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Liquid Biopsy Approach in Precision Oncology

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Liquid Biopsy Approach in Precision Oncology

Abstract:

Liquid biopsy serves as a tool to analyse cell-free nucleic acids (cfNAs) and circulating tumour cells (CTCs) in blood or other body fluids in individuals with cancer. These are considered promising diagnostic and prognostic biomarkers but new discoveries are still being made in regard to their heterogeneity, therefore it is critical to continue researching to demonstrate a clinical advantage in precision oncology. For now they promise to reveal molecular characterisation of tumours which can change the prognosis, monitoring and post-therapy of cancer care. This is challenging because the technologies and methodologies are still not mature enough for capturing and isolating viable cells. Higher affinity and sensitive techniques including a combination of these may result in an efficient characterisation of CTCs and cfNAs. From pre-treatment of blood samples to post-analysis of isolated markers it is required to standardize and regulate methods in order to prove clinical utility, as of now this remains in the experimental phase. Microfluidic systems have become an influential tool in cancer studies for their capability to analyse single cells or molecules and its integration with other techniques, miniaturization and fast analysis which will help overcome challenges these biomarkers face to eventually replace traditional biopsy with liquid biopsy.

Keywords:

Liquid biopsy, cancer diagnosis, circulating tumour cells, circulating cell-free nucleic acids

CERCS: B200, Cytology, oncology, cancerology

Vedelbiopsia Meetodid Onkoloogias

Lühikokkuvõte:

Bakalaureusetöö on kirjutatud inglise keeles, kirjanduse põhjal ning on klassikalise ülesehitusega koosnedes sissejuhatusest, kirjanduse ülevaatest, töö eesmärkidest, diskussioonist ja kokkuvõttest. Töö on esitatud 40-l leheküljel, sisaldades ka ühe joonise ja neli tabelit. Töö koostamisel on kasutatud 48 kirjanduse allikat.

Töö põhisisuks on ülevaate andmine erinevatest vedelbiopsia meetoditest, mille abil saab vähi patsientidel analüüsida veres või muudes kehavedelikes ringlevaid rakuvabasid nukleiinhappeid (cfNAd) ja ringlevaid vähirakke (CTCd). Neid peetakse paljutõotavateks diagnostilisteks ja prognostilisteks biomarkeriteks, mis võimaldavad senisest paremini ning mitte-invasiivselt iseloomustada kasvajaid molekulaarsel tasemel. See on väga oluline vähi patsientide personaalses ravis, võimaldades kasvajate tekke ja arengu paremat jälgimist ning ravi. Töös on kirjeldatud cfNA-de ja CTC-de olemust, päritolu, eraldamist ning analüüsiks kasutatavaid tehnoloogiaid. Samuti on võrreldud vedelbiopsia kasutatavust ja perspektiive tänapäeval traditsiooniliselt rakendatava klassikalise biopsia meetoditega. Arutelu osas on antud ülevaade meetodite hetkeseisust ja võimalikest arengutest tulevikus.

Võtmesõnad:

Vedelbiopsia, vähidiagnoos, veres ringlevad vähirakud, veres ringlev rakuvaba DNA

CERCS: B200, Tsütoloogia, onkoloogia, kantseroloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

cDNA	Complementary DNA
cfDNA	Circulating cell-free DNA
cffDNA	Circulating fetal DNA
cfNAs	Circulating cell-free Nucleic Acids
cfRNA	Cell-free RNA
CK	Cytokeratin
CRC	Circulating rare Cells
CTCs	Circulating tumour Cells
ctDNA	Circulating tumour DNA
CTM	Circulating tumour microemboli
ECM	Extracellular matrix
EMT	Epithelial to mesenchymal transition
EMVs	Extracellular membrane vesicles
EpCAM	Epithelial cell adhesion molecule
FDA	US Food and Drug Administration
IARC	International Agency for Research on Cancer
lncRNAs	Long non-coding RNAs
LOD	Limit of detection
miRNAs	microRNAs
mRNA	Messenger RNA
ncRNAs	Non-coding RNAs
NGS	Next Generation Sequencing
NSCLC	Non-small cell lung cancer
PCR	Polymerase Chain Reaction
POC	Point-of care
PSMA	Prostate-specific membrane antigen

RHD	Rheumatic heart disease
RNP	Ribonucleoprotein
SERS	Surface Enhanced Raman Spectroscopy
siRNAs	Small interfering RNAs
SNPs	Single nucleotide polymorphisms
WHO	World Health Organization

INTRODUCTION

This thesis focuses on helping understand the current advancements on precision oncology. It informs about the advantages and challenges liquid biopsy has as a tool for analysing cancer biomarkers such as circulating cell-free nucleic acids (cfNAs) and circulating tumour cells (CTCs) in blood samples. The method may be useful for cancer research physicians and oncologists who are investing in developing or improving the diagnosis and/or therapy of cancer. Tumorigenesis can be associated with genetic and epigenetic modifications, some of which could be detected in plasma or serum as cfNAs, for this reason these biomarkers may be excellent. However, currently there is not a consensus on protocols, methods and reporting results and very little is known about their heterogeneity. For this reason, this thesis focuses on CTCs as it has been proven that they are the precursors of cancer metastasis and although their true nature is not completely understood they are able to survive and adapt in the circulation system inducing angiogenesis in the target tissue. Although with present knowledge, it is biased to name a better biomarker, cfNAs and CTCs have similar advantages and challenges. In general, the strategies to capture CTCs and differentiate them from other haematological cells are based on their physical (size, density, deformability, dielectrophoresis) and biological (expression of biomarkers) properties, which allows analysing them afterwards. At the same time microfluidic systems have been incorporated in other methods to increase the capture, isolation and analysis of CTCs in a faster and reliable way at a single-cell executability. In general two methods are employed in microfluidic devices, based on the beforementioned. Neither have proven to beat the challenges and be the preferable method of work and the literature suggests that an actual combination of these two may get physicians closer to their goal of analysing these biomarkers for their characterization. The goal of this literature based thesis is to give an overview of the possibilities a liquid biopsy can carry as a test to analyse biomarkers, primarily CTCs and cfNAs.

1 LITERATURE REVIEW

1.1 CANCER IN A NUTSHELL

Cancer has always been a very serious health issue. We all know someone who has survived it or that unfortunately has passed away fighting it. According to the World Health Organization (WHO), cancer is ranked as the second leading cause of death in the world, being responsible for an estimated 9,6 million deaths in 2018. That makes, globally, 1 out of 6 deaths be due to cancer, so this data is highly relevant and has to be taken seriously (Reboux, 2018). Cancer is killing people across the globe on a daily basis and the numbers are increasing every year. Thanks to this information, or more likely, by the concern it arises socially, a lot of research and effort has been put out by governments, scientists and associations who are trying to understand 'cancer' to provide novel solutions that will save the lives of future cancer patients.

Cancer can affect any part of our bodies, growing abnormal cells that proliferate without control or limit at an exponential rate. Since the cells are not encapsulated, it can easily spread or adjoin to the organs closest to where it started and so on; this is known as metastasis and it is the reason cancer gets tougher to fight, not only because a secondary malignant site(s) is growing apart from the primary site but because this is the major cause of death from cancer (Yin *et al.*, 2019). Cancer does not know race, age or social status. Although there are certain factors that will trigger tumorigenesis, the conditions that provoke cancer can be both dependent and independent of our lifestyle. Through research, the WHO and the International Agency for Research on Cancer (IARC) classify cancer causing agents. These can be both extrinsic and intrinsic agents, shown in table 1.

