7.2 Circulating tumor cells, circulating tumor DNA and exosomes. Liquid biopsy associated biosourse review

From the historical point of view, circulating tumor cells (CTCs) should be considered as the only biosourse that can be detected with use of liquid biopsy. This is justified by two facts. First, the work of Thomas Ashworth's, published in 1869. It is based specifically to the detection of CTCs and retrospectively maybe consider to be the first study using liquid biopsy. [471] Another fact is that the term "liquid biopsy" was first used by Alix-Panabieres and Klaus Pantel to define solely the analysis of CTCs isolated from the blood of a cancer patient. [472] But as a result of additional researches in this field and due to the development of the new and the improvement of the already existing technologies, the meaning of the term has significantly expanded. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have defined liquid biopsy as a term for a broad category of minimally invasive tests based on the analysis of any biomarkers derived from blood or other body fluids of the cancer patient. [473] The list of possible biosourses had increased with circulating free DNA (cfDNA) or RNA (cfRNA), circulating tumor DNA (ctDNA), exosomes, protein molecules, tumor markers, tumor-educated platelet (TEPs) and tumor-derived endothelial cells (CTECs). [473,474] This list can be incomplete as it is constantly updated with new data. But one way or another, parts of the new knowledge scattered over different studies must be collected and arrange. It is necessary for understanding of what researches have been already conducted and determine promising directions for new projects. This work aims to provide an overview of the most popular biosourses of liquid biopsy and their main features.

So, as already mentioned, the CTCs has been known since 1869. But due to the lack of effective circulation cell detection, enrichment and isolation technologies at that time, the active research in this area was not conducted. The increased attention of researchers returned to CTCs only at the beginning of the 2000s. [474] Since that time numerous studies have been performed, a lot of already known information have been updated and a lot of new information have been published.

CTCs are considered to be cells that have separated from the primary tumor or metastases and continue to circulate in the blood for a certain period of time. [474] There are two working hypotheses about how the CTCs get into the vascular system. The first theory is based on epithelial-to-mesenchymal transition (EMT). In the published works, it is also called as EMT-mediated release. EMT is a complex process of cell differentiation which results into loss of intercellular contacts and obtaining by the cell numerous characteristic that make it similar to the mobile cell in the developing embryo, or in other words, similar to the stem cell. [475-477]. Another option is respectively called non-EMT-mediated release. This hypothesis is based on the fact, that a group of cells separates from the tumor and travels through the circulation system in clusters. Unfortunately, the exact mechanism by which this process takes place and how the cluster of tumors is actually formed has not been studied yet. [476]

CTCs can be found in blood in 3 different forms. The first is a single CTC, the second is a tumor cluster, which is considered to consist of 2 or more CTCs, and the third is a circulating tumor microemboli (CTM), which can consist of more than 50 CTC. [478,479] CTC clusters and microemboli have been shown to have advantages over single CTC in the vascular system. The fact is that they do not contain apoptotic cells, which prolong their half-life and have lower proliferative activity, which probably makes them less sensitive to chemotherapy. [478] In addition, clusters have a higher metastatic potential. The presence of clusters and emboli in blood of the breast cancer patient is associated with a poor prognosis and corresponding decline of the overall survival. [480]

Biological features of CTCs including cell size and shape are different depending on tumor localization and type, cancer stage and therapy course. [476] But despite the differences, researchers managed to find common for all cells features. This allowed to create a classification of CTCs, which divided them into three subpopulations. Depending on the expression of epithelial cell adhesion molecule (EpCAM) or the mesenchymal cell marker vimentin or both of them simultaneously, CTCs can be classified into E-CTCs, M-CTCs or biphenotypic E/M-CTCs. This distribution, in addition to a purely scientific approach, is also of the practical importance. It was found

that the presence of a subpopulation of M-CTCs in patients with breast cancer is most often associated with the presence of tumor metastases. [481] In patients with colorectal cancer, the proportion of M-CTCs is significantly higher among patients with a tumor size of 5≥cm. In 100% of cases, M-CTCs were detected in patients with T4 stage and TNM stage 4 in the same group of patients. [482]

The most important issue related to CTCs is the metastatic process. Once entered the circulation system, CTC tolerate the influence of factors persisting in the blood, such as, for instance, oxidative stress, shear force or immune system impact. Most of the released cells will be destroyed. It is known that the half-life of CTCs is about of 1 to 2.4 hours and the average concentration among the vast majority of the cancer patients is usually less than 10 cells per milliliter. [476] Only those cells that have specific features and phenotype, which make them capable to initiate metastasis, will pass through transition cascade. This pathway includes the CTCs separation from the primary tumor, its transfer along vessels to the site of attachment, the passage through the vascular membrane into the tissue, and subsequent proliferation of cells until metastasis appearance. But even then, CTCs should adapt to surrounding environment and escape from the destruction. It is also important to remember about another metastasis development hypothesis, according to which CTCs that separate from the primary tumor simply do not have the ability to metastasize. This characteristic appears over time and in the process of changing of the tumor genome under the influence of various factors. This theory is confirmed by the fact that CTCs is found even in the early stages of tumor development. [475,476]

CTCs already took root in practical oncology. In 2013 Food and Drug Administration (FDA) approved CellSearch® - the first liquid biopsy test adapted for the routine use. It is created for monitoring of the disease status of the patients with the advanced stage colorectal, breast and prostate cancer. The evaluation of the course of the disease is based on CTCs number. [483] Numerous studies using this test have already led to the creation of the classification of patients according to the number of CTCs per 7.5 ml of blood. Patients with the amount of CTC less than 5 cells per 7.5 ml of blood are considered as CTC-negative, and patients with number of CTC that equal

or more than 5 are considered as CTC-positive. [475] Research conducted by the J.-Y. Pierga and co-authors has shown that the breast cancer patients that have CTC-positive status before the treatment initiation and remain CTC-positive after 3 or 4 weeks of treatment or become CTC-positive during treatment have poorer prognosis. And vice versa, patient that have CTC-negative status before, stay CTC-negative during or become CTC-negative in course of treatment have much better prognosis and overall survival. [484]

