines by reflectance.

3.9. Statistical analysis

Data were analysed using SPSS 24. The Welch robust test of equality of means was used to analyse differences between the different groups of patients in the size, concentration, protein content, and the ratio of CD147/CD9 of the isolated EV. Statistical significance was set at $p < 0.05$.

4. RESULTS

4.1. Methodology standardization

The study of biomedical uses of extracellular vesicles is a relatively unknown field and one in which rapid advances and interesting discoveries have been made in the last two decades. However, there is great heterogeneity among the methods used by researchers for their isolation from biological fluids. This fact makes it difficult to compare results and compromises their reproducibility. In addition, there is a lack of optimized methods and extracellular vesicle studies present a great inherent variability. To compensate for this, some journals have proposed protocols, given recommendations, created guidelines, in an attempt to create a standardized framework (Théry et al., 2018).

All these indications have been followed throughout the work to achieve meaningful and reproducible results (Figure 10): once taken, the plasma samples were stored at -80ºC, which is by consensus the most suitable temperature for their storage; the NTA is one of the recommended techniques for the characterization of large numbers of EVs, in this work, it was used to measure the concentration of EVs and their size in each sample; the same batch of ExoQuick was used for the isolation of all the samples, in order to prevent any further variability; BCA tests are recommended for knowing the protein content of enriched EVs samples; in LFIAs, the optical signal increases as the strip dries, this is usually considered a source of variability, so it was decided to leave all the strips running for the same time (10 minutes) before reading the optical signal; for the NTA and LFIA studies, freshly isolated EV fractions were used, whereas for the BCA tests, the samples were stored at -80ºC (although their storage was prevented from being prolonged for more than two weeks).

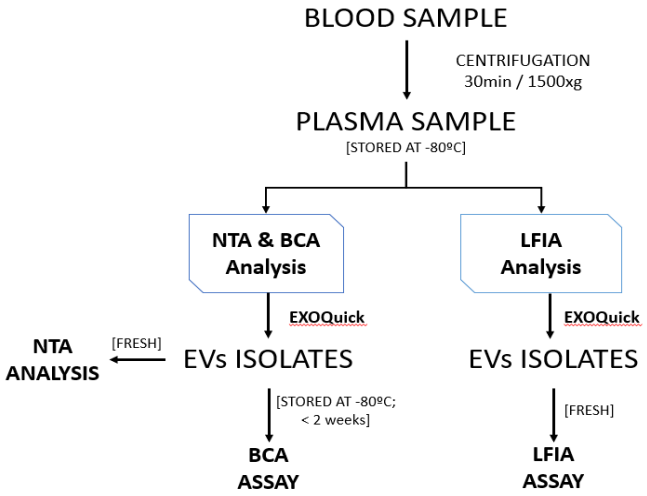


Figure 10 Workflow chart of the experiment

4.2. Characterization of extracellular vesicles

4.2.1. Characterization and size

The size distribution of the isolated EV was obtained by NTA, based on the measurement of Brownian motion relating it to the hydrodynamic diameter through the Stokes–Einstein equation. The NTA studies the light scattering of each individual particle, thus providing both the number of particles and their size. Various articles have shown that high levels of EV concentration are associated with colon cancer. In this work, a decreasing trend can be observed in patients with AdH (mean: 5.08×10^{14} particles/ml) and CRC (mean: $4{,}25 \times 10^{14}$ particles/ml), compared to the control (mean: $5{,}89 \times 10^{14}$ particles/ml) and AdL (mean: $7{,}87 \times 10^{14}$ particles/ml) groups, with particularly higher values in AdL patients (Figure 11). Despite the differences, no statistical significance was reached (p=0,099).

A summary of the mean sizes and mode (+/- SD) of the EV analysed in each group is shown in Table 2. There are no significant differences between the groups (p=0,069).

