



Article

Exploring Optimal Biomarker Sources: A Comparative Analysis of Exosomes and Whole Plasma in Fasting and Non-Fasting Conditions for Liquid Biopsy Applications

Masaki Nasu ^{1,*}, Vedbar S. Khadka ¹, Mayumi Jijiwa ¹, Ken Kobayashi ¹ and Youping Deng ^{1,*}

Department of Quantitative Health Sciences, John A. Burns School of Medicine, University of Hawaii, 651 Ilalo Street, Honolulu, HI 96813, USA; vedbar@hawaii.edu (V.S.K.); jijiwa@hawaii.edu (M.J.); kendk@hawaii.edu (K.K.)

* Correspondence: mnasu@hawaii.edu (M.N.); dengy@hawaii.edu (Y.D.);
Tel.: +1-808-692-1670 (M.N.); +1-808-692-1664 (Y.D.)

Abstract: The study of liquid biopsy with plasma samples is being conducted to identify biomarkers for clinical use. Exosomes, containing nucleic acids and metabolites, have emerged as possible sources for biomarkers. To evaluate the effectiveness of exosomes over plasma, we analyzed the small non-coding RNAs (sncRNAs) and metabolites extracted from exosomes in comparison to those directly extracted from whole plasma under both fasting and non-fasting conditions. We found that sncRNA profiles were not affected by fasting in either exosome or plasma samples. Our results showed that exosomal sncRNAs were found to have more consistent profiles. The plasma miRNA profiles contained high concentrations of cell-derived miRNAs that were likely due to hemolysis. We determined that certain metabolites in whole plasma exhibited noteworthy concentration shifts in relation to fasting status, while others did not. Here, we propose that (1) fasting is not required for a liquid biopsy study that involves both sncRNA and metabolomic profiling, as long as metabolites that are not influenced by fasting status are selected, and (2) the utilization of exosomal RNAs promotes robust and consistent findings in plasma samples, mitigating the impact of batch effects derived from hemolysis. These findings advance the optimization of liquid biopsy methodologies for clinical applications.

Keywords: liquid biopsy; fasting; exosomes; sncRNA; metabolites



Citation: Nasu, M.; Khadka, V.S.; Jijiwa, M.; Kobayashi, K.; Deng, Y. Exploring Optimal Biomarker Sources: A Comparative Analysis of Exosomes and Whole Plasma in Fasting and Non-Fasting Conditions for Liquid Biopsy Applications. *Int. J. Mol. Sci.* **2024**, *25*, 371. <https://doi.org/10.3390/ijms25010371>

Academic Editor: Bruna Scaggianti

Received: 2 December 2023

Revised: 21 December 2023

Accepted: 22 December 2023

Published: 27 December 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Liquid biopsy, which is preferentially performed on circulating nucleic acids extracted from blood plasma, can be a tool to overcome limitations faced by conventional tissue biopsies [1–3]. In plasma samples, biological elements such as circulating cell-free DNA, mRNA, non-coding RNA, proteins, metabolites, and lipids have been detected [1,3–5]. Among these biological materials used in liquid biopsies, circulating cell-free micro-RNAs (miRNAs) have been studied as promising cancer biomarkers [6–9]. Many cancer studies correlate the presence of specific miRNAs in plasma with different types of cancer. However, there are discrepancies in the reported miRNA species among studies of plasma biomarkers. Recent reviews have reported tremendous heterogeneity and inconsistency in cancer-related plasma miRNA markers [7–10]. Although many miRNAs have either shown conflicting results or have been reported in a single study, few miRNA biomarkers have been validated in multiple independent studies [7–9,11,12]. This is due to a lack of standardization in the source of circulating miRNAs (serum/plasma/urine/saliva/etc.), isolation protocol, analytical approach (e.g., using microarray versus RT-qPCR), or sample size. Most of the studies focus on using plasma as the miRNA source. Plasma that is separated from blood samples is supposed to be cell-free, and thus any contamination in plasma with cellular RNAs is supposed to be reported [13]. Recent studies have shown that hemolysis

(the rupture of red blood cells) occurring during blood collection or sample processing can cause the release of intracellular biological materials into plasma specimens [14–16]. Hemolysis is a very common cause of error in the clinical laboratory, accounting for 40% to 70% of unsuitable samples [14,16]. Release of intracellular components such as erythrocytes and platelets upon hemolysis can lead to an increase in the concentration of intracellular analytes. Recent studies have shown that the frequency of hemolysis is highly variable among institutions because it is influenced by the experience level of the phlebotomist and the training of personnel obtaining the specimens [15–19]. Therefore, the degree of hemolysis among labs and clinics can cause a difference in the quality of plasma.

New studies have revealed that cancer-derived exosomes (extracellular vesicles, or EVs) are enriched with sncRNAs signatures of their cell of origin [9,20,21]. The analysis of exosomal miRNAs is superior to whole plasma analysis because exosomes contain more meaningful biomarkers compared to miRNAs in whole plasma [1,9,13,20]. Exosomes are endosomally derived small membrane vesicles (50–100 nm) which are released from cells into the extracellular environment, such as plasma. Recently, many studies have shown that exosomes contain proteins, lipids, metabolites, functional mRNA, sncRNAs, and other non-coding RNAs (such as long non-coding RNAs (lncRNAs)) [1,2,13,22,23]. Exosomes act as a cargo, transferring these biologically active components into targeted cells, playing the role of a cell-to-cell communicator [2,13,20,24]. The content of exosomes, such as mRNA and miRNA, is dependent on the cell of origin and can be transferred into adjacent or distant cells [2,23,25,26]. This suggests that exosomes contain highly specific RNA biomarkers. The membrane of the exosome protects the content RNAs from degradation in the bloodstream (Figure 1A). The RNA content in the exosome is thus relatively stable compared to cell-free RNA floating in plasma [9,20,27]. These advantages suggest that the investigation of exosomal RNAs could provide consistent data to produce reliable cancer biomarkers compared to those from whole plasma RNA contaminated unnecessary with cellular RNAs. Exosomes are therefore an attractive source for diagnostic and prognostic biomarkers [9,20,27–31]. We hypothesize that the exosomal RNA is the most consistent and reliable source of cancer biomarker for precision medicine. Further investigation of the comparison between exosomal RNAs and RNAs from whole plasma is required.