Table 1. Cancer causing agents (Modified after Reboux, 2018).

Intrinsic Agents	Extrinsic Agents
-Genetic factors. -Ageing.	-Physical carcinogens, like UV and ionizing radiation. -Chemical carcinogens, such as asbestos, components of tobacco smoke, aflatoxin (a food contaminant), and arsenic (a drinking water contaminant). -Biological carcinogens, such as infections from bacteria, parasites or viruses.

It might be redundant to include that tobacco use, alcohol abuse, not being physically active and having an unbalanced and unhealthy diet are major risk factors not only for cancer but for other noncontagious diseases.

Diagnosing cancer requires different modes of activity or occurrence. It is absolutely necessary for cancer management to get an early, sensitive and accurate diagnosis. Not only will it make the difference for the patients chance of survival but it will reduce the costs overall. For these reasons, it is important that novel technologies and approaches to identify and study cancer are brought up by scientists and researchers. In body fluids like blood, urine, cerebrospinal fluid and saliva there are cancer biomarkers that can be isolated to analyse tumours (Geeurickx and Hendrix, 2019). This has given physicians the opportunity to study cancer in a non-invasive way known as liquid biopsy, contrary to the traditional biopsy which is invasive and currently the efficient way to extract and study tumour material. In this regard, there has been a lot of effort in enabling better diagnostics and treatments for cancer patients. Microfluidic technology is an auspicious tool that opens the door to new possibilities to analyse tumours. The benefits of this technology expands to, not only the previously mentioned, but economically too. According to the WHO, in 2010, the estimated total annual cost of cancer was US$ 1,16 trillion (Reboux, 2018). The economic impact of cancer is also to be taken note of, as the amount is significantly high and this money can go towards other social branches of improvement.

1.2 WHAT IS LIQUID BIOPSY?

A relatively novel method for cancer diagnosis that is defined as the analysis of tumour material (for example, cells or nucleic acids) taken in a minimally invasive or non-invasive way through the sampling of blood or other body fluids (Wan *et al.*, 2017). From the blood sample scientists are interested in specific biomarkers, particularly in CTCs and cfNAs (circulating tumour DNA, RNAs and exosomes) (Sánchez *et al.*, 2016), shown in figure 1. It is promising that a blood sample can be the key to saving many lives from cancer, however, it is not that straightforward and it will require more years of research and new technology to get to this point. The level of importance in these biomarkers relies in comprehending the

origin of tumours and of metastasis, therefore offering scientists a better perception of tumour behaviour, for both, applying a better and a more effective treatment and for keeping a close eye on patterned advance.

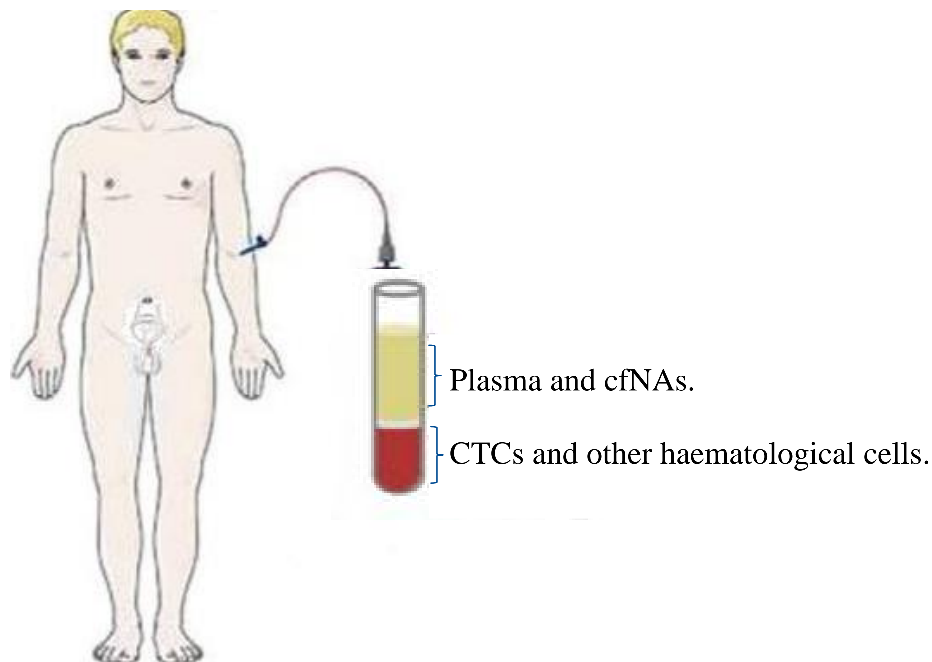

Figure 1. Liquid biopsy test in a cancer patient. Modified from (Lowes *et al.*, 2016) and (Yin *et al.*, 2019). CfNAs can get isolated from plasma or serum and CTCs from cellular fraction of blood samples.

1.2.1 DIFFERENCES TO TRADITIONAL BIOPSY

Traditional biopsy consists of the extraction of cancerous or suspected cancerous tissue for identifying its nature or cause (i.e., needle biopsy, bone marrow biopsy, etc.) (Law *et al.*, 2020). The thing about tumours is that they are heterogenous and taking a sample from one specific point gives only a result for that part of the tumour. Taking many samples is not an option either because tissue extraction is hazardous and painful for the patient; not to mention that depending on the type of cancer or its location, removal could not be possible at all. Moreover, this method is time consuming and pricey. Today, this is performed as the effective way to access the molecular information of the tumour. It provides essential information, i.e.: the histology and description of the tumour characteristics necessary to apply an efficient treatment. For lung cancer, for example, the biopsy followed by histology analyses will enable physicians to tell which type of tumour it is, such as squamous cancer, adenocarcinoma, small cell lung cancer, non-small cell lung cancer (NSCLC), or another, less common type (Shtivelman, 2017).

1.2.2 COMPARISON AND ADVANTAGES

In comparison with traditional biopsy, liquid biopsy offers a reduced risk of complication being non-invasive (or minimally), an increased ability for monitoring over an extended period of time, the possibility to identify clonal evolution and the developments of resistances in cancer cells (Law *et al.*, 2020). However, compared to traditional biopsies, liquid biopsies lack the advantage to determine the histology of tumours with precision, at least for now. Hopefully within the next couple of years liquid biopsy will be at the point of giving scientists this information as for today, the available tests are limited. This defines one of the current limitations of liquid biopsies (Shtivelman, 2017). On a brighter note, liquid biopsies do not require surgery, which reduces costs. This also allows diagnosis to be more time efficient, and patients will avoid a traumatic experience that is sometimes a slow recovery with a needle stick that takes a small amount of blood. There is no comparison on that note considering the well-being of the patient. Furthermore the promises it has once the analysis results come up will overpass traditional biopsies by miles. Another important difference is that when a tissue sample is taken from a tumour, call it a bone marrow biopsy, for example, doctors are only getting a limited amount of information. Considering that by this result, they will determine how the cancer will be treated and give the patient an overall diagnosis of their situation and that tumours are in constant change, everyday evolving, the level of accuracy is not high. Metastatic tumours may be even more divergent from the tumour that seeded them, this implies that mutational analysis of solid tumours is fallible (Wan *et al.*, 2017).