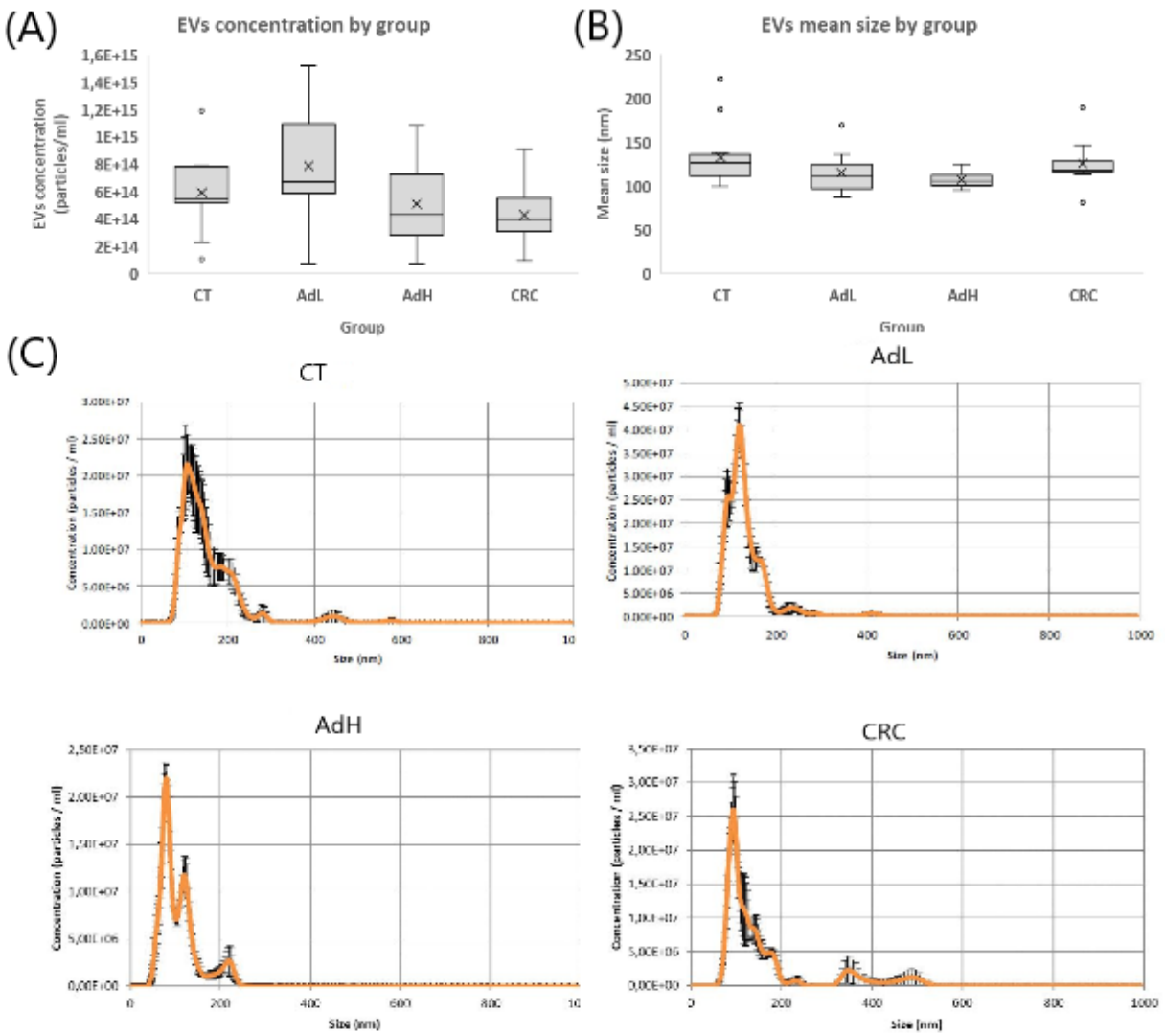


Figure 11 Box plots for the particle concentration (A) and diameter sizes (B) of the isolated EVs in the different groups of patients. (C) Representative graphs depicting the hydrodynamic size distribution profiles of a sample chosen from each group of patients. Patients with AdH and CRC showed lower levels of EV in blood; there are no differences in size between the different groups.

Table 2 Mean size and mode of the EVs of each group.

	Size (nm)	Mode (nm)
CT	133± 34	113 ± 39
AdL	115 ± 22	100 ± 27
AdH	107 ± 10	91 ± 13
CRC	125 ± 31	109 ± 30

4.2.2. Protein content

The BCA test is a colorimetric test capable of estimating the concentration of proteins within the sample. By itself, the protein concentration does not correlate with the number of EVs in the sample, but in conjunction with the concentration obtained by the NTA it can be used to estimate the purity of the sample (Webber & Clayton, 2013). There are no significant differences between groups (p=0, 953), although the CRC group exhibits slightly lower values (Figure 12).

