Review

Stefan Holdenrieder*, Vida Ungerer, Angela Oberhofer and Abel Jacobus Bronkhorst

Pan-cancer screening by circulating tumor DNA (ctDNA) – recent breakthroughs and chronic pitfalls

https://doi.org/10.1515/labmed-2022-0029
Received March 8, 2022; accepted July 4, 2022;
published online July 18, 2022

Abstract: Early detection is crucial for optimal treatment and prognosis of cancer. New approaches for pan-cancer screening comprise the comprehensive characterization of circulating tumor DNA (ctDNA) in plasma by next generation sequencing and molecular profiling of mutations and methylation patterns, as well as fragmentation analysis. These promise the accurate detection and localization of multiple cancers in early disease stages. However, studies with real screening populations have to show their clinical utility and practicability.

Keywords: cancer; ctDNA; early detection; fragment analysis; liquid profiling; methylation; mutations; next generation sequencing; screening.

The concept of cancer screening and its challenges

Many cancer screening programs aim at the detection of cancer in early disease stages to enable optimal–if possible, curative–treatment with best prognosis and high quality of life. Thus, they focus on specific risk groups like smokers or individuals older than 50 years because they have a higher probability of developing cancer. There are many local and national programs running to regularly monitor individuals for increased risk or presence of cancer based on genetic (BRCA1, 2 etc.), biochemical (e.g., fecal immunologic blood test), radiological (computed tomography, ultrasound etc.), or invasive methods (e.g., colonoscopy) [1–6].

On the other hand, screening exams are often critiqued: While they enable the early detection and prompt treatment of cancer in many cases, they also often cause "false alarms". Suspicious findings in false-positive tested persons have to be verified by additional, often invasive exams accompanied by considerable risk of complications and psychological distress [7–9].

Furthermore, several types of bias have to be considered [10]: The "lead-time bias" suggests a longer survival by screening that is overestimated due to an earlier diagnosis in the screened group although the time of death is possibly the same in both groups (Figure 1A). The "length-time bias" leads to distorted perceptions because the probability of early detecting indolent tumors with a low growth rate, late occurrence of symptoms and long survival is higher than detecting aggressive, rapidly growing tumors that become early symptomatic, have a shorter screening interval and are associated with a poor outcome. Thereby, the true screening effect is overrated (Figure 1B). The "overdiagnosis bias" describes the fact that a considerable number of tumors irrelevant to the survival of the patient are detected and eventually treated by aggressive therapies which is known as "overtreatment" (Figure 1C). This means that individuals die later with the tumor but not because of the tumor and are therefore treated unnecessarily [10]. Finally, the development of new therapies and multiple lines of therapy sequences during the course of cancer have made it more difficult to estimate the influence of screening methods for overall survival.

Requirements for blood biomarkers in cancer screening

There are specific challenges related to the early detection of cancer via blood-based markers. For optimal decision-making on the necessity and type of treatment, blood

***Corresponding author: Stefan Holdenrieder**, Institute for Laboratory Medicine, German Heart Centre, Technical University Munich, Lazarettstr. 36, 80636 Munich, Germany,
E-mail: holdenrieder@dhm.mhn.de
Vida Ungerer, Angela Oberhofer and Abel Jacobus Bronkhorst, Institute for Laboratory Medicine, German Heart Centre, Technical University Munich, Munich, Germany