On the contrary, the advantage of liquid biopsy, is that it analyses cells that are shed into the bloodstream by all tumours present in a patient with metastatic cancer. Also, it is expected to give a complete picture of all mutations found in all tumours (Shtivelman, 2017). At least this is the goal, as for now, more research is needed to even assume that all tumours release the same amount of cells into the bloodstream and that taking a blood sample could not pick up this information equally. It is a turning point for cancer patients, as the time continues to pass, this technology will eventually make it feasible to experience cancer differently. The limitations liquid biopsy currently has, mainly, the isolation of the desired biomarkers from the bloodstream, will get better as genetic sequencing technology improved with the arrival of polymerase chain reaction (PCR) and eventually next generation sequencing (NGS) technologies, cancer cells are more thoroughly characterized (Law *et al.*, 2020). In short, liquid biopsies will be beneficial for physicians to monitor the evolution of the tumour, attack it in the best way possible and access information when tissue samples are not accessible. Roche

Diagnostic's Cobas EGFR Mutation Test version 2 is currently the only US Food and Drug Administration (FDA) approved liquid biopsy, and serves as a case study for the successful translation of this technology. The test is in adjustment of an existing tissue biopsy system for identifying specific mutations in NSCLC (Law *et al.*, 2020).

1.3 CELL-FREE DNA

We owe two French physicians the acknowledgement of circulating cell-free DNA (cfDNA) in human blood. In 1948 Mendel and Métais first noticed it, but it was until 1977 when the discovery of elevated levels of cfDNA in patients with cancer was made. This discovery allowed physicians and scientists for further explorations into the connection between the two (Law *et al.*, 2020). Circulating cell-free DNA is defined as DNA circulating in the blood-stream that is not associated with cells (Heitzer *et al.*, 2019). cfDNA are DNA fragments that can be isolated from blood plasma or serum in mammals (Grabuschnig *et al.*, 2020) and it is primarily constituted of monomers that range between 150 and 200 base pairs in length (Raymond, 2019). Its estimated half-life may vary from several minutes to an hour or two giving the scenario in which it is assessed (Kustanovich *et al.*, 2019).
The potential of cfNAs was not of much interest in oncology at early stages of discovery. It was until it was demonstrated that circulating fetal DNA (cffDNA) in maternal plasma, perceived the presence of Y chromosome-specific complementary DNA (cDNA) fractions and a gene which determines Rheumatic heart disease (RHD) in 1997, which is the non-invasive prenatal diagnostic (Grabuschnig *et al.*, 2020). Moreover, it took another 18 years for technology to reach the point of testing in a non-invasive way for trisomy 21, when next-generation sequencing (NGS) became available (Pös *et al.*, 2018). By today non-invasive prenatal testing of most common aneuploidies including trisomy's 21, 18 and 13 became a well-adapted screening method in many countries (Wan *et al.*, 2017).

1.3.1 CIRCULATING TUMOUR NUCLEIC ACIDS

Under the term of cfNAs usually it makes reference to DNAs and RNAs. A great variation of cell-free nucleic acid molecules take part of multiple physiological processes which are not fully understood and will require years of further investigation to understand their roles. For this reason, cfNAs are not the biomarker of interest in this thesis, however, the most relevant finds are noted. cfNAs are contained in extracellular membrane vesicles (EMVs) or

they form ribonucleoprotein (RNP) complexes where they are protected from degradation. EMVs that are released from tumour cells are known to carry oncogenic factors, like nucleic acids, and thus are named oncosomes. When oncosomes are introduced into a recipient cell, it transports the cancer cells and transfers its oncogenic message across idle cells (Pös *et al.*, 2018). This is believed to be a propagation form of cancer and thus serving as an appropriate field of study in precision oncology. For this reason, nucleic acids have gained their field of study as biomarkers for various diseases like: neurodegenerative, infectious diseases and cancer (Gilboa *et al.*, 2020). Studies demonstrated that nucleic acid biomarkers can be used for diagnosis, determining optimal therapy and understanding the severity of the illness (Salvianti *et al.*, 2020).

Regardless that technology has now enabled the possibility to examine forward cfNAs, there are still important challenges to consider with its analysis. First, they are also, like other biomarkers, present at a very low concentration in blood samples; this means that ultra-sensitive techniques are necessary. Second, since the sample is heterogenous, it will contain multiple off-target nucleic acids; so there needs to be specific target molecule technologies. Another challenge because of this is that the measurements can mask nucleic acids of interest, so ultra-sensitive and single-molecule technologies are needed for analysis (Gilboa *et al.*, 2020). The immune system also prevents cfNAs to be elementary biomarkers. The quantity will be influenced by degradation or the lymphatic system overall. Another natural process by which nucleic acids are drained out of the circulation is by the kidney and the liver; studies have not been too precise about the half-life of cfNAs, as it ranges from 15 minutes to several hours (Schwarzenbach *et al.*, 2011). Other studies show that the levels of cfNAs may also point out to other pathological or physiological processes that are not tumour related, like inflammations. The production of cfNAs has shown to raise in patients with cancerous lesions compared to not having a tumour, all the same, an increased level has also been reported in people with harmless lesions (Schwarzenbach *et al.*, 2011). Thanks to novel technologies, today it is possible to examine further in this area, however, it was not until very recent that this was possible and thus there are a lot dead ends. Soon, as collective results come up in the databases it will be possible to use cfNAs as a complimentary, or even replace it, with conventional cancer diagnosis (tissue biopsy). As of now, there has not been many efforts to develop microfluidic tools to evaluate and isolate cfNAs (Sun *et al.*, 2018). cfNAs hold a promising future in screening, diagnosis, medical prognosis, therapy and outcome of cancer and other diseases (Pös *et al.*, 2018). Furthermore, experiments on graft hybrids showed that cfNAs differentiated from somatic cells to germinal cells and pass along

to offspring. This made a reality the heritage of new features through cfNAs and can signify that they may take part in evolution mechanisms (Pös *et al.*, 2018). This demonstrates that the nature of cfNAs yield in the bloodstream is not yet understood.

1.3.2 ANALYSIS OF DNA

DNA biomarkers include deletions, insertions, single nucleotide polymorphisms (SNPs), short tandem repeats or other variations. Other DNA biomarkers are translocations, pathogenic DNA and mitochondrial DNA (Gilboa *et al.*, 2020). Since cancer affects the nature of DNA directly at the cellular level, it is of relevance to study these changes, as they can be measured in the tumour (Ziegler *et al.*, 2012). DNA analysis has major challenges to overcome. First, the human genome was sequenced 17 years ago and is very complex, from this we know that there are gene sequences longer than 1000 nucleotides; this means that precise mapping requires long read capabilities and tools (NHGRI, 2015). Furthermore, we now know that the human genome has approximately three billion nucleotides, to fully understand how an individual will respond to treatment it requires to analyze a large indefinite number of genes; which will be possible as technologies for genetic sequencing rise. For these biomarkers it also implies difficulties that the samples are highly diversified, as the set of molecules may superimpose the sequences of interest, which is why single-molecule methods are required to overcome and identify individual molecules.