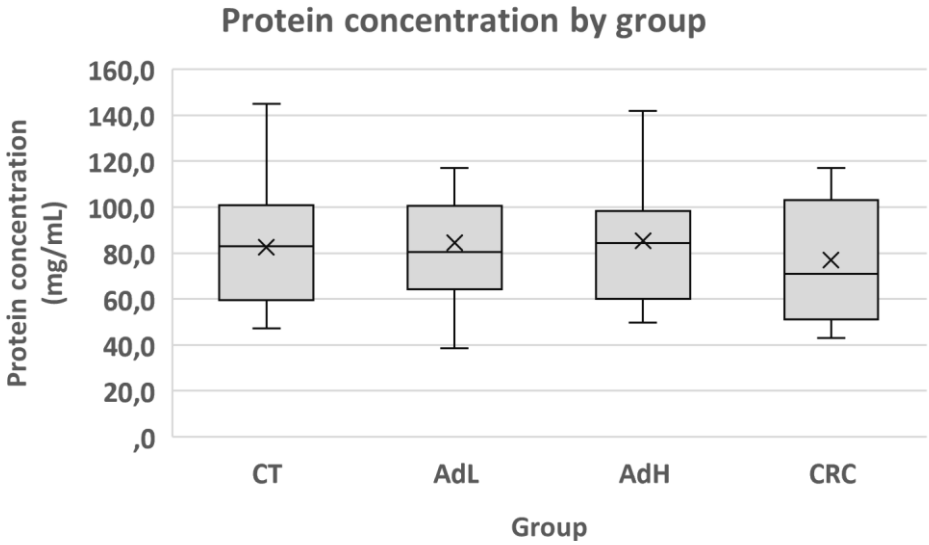


Figure 12 Protein concentration obtained by BCA assay: all groups show similar results though colorectal patient samples were slightly more variable.

The ratio EV concentration/protein content is used to estimated the purity of the samples (Table 3) (Serrano-Pertierra et al., 2019; Webber & Clayton, 2013).

Table 3 EV/protein ratio for each group

	CT	AdL	AdH	CRC
EV Conc. (particles/ml)	$1{,}0\times 10^{15}$	$7{,}87\times 10^{14}$	$5{,}08\times 10^{14}$	$4{,}25E\times 10^{14}$
BCA (protein mg/ml)	82,51	84,43	85,37	77,01
EV/protein ratio (particles/protein mg)	$1{,}29\times 10^{13}$	$9{,}32\times 10^{12}$	$5{,}95\times 10^{12}$	$5{,}52\times 10^{12}$

It is interesting to study the pattern of vesicle concentration and the protein content of the sample to see if there is a correlation (Figure 13). The correlation line has been calculated, by the R value it can be assumed that there is no correlation between the concentration of proteins and EVs of the samples. This was intended to detect changes in the composition of EVs depending on the state of the disease.

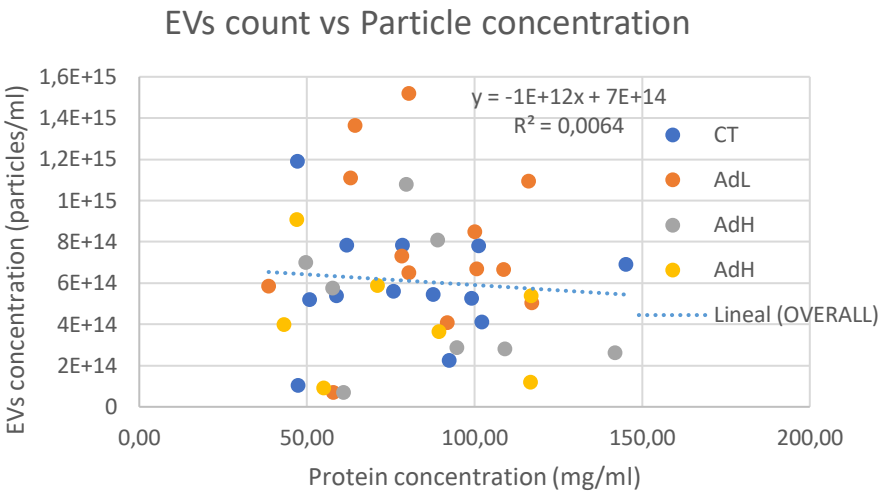


Figure 13 EVs concentration vs protein concentration : the protein concentration obtained by the BCA assay is represented on the horizontal axis and the results of the concentration of NTA vesicles on the vertical axis.

