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Review Article

Virtosome: Is it the Answer to Liquid Biopsy Dilemma?

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Abstract

Liquid biopsy including circulating tumor cells (CTC), circulating tumor DNA (ct DNA) and exosomes and has been an attractive concept to both researchers and clinicians due to its convenience, dynamicity, universality, and cost-effectiveness. However, when it comes to real life application, it was faced by so many challenges that delayed taking this practice to a more widespread scale and involving it in cancer guidelines. Perhaps one of the most important points made was that the controversial origin of different types of these biopsies and its effect on specificity. That point was greatly overcome by advancements in genetic material isolation and the sequencing techniques. Next-generation sequencing (NGS), a technology used to sequence DNA templates [1], has greatly helped overcome that point. Another crucial point was the representation of the cell dynamic that this biopsy conveys to us; does it represent necrosis, apoptosis, normal secretory mechanism that is part of the basic biologic functions, or a mixture of all that varies with the different cellular inputs.

Virtosomes are DNA-RNA-lipoprotein complexes, which are synthesized exclusively by living cells. They have released in an energy-dependent, highly regulated manner within few hours of their formation. These unique characteristics that distinguish them from other forms make it a possibly highly specific and sensitive future biomarker.

What is a Liquid Biopsy?

Histopathological analysis of tumor tissues and cells is a cornerstone in the diagnosis of malignant conditions. This practice has been done unchanged for past decades except for the introduction of improved quality to treat and interpret the samples. The National Cancer Institute definition of a biopsy is the removal of cells or tissues to be examined by a pathologist [2]. Recently, the introduction of liquid biopsy concepts gave a novel perspective to this definition.

Liquid biopsies are samples obtained from non-solid biological tissue, mainly the blood, and derived from the primary tissue of interest [2]. The literature describes three main categories: circulating tumor DNA (ctDNA), circulating tumor cells (CTC) and exosomes.

The reason why DNA, cells, and exosomes are shed from tumors and healthy tissues are not fully known yet [3,4]; intercellular communication has been offered as a major cause, but other factors such as apoptosis, necrosis, and disposal of excess compounds were suggested as well. While considering liquid biopsies as cellular messengers confer depth to their role in disease development and body hemostasis, other theories lean towards assuming that they are the byproduct for more important process [5-9].

ctDNA is the free DNA released by tumor cells and detected in the blood, hence the name circulating. While most research is conducted on blood samples, researchers were able to isolate ctDNA from other body fluids such as urine and cerebrospinal fluid [8]. Physiologically, free DNA is released from the cells of healthy individuals and can be detected in their blood [4], but the levels of free DNA detected in diseased individuals are higher [10,11].

As all liquid biopsies, the mechanism of DNA release from the cells is still controversial. One theory attributed DNA release to be a byproduct of necrosis as a result of increased cell turnover which over saturates the phagocytic capacity to remove debris [12]. This theory was supported by the length range of the DNA strands detected in the blood. Free DNA fragments length were also the reason why some researchers think apoptosis can be their primary source since recent reports mentioned fragment length range of 180-200 bp that displayed the ladder pattern indicative of an apoptotic activity [9,13]. This can explain the surge in ctDNA levels in plasma after treatment administration. Another theory suggested that ctDNA is released from other circulating components in the blood such as CTCs, exosomes and blood macrophages [12,14] but it has many shortcomings since the DNA content of these structures cannot correlate with the levels of ctDNA detected in blood. Also, the free DNA sequences do not necessarily reflect the same sequences obtained when DNA from CTCs, exosomes, and leukocytes is sequenced. In fact, it shows more dynamic potential compared to them [15,16].

CTCs were first described as a tumor biomarker in 1869 by Ashworth TR [17]. The fact that the only source of CTCs can only be a tumor site, whether it is primary or secondary, making them a reliable biomarker. CTCs contain the whole tumor genome, giving flexibility for analysis and verification of the cell source. They are detected using antibodies that recognize surface markers specific to a tumor and its cellular origin [18], and 5 or more tumor cells in 7ml blood are considered an indication for poor prognosis [15].

Exosomes are a highly specialized category of extracellular vesicles (ECVs) that are formed during the process of autophagy. They are secreted in the extracellular environment when late endosomes fuse with the plasma membrane and not lysosomes for degradation in a complete autophagy scenario [19,20]. These nanovesicles have a distinct and complicated structure that distinguish them from other ECVs [21]. They are membrane-bound that is derived from the plasma membrane during the formation of an endocytic vesicle of early endosomes. So, identification of their source cells can be achieved by surface markers recognition [22,23]. Though they are not a rich source for cellular DNA, they were found to contain specialized RNA molecule: microRNA (miRNA) [12,24]. miRNAs are believed to have an important in intercellular communications. This role probably affects metastatogenesis [22,25,26], immunological response of the host [27] and even the treatment process [24, 27].