1.3.3 ANALYSIS OF RNA

In regard to RNA, there is a vast list of types but the most common and the ones that will be described are: messenger RNA (mRNA) and non-coding RNAs (ncRNAs), like microRNAS (miRNAs), long non-coding RNAs (lncRNAs) and small interfering RNAs (siRNAs). All these are involved in gene expression control and regulations which makes them appropriate biomarkers for cancer.

The biological process of mRNA gives it a high value for cancer diagnosis, although little is known, analyzing mRNA can be used for gene expression identification and illness diagnostics (Le Rhun *et al.*, 2020). However, the ratio of mRNA in samples is low and unstable due to degradation, which complicates its analysis (Sun *et al.*, 2018). Technologies with high sensitivity are preferential, this helps overcome the low copy number of mRNAs, which does not surpass the hundreds of copies per cell (Gilboa *et al.*, 2020).

miRNAs are involved in gene regulation and it is known that they activate or repress translation of proteins. They are short strands of ncRNA and they become abnormal in cancer, causing it to become more aggressive by proliferation, apoptosis, metastasis and angiogenesis. For this reason, scientists believe that they can give insight for diagnosis, monitoring and healthful remedies in cancer patients (Sohel, 2020). Due to their size, 17-25 nucleotides, and low levels found in samples, with reports showing femtomolar ranges, it is difficult to analyze them with conventional tools (Gilboa *et al.,* 2020). In contrast, there are longer nucleotide RNAs, which are >200 nucleotides. However, this does not simplify their analysis. lncRNAs are non-coding as well and regulate intercellular trafficking, transcriptome, chromosome remodeling, etc.. They have a cancer biomarker potential because they show to mediate epigenetic alterations by recruiting chromatin modification factors, this leads to gene silencing (Ghafouri-Fard *et al.*, 2020). Although they have promising qualities and will contribute to precision oncology in the future, together with siRNAs, at present days, there is not enough information on how they can influence the development in cancer (Ahmadzada et al., 2018). It must also be taken into consideration that RNA quantification requires to reverse transcribe the RNA to cDNA form, traditional technologies still complicate this process; Another complication is that biologically RNA will amplify and this can sabotage the original amount of RNA that was intended to get analyzed. Finally, the great varieties of RNA come in different proportions, so it is more likely that other abundant RNA will complicate the detection of the RNA of interest (Sun *et al.*, 2018).

1.4 CIRCULATING TUMOUR DNA AS A BIOMARKER

Primary tumour cells undergo necrosis, apoptosis, phagocytosis, or cell detachment, and release tumour-derived cell-free DNA, otherwise known as circulating tumour DNA (ctDNA), into the bloodstream. Early detection of ctDNA via simple blood draw offers the potential for population-level screening, early detection, intervention, and progressive monitoring throughout treatment. Most of the cfDNA originates from cells of hematopoietic lineage but in patients with cancer it contains circulating tumour DNA which has the potential of being a biomarker in precision oncology. Evidence shows that ctDNA provides a well-rounded view of the tumour genome as it reflects DNA released from multiple tumour regions or different tumour foci (Heitzer *et al.*, 2019). Table 2 points out to some favourable approaches and limitations ctDNA currently face.

Table 2. ctDNA pros and cons.

Approaches	Limitations
Suitable for analysis of specific mutations and copy number alterations.	Without standardized or validated techniques.
Viable long-term storage for posterior analysis.	Possible clinical implementation, but still at risk of cfDNA dilution bias. It is currently impossible to know if the lack of ctDNA is real or whether it is bias due to dilution in the cfDNA in a sample that does not show copy number alterations or mutations in the analysed gene (Puche-Sanz *et al.*, 2020).
Useful for minimal residual or localized disease.	Very low levels, often <0.1% (Zhang *et al.*, 2019).

ctDNA as a biomarker can be revealing about tumour mass, giving an overall extent of the disease to physicians to understand the appropriate approach to treat cancer. It has also shown to have prognostic value, as the presence of ctDNA after treatment, whether by extraction of the tumour in surgery and/or with chemotherapy will result in predictive analysis associated with recurrence and poor outcome, enabling patients to be treated more effectively and being able to trace their health in a timely manner (Salvianti *et al.*, 2020). The idea is that cancer patients that went through chemotherapy and/or surgery to treat their cancer are able to know how well they are doing by analysing their ctDNA levels. As studies show that there is a correlation between the size of the tumour and the amount of ctDNA found in plasma. The magnitude of ctDNA that originates from individual cancer lesions depends on the anatomic location and size of the tumour; It has been estimated that, for a patient with a tumour of 100g in weight, 3.3% of its DNA can be released in the bloodstream every day. Also, the presence of CTCs and metastatic lesions can contribute to ctDNA yields (Zhang *et al.*, 2019).

An important thing to take into account is the process of drawing blood. It has to be done considering important factors about ctDNA. Research suggests that ctDNA has a half-time in the circulation, with a reported average around 15 minutes, due to rapid clearance by lymphatic circulation via kidneys, spleen and liver (Siravegna *et al.*, 2019). During blood

collection and processing it is essential to avoid white blood cell lysis which increases dilution of tumour circulating fragments from genomic DNA derived from white blood cells (Siravegna *et al.*, 2019). During enrichment, research has demonstrated that sensitivity can be increased on the basis of their length, which is reported to be around 132-145 base pairs, compared to cfDNA from normal cells being approximately 166 base-pairs (Heitzer *et al.*, 2019). On a brighter note, since it is known that there is low concentration of ctDNA in the blood, a lot of research has been done to improve analytical specificity and sensitivity. In recent times, the technologies for DNA isolation have been of major improvement and more is understood to treat the blood accordingly to get consistent results. Apart from knowing that ctDNA is time sensitive; transportation, processing and storage of the sample play an important role in the consistency of the results too, as they also affect ctDNA directly either by degrading it or in quantification itself. However, it is too soon to have a common procedure across all laboratories, but this information has helped most research work conclude that it is essential to isolate plasma within an hour after blood draw to prevent cfDNA degradation due to DNase activity. Purposely designed blood collection tubes for cfDNA are accessible from multiple providers which minimize cell lysis and stabilize the total cfDNA pool by the inclusion of various additives and preservatives (Siravegna *et al.*, 2019). After blood draw, the procedure is a pretreatment to improve separation efficiency and this can be done by centrifugation or filtration, and studies show that this step may be reducing the count of ctDNA (Gao *et al.*, 2020).

All the above mentioned is great advancement for using ctDNA as a biomarker, but on the contrary the true nature and source of cfDNA is still unknown and open for researchers discussion. A lot of research suggests that it might be derived from dying tumour cells being released from apoptosis or other forms of cell death, but recent ctDNA analyses have identified somatic modifications associated with aggressive, proliferative cancer cell clones. Determining to what degree different tissues contribute to the composition of plasma in both health and disease will be critical to understanding the physiology of DNA release from cells into the bloodstream (Heitzer *et al.*, 2019). In fact, there is considerable inconsistency between the studies and methodologies done to understand cfDNA composition. So far, most of the results have not been able to prove the exact origin or correlation between tumour tissue contribution and levels of ctDNA which concludes that there is very limited knowledge in this area and why ctDNA is not quite the favourite biomarker when it comes to liquid biopsy.