Recently, metabolites, including lipids, are brought to attention as liquid biopsy biomarkers [32–34]. Aberrant metabolism, including lipid metabolisms and fatty acid metabolism, have been reported in cancers and are known to be one of the hallmarks of cancer [35,36]. However, the findings in cancer metabolites studies are extremely varied, depending on the application of study designs, methods, and analyses [32,36]. Our hypothesis is that the difference in fasting status during these studies is the cause of difficulties in metabolite biomarker studies. We would like to identify reliable metabolites which are not affected by fasting/non-fasting status. Very few papers from metabolite biomarker studies mention whether fasting status was taken into consideration or not. Because fasting has previously been the standard before blood sampling for a lipid profile in most countries, lipid profiles are conventionally measured in the fasting state [37]. However, in recent times, the shift toward using non-fasting rather than fasting lipid profiles has been increasingly debated [38,39]. It has been proposed that after normal food intake, concentrations of lipids, lipoproteins, and apolipoproteins differ only minimally from the fasting to the non-fasting state in the general population [37,40].

In this project, we would like to prove that the contamination of cell-free RNA profiles with degraded cellular RNAs is the main cause of the discrepancy and inconsistency in blood plasma biomarker studies (Figure 1A). We hypothesize that the biological materials in exosomes were not contaminated by other materials in blood. The other hypothesis is that the contents of exosomes are not affected by fasting/non-fasting status compared to other cell-free components in whole plasma. Therefore, exosomes are the better source for a biomarker study compared to the biological materials found in whole plasma. To prove these hypotheses, we compared the RNA profiles in the exosome with the RNA profiles in whole plasma. We analyzed small RNA expression levels in plasma and exosome

samples from four healthy individuals using small RNA-Seq based on NGS technology. We also compared fasting profiles and non-fasting profiles (Figure 1B–D). We collected blood samples from participants immediately following overnight fasting and again after breakfast (non-fasting sample). Our data showed clearly that RNA content is not affected by fasting and non-fasting status. We also showed the differences between exosomal RNA and whole plasma RNA. Our data suggested that the exosomal RNA is not contaminated with cellular blood RNA. In contrast, the whole plasma RNA can be contaminated with cellular blood RNA. Therefore, the exosomal RNA is suitable for cancer biomarkers to avoid batch effects. We had a difficult time comparing the differences in metabolites between plasma and exosome. However, we found some metabolites which are not influenced by fasting/non-fasting status in plasma.

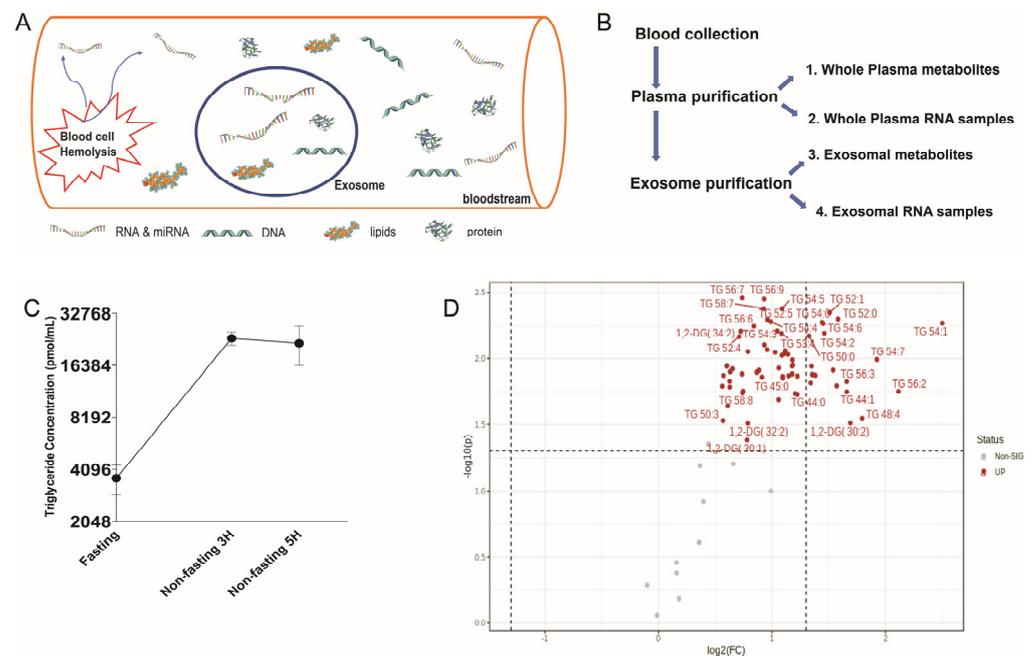


Figure 1. Hypotheses and experimental designs. **(A)** Exosomal RNAs are protected from bloodstream environment. Cell-free RNAs in blood are not only derived from exosomes but also from hemolysis of blood cells. The hypothesis of this study is that blood cell RNAs resulting from hemolysis can lead to batch effects in RNA expression levels across various cohorts. **(B)** Experimental scheme. We compared biological material in exosomal samples and whole plasma samples: Plasma samples were used to purify (1) whole plasma metabolites, (2) whole plasma RNAs and exosomes. Exosomes were used to further purify (3) exosomal metabolites and (4) exosomal RNAs. **(C)** Blood collections were conducted at three different time points to compare the profiles of individuals while fasting and not fasting. (1) Following an overnight fast. (2) Three hours post-breakfast. (3) Five hours after breakfast. The graph displays the fluctuation in concentration of triglyceride (TG 56:2) over time. **(D)** A volcano plot was created, using a fold change threshold of 1.4 and a t-tests threshold of 0.05, to demonstrate the impact of fasting/non-fasting status. Triglyceride (TG) and diglyceride (DG) concentration at non-fasting were normalized by fasting status. Red dots indicate significantly increased TG and DG 3 h after consuming breakfast. Out of 80 species, 68 exhibited a significantly increased concentration.

2. Results

2.1. RNA Profile Is Not Affected by Fasting/Non-Fasting Status

Exosomal RNA samples showed the presence of 2400 miRNA species, 68 piRNAs, 233 tRNAs, and 85 other small RNAs, including snoRNAs. In contrast, whole plasma RNA samples contained 2425 miRNAs, 92 piRNAs, 252 tRNAs, and 96 other small RNAs. Correlation analysis showed that fasting/non-fasting status did not substantially affect RNA profiles, with a high correlation coefficient of 0.9994 for exosomal small RNAs and a

slightly lower coefficient of 0.9868 for plasma small RNAs (Figure 2A,B). These findings highlight that fasting is not a prerequisite for discovering RNA biomarkers, either from plasma or exosome. Notably, exosomal miRNA profiles showed the correlation coefficient of 1.00 (Figure 2C). Although the stability of exosomal profiles was higher than that of plasma profiles, the overall difference was marginally significant ($p = 0.0618679$). Correlation coefficients for piRNA and tRNA in exosomes also exceeded those in plasma (Figure 2D,E), while other small RNA samples show strong positive correlations (Figure 2F).