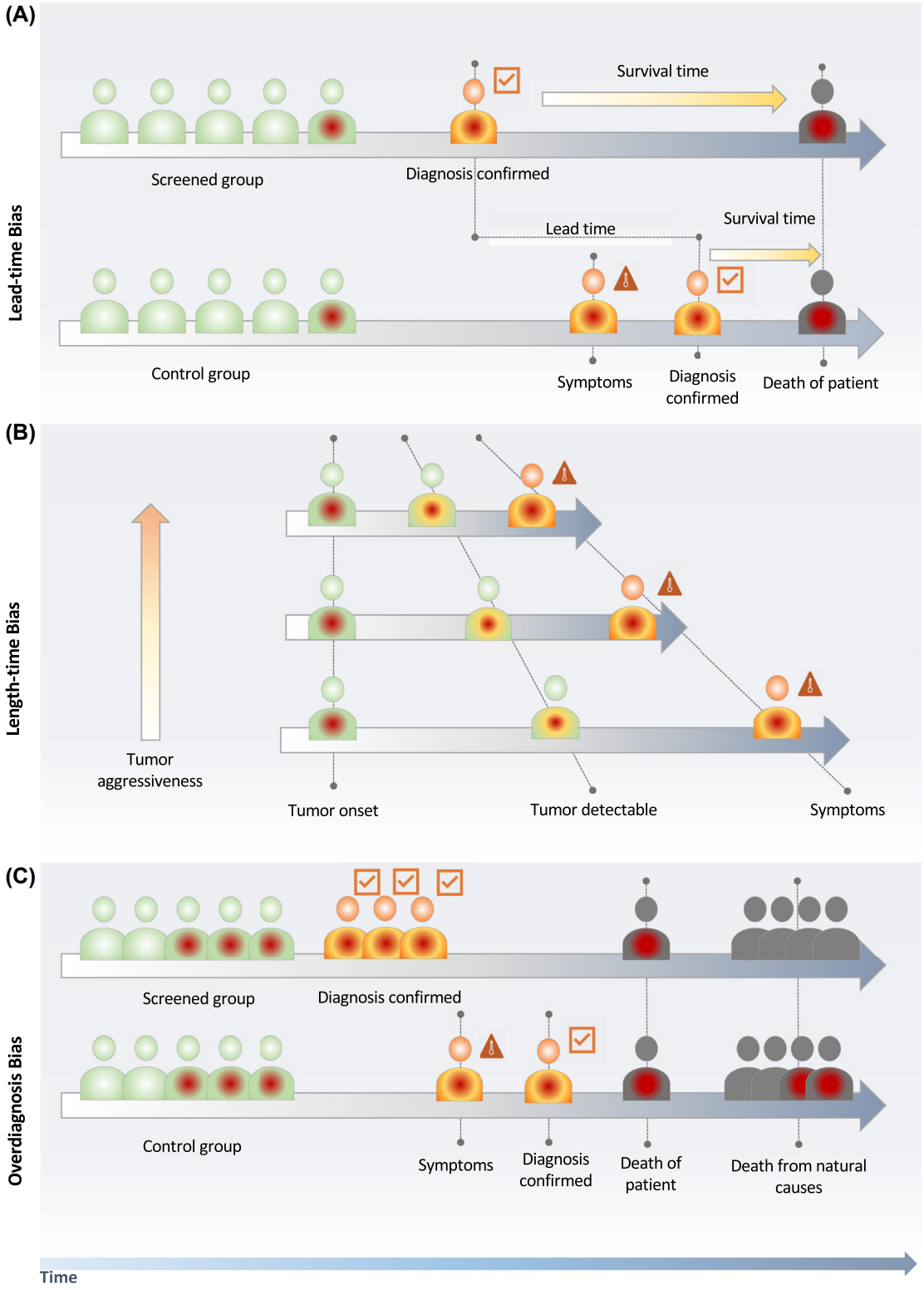

Figure 1: Biases that may result in systematic overestimation of the benefits of cancer-screening.
Lead-time bias (A), length-time bias (B), and overdiagnosis bias (C) may arise when the natural history of asymptomatic disease is not taken into consideration (Figure adapted from Gates, 2014 [10]).

diagnostics should inform about the presence of cancer, the location of the tumor, and the aggressiveness and invasiveness of the tumor.

To maximize the chances of curative outcome, therapy should be applied as early as possible, ideally when lesions are still very small and barely detectable by radiological methods. However, in such early stages of disease most tumors do not release a detectable number of biomarkers into circulation, making it very difficult to quantify minor biochemical changes in blood plasma and serum [11–13].