1.5 CIRCULATING TUMOUR CELLS

Thomas Ashworth documented the first observations or findings of CTCs in 1869, where he identified microscopically, from a cancer patient who died due to metastatic illness what today is classified as circulating tumour cells (Geeurickx and Hendrix, 2019). Since then it was known that someday this discovery was going to help identify tumorigenesis, and it has taken more than a century for these efforts to reflect in something tangible.

1.5.1 ORIGINS AND FREQUENCY OCCURRENCE

The origin of CTCs remains unknown and is not fully understood. The most realistic explanation is that they emerge from a primary or even secondary metastatic tissue and enter the bloodstream. The exact mechanism has not been proven but there are various events that add up to this theory, including the hypoxic tumour environment, extracellular matrix (ECM) remodelling, active proliferation and epithelial to mesenchymal transition (EMT) (Sun *et al.*, 2018). In the actuality this model suggests that CTCs travel from the tumour or the metastasis itself into the blood circulation and accesses nearby organs and causes the development of malignant growths that is, propagating it to other body parts. This makes the cancer more aggressive and harder to fight. This can happen at any stage of the cancer but the more advanced the state the more CTCs seem to be released; It is important to note that CTCs are extremely rare, with 1 CTC present for 1x10E6 – 1x10E9 blood cells (Geeurickx and Hendrix, 2019). They have a half-life of 1 to 2.4 hours and can be differentiated from mesenchymal blood cells by the expression of epithelial surface proteins, such as epithelial cell adhesion molecule (EpCAM) or proteins of the cytokeratin family (CKs 8, 18 and 19), or by their epithelial morphology (Geeurickx and Hendrix, 2019). After the CTCs are shed into the bloodstream they will be present for a relatively limited time, giving physicians a time frame to take samples. It is possible that the lasting of the CTCs is only a short time due to immune wipe out or apoptosis.

In studies, it has been proven that there is already a small percentage of CTCs in blood analyses even before clinically diagnosed metastasis. Nonetheless, studies are not pursuant at all times given that cancer behaves differently in all patients, not to mention that the methods nowadays have a low sensitivity and accuracy (Sun *et al.*, 2018). Comparatively, studies also demonstrate that the relation between the amount found of CTCs in the blood is proportionate to the cancer status of the patient; the number can spike as the primary tumour site expands or alternatively downsize in response to tumour shrinkage with efficient therapy

(Hiltermann *et al.*, 2012). To date, the FDA has approved one system for clinical use that can detect CTCs in the blood; in 2004 the system was authorized to predict outcomes on breast cancer, in November 2007 and February 2008, the system was granted as well approval for colorectal and prostate cancers (Millner *et al.,* 2013). The name of the system, by Menarini Silicon Biosystems (Bologna, Italy), is called CellSearch® and it is for identification, isolation and enumeration of CTCs, taking 15 years of research and technical innovation to reach this standard.

1.5.2 PREDICTIVE INFORMATION IN CTCs

It already says something that there is an approved system for CTCs detection, and as all new technologies and methods, the price at first is high but eventually it will become more accessible as the understanding of its current results are used to make it more efficient, sensitive and simple. CTCs are important because they render towards phenotypic and genetic heterogeneity of tumour tissue and scientists have been able to grow them *in vitro*, which gives them a significant value for individualized analysis; like learning the effects of drug reactivity (Puche-Sanz *et al.*, 2020). The true value of CTCs relies on their promising diagnosis. Another good reason to study CTCs is that they range in size from 4 to 20 μm, they can also be found in clusters of 2 to 100 cells and range in size from 10 to 100s μm in diameter (Sun *et al.*, 2018). Since CTCs are a heterogenous population this can be relevant for their characterization and make a difference when a method is used for their isolation. CTCs stain positive for epithelial cell adhesion molecule. In table 3 there is a comparison between the advantages and difficulties CTCs face.

Table 3. CTCs pros and cons.

Approaches	Limitations
Competent for RNA and protein expression.	Problematic long-term storage for analysis later on.
Categorization of tumour heterogeneity.	Complex and pricey detection but has a standardized and validated system.
Expectation of cultures and susceptibility testing.	Challenging clinical execution due to high technical and economic costs and low-level detection rate.

When cancer cells are shed into the bloodstream they can undergo EMT, this will make epithelial cells lose their phenotype, losing their cadherins and eventually making them lose into the blood circulation. What happens next is that they transition to mesenchymal phenotype. Along this transition, the expression of CKs and EpCAM is lost together with the morphology of epithelial cells (Geeurickx and Hendrix, 2019). Some studies account to mesenchymal CTCs to induce the formation of metastases (Bourcy *et al.*, 2016). Physicians have come to the conclusion that EMT is required in CTCs to adapt into the bloodstream environment and therefore survive in it enough to metastasize in other organs (Puche-Sanz *et al.*, 2020). Some studies have found that CTCs in circulation have a much longer durability than blood cells (Bagnall *et al.*, 2015). This gives another point in favor to studying and isolating CTCs efficiently.

Circulating tumour cells may be the most constituted detective target in liquid biopsy. First, they can be of significant value to detect early cancer along with some directional hints for treatment and insight to precise location of tumorigenesis. The presence of even one single CTC cluster in blood when taking samples has proven that survival rates reduces in cancer patients, giving it a high value for studying precision oncology (Sun *et al.*, 2018). However, the challenges that CTCs detection encounters are still relatively relevant. First, the amount found is considerably low and therefore needs methods of higher affinity and sensitivity, not to mention that there needs to be enrichment to improve the before mentioned. Secondly, there is a time gap to complete the sampling in a highly efficient manner because of its life time.

1.5.3 TECHNOLOGICAL CONSIDERATIONS

Circulating tumour cells detection is of extreme importance to make precision oncology a reality. In the last decade one can see the increment of research published online of new ways to detect CTCs with different technologies. In part, time has allowed CTC detection to become promising and attractive to physicians and scientists but the truth is that with time advances came in medicine, biology, chemistry, oncology and material sciences that have made it possible to see advancement in this field. Presently, CTCs are being detected using epithelial markers. For this reason, CTCs which do not have an epithelial constitution are not being detected, and as mentioned before, that is probably because it has undergone EMT.