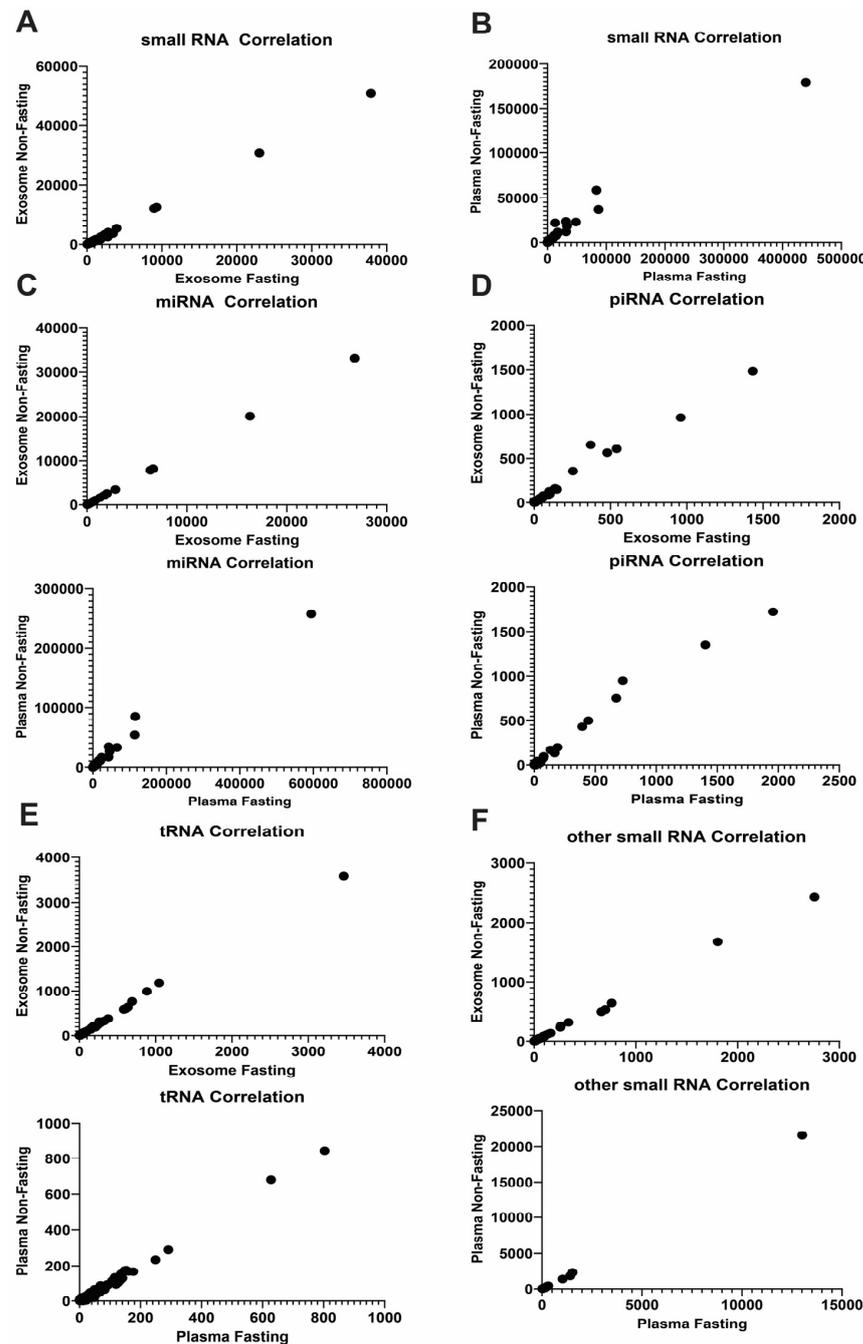


Figure 2. Correlation assay to compare differences between fasting and non-fasting RNA profiles. (A) The correlation coefficient for exosomal small RNAs is 0.9994. (B) The correlation coefficient r for small RNAs in whole plasma is 0.9868. Correlation assay in different type of small RNAs (C–F). (C) miRNA, Exosome $r = 0.9999$, whole plasma $r = 0.9903$. (D) piRNA, Exosome $r = 0.9896$, whole plasma $r = 0.9825$. (E) tRNA, Exosome $r = 0.9992$, whole plasma $r = 0.9889$. (F) Other small RNA, Exosome $r = 0.9987$, whole plasma $r = 0.9996$.

2.2. Exosomal RNA Profile Is Different from Whole Plasma RNA Profile

The principal component analysis (PCA) plot, utilizing normalized RNA-seq data, uncovered marked differences between exosomal and plasma RNA profiles (Figure 3A). While exosomal RNA profiles demonstrated remarkable consistency among different healthy individuals (blue dots are hidden by orange dots), plasma RNA profiles showed considerable heterogeneity, with the dots scattered around the plot. This observation indicates that the expression levels of exosomal small RNA remain stable regardless of whether the individual is fasting or not, thereby implying their stability compared to whole plasma RNAs. Interestingly, some important RNA variants, such as miR-486-5p, miR-92a-3p, has-miR-16-5p, and has-miR-451a, which are known to be enriched in blood cells, contributed to the heterogeneity in plasma RNA profiles (Figure 3B). Plasma RNA profiles have this heterogeneity because concentrations of these blood cell miRNAs are different among different individuals.

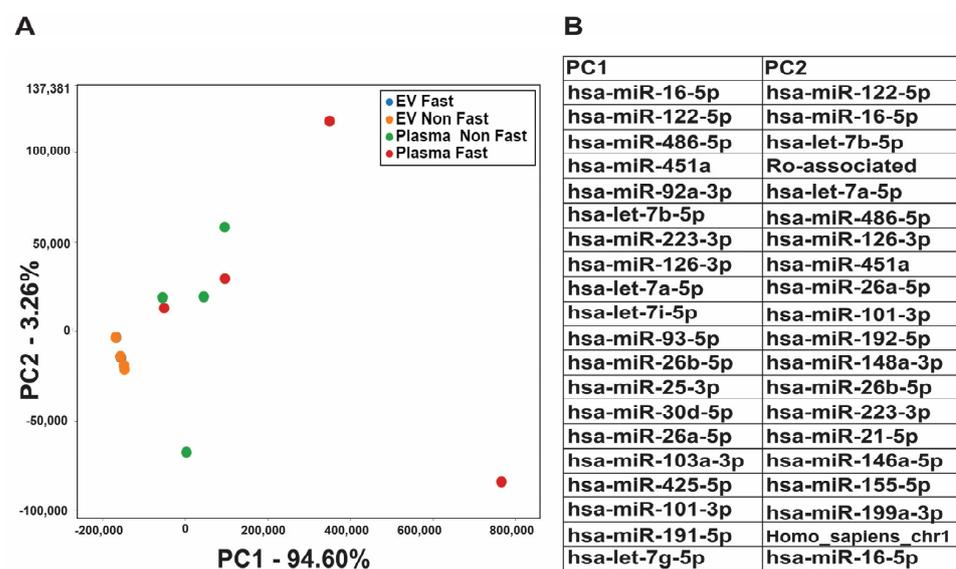


Figure 3. Principal component analysis (PCA) to compare differences between exosomal RNA profiles and whole plasma RNA profile. (A) PCA plot comparing exosomal (EV) RNAs at fasting (blue), exosomal (EV) RNAs at non-fasting (orange), plasma RNAs at fasting (red), and plasma RNAs at non-fasting (Green). PC1 captures the most important variation, PC2 captures the second most important variation. (B) Top 20 small RNA species in PC1 and PC2. These RNAs were differentially expressed with high variance between these two profiles.