Many attempts to identify and use single tumor- and organ-specific protein markers for the early detection of cancer often failed because many so called "tumor markers" are not tumor-specific but are also released from non-malignant cells in small amounts [14, 15]. In addition, there are only few organ-specific markers known. One exception is the prostate specific antigen (PSA), which has shown a considerable reduction of mortality in some large screening trials for early detection of prostate cancer both in the European ERSPC and the Goteborg trial [16, 17]. Successful implementation of these findings into practical workflows necessitates the use of uniform methods, accurate decision thresholds for biopsies, and well-defined time intervals between serial determinations and reflex testing e.g., with free PSA for results in the "grey zone". If these aspects are not taken into careful consideration, if the screening cohort is contaminated, treatment methods and outcome measures are different, then opposite results with poor value of PSA for screening purposes may be found – even in large-scale and long-running trials [18].

For diagnostic industries, the blood-based screening for cancer is highly attractive because testing a large number of "patients at risk" represents an enormous market potential with considerable estimates of compound annual growth rates (CAGR) of around 8–9% during upcoming years [19, 20]. However, as Covid-19 screening tests have recently shown very clearly, it is crucial that assays used for screening purposes do not only demonstrate high sensitivity and specificity, but that they are also validated in the proper screening setting like in defined risk cohorts: For meaningful information they should provide a high positive or negative predictive value that depends strongly on the prevalence of the disease in the tested population. At an overall prevalence for some cancer types of around 1:100 to 1:1,000, the rate of false-positive results will significantly outnumber the correct-positive ones! [11–14].

An improvement of the predictive values can be achieved by individual monitoring of serial biomarker determinations, their dynamic changes over time, and the parallel testing of multiple markers in order to increase the sensitivity and specificity for cancer detection. Furthermore, biomarkers may be used in conjunction with other screening methods, such as colonoscopy or low-dose computed tomography (LDCT) to reduce the false-positive detection rate in sequential screening scenarios [11, 12, 14, 21–23]: In "prescreening" approaches, individuals with higher risks of cancer can be identified first by sensitive biomarkers followed by regular or more frequent check-up exams like colonoscopy. "Post-screening" use of biomarkers or biochemical risk scores can help to better estimate the relevance of small suspicious lesions detected by screening exams–e.g., by LDCT screening in smokers–and determine the necessity of further invasive follow-up investigations. Both approaches are intended to precisely identify individuals with the highest cancer risk for more intensive investigations and leave others for regular monitoring, thereby reducing overall costs and capacities needed for check-up exams [12, 22, 23]. It has to be emphasized that all these considerations are valid for cancer screening with already established protein markers as well as with newly developed blood-based nucleic acid biomarkers.

Pan-cancer screening through plasma ctDNA analyses

The use of next generation sequencing (NGS) technologies enables comprehensive molecular characterization of cancers in tumor tissue as well as in blood plasma derived ctDNA – also known as liquid profiling [23–25]. This means that also in blood plasma, the presence of typical molecular patterns for a specific tumor or even a multitude of different tumors can be assessed. This so-called "pan-cancer screening" is based on diverse principles: (i) the molecular profiling of mutations on ctDNA in combination with protein markers, (ii) the profiling of methylation pattern on ctDNA, and (iii) the profiling of ctDNA fragmentation patterns. All of these approaches focus on the sensitive detection, specific characterization and localization of cancer [11, 23–28]. There are currently several takeovers and fusions of big companies in the United States (Illumina/Grail, Exact Sciences/Thrive) who have developed ctDNA-based screening products and launched large prospective screening studies, mirroring the enormous dynamics and the high market potential in this diagnostic field [29].