Research is working towards making sure that all CTCs are detected, independent of epithelial, in novel techniques. Another thing that current methods need to improve is capturing viable CTCs, as many are lost in the process and the count is already very low in circulation (Millner *et al.,* 2013). There is heterogeneity within CTC populations, so far some of the cancer cells identified are: mesenchymal tumour cells, stem tumour cells, epithelial tumour cells, epithelial to mesenchymal hybrid tumour cells and clusters of tumour cells known as circulating tumour microemboli (CTM) (Laget *et al.*, 2017). However, recent studies point to this heterogeneity being greater and different between patients and even from a single blood sample (Bankó *et al.*, 2019). Apart from this complicating the capture of viable CTCs it requires new technologies and approaches to properly isolate them. Learning and identifying the subtypes for CTCs will give physicians a detailed status of the patients risk for metastasis as well as an individualized approach to fight the illness; not to mention that it could have the potential to reveal the site where the metastasis will form next (Millner *et al.*, 2013). To date, the quantification of CTCs is used to evaluate the efficacy of therapy. Since the correlation between disease state and CTCs numbers are proportionate. Therefore, if the analysis shows a lower number of CTCs on the blood after treatment, physicians can assume that the treatment is indeed working. To prevent EpCAM mediated cell capture from a partial issue or situation, that is, to improve its affinity, there can be used specific biomarkers like antibodies or aptamers targeted toward specific markers (Sun *et al.*, 2018). CTCs can be isolated using density gradient centrifugation, like Ficoll™ or Percoll® gradients, and they fractionate together with peripheral blood mononuclear cells; their estimated density ranges approximately from 1,064 to 1,065 g/cm^3 (Sun *et al.*, 2018).

Detection technologies use indicators for CTCs detection, such as: purity, recovery rate and limit of detection (LOD) (Gao *et al.*, 2020). Purity makes reference to the percentage of enriched CTCs to the total number of cells in the enriched samples. The recovery rate will be the number of the enriched CTCs to the total amount of CTCs in the blood in percentage, it is sometimes referred to as enrichment efficiency or capture efficiency in other literatures. The LOD sets the limit of the CTC concentration that could potentially be detected in the blood. Due to their extreme rarity, most methods require an enrichment step antecedent to detection of CTCs, this step is meant to separate in fractions the CTCs from unwanted cellular components of blood and blood plasma and ready for detection techniques. Cells can be separated by their physical properties such as cell size, migratory properties, density and protein expression (O'connor, 2016). Cancer care institutions have not yet acquired CTC technologies into their guidelines for clinical care, because although they have prognostic

value, there has not been any improvement towards survival rate in cancer patients (Yin *et al.*, 2019). Presently, the clinical function of CTCs is based on enumeration of CTCs with an epithelial phenotype, with research clinicians have discovered that instead of just focusing on enumeration technologies they can expand to characterization technologies and get something that is more concrete for clinical utility (Ferreira *et al.*, 2016). With the characterization of CTCs clinicians could instead provide the patients with a relative distribution of CTCs and their metastatic potential, allowing physicians to veraciously predict outcome both selecting the most personalised treatment and for future metastatic sites (Millner *et al.*, 2013). CTC detection methods are currently being used mainly for research purposes despite their potential role in cancer well-being. Unfortunately the lack of standardized technologies prevents physicians to obtain congruent data as the techniques used are different as well as the technological platforms (Bankó *et al.*, 2019).

1.5.4 ENRICHMENT

It is most common that CTCs are enriched to be isolated for the subsequent phenotype identification and molecular analysis. Enrichment of CTCs can be done in two different ways, positive and negative enrichment. In 2004 the first negative enrichment technology was developed to enrich rare cells in blood (Kowalik *et al.*, 2017). The negative enrichment consists in capturing non-target cells or healthy blood cells and wash out with a solvent target cells or CTCs in this case. Contrary to positive enrichment, which does the opposite, it elutes healthy blood cells and captures CTCs. Moreover, positive enrichment can be divided into two categories: *in vitro* enrichment and *in vivo* enrichment. CTC detection without an enrichment step is known as direct detection and it can be done with two technologies, surface enhanced Raman spectroscopy (SERS) and line-confocal microscope (Shen *et al.,* 2017). Understanding the explicit partiality that prevents different enrichment methods to get standardized will help significantly towards working on technologies that focus on different CTC subpopulations. Antigen-free approaches have been shown to be more favorable in isolating CTCs now that clinicians have discovered that the heterogeneity in CTCs is greater than first thought (Salvianti *et al.*, 2020). The presence of CTMs or CTC clusters, is another biological sign that gave clinicians insight that enrichment technologies had to peruse another direction, researchers got their attention to this when they found that CTMs have the ability to resist apoptosis and form metastasis at a significantly higher rate than individual CTCs (Ferreira *et al*., 2016).

1.5.5 DETECTION

To compare different detection methods and describe device execution, the following parameters are taking into account in table 4.

Table 4. Parameters for CTC detection (Modified after Bankó *et al.*, 2019).

Capture efficiency	Represent the efficiency by which a device captures CTCs from a sample.
Purity	Ability of the device to specifically capture CTCs within a background of contaminating cells.
Enrichment	Refers to the factor increase of tumour cells in the sample volume relative to a background of other cells (primarily leukocytes) before and after running the sample.
Throughput	Volume or number of cells in a sample that a device can process in a unit of time.
Cell viability	Percentage of CTCs that remain alive after enrichment.
Release efficiency	Percentage of cells that are recovered from the device.

The technologies for CTC detection must be repeatable, rapid, reliable, cost-effective and capable to wide-ranging use and extensive production. They also need to be able to capture the extremely rare cells, the sample for that success should be around 7,5ml (Ferreira *et al.*, 2016). The beforementioned is what the technologies are stating as the basic requirements to evaluate and compare the outcomes of CTC detection. However, the identification of cancer cells in blood raises a challenge because of its similarities to non-tumour circulating rare cells (CRC) also found in samples, like: epithelial-atypical cells, normal stem cells, epithelial-normal cells, endothelial cells and physiological-state dependent cells (i.e., fetal cells in women during and after pregnancy or giant monocytes) (Laget *et al.*, 2017). Another challenge is the pre-treatment done to blood prior to measuring it, recent studies have shown that lysis or centrifugation may considerably lower the number of CTC in the samples. For this reason technologies that use whole blood samples are rising but still have to overcome some of the challenges that come without pre-treatment, devices must come in various pore sizes and shapes or have fluid-assisted separation technology (Bankó *et al.*, 2019).

The more clinicians are testing and trying different methods they realise how this topic is everchanging and giving new insight on approaches required for efficient detection of CTC

and their subpopulations. For example, in measurements of cell deformability and drug response they realised that it is relevant to have viable cells, thus two supplementary criteria were added to the parameters for CTC detection; release efficiency and cell viability (Ferreira *et al.*, 2016). Also, the change from CTC enumeration to molecular characterization will require techniques to provide a greater recovery rate. For now, CTC capture focuses on strategies to sort cells by their size, deformability, electrical charge, density and tumour cell surface marker expression (Ferreira *et al.*, 2016). In reality, there is not a technology that is suitable for or used in all circumstances and the different approaches should be taken into account according to the type of cancer being studied.

1.5.6 CTC MICROFLUIDIC-BASED APPROACHES

In the last decade, microfluidic-based approaches have significantly improved the applications of sample preparation, enrichment and point-of care (POC) diagnostics. They are first-class miniature tools for studying tumours and they are ideal for single cell sequencing applications for ultimately understanding cancer (Menon *et al.*, 2019). In diagnostics it makes it possible for extremely accurate fluid manipulation and control, this allows for higher throughput and faster sample processing. Microfluidic-based technologies have thus been attractive for having the quality for early diagnosis and real-time monitoring together with a remedial efficacy of the illness. The technology is used to sort through liquid biopsy samples and help identify specific cancer biomarkers, it can discriminate between cells at the single-cell level and enable separation and detection of CTCs, which is not possible with bulk techniques (Sun *et al.*, 2018).