4.3. Lateral flow immunoassay and biomarker analysis in extracellular vesicles

After dispensing the antibodies, the membranes were dried for 20 minutes to ensure the immobilization of the antibodies. Next the conjugate pad and the adsorbent were assembled onto the backing card overlapping with the nitrocellulose paper by 2 mm. Last, the card was cut into smaller strips 5 mm wide. The assay consisted of using anti-CD63 functionalized gold nanoparticles as capture antibody. The EVs would bind to these nanoparticles and move up through the sample. At a certain height, they would meet the test line, were anti-CD9 or anti-CD147 antibodies were immobilised. Finally, the nanoparticles would pass the control line, with anti-human IgG antibodies, and the assay would end. The LFIA tests were satisfactory, the EVs isolates were compatible with the test. EV-depleted plasma and the running buffer were used as negative controls.

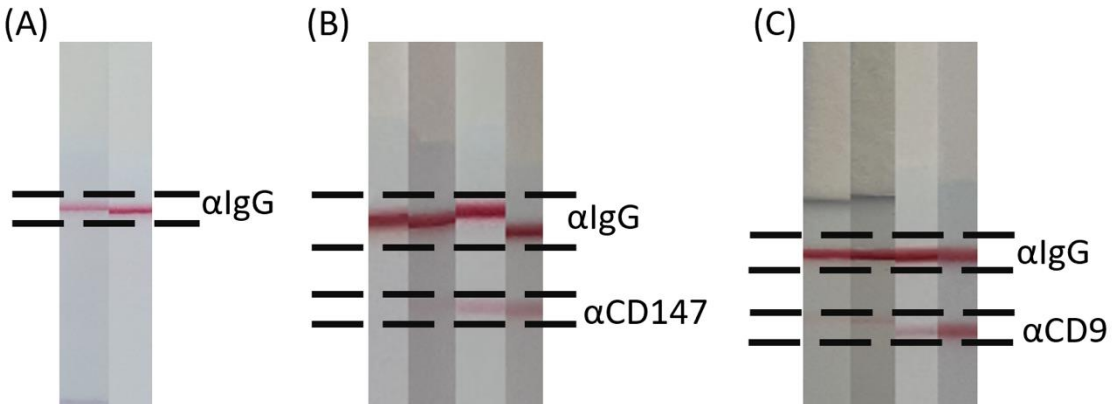


Figure 14 Images of strips: (A) Running buffer as blank (left) and EV-depleted plasma as negative control (right); (B) Different strips for the detection of CD147; (C) Different strips for the detection of CD9.

The ratio between both signals (CD147-positive and CD9-positive EV) is the analytical value of interest. The CD147 / CD9 ratio, result of dividing the signal obtained in CD147 LFIAs by that obtained in CD9 LFIAs, can be considered an estimation of the proportion of CD147-positive EVs in relation of the total number of EV (CD9-positive EV). Double positive CD147 / CD9 EVs, according to the bibliography, are notably more numerous in CRC patients, returning to normal values after surgery to remove the tumours (Tian et al., 2018; Xin et al., 2016, p. 1; Yoshioka et al., 2014). In the experiment (Figure 15), the group of patients with AdH shows a higher CD147 / CD9 ratio than the rest of the groups, although it did not reach statistical significance (p=0,373).

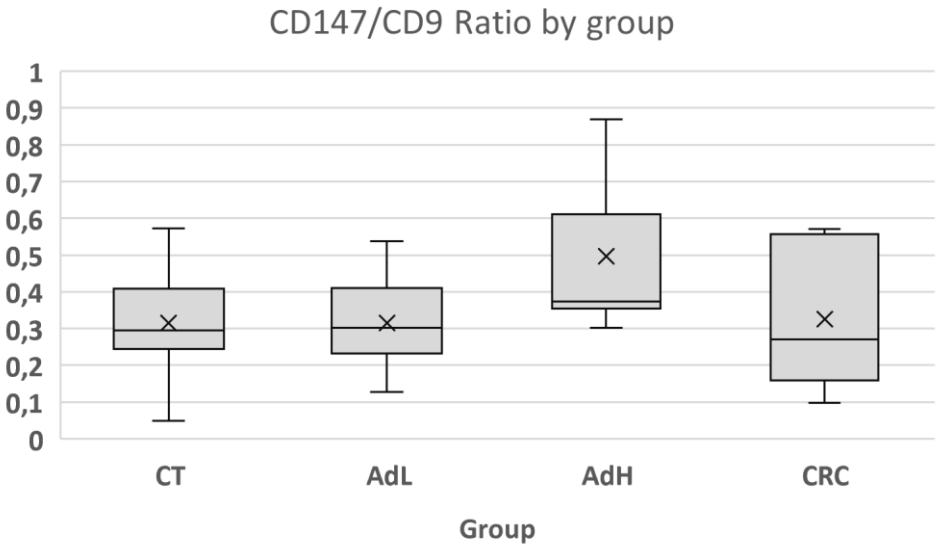


Figure 15 CD147/CD9 ratio on each group: healthy patients (CT) and low risk adenomas (AdL) show a low ratio and similar variability; high-risk adenoma patients (AdH) have a remarkable high ratio; finally, colorectal cancer patients (CRC) have a ratio slightly above average.