Profiling of plasma ctDNA mutations and protein patterns

The principal challenge in cancer screening by ctDNA is the low amount of tumor DNA in blood plasma, particularly in

early tumor stages, which makes the detection of single molecular markers very difficult [11–13]. Given the fact that in cancers with a diameter of 10 mm, less than 1 genome equivalent (GE) is expected in 10 mL of the blood and proportion of ctDNA among total DNA is lower than 0.01%, the probability of detection by a single marker is quite low [13]. However, recent technical improvements such as pre-enrichment of ctDNA, error reduction in NGS analyses by unique molecular identifiers (UMIs) as well as inclusion of multiple gene targets have shown some promising results in the early detection of some cancers [12, 23–25]. Yet the sensitivity for detecting pancreatic cancer was quite limited with 30% at a 99% specificity when diverse ctDNA markers were solely used in the CancerSeek study; only with the addition of protein markers CA 19–9, CEA, HGF and OPN it could be increased to 64% [30].

In a subsequent study, 61 amplicons from 16 genes and 8 protein tumor markers were used in order to early detect a variety of gastrointestinal, gynecological and lung cancers in 1,005 tested patients [31]. Sensitivities of 69–98% were achieved for the detection of ovarian, liver, stomach, pancreas and esophagus cancers at a specificity of more than 99% versus a healthy control group (n=812). For colorectal, lung and breast cancer the sensitivities were somewhat lower. In cancer stages I to III, median sensitivities were at 70% but in the very early stage I they were considerably lower with only 43%. Most remarkably, tumors could be localized by the combination of ctDNA and protein markers in up to 83% of cases wherein protein markers made the main contribution [31]. Although the conclusions of the study were limited due to the lack of a "real screening cohort", the selection of tumor patients and healthy control groups, and the lack of an independent validation, new studies were encouraged by the promising results.

In the prospective intervention study DETECT-A, 10,005 women aged 65–75 years and without any known tumors were examined by a stepwise diagnostic workflow, including double blood exams and positron emission tomography-computed tomography (PET-CT) [32]. In this scenario, suspicious ctDNA findings at the initial basis blood draw applying the original CancerSEEK method had to be confirmed in a second blood draw wherein only particular DNA mutations or proteins that were abnormal in the baseline test were assessed. In addition, potential molecular contaminations by clonal hematopoiesis were excluded rigorously by analyzing a larger amount of white blood cell DNA. Only confirmed positive patients subsequently underwent PET-CT analysis.

By use of this differentiated procedure, critical results were obtained in 490 persons in the basis blood draw that were confirmed in 134 cases. After PET-CT, 64 individuals were suspected of having cancer which was finally confirmed in 26 cases. Among them 9 had lung cancer, 6 ovarian cancer, 2 colorectal cancer but none had breast cancer. Tumors were detected in these 26 women by use of ctDNA in 14 cases, by use of protein markers in 11 cases and by both methods in one case. An additional 24 tumors were found by standard screening procedures, and 46 tumors by the presence of symptoms [32].

As shown by this detailed description, blood-based screening enabled the detection of 27% of the 96 tumors in a total of more than 10,000 tested women. Double blood exams narrowed the number of women with tumor suspicion down to 1.2% who were chosen for PET-CT analysis. Finally, only 59% of the remaining 64 suspicious women after PET-CT (in total 0.4% of all screened subjects) were unnecessarily subject to further diagnostic procedures. Although the procedure was extremely laborious and expensive and absolute numbers seem to be small, the positive predictive value (PPV) was considerably higher with 40,6% (26 out of 64) as found by many other screening methods [32].

The design of the DETECT-A study is an exemplary demonstration of how the strategy how PPV can be improved in screening trials (i) by identifying patients with the highest cancer risk to increase the pretest-probability, (ii) by multimarker testing with an orthogonal design, (iii) by repetitive, serial testing and exclusion of non-specific results, and (iv) by inclusion of subsequent sensitive radiological exams – e.g., by use of PET-CT analyses. This approach is also supported by the proposition of integrative cancer diagnostics of the EFLM that also suggests the use of highly sensitive technologies like PET-MRI and artificial intelligence-based interpretation tools [25].