It was in 2007 when CTC isolation was first demonstrated using a microfluidic-based approach with the CTC-chip and it was because of its success in identifying CTCs in >99% of the patient samples that it gave clinicians a focus in improving and developing further microfluidic-based systems to isolate and detect CTCs (Sun *et al.*, 2018). The focus clinicians can use for CTC isolation strategies rely on biological and physical properties of cells, that can provide unique information to distinguish malignant cells from other haematological cells present in the heterogeneity of blood samples, like white and red blood cells. Techniques can be based on immune-affinity capture of CTCs, i.e., on antibody-antigen interactions using EpCAM as target; working on exploiting cell surface markers making it possible to identify CTCs related to tumorigenesis. Or the other technique, known as label-free, focus on unique physical characteristics like shape, size, deformability and conditions of microscale flow (Silva *et al.*, 2020).

1.5.6.1 LABEL-FREE BASED APPROACH

The detection of biomolecules or cells at low abundance is extremely difficult. Label-free techniques are a promising option for biomolecule detection based on its advantages, including low-cost and easy operation. Meanwhile, microfluidic systems have been utilized for analyzing single molecules or cells at low sample volumes. Combining these two techniques may therefore offer a promising strategy to separate and detect low-abundance biomolecules or cells in complex samples (Leung *et al.*, 2019). There has been demonstrated that the label-free path can separate CTCs by hydrodynamic chromatography, filtration and dielectrophoresis methodologies with the downsize that they do not attain a high purity of CTCs and cell viability is compromised for further analysis (Chen *et al.*, 2020). The separation of CTCs in a label-free microfluidic system depends on physical differences of the cells, such as its shape, size, electrical impedance or polarizability. These at the same time can be classified into three methods, kinetic separation, equilibrium separation and namely elution separation (Leung *et al.*, 2019). Nevertheless, this technique comes with many challenges and still requires a covalently-attached label (can be an enzymatic or a fluorescent label), to detect the targeted cell or molecule; at the same time this poses disadvantages like the alteration of binding affinity and electrophoretic mobility change, interference of fluorescence signal or loss of numerical response; moreover, fluorescent labels could have damaging effects on the assay detection time (Leung et al., 2019).

1.5.6.2 IMMUNO-AFFINITY BASED APPROACH

The biological differences between cells can also be used to differentiate normal cells from CTCs. Incorporating microfluidic systems to this separation technique can increase the isolation of CTCs from millions of normal cells in a blood samples due to the force generated by blood flow in the microchannel allowing the separation of intact CTCs for post-analysis and culture (Silva *et al.*, 2020). Typically, the devices to capture CTCs with immune-affinity approaches need to get stained and evaluated on-chip or lysed to extract nucleic acids or proteins, the final purpose being the analysis of intact isolated CTCs. From 2007 clinicians have been incorporating techniques and technologies to improve and enhance previous designs. Recently, the rise to incorporate more than two approaches became trending to accomplish a more specific immune-affinity capture (Sun *et al.*, 2018). Currently, a novel 3D

printed microfluidic device that can increase the surface area and fluid flow manipulation which results in higher CTC capture efficiency has been proposed. This device works with anti-EpCAM antibodies to isolate CTCs from blood samples, despite it is quite complicated to efficiently fabricate 3D structures with these beforementioned features to isolate CTCs, Chen et al. demonstrated that it is possible (Chen *et al.*, 2020).

1.5.6.3 FUTURE DIRECTION FOR CTCs SEPARATION USING MICROFLUIDIC SYSTEMS

Despite the advantages that incorporating microfluidic systems with isolation techniques has increased the efficiency of CTCs capture it has not been widespread for clinical or commercial applications. This can be judging from the complexity to translate laboratory standardization to pre-clinical or clinical devices followed by the unwillingness of places to adopt novel and unacquainted technologies (Sun *et al.*, 2018). A combination between immune-based and label-free based techniques in microfluidic devices has been looked into and results from several studies point out to the potential it has to achieve a more specific selection of CTCs, a higher efficiency and faster categorization with increased recovery rate and purity (Silva *et al.*, 2020). Additionally, these biological and physical principals can be integrated into multi-step procedures on progressive nanotechnologies and microfluidic systems to accomplish high-standard capture and analysis of CTCs (Yin *et al.*, 2019). With the development of new devices and methods, it may be that label-free technologies could compete with affinity-based methods providing superior performances, regarding a higher throughput. A limitation related to microfluidics is primarily low throughput and the ability to process large amounts of samples and detect viable number of CTCs for post-analysis (Sun *et al.*, 2018). One disadvantage of label-free separation is the low selectivity achieved compared to affinity-based methods (Leung *et al.*, 2019) but on a brighter note it carries the potential to isolate a larger number of cells (Silva *et al.*, 2020). Thus, these setbacks are the reason a combination of techniques may overall work best for CTC capture and analysis.

2 AIM OF THE THESIS

- Review the potential of liquid biopsy as a test for cancer diagnosis.
- Overview of biomarkers, such as CTCs and cfNAs to analyse tumorigenesis.
- Technological approaches and research on better understanding cancer.
- Identification of novel diagnostic tools for cancer.

3 DISCUSSION

From venous blood clinicians can isolate, identify and measure biomarkers such as CTCs and cfDNA; this at the same time contains in patients with cancer ctDNA and cell-free RNA (cfRNA). cfRNA is a mixture of small RNAs but it also contains EVs. These analytes carry genetic and epigenetic information about tumorigenesis or metastases which can be studied by scientists to understand the cause and effect of cancer. The studies are carried out by analyzing a blood sample of around 7,5ml through a test known as liquid biopsy (Marrugo-Ramírez *et al.*, 2018). A relatively high amount of blood is required giving the extreme rarity of these biomarkers, where the occurrence or ratio can be from one to a million or even a billion if we compare it with other blood cells (Dive and Brady, 2017). However, the clinical utility of liquid biopsy has not reached to something concrete to date, as many challenges are presented by the everchanging understanding of the biomarkers. Techniques and prototypes continue to surge as research continuously increases throughout the years, which indicates that this test will reach fruition in the coming years, transforming overall the healthcare system for people with cancer. The goal of liquid biopsy is to determine patient prognosis, be able to monitor tumour recurrence and responses to therapy in real-time, identify new drug target mechanisms and make clear and comprehensible in this way drug resistance; in general learn about tumorigenesis and metastasis progression (Siravegna *et al.*, 2019). While traditional biopsies are still clinically relevant and is the test being carried out currently, it may be obsolete in the future once liquid biopsy reveals the beforementioned including that it is a non-invasive (or minimally) test, contrary to traditional biopsy which is not always feasible giving the nature or location of the tumour.