To delve deeper, we evaluated the expression level of 28 miRNAs which are reported to derived from platelets and erythrocytes, including has-miR-1-3p, miR-10a-5p, miR-10b-5p, miR-126-3p, miR-144-3p, miR-144-5p, miR-145, miR-150-5p, miR-16-5p, miR-183-5p, miR191-5p, miR-197-3p, miR-21-5p, miR-223-3p, miR-23a-3p, miR-24-3p, miR-26a-1-3p, miR-27b-3p, miR-28-3p, miR-29b-3p, miR-320a, miR-33a-5p, miR-411-5p, miR-423-3p, miR-423-5p, miR-451a, miR-486-5p, and miR-92a-3p [41–44]. Most of these miRNAs exhibited higher expression in whole plasma RNA samples than in exosomes (Figure 4A). The heat map was generated using log₂ fold change values normalized by average of all DEseq2 read. Out of 28 miRNA, 25 showed significant difference with a *p* value lower than 0.01 while has-miR-411-5p had a *p* value of 0.0124. This result explains that blood cell-derived miRNAs can introduce bias in the measurement of miRNA biomarkers' expression levels in whole plasma RNA profiles. (Figure 1A). Expression levels of small RNAs were compared between plasma and exosomes based on their types, including miRNA, piRNA, tRNA, and other small RNAs, including snoRNA (Figure 4B–E). The heat map for miRNAs was constructed using the top 18 upregulated and 18 downregulated miRNAs in exosome compared to plasma log₂ fold changes (Figure 4B). A total of 810 miRNAs exhibited significant

differences in expression between exosomal RNAs and plasma RNAs ($p < 0.05$), confirming distinct miRNA profiles in exosomes and plasma. Among these, 28 out of 36 miRNA showed significant changes in fold change (p value < 0.01), with 5 miRNAs showing p values between 0.04 and 0.01. Notably, has-miR-6731-5p, the most upregulated miRNA in exosomes, had an average NGS normalized read of 465.77 in exosome, compared to 22.11 in plasma. Conversely, the most downregulated miRNA in exosomes, has-miR-192-5p, had 13.46 read in exosome and 1853.93 in plasma. Additionally, 7 miRNAs (has-miR-10a-5p, has-miR-10b-5p, has-miR-126-3p, has-miR-144-3p, has-miR-144-5p, has-miR-451a, has-miR-486-5p) were identified as top upregulated miRNAs in plasma and were reported to have originated from blood (Figure 4A). In the case of piRNAs, the expression level of 30 piRNAs was significantly different between exosomal piRNAs and plasma piRNAs ($p < 0.05$). The heat map featured the top 11 upregulated and 11 downregulated piRNA in exosomes (Figure 4C). Interestingly, some of these piRNA, such as hsa_piR_018573, hsa_piR_001318, hsa_piR_019324, and hsa_piR_018849 have been reported as downregulated piRNAs in prostate cancer urine [45], suggesting their potential as consistent exosomal cancer biomarkers. Similarly, tRNA expression levels showed significant difference between exosomal tRNAs and plasma tRNAs, with the top 18 upregulated and 18 downregulated tRNA forming distinct patterns (Figure 4D). For other small RNAs (snoRNA), the heat map revealed significant differences in the expression level of 59 small RNAs between exosome and plasma ($p < 0.05$). The top 12 upregulated and 12 downregulated small RNAs formed distinct clusters (Figure 4E). It is noteworthy that only limited research has focused on these other small RNAs in exosomes. Nevertheless, the detection of ACA31 was found to be increased in EVs released from cells which were infected with West Nile virus [46].

2.3. Unstable Metabolites in the Plasma Profiles, Which Is More Reliable than the Exosome for Metabolomics Study

Exosomal metabolites showed limited detection, with only 261 out of 513 total metabolites found in whole plasma samples (Supplementary Table S1). Notably, lipidomic type species (fatty acids, TG-DG, lipids) were detected as being relatively more prevalent than amino acids and bile acids in exosome. Both the correlation assay and the volcano plot assay indicated that exosome metabolites were more affected by fasting status than plasma (Figures 5 and 6 and Supplementary Figure S1). Additionally, the concentrations of exosome metabolites were too low, mostly in the single or double-digit pmol/mL range, to yield a significant outcome. Thus, plasma metabolite profiles were primarily utilized to identify stable or unstable metabolites between fasting and non-fasting conditions. Both plasma and exosome metabolites profiles responded to the fasting, non-fasting status (Figure 5A,B), with exosomes exhibiting a higher sensitivity to these conditions compared to whole plasma. The correlation coefficient was 0.9435 in exosome, and slightly higher at 0.9634 in whole plasma. Additionally, correlation analysis between fasting and non-fasting in plasma revealed that bile acids were relatively unstable compared to other type of metabolites (Figure 5C–F). These findings emphasize the significance of choosing the suitable metabolite species for metabolomics research.