Why Liquid?

The convenience of a liquid biopsy over solid biopsy included clinical, research and practical reasons. The fact that liquid biopsies are systemic biopsies can be pivotal. This sole criterion overcomes the heterogenicity of the tumor cellular clones that cannot be avoided with solid biopsy samples. Also, it does not neglect the distant and inaccessible tumor sites. Based on that a tumor can test positive to a treatment that can test negative on solid biopsy especially in cases of recurrence or metastases after removal of the primary growth [28].

Liquid biopsies, in general, have short half-lives, which means that they are more likely to represent a more recent state of the tumor. This makes them an ideal method to track clone selections that can be caused by drugs, or happen with metastases or spontaneously. The concentration/number of the liquid biopsy was found to correlate with the tumor burden in cancer patients [16,29-31]. Thus, it can be a good method to follow up treatment effects[31-33]. This has been extensively studied in many trials. In fact, it was found to have superior results to imaging techniques currently used in practice to evaluate and follow up patients. The effect appears earlier and reflects more sensitivity and specificity since it done through surface markers, in case of exosomes and CTCs, and genetic material, for all of them [10,11,34-38].

Adding to the advantages of a liquid biopsy is its convenience to obtain. Blood, urine and fluid samples can be withdrawn in a timely and cost-effective pattern compared to imaging or re-biopsies if indicated. This also reduces subjecting the patients to increased radiation doses, anesthesia and morbidities that might be associated with them. We have reviewed the causes for the conveniences of liquid biopsy versus tissue biopsy in an article under publication “Circulating Tumor DNA: A review for its role in cancer prevention, diagnosis, and treatment”.

Liquid Biopsy Role as a Biomarker

Screening and Early Detection

In 2017, Phallen *et al* proposed the used ctDNA to diagnose and screen early cancers. The authors have used a panel of somatic mutations and looked for them randomly in the ctDNA of diagnosed early-stage cancer patients including colorectal, breast, lung and ovarian. This displayed high concordance with the corresponding tissue biopsies [39]. Another review article suggested that if these results can be reproduced in asymptomatic cases the potential for using ct DNA as a screening tool can be very much applicable [40].

A Response, Prognosis, and Prediction

However, since ctDNA and CTCs have been researched as biomarkers, their role in the detection of response to treatment by follow up of the identified tumor mutations was the main aim of many studies[31,41-43]. After all, early prediction of response to treatment and avoiding unnecessary treatment after the development of resistance is a principal goal for the treating oncologist. Clonal selection by anti-cancer agents and genetic instability of cancer cells was considered among the reasons for resistance to treatment. Using ctDNA and CTCs to measure the mutational burden and evolving mutations can be indicative of a patient's response to treatment by therapy [29]. Also, some studies reported that pre-operative levels of ctDNA and CTCs were found to be highly predictive for the risk of recurrence. Needless to say, patients with higher baseline ctDNA and CTCs levels were found to have recurrences more frequently than those who had lower levels [36,44].

In our opinion, the most important point in relying on ctDNA and CTCs is their ability to provide an insight to the mutational changes in the tumor cells that resulted from treatment or genetic instability which both imaging and tumor markers are unable to provide. This in itself can be a very strong predictive value in patients who initially found to be responsive to a certain treatment by analyzing the initial tumor tissue biopsied.

If we considered the methods used routinely to monitor patients in oncology clinics, they include imaging and tumor markers. Tumor markers such as carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) in colon cancer, and carbohydrate antigen (CA125) in ovarian cancer are used extensively in clinical practice, though the benefit from them is very limited and the specificity is low and recently the United States Preventive Service Task Force (USPSTF) has recommended against their routine use [45].

The other method to evaluate patients' responses is imaging. Besides the potential hazards from repeated radiation exposure in cancer patients, imaging has proved not to have superior results compared to ctDNA in clinical studies[10,43]. Also, there is risk associated with the technique itself such as contrast dye allergies and ineligibility of really impaired patients. This is without considering the time needed for patient preparation and the resulting in compliance with some patients with treatment.

Challenges of Liquid Biopsy

Despite their many advantages, liquid biopsies did not make their breakthrough in clinical practice. The reasons for this are multifactorial.