CTC-based liquid biopsy is a major trend between all biomarkers as it has demonstrated the detection of early cancer giving the characteristics of CTCs which are the precursors of the illness. In the near future it is expected by repeated sampling that the molecular profile of CTCs can expand for the establishment of CTC cultures which will allow in-depth or intrinsic characterization as of today very little is known at the single-cell level and its mechanisms of survival in the bloodstream. *Ex vivo* analysis will be needed to reach the understanding of CTCs heterogeneity and their specific advantages for precision oncology. The evidence discloses that the enumeration of CTCs can be used to predict, monitor and understand post treatment relapse of cancer (Bankó *et al.*, 2019). CTC isolation and detection is normally done by both enrichment and detection. Enrichment step is focused on approaches based on the size, the density or immunomagnetic and microfluidic properties whereas detection step is protein or nucleic acid based (Lowes *et al.*, 2016). It is true that both CTCs and cfDNA

have the potential to provide prognostic insight to physicians based on the quantity detected in the sample, however cfDNA has a downsize when it comes to differentiating it from ctDNA (cfDNA can originate both from tumour cells or other cell types) and it is key to evolve the study of single gene testing to multigene panel testing which ideally will capture a whole or at least better picture/exposure of the tumour genome. Detection of cfNAs holds a promising approach for tumour diagnosis, however there is not enough knowledge in the field and the lack of clinical studies are preventing the rise of these biomarkers. For now, these rely on literature and future willingness of physicians to invest and examine validated methods for their detections and understanding of their characteristics. With current technologies and knowledge it is hard to name a superior biomarker since as mentioned earlier, both have equivalent and comparable utilities in precision oncology. Further research is required to conclude this but there might be a scenario where one is better than the other according to the type of cancer or stage in which it is required.

In regards to the technological requirements for cfDNA and CTCs, both require ultra-sensitive detection techniques but CTCs also need capture implementation. Additionally, cfDNA has the advantage of being able to get stored for analysing at some eventual time giving it a possibility to have a bank whereas CTCs need to be processed in a timely manner (Lowes *et al.*, 2016). On another note, research showed that unlike cfDNA, CTCs may be evaluated at the single cell level (Lowes *et al.*, 2016). Another fact is that most studies of these biomarkers are observational research, where the methodologies, cancer type and stage vary, steps and interpretations are always a variable thus it is important that in the future results are based on the scientific method. Microfluidic-based systems have effectively demonstrated a fast and trustworthy model for isolating CTCs however a combination of several detection technologies show a higher efficiency as it can overcome individual technique challenges. The focus needs to be on developing technologies that result in high viable cells as of today a great number is lost through both pre-treatment and treatment steps (Vera-Ramirez, 2020). As studies continue to process new outcomes, it will be easier for clinicians to understand that biomarker selection is indispensable along the proper tools and technologies to properly isolate and have success in presenting their clinical advantage.

Microfluidic-based approaches may have a superior approach based on their miniature and unique flow pulsation compared to conventional approaches. However, it still requires to be combined with other techniques and methods that increase purity as of now it shows efficiency in targeting extremely rare cells with high sensitivity. The advantages these systems

will contribute to detection, separation and analysis are such like: capacity for enough volume samples, multiplexing capabilities, time-efficient processing and large surface area-to volume ratio. The integration of microfluidic systems with technologies (electrical biosensors, mechanical sensors and photonic sensors) will allow the development of portable diagnostic devices through parallelization (Leung *et al.*, 2019). Affinity-based approaches mostly focus on EpCAM because this antigen is over-expressed in a variety of cancers like: lung, mammary, bladder, intestine, colon, etc., (Keller *et al*., 2019). Alternatively, specialized markers may also be used for other cancer types, like prostate-specific membrane antigen (PSMA) (Yin *et al.*, 2019). Label-free techniques with integrated microfluidic chips allow a greater throughput compared to affinity-based methods resulting in higher CTCs for post-analysis, this may be due to the physical properties of CTCs where compared to white blood cells (~7-15 µm) and red blood cells (~6-8 µm) they are relatively larger (~17-52 µm) effectively accomplishing separation through size differences as a parameter (Silva *et al.*, 2020). Nevertheless, improvement in this technique is vital to deal with the heterogeneity in CTC subpopulations to avoid non-specific capture. To the best of my understanding, microfluidic technologies are in the majority still deriving from an experiment or concept perspective, several designs are feasible but the translation to these systems are still limited to tangible marketed tools. To reach this point it is essential that scientists, clinics and governments considerably invest for standardization, fabrication and repetition of studies as well as the development of new technologies of genome sequencing, single cell analysis, molecular imaging, nanotechnology and so on.

The CellSearch® system is the only approved FDA method to date for CTC analysis in colon, prostate and breast metastatic cancers but many other approaches are available for testing (Romay et al., 2019). This is given that the translation of these approaches from laboratory to clinical practice requires significant validation and time. The CellSearch® system is not microfluidic-based and instead it focuses on the separation and imaging of target cells by a combination of magnetic nanoparticles that differentiate CTCs from other cells (Menarini Silicon Biosystems, 2020). Also, it is a discrepancy that being from 2004 it has not adapted the new discoveries in the field. In short, there is a wide variety of isolation technologies and products in development, which makes it complicated to pass judgment and compare between each other or even propose a combination of these technologies to my knowledge. Furthermore, there is a vast mixture of techniques and results based on different cancers, patients, methods and interpretations and in order for this to become standardized reliable

data is needed. However, I predict that CTC isolation will continue to evolve according to new discoveries and current techniques will become obsolete or adjust for capturing different cells for other medical fields. Many papers focus on these evaluations and can be looked at into more detail, however, despite the advantages beforementioned in both CTCs and ctDNA changing clinical care in precision oncology, neither tests are used in cancer-care quite yet, but it is something that will come in time once their clinical utility is proven and efficient (Heitzer, 2020).

SUMMARY

The emerging need to reform precision oncology has significantly shown advancements in the field with promising outcomes, from diagnosis to treatment care. While traditional biopsy holds a significant value on the histology of malignant tumours it is not always viable to access them giving their nature or location, so the rise of liquid biopsy will solve these challenges in the coming years once the test is proven to reveal the heterogeneity and characteristics of tumour-specificity. Liquid biopsies are meant to provide early detection information as well as repeated testing during the course of the illness to keep track of therapy response and expand the knowledge of cancer development by allowing further research of specific biomarkers; in the case of CTCs, it can be a source for *in vitro* cultures that allow in-depth molecular characterization. However, the true nature and heterogeneity of CTC populations is still not fully understood and requires extended evaluations to improve clinical care. Evidence points out to a correlation between CTCs surviving in the bloodstream and the formation of metastasis so it has an increased awareness value for prediction, monitoring and progression of cancer patients. At the same time, the analysis of cfNAs, which are released from tumour cells, are also meant for tumour diagnosis. However, this area has insufficiently powered studies carried by small study populations and uncontrolled methods which prevents them from validated and reliable accountability for clinic care. The field of microfluidics continues to improve and offer CTCs isolation for post-analysis purposes, however the current designs still face challenges that prevents the techniques to be completely efficient in capturing and isolating a great number of viable cells. Considering the extreme rarity of CTCs in the blood it is fundamental that a combination of several detection technologies are used to overcome these challenges.

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Appendix

I. Glossary

Adenocarcinoma A malignant tumour formed from glandular structures in epithelial tissue.	Cadherins Group of proteins that mediate the calcium-dependent adhesion of cells with each other.
Aneuploidy The condition of having an abnormal number of chromosomes in a haploid set.	Graft hybrid A hybrid plant that is produced by grafting and that exhibits characters of both the stock and the scion.

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