We have identified metabolite species which were significantly affected by fasting/non-fasting status in whole plasma profiles (Figure 6 and Supplementary Table S2). Among 30 amino acids species examined, only beta alanine showed a slight increase following food consumption (\log_2 FC = 1.055, p value = 0.0027) (Figure 6A and Supplementary Table S2). The \log_2 fold changes of the remaining 28 amino acid species ranged from 0.37 to -0.27 , indicative of minimal variations. The concentration of 10 out of 35 bile acids were increased with \log_2 FC > 1 , while 7 bile acids experienced reduction with \log_2 FC < 0.48 . Notably, bile acid such as cholic acid (NorCA), glycochenodeoxycholate (GCDCA) and glycodeoxycholic acid (GDCA) significantly increased post-food consumption, boasting \log_2 FC values of 1.62, 1.59, and 1.41 respectively (Figure 6B). On the other hand, the concentrations of deoxycholic acid (DCA), and allolithocholic acid (alloLCA) were significantly decreased with \log_2 FC of -0.73 , and -0.89 , respectively (Figure 6B). The concentration of most fatty acids (24 out of 39) decreased after

food consumption (Figure 6C). However, only 2 fatty acids, caprylic acid (C8:0) and hexanoic acid (C6:0), significantly increased in concentration with log2FC of 0.87 (p value = 0.0046), and 0.69 (p value = 0.014), respectively (Supplementary Table S2). This phenomenon, fatty acids concentration rising during fasting and subsequently decreasing after refeeding, is consistent with findings reported by Whitehurst et al. [47]. Our study also revealed a significant decrease in various fatty acids, including arachidonic acid (C20:4(cis_5,8,11,14)), eicosapentaenoic acid (EPA) (C20:5(cis_5,8,11,14,17)), adrenic acid (docosatetraenoic acid, C22:4(cis_7,10,13,16)), and docosahexaenoic acid (C22:6(cis_4,7,10,13,16,19)) (Figure 6C and Supplementary Table S2). Notably, caprylic acid (C8:0) showed the most significant change (p value = 0.0046), increasing after refeeding.

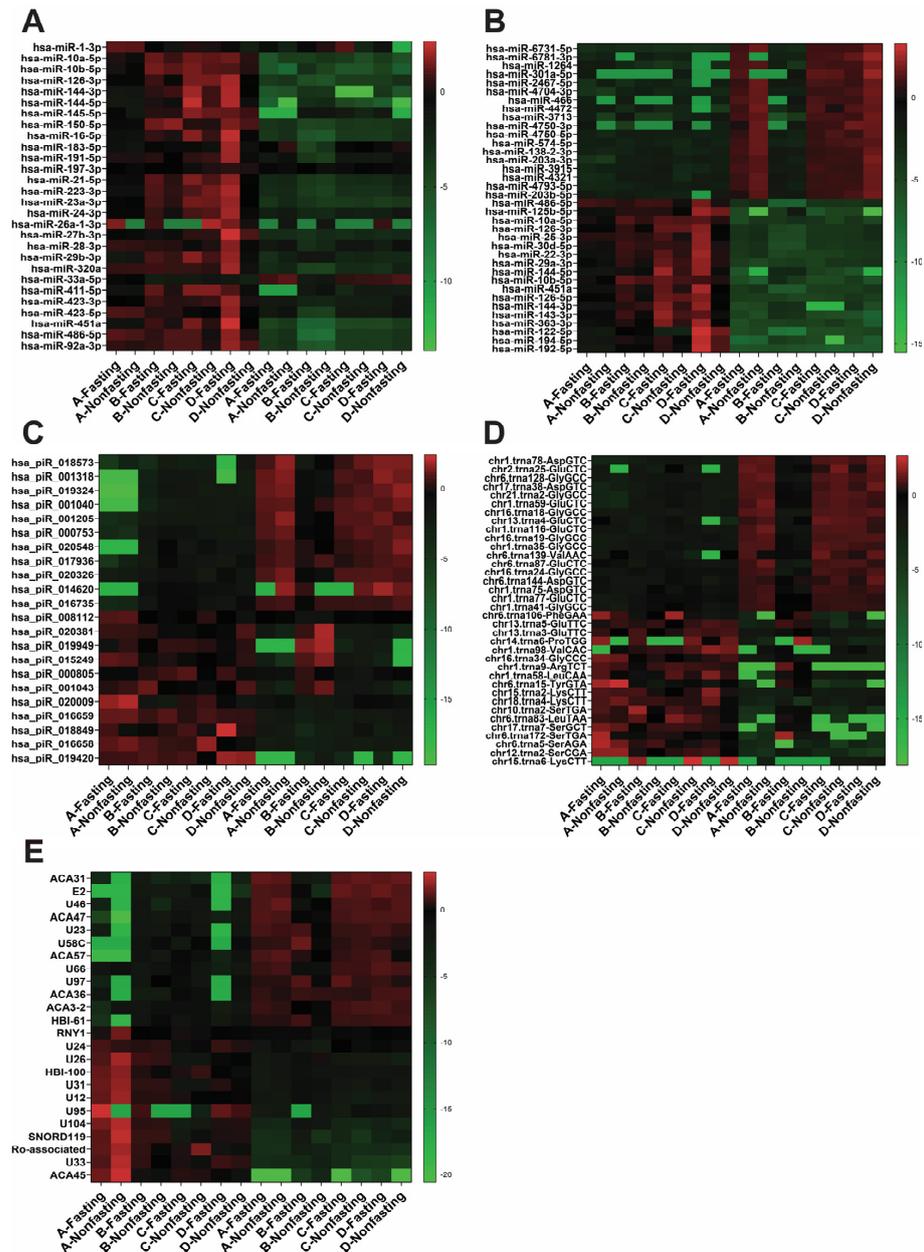


Figure 4. Heat maps showing differences between exosomal RNA profiles and whole plasma RNA profiles. (A) Heat map to compare expression level of platelet- and erythrocyte-derived miRNAs. Highly expressed miRNAs in blood cells are also highly expressed in plasma, but not in exosomes. Heat maps display the differentially expressed small RNA in miRNA (B), piRNA (C), tRNA (D), and other RNAs including snoRNA (E) between exosomal and whole plasma profiles.