Protocols Inconsistencies

Protocols used by to process the samples of the patients report multiple differences that might affect the results of the samples, such as pre-analysis processing and storage methods. Since it is a liquid phase sample and the biomarker have a short half-life, processing time, temperature and time of sample withdrawal has to be consistent to avoid sample losses. Besides, researchers are required to provide a consistent specification for the methods used to extract and analyze the samples, starting from sample collection down to sequencing methods used to interpret the final results. So far, different institutions reproduce their results based on their own methods without enough inter-institutional evaluations.

When we consider CTCs and exosomes, their primary isolation depends on the identification of surface markers. This increases the frequency of false positives and negatives. Since commercially base isolation kits do not consider tissue dysplasia from epithelial to mesenchymal [46,47]. Though false positives can be excluded by sequencing of their DNA/RNA content, false negative samples will remain unidentified. Some researchers might need to consider a second run for the identification method to identify missed samples on the first run.

Immune identification of exosomes and CTCs is not as cost-efficient when compared to ctDNA regarding the data that can be obtained from one sample of each. However, sequencing for all of them especially with the introduction of second-generation sequencing techniques to improve specificity is an expensive procedure to be carried on in routine practice, but many researchers assume that this cost will drop with the increased utilization of these techniques.

Also, CTCs and exosomes can be more heterogeneous compared to ctDNA. CTCs number for early tumors can be as low as less than 5 cells/7.5ml of blood [15]. This is a narrow margin to differentiate between high and low risk disease.

Exosomes derived from single cell line showed heterogenicity in their sizes, protein content, RNA species and macromolecular composition [48]. This is possibly related to different functional roles that need to be further investigated to decide on the value of their use as a biomarker.

As a result, CTCs and exosomes became more and more a primary target for research, while it plays in favor of ctDNA to be used as a biomarker in routine clinical practice.

Virtosome

Since the genetic material was first isolated from the blood in 1948 [4], researchers have been trying to identify how and why they are released into the blood. CTCs, exosomes, active secretion, necrosis, apoptosis, and leukocytes have been included as a source for DNA and RNA in the blood as mentioned before.

However, with the recent advancement in sequencing techniques, ctDNA use in research escalated rapidly. It became increasingly clear that its source can, in fact, be more than one. Identifying the source that can overcome its challenges can speed its use in clinical practice.

Origin and Structure

One of the sources of ctDNA is active secretion by living cells. This was first described in bacterial cells [49]. Later, this concept was found to apply to both eukaryotic and prokaryotic cells [50]. Further research identified this genetic material as a non-membrane bound structure formed of a DNA/RNA lipoprotein complex [51]. The DNA part is formed in the nucleus and is later assembled with the protein and lipid components in the cytoplasm [52-54]. Its RNA part is finally added before it is released from the cell [55]. Its lipoprotein element is believed to play a role in protecting the genetic material from digestion by nucleases. This complex was identified as virtosome.

A recent report by Cataldi *et al*. provided a detailed description of the virtosome structure. The researches isolated the DNA/RNA lipoprotein complex from human lymphocytes from both the cytoplasm and from the cell culture medium. They used a multistep centrifugation/ultracentrifugation method to isolate the lipoprotein complex then analyzed its protein, lipid, and nucleic acid contents. Other researchers used agarose gel column chromatography to isolate the complex but reported same similar composition though less specific. According to Cataldi *et al.*, DNA/RNA content represented approximately 40% of the virtosome structure which can be considered as a significant part of its composition. The complex is freshly synthesized and released in the extracellular environment. And its synthesis is regulated by what seems like a negative feedback to prevent the cell from accumulating older complexes [51,52,55,56].

Role and Functions of Virtosomes

Virtosome is a novel complex, so many of its aspects are still be identified. However, thanks to increased research for ctDNA and CTCs, researchers became more aware of what to look for when approaching a liquid biopsy.

Role as a Messenger

Virtosome is capable of entering other cells after its release from the primary cell of origin. It was also found that the complex was not digested by the lysosomes of the hosting cells. It is then capable of entering the nucleus of the host cells without noted changes. How this is achieved is not clear yet, however some researchers think that it might be happening by a process similar to transformation in which DNA plasmid affect the pH to alter the lysosomal activity [52,55]. Only few research was done to study the effect of virtosomes on host cells, even though genetic material entry into other tissues and its effects was the scope many studies [57]. However, many of these studies used DNA from the supernatant of cell culture that included released virtosomes [58]. Cataldi *et al*., have reported the effect of virtosomes on lymphocytes. The authors created two groups of stimulated and unstimulated lymphocytes and extracted the virtosomes from the cell culture media and the cytosol of both types.