The ctDNA is the next popular liquid biopsy biosource that will be reviewed. According to the definition made by ASCO and CAP ctDNA is a portion of the overall cfDNA containing in blood. [473] The observation about the exploring of ctDNA should start from the moment of the cfDNA discovery. It happened in 1948, when Mandel and Metais first detected the fragments of DNA circulating in blood. [485] But unfortunately, in that time this work did not become popular among researchers, so further studies were deferred for almost 30 years. The next significant research was performed in 1977 by Leon and co-authors. They showed that the cancer patients, especially those with the advanced stage, have much higher cfDNA levels than the healthy volunteers. Misconception that persistence of the cfDNA associated strongly with malignant tumor was based on these results. [486] This conception lived until the 1989, when the sources of cfDNA formation were reported, and it was informed that only part of circulating DNA has tumor origin. Due to the lack of effective technologies for the research conduction in this theme, there were a little of studies performed until 2005. But since that time the new era of clinical research of ctDNA has started. [487] Scientists put a large hope to the wide implementation of the ctDNA based tests in clinical practice, and new data obtained from the latest research prove that hope.

First practical question about ctDNA to be asked is how to differ cfDNA from ctDNA? It is obvious that they have different origin. cfDNA release mainly after somatic or blood cells destruction, while the ctDNA appears in the blood after necrosis or apoptosis of tumor cells or CTCs. They also can have different fragments length. ctDNA fragments, usually shorter, with the common length of 134 bp and 144 bp, while the length for cfDNA is usually close to 167 bp. The strict periodicity of base is

the characteristic of ctDNA, unlike cfDNA. [488] They can differ by other fragments associated features, but the main difference between them is in the genetic material they consist of. The point mutations, that can be found only in ctDNA and provide main information about tumor features, are the main targets of the large number of researches. [474]

It is already known that, the presence of KRAS, NRAS mutations in ctDNA of colorectal cancer patient is associated with insensitivity to EGFR-inhibitors (cetuximab or panitumumab). It means that obligatory analysis for these mutations is needed before treatment initiation to prevent patient from ineffective treatment prescription. [489] BRAF V600E mutation indicates poor response to standard chemotherapy. [490] PIK3CA-mutant ctDNA observed before treatment initiation associated with shorter overall survival. [491]. This is only a small part of most commonly mentioned in articles, already formed regularities of ctDNA point mutations that can be used in clinic.

Unlike CTCs, there are multiple already existing ctDNA-based liquid biopsy tests that have been approved by the FDA. The names of these tests are the cobas EGFR Mutation Test v2, Guardant360 CDx and FoundationOne Liquid CDx. All of them were created to perform pre-treatment assessment of the possibility for targeted therapy assignment. The cobas EGFR Mutation Test v2 was approved in 2016 as a companion diagnostic for Osimertinib. Later, FDA approved this test to support EGFR tyrosine kinase inhibitor therapy as well. This test based on EGFR mutation detection in ctDNA in blood of non–small cell lung cancer patients. Guardant360 CDx and FoundationOne Liquid CDx were approved by FDA in 2022. Both of them are comprehensive genomic profiling liquid biopsy tests, which use next-generation sequencing (NGS) for mutation detection. They can be used for different cancer localization to identify patients who will most likely benefit from checkpoint inhibitors prescription. [483]

Researchers believe that CTCs- and ctDNA based tests have the potential to turn into reality the concept of personalized medicine, which mean that all treatment decisions will be based only on the patient specific, genome-specific diagnosis results. One of the conducted studies show the possible way to implementation of this idea.

Investigators use NGS for diagnostic cancer sequencing. Then they create dPCR based personalized tests, which allow them to monitor ctDNA levels and to correlate them with the disease course. It was performed among metastatic patients with breast, lung, colon, melanoma and cholangiocarcinoma cancer. The results of the research proved that monitoring based on personalized tests can be used and have significant clinical importance. [492]

Extracellular vesicles (EVs) should be considered next, both from the historical and clinical point of view. EVs it is small cell particles that have two layers of lipid membrane, don't have replication activity and can be released from nearly all somatic cells including tumor cells. By the origin, main features and diameter, they can be divided into 3 subtypes: the smallest - exosomes, medium size - apoptotic bodies and the biggest - micro-vesicles. [493] Exosomes are the most valuable in a case of liquid biopsy. Therefore, they will be discussed below.

Exosomes were first represented by EG Trams in 1981 during sheep reticulocytes description. But then these particles were not given a name. It happened only 6 years later, when they were named by Johnstone RM. [494] Later in 1996 results obtain from research provided by G. Raposo show that exosomes derived from B cells can take part directly in the antitumor response. [495] In 1998 L. Zitvogel et al. also explored those dendritic cells can release exosomes which enlarge T cells influence in antitumor response. [496] Since that moment, researchers have not weakened their scientific interest in relation to exosomes. It was discovered that they extremely important during antitumor response, antigen presentation, tumor cell migration and invasion. [497] But unfortunately, this biomarker has not found it clinical application yet. More studies should be performed for search of exosomes practical application. [474]

CTCs, ctDNA and exosomes are perspective biosources for liquid biopsy research. But for wide clinical implementation of CTCs-, ctDNA- or exosomes - based tests they need to prove to be reliable, repeatable, be east to perform and have affordable price. In order to fulfill these conditions, more research should be provided, especially those aimed at solving the standardizing problem.