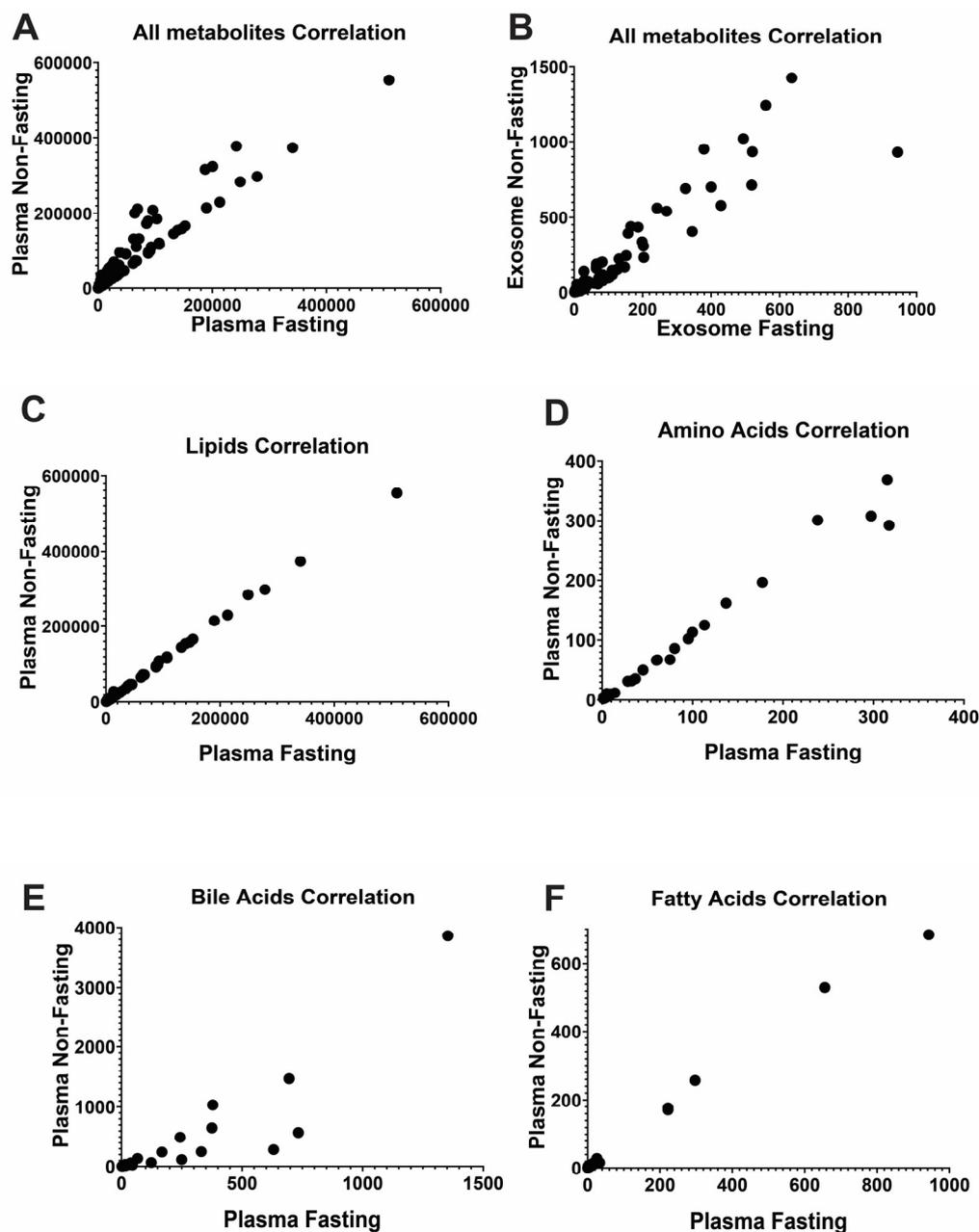


Figure 5. Correlation assay comparing metabolite profiles between fasting and non-fasting conditions. (A) Correlation between fasting and non-fasting for all plasma metabolites. r : Correlation coefficient. $r = 0.9634$. (B) For all metabolites in exosome $r = 0.9435$. (C) Only for lipid species in plasma $r = 0.9996$. (D) Only for amino acids $r = 0.9911$. (E) For bile acids $r = 0.8936$. (F) For fatty acids $r = 0.9979$.

Out of 329 lipid species examined the majority (296) showed no significant changes (Figure 6D), indicating that only a (10%) of lipid species were influenced by fasting. Log2 fold change for 281 lipids fell within the range of -0.5 to 0.5 . However, 20 lipid species including 12 phosphatidylethanolamines (PE), 4 lyso Phosphatidylethanolamines (LPE), 2 ceramides (Cer), and 2 phosphatidylserines (PS), exhibited significant upregulation (Supplementary Table S2). Additionally, 13 lipids species such as C8 Octanoylcarnitine, C10 Decanoylcarnitine, C12 dodecanoylcarnitine, C12:1, C14:1 (cis-5-Tetradecenoylcarnitine), C14:1-OH, C14:2, C16:2, C18:1-OH. C14:1 and C14:2 were reported to be elevated during fasting [48,49]. These findings suggest that the significantly altered lipids may not be appropriate as cancer lipid biomarkers. The volcano plot analysis of exosome profiles showed that only certain TG-DG and lipid species had significant up- or downregulation

reactions, which cause the release of EVs from platelets and can alter the profiles of EVs. The presence of platelet-derived EVs in serum may compromise the accuracy of cancer biomarker studies. These studies support the idea that focusing on EVs purified from plasma, rather than whole plasma or serum, is the first step in reducing contamination and background information for RNA biomarker studies.

Compared to RNA profiles, metabolites were affected more by fasting/non-fasting status. The extent to which the metabolites were affected by fasting status had variable results, with different metabolite species yielding different profiles. The effect of fasting on lipid species was minimal; 90% of lipid species did not show significant changes. These results suggest that fasting is not necessary for plasma biomarker studies, but it is important to choose lipid species that are not affected by fasting. The concentration of some metabolites in plasma can be changed by fasting itself [48,54–56]. Studies have shown that 20% to 50% of metabolites can be affected by fasting/non-fasting status [55,57]. For some metabolites, the effects of fasting status are significant, although for most metabolites the effects are modest. The results from our study can also support previous studies. For example, fatty acids such as palmitic acid (C16:0) are proposed to regulate metastasis [58], and they have a relatively stable baseline in our data (FC 0.8088, Log₂ FC −0.30614, *p* value 0.1131). This suggests that palmitic acid could be a good biomarker. One NSCLC study identified that LysoPC 20:3 (lysoPC a C20:3 in our lipid data), PC ae C40:6, and citric acid (amino acids) were significantly different between healthy controls and stage I/II NSCLC [34]. These are reliable biomarkers since the effect of fasting/non-fasting is very small in our data; LysoPC 20:3 (fold change 0.9245, log₂FC −0.11312, *p* value 0.06), PC ae C40:6 (fold change 1.0607, log₂FC 0.085, *p* value 0.71), and citric acid (amino acids) (fold change 1.08, log₂FC 0.114, *p* value 0.41). Our data revealed that bile acids and fatty acids may not be a good candidate for biomarker studies. The concentration of these metabolites decreased significantly after food consumption. This effect in which the concentration of metabolites increased during fasting and decreased after refeeding is observed in Whitehurst et al. [47]. It is reported that fasting can increase the concentration of free fatty acid and glycerol in blood plasmas suggesting the abnormal condition during fasting [47]. Fasting itself may cause drastic effects on these metabolite biomarkers. Fold change analysis alone revealed that TG, DG fatty acids and bile acids were unstable (48.72%, 48.57%) (Supplementary Table S3). 19 out of 39 fatty acids, 28 out of 30 amino acids and 292 out of 329 lipids were within the fold change threshold. Out of 35 bile acid species, 17 showed drastic changes (10 increased and 7 decreased). A similar report was found in a study by Townsend et al. [59]. Bile acid had the most variability among other metabolites such as amino acid derivatives, amines, amino acids, lipids and lipid metabolites [60]. Therefore, bile acids may not be appropriate to use as a liquid biopsy biomarker. Some advantages to using non-fasting samples rather than fasting samples are proposed: patients do not need to fast, and clinicians can have the lipid profile determined at any random time of day [37]. Fasting can be a barrier to population screening. It is unpopular with children, and it is often unsuitable for patients with diabetes [61]. It is difficult to conduct a large-scale biospecimen collection when participants are spread across a wide geographic area. The standardization of fasting and blood collection protocol is difficult to achieve. For instance, some patients may be unable to adhere to the rigorous fasting protocols necessary for a blood draw [59,62]. Therefore, we conclude that fasting is not necessary, but it is important to use metabolite species with stable baseline concentration regardless of fasting status.