Profiling ctDNA methylation patterns

An alternative approach is the comprehensive analysis of unique ctDNA methylation marks which are found much more frequently than tumor-specific mutations. Cancer-associated patterns comprise the methylation of CpG islands in promotor areas of suppressor genes, such as MLH1 in colorectal cancer, that lead to the inhibited suppression of tumor growth or DNA repair [33]. Promotor hypermethylation of the septin-9 gene was used as the basis of the Epi proColon® test from Epigenomics that achieved a ca. 70% sensitivity at 80% specificity for colorectal cancer detection and was approved as the first methylation assay by the U.S. Food and Drug

Administration (FDA) despite a positive predictive value of only 2–3% [34, 35].

Beyond hypermethylation of specific sites, a general hypomethylation and numerous irregular methylations are present over the whole genome in cancer [23–28, 36]. These altered tumor and tissue-specific methylation patterns can be analyzed in a targeted or genome-wide manner by chromatin-immunoprecipitation of cell-free methylated DNA and high-throughput sequencing (cfMeDIP-Seq) or by sequencing after bisulfite conversion. There are pros and cons of the different approaches, like the loss of DNA after bisulfite conversion and the dependency on antibodies in ChIP during MeDIP-Seq that are also reviewed in detail elsewhere [23, 28]. Then, classifiers are built on the basis of tens of thousands of methylation signals differing between tumor and normal tissue, followed by validation and use for prediction of cancers in early stages in plasma samples as reviewed in [23, 26, 28, 36–38].

The potential diagnostic power of methylation patterns has systematically been investigated in several studies of the CCGA consortiums with more than 15,000 participants. The so-called multi-cancer early detection (MCED) classifier was validated in 6,689 individuals and reached a specificity of more than 99% and a sensitivity of 67% for 12 cancer types in stages I–III. Thereby, sensitivity increased from 39% in stage I to 69% in stage II and 83% in stage III. However, the detection of some tumor types like breast and prostate cancer remained difficult particularly in the early tumor stage I. Most remarkably, tissue of tumor origin was localized in 96% of the cases by a specific methylation signature [37]. In another recent study, the high specificity and sensitivity of the MCED classifier was confirmed in an independent validation cohort [38]. The so called Galleri® test from Illumina/Grail is now awaiting approval by the FDA.

Similarly to the ctDNA mutation approach, there is a lively debate on the clinical utility of the Galleri® test with respect to the limited sensitivity and low PPV particularly in early stages and for rare cancers, the stability of the marks and the algorithm as well as the need for validation studies in real screening settings [13, 39, 40]. Obviously, several prospective population-scale trials with diverse studies designs have been initiated with more than 300,000 planned participants that should be able to answer current open questions in the next years [40].

Fragmentomic analyses of ctDNA

A relatively new screening approach is the analysis of ctDNA fragmentation patterns in blood [26, 28]. This arises from the fact that 146 bp of DNA are associated with histone proteins in so-called nucleosomes that protect DNA from spontaneous degradation by nucleases [41]. In the case of regulated chromatin breakdown during apoptotic cell death in the cell nucleus or in the case of unregulated cleavage after release of DNA into the blood stream, DNases will preferably bind at the easily accessible linker-DNA-sites between nucleosomes, resulting in different fragmentation patterns [26, 41]. This affects the length of fragments, which are in tumor patients slightly shorter than in healthy controls (peak at 146 bp vs. 166 bp) as well as the type of fragmentation with different breaking patterns, orientation, and end-point sequence motifs [26, 42]. An additional diagnostic feature is the "nucleosomal footprint" of ctDNA, i.e., the portion of DNA sequences that are conserved by nucleosomal protection and of degraded DNA sequences. This footprint provides functional evidence for the binding of transcription factors which is informative for the presence of cancer as well as for the tissue of tumor origin [26, 28, 43].

A recent study compared the diagnostic power of a genome-wide DNA fragmentation profiling in combination with clinical risk factors, carcinoembryonic antigen and CT-scan for the presence of lung cancer. Although the study was small, the sensitivity of the DELFI fragmentation score reached a remarkable 91% in the early stages I and II, and 96% in stages III and IV, however, at a specificity of only 80% [44]. Beyond size, there are plenty of further plasma cfDNA fragmentation characteristics that may be exploited for diagnostics purposes in the future [26].