Then they used it to treat the other cell group and recorded its effect on their numbers. While the virtosomes from the unstimulated group produce little effect on the stimulated cells number, the complex extracted from the stimulated cells resulted in about 6.5 times increase in number for the same time of incubation [59]. In another experiment by Anker *et al*, the complex was extracted from pre-sensitized T-lymphocytes to herpes and polioviruses and used to treat nude mice. The serum of these mice was found positive to antibodies against these viruses depending on which was used to sensitize the T-cells [58].

Role as a Biomarker

Free DNA role as a biomarker has been established in many studies [60-62]. The discussed aims were targeted toward using it for early detection, prognosis, prediction of response, evaluation and follow-up of patients [10,36,39,63,64]. Virtosomes have the potential to carry the same role since they represent part of free DNA, thus maintaining the same advantages they offer as a biomarker. They provide more to the quality of the biomarker since they present the living population of the tumor. This will reduce the fluctuations in the circulating levels of virtosome that naturally occur in other forms of liquid biopsy following treatment. This also will make their reliability in reflecting the mutational changes in the survival cell population, thus giving a more accurate prediction for tumor response to therapy. It also has a good potential to reflect resistant clones in the tumor. Since it is secreted from living cells regardless of the cell-cycle phase and differentiation, it can be applied to a wide range of abnormal cellular growths, whether it is aggressive malignancies such as acute leukemias or chronic and borderline malignancies. A Virtosomal structure is freshly synthesized and secreted and it takes eighteen hours at most to release all synthesized complexes from the cells. This time included the time needed by the cell line to uptake the nucleotides from the medium [50]. It is not a hybrid structure like exosomes, partially made from the cell plasma membrane, and it does not include DNA formed by unwanted sources such as necrosis.

Isolation and Characterization

Several methods for virtosomes isolation were described: Stroun *et al*. used $Cs2SO_4$/CsCl gradient centrifugation. The DNA nucleotides were radio-labeled and the density was compared to DNA density of the producing cells, frog auricles [54]. This technique cannot be applied to extract virtosomes from the plasma of humans for many reasons. VitosomeDNA is normally not radio-labeled, also, plasma contains a vast majority of compounds and lipoprotein complexes that probably have the same density. Besides, the density of the specific virtosome released from a tumor will depend on cell origin which there is no reference to. The same principle applies to extraction by sucrose gradient centrifugation [65]. Agarose gel or hydroxyapatite column chromatography were used in few studies conducted in the eighties and nineties of the last century [52,53]. Considering the advances introduced since that time, it holds a lot of promise since agarose bead quality, sizes and coating all can produce a highly purified compound.

Free DNA Secretion Forms

In our attempt to study released DNA from cells, we used 293Tcells transfected with DsRed containing plasmid. The cells were incubated in 10% fetal bovine serum DMEM medium. Samples from the media were collected after 24h, 48h, 72h, up to 6 days. The samples were centrifuged at 17000g to remove debris and then ran by electrophoresis on 1% agarose gel.

We used three different sets of primers that were designed to detect different portions of the DsRed sequence. We noticed that DsRed was detected at all-time points for all primer sets; however, the detection was inconsistent at 24- and 48-hours samples. This suggested that released DNA are in the form of fragments that might include a certain sequence at one point but not another. This was proved by the consistent detection of DsRed in all the samples beyond 48h when there is sufficient release of DNA to cover all the whole sequence of DsRed. We did not use any DNA isolation methods or kits to extract the DNA from the media.