Our study encountered two notable limitations. First, our results suggested that 500 µL of plasma to isolate EVs was adequate for RNA profiles but was not sufficient to isolate enough EVs for metabolite study. It was not possible to obtain enough data to discuss the effect of fasting on exosomal metabolites. It was difficult to detect enough metabolites to make a conclusion for exosomal metabolites. It is also important to discuss the fact that no single standardized method exists yet for the perfect isolation of exosomes for their use in cancer biomarker studies [63–65]. There is still some debate as to whether it

is even possible to isolate the intact and pure exosomes. The ultracentrifugation-based technique was initially the commonly used method for isolating EVs. This technique does have its limitations; it is time-consuming and requires specialized equipment that is not available at clinical labs. This can therefore pose significant challenges for their use in clinical diagnostic tests [66]. Another difficulty is the optimization of centrifuge speed and time: the excessive spinning results in damage of EVs, and inadequate centrifugation may result in a high level of impurities [65]. In our study we used a spin column kit to separate any large particles such as membrane fragments, apoptotic bodies, and other cell fragments from small EVs (which have the size range between 30 and 100 nm). This kit also utilizes another membrane with an immobilized lectin-based compound to capture the glycosylated EVs [67]. The presence of glycoconjugates is involved in EV biogenesis, and in the efficient uptake of EVs by recipient cells [67]. This Capturem Extracellular Vesicle Isolation Kit (Mini) consists of these different spinnable membrane filter columns to capture EVs. This provides a unique solution for isolating concentrated, high-purity EVs in the range of 30–100 nm. (Supplementary Figure S2). The data that were collected using this kit were satisfactory to examine exosomal RNA profiles. However, we understand that it is still challenging, and there is debate about exosome (extracellular vesicles, or EVs) research [29,68,69]. Current isolation techniques may still contain some other vesicles and lipoprotein contaminant [64]. The detailed biogenesis of exosomes and their basic functions and the lack of specific biomarkers are still under investigation [69]. Standardized isolation and characterization methods must be developed. The ultimate goal is to identify exosomes released directly from cancer cells and to evaluate the content of exosomes to determine specific personalized cancer therapy.

Secondly, our sample size was small. It was suggested that metabolite liquid biopsy needs a larger number of participants, as a large sample size is critical for reaching any conclusion with moderate confidence [56]. Regarding RNA profiles, we observed consistent effects between individuals of fasting/non-fasting status, despite the small sample size. This observation was explained with the PCA plot (Figure 3). However, it is important to note that this study would benefit more from a larger sample size.

4. Materials and Methods

4.1. Plasma Samples and Whole Plasma RNA Purification

We used blood samples from 4 healthy volunteers to understand the baseline expression level of biomarkers (Supplementary Table S4). These donors gave written informed consent for the use of their blood samples in accordance with IRB protocol number 2021-01033. The volunteers underwent an overnight fast and were subsequently subjected to a blood test (referred to as “fasting samples”). After the fasting blood samples were collected, the participants ate a nutritious breakfast. Following this, they were instructed to avoid any food or drinks except water. Additional blood samples were collected from the individuals three hours and five hours after the breakfast (termed “non-fasting samples”). Blood samples were collected into BD K2 EDTA tubes. Plasma samples were isolated right after the blood collections via centrifuging at $1500\times g$ for 15 min. Total RNA, including small RNA, was extracted from 200 μL of whole plasma using miRNeasy Serum/Plasma Kit (Cat. No.217184, Qiagen, Hilden, Germany), following the manufacture protocol.

4.2. Exosome Preparation and RNA Samples

Exosomes were purified from 500 μL of plasma by using Capturem™ Extracellular Vesicle Isolation Kit (Cat. No. 635741, Takara Bio USA, Inc., San Jose, CA, USA) Exosomes were eluted with 200 μL elution buffer. Total RNA, including small RNA, was subsequently extracted from these exosome samples using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany).

4.3. Small RNA Sequencing

Library preparation and small RNA sequencing were conducted by the Genomics and Bioinformatics Shared Resources (GBSR) at the University of Hawaii Cancer Center (UHCC). The construction of the small RNA sequencing library employed the QIAseq miRNA Library Kit (Cat. No.331505, Qiagen, Germany) and the QIAseq miRNA NGS 12 Index IL (Cat. No.331582, Qiagen, Germany) according to the manufacturer's instructions. The quality of libraries was validated on the Agilent 2100 Bionalyzer using a high-sensitivity DNA chip. The next-generation sequencings were carried out on the Illumina NextSeq 500 platform. The analysis of sequencing data was conducted by GeneGlobe (QIAGEN). The sequencing data were normalized using the DESeq2 method to evaluate RNA expression level.

4.4. Metabolic/Lipidomic Analysis

Metabolite and lipid concentration analysis were carried out using ultra-performance liquid chromatography (LC) coupled with tandem mass spectrometry (MS) at the UHCC Metabolomics Shared Resource. The normalization standards and stable isotope-labeled standards were used for the measurements. We checked a total of 513 metabolites, including 329 Lipid species, 35 bile acids (BAs), 55 triacylglycerol (TGs), 25 diacylglycerol (DGs), 39 fatty acids, and 30 amino acids. The lipidomic and metabolomic concentration measurement were conducted following the protocol published in reference [70]. To distinguish between fasting/non-fasting states, we focused on TGs and DGs. These species are well known to show clear increase when people eat after overnight fasting [39,61]. Out of 80 TG and DG species, 60 were significantly increased after eating (Figure 1D). As an example, the concentration of Triglyceride (TG 56:2) exhibit a substantial 4.32-fold change three hours after the breakfast, with two-sided p value of 0.017 (Figure 1C). Building upon these findings, we explored the changes in other metabolites and small RNA species. Statistical analyses were carried out using Prism 9 (Graphpad Software Inc., LA Jolla, CA, USA), and MetaboAnalyst (McGill University, Montreal, CA, USA). We duplicated samples from each volunteer, resulting in a total of 8 experiments for this metabolites experiments.