Perspectives

Various NGS-based pan-cancer-screening approaches yield promising first results in initial studies that have to be confirmed in independent, multicentric and prospective trials. Most importantly, new tools for early cancer detection have to be evaluated in "real screening populations settings" mirroring the real prevalence of different cancer types and confounding pathologies, considering long observation times and correction for all biases that occur under screening circumstances [10]. Earlier screening trials have shown how difficult, laborious and expensive it can be to show the real benefit of a screening method [16–18].

Although the overall sensitivity of several pan-cancer-screening methods seem to be promising, detection in preferred early stages remains challenging as sensitivity is then frequently below 50% and for some tumor types like

breast and prostate cancer as low as 10–20%. This illustrates that in tumors with small volumes minor traces of plasma ctDNA may be missed even if multitarget approaches are applied [13, 39]. Given the low prevalence of some cancers even in risk groups, detection rate and the positive predictive value appears to be too low for successful implementation in clinical patient care [13, 39].

Future strategies therefore have to focus on (i) improving technologies and better understanding the biology and metabolism of the markers investigated, (ii) increasing the pretest-probability by addressing persons with highest cancer risk, (iii) applying multimarker testing with orthogonal designs, (iv) serial testing and interpretation of individual marker dynamics, (v) including subsequent highly sensitive radiological exams and (vi) developing smart, artificial intelligence-based interpretation tools, some of these aspects also being recently suggested by the EFLM [25], to finally develop integrative, step-wise screening programs.

Other essential criteria for a broad acceptance and use of screening tools in patient care is data that is convincing and easy-to-interpret and understand, the availability of diagnostic capacities, the simplicity, robustness and speed of analysis, integration into existing workflows, the cost-benefit efficiency, reimbursement issues and the general acceptance by clinicians, patients and society. All of these issues and challenges have to be addressed by the new approaches.

Finally, it has to be considered that the use of screening tools is only beneficial if it leads to therapeutic consequences. If a tumor is detected by molecular screening already in an asymptomatic stage, or possibly even before it becomes visible by imaging methods, the question will arise as to when and how to treat it. Both alternatives – watchful waiting as well as preemptive therapies – will confront the patient, the clinician and the whole health care system with new challenges [45]. Therefore, all screening approaches – old or new – should always bear in mind the main bioethical principle of medicine: "primum non nocere – first, do not harm"!

Acknowledgments: The manuscript has been adapted with permission of the publisher from Trillium Diagn 2021; 19: 234–236.
Research funding: None declared.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Informed consent: Not applicable.
Ethical approval: Not applicable.