5. DISCUSSION

The results obtained from the NTA regarding the concentration of EVs show that there is a trend in the patients of AdH and CRC to lower levels of EVs in blood, although these differences are not enough to show statistical significance (Figure 11). This contradicts the results obtained in most of the literature on the subject. According to it, the number of EVs can be considered by itself as a biomarker (Cappello et al., 2017; Johnsen et al., 2019; Verma et al., 2015). This has been observed many times that different types of cancer are characterized by abnormal levels of EVs in plasma. The total EV number has been studied in melanomas by using immunocapture techniques (Logozzi et al., 2009; López-Cobo et al., 2018). Later, new results in other histological types of cancer, such as colorectal cancer would reinforce this idea (Silva et al., 2012). The most widely accepted hypothesis about this phenomena is that cancer cells, through mechanism such as the Warburg effect and proton pumps, acidify the microenvironment in which they are found, promoting the release of EVs (Fais et al., 2014; Spugnini & Fais, 2017).

As it was already indicated before, it is difficult to compare results with other experiments due to the great variety of methods that exist for the isolation and analysis of EVs, in addition to the inherent variability that they exhibit. The particle count for all groups of this work was several orders of magnitude greater than that one obtained in other papers, with the same analytical method, and even with the same isolation method (Johnsen et al., 2019; Serrano-Pertierra et al., 2019; Tian et al., 2018). It is known that polymer-based purification, size exclusion-chromatography purification, tend to cause an overestimation in the number of particles detected by NTA, showing greater counts than ultracentrifugation, which is considered the "gold standard" of EVs purification. This is because, in the blood, there is a population of particles with characteristics very similar to EVs, the lipoproteins (Cappello et al., 2017; Webber & Clayton, 2013).

The literature shows that lipoproteins, integrated by low-density (and very low-density) lipoproteins (LDL and VLDL), high-density lipoproteins (HDL), and chylomicrons (CM) are a great source of error when counting EVs (Johnsen et al., 2019). Polymer-based purification methods and size-exclusion chromatography are known to be unable to properly separate the low-density lipoproteins and chylomicrons (65) due to their similar nature and size to EVs (Figure 16). Furthermore, their levels in blood plasma were found several orders of magnitude above those of extracellular vesicles, so lipoprotein contamination usually leads to an important overestimation in the number of particles when the samples

are analysed with NTA (Johnsen et al., 2019; Sódar et al., 2016). Ultracentrifugation, being a density-based separation, can exclude low-density lipoproteins and chylomicrons. High-density lipoproteins still contaminate the isolate. However, the diameter of the high density lipoproteins is close to 10 nm (Figure 16), much lower than their detection limit by NTA, so they do not contribute significantly to the particle count (Johnsen et al., 2019). Hence the reason for ultracentrifugation protocols exhibiting lower particle counts when studied on NTA. According to this, some reviews, based on the concentrations of EVs detected in multiple studies, have estimated that the number of EVs in blood plasma would be of the order 10^{10} vesicles per millilitre (Cappello et al., 2017).

Assuming then that the particle count corresponds mainly to lipoproteins, an explanation could be made as to why there are fewer particles among patients with AdH and CRC. Multiple studies have revealed that there is a strong association between low blood cholesterol levels (and therefore also lipoproteins count) and colon cancer (Benn et al., 2011). Confirming these hypotheses would require retesting the samples making sure to remove lipoproteins by ultracentrifugation. If much lower values of particles / ml were detected in the NTA, it could be assumed that there has been a strong contamination with lipoproteins. Another alternative would be to confirm the presence of lipoproteins in the isolates.