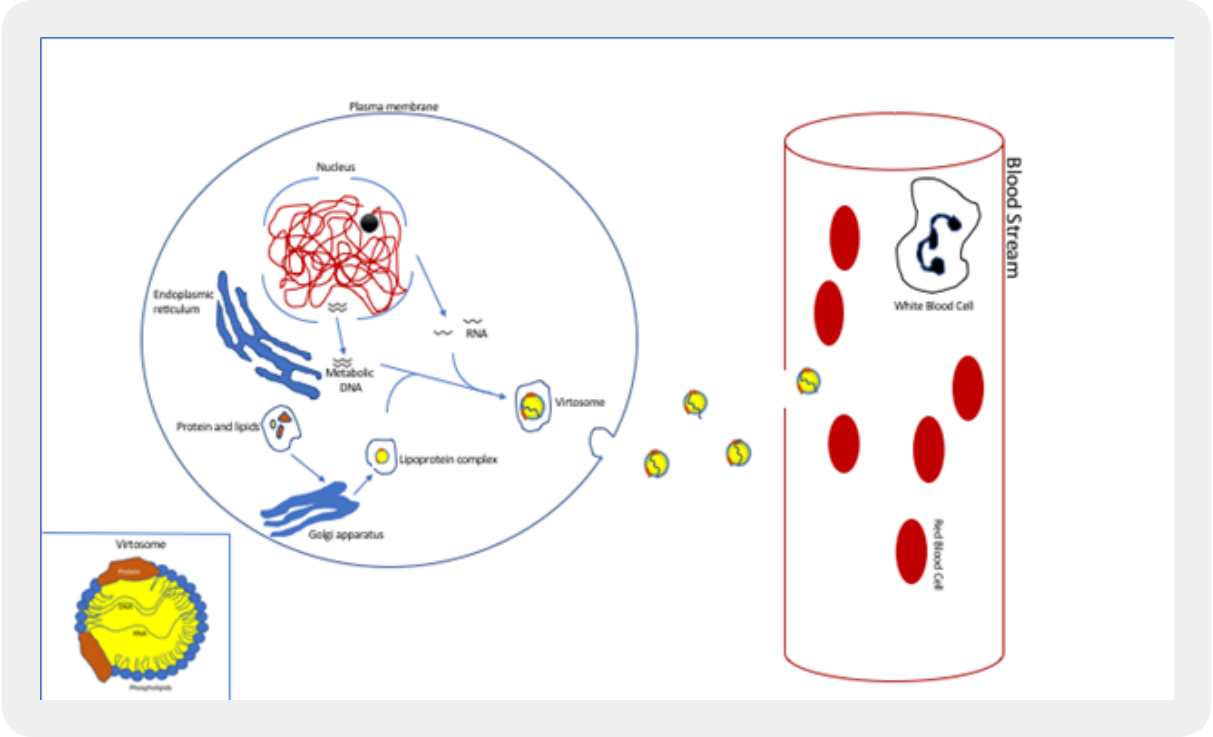

Figure 1: *shows the suggested hypothesis of a virtosome formation. The DNA component is formed in the nucleus then transported to the cytosol to be incorporated into the lipoprotein part. Finally, the RNA is added to the lipoprotein/DNA complex. The final structure is released outside the cell through exocytosis.*

What Needs to be Answered?

Since virtosome is a novel complex, research done is very limited. Thus, a lot of questions need to be answered.

For example, it is not clear if the ctDNA research done recently included virtosome as part of their DNA assays. Does the genetic material in the virtosome need to be isolated from their lipoprotein part in order be detected? And are the DNA isolation protocols and commercially available columns capable of isolating virtosome DNA.

DNA in the virtosome was suggested to be a metabolic DNA. It is the DNA synthesized by the cells for non-replication or differentiation purposes and their synthesis occurs even during the resting phases. Some reports have indicated that metabolic DNA is associated with RNA and lipoproteins [66]. So, how close are these reports close to the virtosome description? And do we need to consider these studies as potential effects for virtosomes.

Virtosome contains both RNA and DNA components. Some reports suggested that RNA is messenger RNA (mRNA) since it is formed by a DNA-dependent RNA polymerase. However, others believe that this RNA was capable of altering the biological functions and thus, siRNA is more appropriate [55]. So, characterization of the type and function of RNA in the complex still need to be done.

Bibliography

1. Naik, A., Rahouma, M., Eldesoki, I., Gray, K. D., Kamel, M., *et al.* (2018). 50 Gene Panel Testing in Non-Small Cell Lung Cancer and Options for Targeted Therapy. *CPQ Cancer., 1*(2), 1-10.

2. Lowy, D. NCI Dictionary of Cancer Terms - National Cancer Institute. National Cancer Institute - at the National Institutes of Health.

3. Fader, C. M. & Colombo, M. I. (2009). Autophagy and multivesicular bodies: two closely related partners. *Cell Death & Differentiation, 16*(1), 70-78.

4. Glazova, M. (1984). Mandel'štam and Dante: The Divine Comedy in Mandel'štam's poetry of the 1930s. *Studies in Soviet Thought, 28*(4), 281-335.

5. Kucharzewska, P. & Belting, M. (2013). Emerging roles of extracellular vesicles in the adaptive response of tumor cells to microenvironmental stress. *Journal of Extracellular Vesicles, 2*(1), 20304.

6. Filimonenko, M., Stuffers, S., Raiborg, C., Yamamoto, A., Malerød, L., *et al.* (2007). Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. *Journal of Cell Biology, 179*(3), 485-500.

7. S.A., J. (2005). Pakistan Journal of Medical Sciences: Editor's note. *Pakistan Journal of Medical Sciences, 21*(2), 117-118.