References

1. Bayerische krebsgesellschaft. Available from: http://www.bayerische-krebsgesellschaft.de [Accessed 03 Jul 2022].
2. National Cancer Institute. FDA approved tests and devices. Available from: https://edrn.nci.nih.gov/about/fda-approved-tests [Accessed 03 Jul 2022].
3. Cardoso LR, Guo F, Heisser T, Hackl M, Ihle P, De Schutter H, et al. Colorectal cancer incidence, mortality, and stage distribution in European countries in the colorectal cancer screening era: an international population-based study. Lancet Oncol 2021;22: 1002–13.
4. Russo A, Incorvaia L, Capoluongo E, Tagliaferri P, Gori S, Cortesi L, et al. Implementation of preventive and predictive BRCA testing in patients with breast, ovarian, pancreatic, and prostate cancer: a position paper of Italian Scientific Societies. ESMO Open 2022;7:100459.
5. Wait S, Alvarez-Rosete A, Osama T, Bancroft D, Cornelissen R, Marušić A, et al. Implementing lung cancer screening in Europe: taking a systems approach. JTO Clin Res Rep 2022;3:100329.
6. Becker N, Motsch E, Trotter A, Heussel CP, Dienemann H, Schnabel PA, et al. Lung cancer mortality reduction by LDCT screening-results from the randomized German LUSI trial. Int J Cancer 2020;146:1503–13.
7. Canelo-Aybar C, Ferreira DS, Ballesteros M, Posso M, Montero N, Solà I, et al. Benefits and harms of breast cancer mammography screening for women at average risk of breast cancer: a systematic review for the european commission initiative on breast cancer. J Med Screen 2021;28:389–404.
8. Paschen U, Sturtz S, Fleer D, Lampert U, Skoetz N, Dahm P. Assessment of prostate-specific antigen screening: an evidence-based report by the German institute for quality and efficiency in health care. BJU Int 2022;129:280–9.
9. Jiao B, Gulati R, Katki HA, Castle PE, Etzioni R. A quantitative framework to study potential benefits and harms of multi-cancer early detection testing. Cancer Epidemiol Biomarkers Prev 2022; 31:38–44.
10. Gates TJ. Screening for cancer: concepts and controversies. Am Fam Physician 2014;90:625–31.
11. Duffy MJ, Diamandis EP, Crown J. Circulating tumor DNA (ctDNA) as a pan-cancer screening test: is it finally on the horizon? Clin Chem Lab Med 2021;59:1353–61.
12. Bronkhorst AJ, Ungerer V, Holdenrieder S. Early detection of cancer using circulating tumor DNA: biological, physiological and analytical considerations. Crit Rev Clin Lab Sci 2019:1–17.
13. Pons-Belda OD, Fernandez-Uriarte A, Diamandis EP. Can circulating tumor DNA support a successful screening test for early cancer detection? The grail paradigm. Diagnostics 2021;11: 2171.
14. Holdenrieder S. [Trends in the use of tumor markers in clinical routine application]. Med Welt 2013;64:12–9.
15. Stieber P. [Sensible use of tumor markers]. J Lab Med 2008;32: 339–60.
16. Schroeder FH, Hugosson J, Roobol MJ, Tammela TLJ, Ciatto S, Nelen V, et al. Prostate-cancer mortality at 11 years of follow-up. N Engl J Med 2012;366:981–90.
17. Hugosson J, Carlsson S, Aus G, Bergdahl S, Khatami A, Lodding P, et al. Mortality results from the Göteborg randomised population-