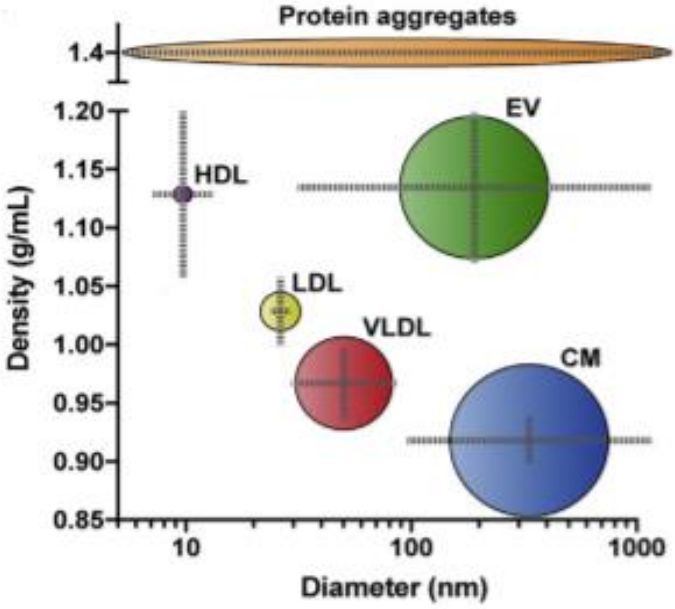


Figure 16 Particle populations in human plasma ordered by density and diameter: HDL and EVs, since they have similar densities, are isolated together in ultracentrifugation; for SEC and polymer-based methods, EVs tend to be isolated with LDL,VLDL and CM, which due to their size, can be detected on NTA. [Source: (Johnsen et al.,2019)]

There are enzymatic methods that could be used for this (Steele et al., 1980). To reduce lipoprotein contamination, it would be advisable to perform blood draws on fasting, since it has been observed that lipoprotein levels increase after meals (Mørk et al., 2018).

Regarding the size observed in the NTA, they are within the range to be considered small EVs, exosomes (Table 2) (Serrano-Pertierra et al., 2019). No group shows significant differences in comparison to the rest (Figure 12). In the literature, the size of the EVs is not considered a biomarker (Serrano-Pertierra et al., 2019). When comparing the results with those of experiments with the same isolation and analysis method, it can be observed that they are markedly smaller (Serrano-Pertierra et al., 2019). One way to assess the purity of EV isolates is by calculating their particle count / protein concentration ratio (Webber & Clayton, 2013). According to the literature, ratios above 3×10^{10} can be considered good quality samples. The ratio EV/protein of all samples was found above this number, meaning they were of considerable purity (Table 3). However, because the concentration of EVs is so high (probably due to an overestimation caused by contamination with lipoproteins), these results should be taken with caution.

The LFIAs were carried out with two clear objectives: to verify their viability as a screening method and to measure the presence of CD9 and CD147 in the samples. Regarding the latter, the LFIA results show an increase in the CD147 / CD9 ratio in patients with AdH and CRC, although these are not high enough to reach statistical significance (Figure 15). The literature shows that high ratios of CD147 / CD9, corresponding to high numbers of double positive CD147 CD9 EVs are associated with the development and presence of colorectal cancer (Tian et al., 2018). After surgical treatment, the levels returned to normal, showing an association between cancer and these EVs. However, EVs variation in each stage of cancer development has never been studied before. The results of this work support the previous literature, although they also seem to indicate that there is a peak in CD147 / CD9 positive EVs in the high-risk adenocarcinoma stage. The results of the experiment support the LFIA as a candidate for colon cancer screening. The strips gave no signal when they were analysed with blanks (only running buffer) or with negative controls (EV-depleted plasma obtained in the isolation procedure), while they gave mixed results in the samples (Figure 14). Furthermore, it would be a much faster method of diagnosis when compared to colonoscopy and FOBT, whose results can delay for weeks (*Fecal Occult Blood Test - Mayo Clinic*, n.d.). The time needed to get the results from the strip would be approximately 15 minutes, and it is possible to make multiple tests at the

same time. It would also be cost-effective, since manufacturing the strips would be simple. The main drawback would be the variability of results, which would require further refinement.

6. CONCLUSIONS

- It is important to consider the purification strategy for EVs, using the most appropriate one according to the analysis that will be carried out and the characteristics to be studied, to prevent the influence of lipoproteins.
- It has been possible to manufacture LFIA for the detection of CD147-positive EVs.
- LFIAs are a suitable technique for the study of EVs isolated from blood plasma using ExoQuick, offering a potential diagnosis method for CRC. Providing results quickly and with a simple manufacturing process.
- The presence of double positive CD147 / CD9 EVs seems to increase in high-risk adenocarcinomas, and to be reduced again in the colorectal cancer stage. This finding needs further experiments with a large cohort for statistical significance.

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