8. Li, Y., Elashoff, D., Oh, M., Sinha, U., St John, M. A. R., *et al.* (2006). Serum circulating human mRNA profiling and its utility for oral cancer detection. *Journal of Clinical Oncology, 24*(11), 1754-1760.

9. Derakhshan, M. & Mohammad Derakhshan, C. (2007). Apoptosis at a Glance: Death or Life? *Pak J Med Sci., 23*(6), 979-982.

10. Manuscript, A. & Malignancies, H. (2014). Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies. *Sci Transl Med., 6*(224), 224ra24.

11. Alix-Panabieres, C. & Pantel, K. (2016). Clinical applications of circulating tumor cells and circulating tumor DNA as a liquid biopsy. *Cancer Discovery, 6*(5), 479-491.

12. Gahan, P. B. & Stroun, M. (2010). The Biology of Circulating Nucleic Acids in Plasma and Serum (CNAPS). *In Extracellular Nucleic Acids, 25,* 167-189.

13. Diehl, F., Li, M., Dressman, D., He, Y., Shen, D., *et al*. (2005). Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proceedings of the National Academy of Sciences, 102*(45), 16368-16373.

14. Guescini, M., Genedani, S., Stocchi, V. & Agnati, L. F. (2010). Astrocytes and Glioblastoma cells release exosomes carrying mtDNA. *Journal of Neural Transmission, 117*(1), 1-4.

15. Dawson, S. J., Tsui, D. W. Y., Murtaza, M., Biggs, H., Rueda, O. M., *et al*. (2013). Analysis of Circulating Tumor DNA to Monitor Metastatic Breast Cancer. *New England Journal of Medicine, 368*(13), 1199-1209.

16. Heidary, M., Auer, M., Ulz, P., Heitzer, E., Petru, E., *et al.* (2014). The dynamic range of circulating tumor DNA in metastatic breast cancer. *Breast Cancer Research, 16*(4), 421.

17. Thakur, R. & Mishra, D. P. (2012). Modulation of cell death pathways in cancer stem cells: Targeting histone demethylases. *Advances in Bioscience and Biotechnology, 3*(6), 720-730.

18. Maheswaran, S., Sequist, L. V, Nagrath, S., Ulkus, L., Brannigan, B., *et al*. (2008). Detection of Mutations in EGFR in Circulating Lung-Cancer Cells. *New England Journal of Medicine, 359*(4), 366-377.

19. Baixauli, F., Lopez-Otin, C. & Mittelbrunn, M. (2014). Exosomes and autophagy: Coordinated mechanisms for the maintenance of cellular fitness. *Frontiers in Immunology, 5*(403).

20. Deretic, V. (2008). Autophagosome and phagosome. *Methods in Molecular Biology, 445,* 1-9.

21. Raposo, G. & Stoorvogel, W. (2013). Extracellular vesicles: Exosomes, microvesicles, and friends. *Journal of Cell Biology, 200*(4), 373-383.

22. Beckham, C. J., Olsen, J., Yin, P. N., Wu, C. H., Ting, H. J., *et al*. (2014). Bladder Cancer Exosomes Contain EDIL-3/Del1 and Facilitate Cancer Progression. *The Journal of Urology, 192*(2), 583-592.

23. Mizutani, K., Terazawa, R., Kameyama, K., Kato, T., Horie, K., *et al*. (2014). Isolation of prostate cancer-related exosomes. *Anticancer Research, 34*(7), 3419-3423.

24. Prathipati, P., Nandi, S. S. & Kumar Mishra, P. (2015). Stem Cell-Derived Exosomes, Autophagy, Extracellular Matrix Turnover, and miRNAs in Cardiac Regeneration during Stem Cell Therapy. *Stem Cell Rev., 13*(1), 79-91.

25. Brinton, L. T., Sloane, H. S., Kester, M. & Kelly, K. A. (2015). Formation and role of exosomes in cancer. *Cellular and Molecular Life Sciences, 72*(4), 659-671.

26. Li, M., Zeringer, E., Barta, T., Schageman, J., Cheng, A. & Vlassov, A. V. (2014). Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 369*(1652).

27. Greening, D. W., Gopal, S. K., Xu, R., Simpson, R. J. & Chen, W. (2015). Exosomes and their roles in immune regulation and cancer. *Seminars in Cell & Developmental Biology, 40,* 72-81.

28. Crowley, E., Di Nicolantonio, F., Loupakis, F. & Bardelli, A. (2013). Liquid biopsy: monitoring cancer-genetics in the blood. *Nature Reviews Clinical Oncology, 10*(8), 472-484.