based prostate-cancer screening trial. Lancet Oncol 2010;11: 725–32.
18. Andriole GL, Crawford ED, Grubb RL, Buys SS, Chia DD, Church TR, et al. Mortality results from a randomized prostate-cancer screening trial. N Engl J Med 2009;360:1310–9.
19. Expert market research. Available from: https://www.expertmarketresearch.com/reports/cancer-screening-market [Accessed 03 Jul 2022].
20. Fortune business insight. Available from: https://www.fortunebusinessinsights.com/industry-reports/lung-cancer-screening-market-101174 [Accessed 03 Jul 2022].
21. Russell MR, Graham C, D'Amato A, Gentry-Maharaj A, Ryan A, Kalsi JK, et al. A combined biomarker panel shows improved sensitivity for the early detection of ovarian cancer allowing the identification of the most aggressive type II tumours. Br J Cancer 2017;117:666–74.
22. Choe W, Chae JD, Lee BH, Kim SH, Park SY, Nimse SB, et al. 9G test TM cancer/lung: a desirable companion to LDCT for lung cancer screening. Cancers 2020;12:3192.
23. Gao Q, Zeng Q, Wang Z, Li C, Xu Y, Cui P, et al. Circulating cell-free DNA for cancer early detection. Innovation 2022;3:100259.
24. Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. Nat Rev Genet 2019;20:71–88.
25. Froelich MF, Capoluongo E, Kovacs Z, Patton SJ, Lianidou ES, Haselmann V. The value proposition of integrative diagnostics for (early) detection of cancer. Clin Chem Lab Med 2022;60: 821–9.
26. Lo YMD, Han DSC, Jiang P, Chiu RWK. Epigenetics, fragmentomics, and topology of cell-free DNA in liquid biopsies. Science 2021;372:eabb3616.
27. Bronkhorst AJ, Ungerer V, Oberhofer A, Holdenrieder S. The rising tide of cell-free DNA profiling: from snapshot to temporal genome analysis. J Lab Med 2022;46:207–24.
28. Oberhofer A, Bronkhorst AJ, Ungerer V, Holdenrieder S. Expanding the scope of liquid biopsies through the profiling of disease and tissue-specific epigenetic signatures in cell-free DNA. J Lab Med 2022;46:283–94.
29. Al Idrus A. Exact sciences snaps up liquid biopsy company thrive in $2.15B deal. Available from: https://www.fiercebiotech.com/medtech/exact-sciences-snaps-up-liquid-biopsy-company-thrive-2-15b-deal [Accessed 03 Jun 2022].
30. Cohen JD, Javed AA, Thoburn C, Wong F, Tie J, Gibbs P, et al. Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. Proc Natl Acad Sci U S A 2017;114:10202–7.
31. Cohen JD, Wang Y, Thoburn C, Afsari B, Danilova L, Douville C, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science 2018;359:926–30.
32. Lennon AM, Buchanan AH, Kinde I, Warren A, Honushefsky A, Cohain AT, et al. Feasibility of blood testing combined with PET-CT to screen for cancer and guide intervention. Science 2020;369: eabb9601.
33. Westwood A, Glover A, Hutchins G, Young C, Brockmoeller S, Robinson R, et al. Additional loss of MSH2 and MSH6 expression in sporadic deficient mismatch repair colorectal cancer due to MLH1 promoter hypermethylation. J Clin Pathol 2019;72:443–7.
34. Potter NT, Hurban P, White MN, Whitlock KD, Lofton-Day CE, Tetzner R, et al. Validation of a real-time PCR-based qualitative assay for the detection of methylated SEPT9 DNA in human plasma. Clin Chem 2014;60:1183–91.
35. Johnson DA, Barclay RL, Mergener K, Weiss G, König T, Beck J, et al. Plasma Septin9 versus fecal immunochemical testing for colorectal cancer screening: a prospective multicenter study. PLoS One 2014;9:e98238.
36. Shen SY, Singhania R, Fehringer G, Chakravarthy A, Roehrl MHA, Chadwick D, et al. Sensitive tumour detection and classification using plasma cell-free DNA methylomes. Nature 2018;563:579–83.
37. Liu MC, Oxnard GR, Klein EA, Swanton C, Seiden MV, Consortium CCGA. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. Ann Oncol 2020;31:745–59.
38. Klein EA, Richards D, Cohn A, Tummala M, Lapham R, Cosgrove D, et al. Clinical validation of a targeted methylation-based multi-cancer early detection test using an independent validation set. Ann Oncol 2021;32:1167–77.
39. Pons-Belda OD, Fernandez-Uriarte A, Diamandis EP. Multi cancer early detection by using circulating tumor DNA-the Galleri test. Diagnostics 2022;12:1244.
40. Klein EA, Beer TM, Seiden M. The promise of multicancer early detection. Comment on Pons-Belda et al. can circulating tumor DNA support a successful screening test for early cancer detection? The grail paradigm. Diagnostics 2021, 11, 2171. Diagnostics 2022;12:1243.
41. Holdenrieder S, Stieber P. Clinical use of circulating nucleosomes. Crit Rev Lab Med Sci 2009;46:1–24.
42. Mouliere F, Chandrananda D, Piskorz AM, Moore EK, Morris J, Ahlborn LB, et al. Enhanced detection of circulating tumor DNA by fragment size analysis. Sci Transl Med 2018;10:aat4921.
43. Ulz P, Perakis S, Zhou Q, Moser T, Belic J, Lazzeri I, et al. Inference of transcription factor binding from cell-free DNA enables tumor subtype prediction and early detection. Nat Commun 2019;10: 4666.
44. Mathios D, Johansen JS, Cristiano S, Medina JE, Phallen J, Larsen KR, et al. Detection and characterization of lung cancer using cell-free DNA fragmentomes. Nat Commun 2021;12:5060.
45. Tempero M. Pan-cancer screening: a dream or a nightmare. J Natl Compr Cancer Netw 2021;19:773.