29. Pao, W., Miller, V. A., Politi, K. A., Riely, G. J., Somwar, R., *et al.* (2005). Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS medicine., 2*(3), e73.

30. Murtaza, M., Dawson, S. J., Tsui, D. W. Y., Gale, D., Forshew, T., *et al.* (2013). Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature, 497*(7447), 108-112.

31. Abdel Karim, N., Eldessouki, I., Taftaf, A., Deeb, A., Gaber, O., Makramalla, A. & Correa, Z. M. (2018). Case Report GNQ-209P Mutation in Metastatic Uveal Melanoma and Treatment Outcome. Hindawi Publishing Corporation.

32. Kumar, S., Guleria, R., Singh, V., Bharti, A. C., Mohan, A. & Das, B. C. (2010). Plasma DNA level in predicting therapeutic efficacy in advanced nonsmall cell lung cancer. *European Respiratory Journal, 36*(4), 885-892.

33. Taniguchi, K., Uchida, J., Nishino, K., Kumagai, T., Okuyama, T., *et al.* (2011). Quantitative detection of EGFR mutations in circulating tumor DNA derived from lung adenocarcinomas. *Clinical Cancer Research : an Official Journal of the American Association for Cancer Research, 17*(24), 7808-7815.

34. Heitzer, E., Auer, M., Hoffmann, E. M., Pichler, M., Gasch, C., *et al.* (2013). Establishment of tumor-specific copy number alterations from plasma DNA of patients with cancer. *International Journal of Cancer, 133*(2), 346-356.

35. August, R. A., Journal, O. & Malignancies, H. (2016). Molecular Monitoring of Cell-Free Circulating Tumor DNA in Non-Hodgkin Lymphoma Advances in Molecular Monitoring Methods for Non-Hodgkin Lymphomas Circulating Tumor DNA in Other Malignancies, (Figure 1), 1-11.

36. Guo, N., Lou, F., Ma, Y., Li, J., Yang, B., *et al.* (2016). Circulating tumor DNA detection in lung cancer patients before and after surgery. *Scientific Reports, 6,* 33519.

37. Kumar, D., Gupta, D., Shankar, S. & Srivastava, R. K. (2015). Biomolecular characterization of exosomes released from cancer stem cells: Possible implications for biomarker and treatment of cancer. *Oncotarget, 6*(5), 3280-3291.

38. Pisitkun, T., Shen, R. F. & Knepper, M. A. (2004). Identification and proteomic profiling of exosomes in human urine. *Proceedings of the National Academy of Sciences, 101*(36), 13368-13373.

39. Phallen, J., Sausen, M., Adleff, V., Leal, A., Hruban, C., *et al.* (2017). Direct detection of early-stage cancers using circulating tumor DNA. *Science Translational Medicine, 9*(403), eaan2415.

40. Diamandis, E. P. & Fiala, C. (2017). Can circulating tumor DNA be used for direct and early-stage cancer detection? *F1000Research, 6,* 2129.

41. Diehl, F., Schmidt, K., Choti, M. A., Romans, K., Goodman, S., *et al.* (2008). Circulating mutant DNA to assess tumor dynamics. *Nature Medicine, 14*(9), 985-990.

42. Warton, K., Mahon, K. L. & Samimi, G. (2016). Methylated circulating tumor DNA in blood: power in cancer prognosis and response. *Endocrine-Related Cancer, 23*(3), R157-71.

43. Pantel, K., Alix-Panabieres, C., Pantel, K., Schwarzenbach, H., Hoon, D., *et al.* (2016). Blood-Based Analysis of Circulating Cell-Free DNA and Tumor Cells for Early Cancer Detection. *PLOS Medicine., 13*(12), e1002205.

44. Warton, K., Mahon, K. L. & Samimi, G. (2016). Methylated circulating tumor DNA in blood: power in cancer prognosis and response. *Endocrine-Related Cancer, 23*(3), R157-71.

45. Schiffman, J. D., Fisher, P. G. & Gibbs, P. (2015). Early Detection of Cancer: Past, Present, and Future. *Am Soc Clin Oncol Educ Book.,* 57-65.

46. Willis, G. R., Kourembanas, S. & Mitsialis, S. A. (2017). Toward Exosome-Based Therapeutics: Isolation, Heterogeneity, and Fit-for-Purpose Potency. *Frontiers in Cardiovascular Medicine, 4,* 63.

47. Ilie, M., Hofman, V., Long, E., Bordone, O., Selva, E., *et al.* (2014). Current challenges for detection of circulating tumor cells and cell-free circulating nucleic acids, and their characterization in non-small cell lung carcinoma patients. What is the best blood substrate for personalized medicine? *Annals of Translational Medicine, 2*(11), 107.

48. Willms, E., Johansson, H. J., Mäger, I., Lee, Y., Blomberg, K. E. M., *et al.* (2016). Cells release subpopulations of exosomes with distinct molecular and biological properties. *Scientific Reports, 6.*

49. Borenstein, S. & Ephrati-Elizur, E. (1969). Spontaneous release of DNA in sequential genetic order by Bacillus subtilis. *Journal of Molecular Biology, 45*(1), 137-152.

50. McIntosh, A. A. G. & Adams, D. H. (1985). Further studies on the extrusion of cytosol macromolecules by cultured chick embryo fibroblast cells. *International Journal of Biochemistry, 17*(2), 147-153.

51. Adams, D. H. & Gahan, P. B. (1982). Stimulated and Non-stimulated Rat Spleen Cells Release Different DNA-Complexes. *Differentiation, 22*(1), 47-52.

52. Adams, D. H. & G Mcintosh, A. A. (1985). Studies on the cytosolic DNA of chick embryo fibroblasts and its uptake by recipient cultured cells. *Tel., 17*(10), 1041-1051.

53. Adams, D. H. & Mcintosh, A. A. G. (1984). The Cytosol Origin of macromolecules extruded by cultured chick embryo fibroblast cells. *Int. J. Biochem., 16*(7), 721-726.

54. Stroun, M. & Anker, P. (1972). Nucleic acids spontaneously released by living frog auricles. *The Biochemical Journal, 128*(3), 100P-101P.

55. Gahan, P. B. & Stroun, M. (2010). The virtosome-a novel cytosolic informative entity and intercellular messenger. *Cell Biochemistry and Function, 28*(7), 529-538.

56. Bronkhorst, A. J., Wentzel, J. F., Aucamp, J., van Dyk, E., du Plessis, L. & Pretorius, P. J. (2016). Characterization of the cell-free DNA released by cultured cancer cells. *Biochimica et Biophysica Acta - Molecular Cell Research, 1863*(1), 157-165.

57. Gahan, P. B., Wyndaele, R., Mantell, S. & Boggetti, B. (2003). Evidence that direct DNA uptake through cut shoots leads to genetic transformation of Solanum aviculare forst. *Cell Biochemistry and Function, 21*(1), 11-17.

58. Anker, P., Jachertz, D., Maurice, P. A. & Stroun, M. (1984). Nude mice injected with DNA released by antigen-stimulated human T lymphocytes produce specific antibodies expressing human characteristics. *Cell Biochemistry and Function, 2*(1), 33-37.

59. Cataldi, S. & Viola-Magni, M. (2016). Components of the cytosolic and released virtosomes from stimulated and non-stimulated human lymphocytes. *Biochemistry and Biophysics Reports, 6,* 236-241.

60. Roschewski, M., Staudt, L. M. & Wilson, W. H. (2016). Dynamic monitoring of circulating tumor DNA in non-Hodgkin lymphoma. *Blood, 127*(25), 3127-3132.

61. Yeh, P., Hunter, T., Sinha, D., Ftouni, S., Wallach, E., *et al.* (2017). Circulating tumour DNA reflects treatment response and clonal evolution in chronic lymphocytic leukaemia. *Nature Communications, 8,* 1-7.

62. Schwarzenbach, H., Hoon, D. S. B. & Pantel, K. (2011). Cell-free nucleic acids as biomarkers in cancer patients. *Nature Publishing Group, 11*(6), 426-437.

63. Chen, K. Z., Lou, F., Yang, F., Zhang, J. B., Ye, H., *et al*. (2016). Circulating Tumor DNA Detection in Early-Stage Non-Small Cell Lung Cancer Patients by Targeted Sequencing. *Sci Rep., 6,* 31985.

64. Holdhoff, M., Schmidt, K., Donehower, R. & Diaz, L. A. (2009). Analysis of circulating tumor DNA to confirm somatic KRAS mutations. *Journal of the National Cancer Institute, 101*(18), 1284-1285.

65. Adams, D. H. & Gahan, P. B. (1982). Stimulated and Non-stimulated Rat Spleen Cells Release Different DNA-Complexes. *Differentiation, 22*(1-3), 47-52.

66. Gahan, P. B., Anker, P. & Stroun, M. (2008). Metabolic DNA as the origin of spontaneously released DNA? *In Annals of the New York Academy of Sciences, 1137,* 7-17.