5. Conclusions

In conclusion, we propose that exosomes (EVs) serve as a superior sources of nucleic acid biomarkers, and we challenge the necessity of fasting in the investigation of RNA and metabolite biomarkers in liquid biopsy studies with plasma. Our study clearly showed that exosomal RNA profiles are different from whole plasma RNA. The importance of utilizing exosomal RNA as a strategy to mitigate batch effects in RNA biomarker studies is highlighted by the potential contamination of whole plasma RNAs with cellular RNAs. Regarding metabolites, our research indicates that it is crucial to carefully select metabolites that are not affected by fasting or non-fasting in order to guarantee the accuracy of biomarkers. Fasting itself may have drastic effects on certain metabolite biomarkers. The findings of our study indicate that TG, DG, fatty acids, and bile acids are inadequate biomarkers, whereas amino acids and lipid species showcase better stability for biomarker applications. Our data suggests that conducting plasma analysis for lipidomics and metabolomics is feasible without imposing fasting requirements. However, clear limitations persist, necessitating the avoidance of metabolites influenced by food consumption. This research highlights the potential to enhance the precision and practicality of liquid biopsy studies for cancer biomarker research. To establish reliable and clinically consistent biomarkers, especially for RNA biomarkers, it is suggested that the first step should be to standardize the use of exosomes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25010371/s1>.

Author Contributions: Conceptualization, M.N. and M.J.; methodology, M.N.; software, V.S.K.; validation, M.N. and M.J.; formal analysis, M.N. and V.S.K.; investigation, M.N.; resources, M.N.

and M.J.; data curation, M.N. and V.S.K.; writing—original draft preparation, M.N.; writing—review and editing, M.J., K.K. and V.S.K.; visualization, M.J., K.K. and V.S.K.; supervision, Y.D.; project administration, M.N.; funding acquisition, Y.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Institutes of Health (NIH), grant number 5R01CA223490 and 5R01CA230514. This work was partially supported by the NIH grants P30GM114737, P20GM103466, U54MD007601, U54GM138062, P30CA071789, P20GM139753, and U54HG013243.

Institutional Review Board Statement: Under an expedited review procedure, the research project was approved by the University of Hawaii Institutional Review Board (UH IRB protocol number 2021-01033, Date of approval 19 April 2022).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: RNA sequence data are deposited at NCBI-GEO under accession number GSE216556. Please contact the authors for further data requests.

Acknowledgments: We would like to thank Orion S. Rivers and Tina Weatherby Carvalho of the University of Hawaii Pacific Biosciences Research Center Biological Electron Microscope Facility for their assistance with the electron microscope.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Goričar, K.; Dolžan, V.; Lenassi, M. Extracellular Vesicles: A Novel Tool Facilitating Personalized Medicine and Pharmacogenomics in Oncology. *Front. Pharmacol.* **2021**, *12*, 671298. [[CrossRef](#)] [[PubMed](#)]
- Pinzani, P.; D'argenio, V.; Del Re, M.; Pellegrini, C.; Cucchiara, F.; Salvianti, F.; Galbiati, S. Updates on liquid biopsy: Current trends and future perspectives for clinical application in solid tumors. *Clin. Chem. Lab. Med.* **2021**, *59*, 1181–1200. [[CrossRef](#)] [[PubMed](#)]
- Gao, W.; Chen, Y.; Yang, J.; Zhuo, C.; Huang, S.; Zhang, H.; Shi, Y. Clinical Perspectives on Liquid Biopsy in Metastatic Colorectal Cancer. *Front. Genet.* **2021**, *12*, 634642. [[CrossRef](#)] [[PubMed](#)]
- Calabrese, F.; Lunardi, F.; Pezzuto, F.; Fortarezza, F.; Vuljan, S.E.; Marquette, C.; Hofman, P. Are There New Biomarkers in Tissue and Liquid Biopsies for the Early Detection of Non-Small Cell Lung Cancer? *J. Clin. Med.* **2019**, *8*, 414. [[CrossRef](#)] [[PubMed](#)]
- Pisapia, P.; Malapelle, U.; Troncone, G. Liquid Biopsy and Lung Cancer. *Acta Cytol.* **2019**, *63*, 489–496. [[CrossRef](#)] [[PubMed](#)]
- Khandelwal, A.; Seam, R.K.; Gupta, M.; Rana, M.K.; Prakash, H.; Vasquez, K.M.; Jain, A. Circulating microRNA-590-5p functions as a liquid biopsy marker in non-small cell lung cancer. *Cancer Sci.* **2020**, *111*, 826–839. [[CrossRef](#)]
- Yu, H.; Guan, Z.; Cuk, K.; Zhang, Y.; Brenner, H. Circulating MicroRNA Biomarkers for Lung Cancer Detection in East Asian Populations. *Cancers* **2019**, *11*, 415. [[CrossRef](#)]
- Filipów, S.; Łaczmański, Ł. Blood Circulating miRNAs as Cancer Biomarkers for Diagnosis and Surgical Treatment Response. *Front. Genet.* **2019**, *10*, 169. [[CrossRef](#)]
- Endzelins, E.; Berger, A.; Melne, V.; Bajo-Santos, C.; Sobolevska, K.; Abols, A.; Rodriguez, M.; Santare, D.; Rudnicki, A.; Lietuviets, V.; et al. Detection of circulating miRNAs: Comparative analysis of extracellular vesicle-incorporated miRNAs and cell-free miRNAs in whole plasma of prostate cancer patients. *BMC Cancer* **2017**, *17*, 730. [[CrossRef](#)]
- Yu, H.; Guan, Z.; Cuk, K.; Brenner, H.; Zhang, Y. Circulating microRNA biomarkers for lung cancer detection in Western populations. *Cancer Med.* **2018**, *7*, 4849–4862. [[CrossRef](#)]
- Rodríguez, J.; Avila, J.; Rolfo, C.; Ruíz-Patiño, A.; Russo, A.; Ricaurte, L.; Ordóñez-Reyes, C.; Arrieta, O.; Zatarain-Barrón, Z.L.; Recondo, G.; et al. When Tissue is an Issue the Liquid Biopsy is Nonissue: A Review. *Oncol. Ther.* **2021**, *9*, 89–110. [[CrossRef](#)] [[PubMed](#)]
- Bagheri, A.; Khorshid, H.R.K.; Tavallaie, M.; Mowla, S.J.; Sherafatian, M.; Rashidi, M.; Zargari, M.; Boroujeni, M.E.; Hosseini, S.M. A panel of noncoding RNAs in non-small-cell lung cancer. *J. Cell. Biochem.* **2018**, *120*, 8280–8290. [[CrossRef](#)] [[PubMed](#)]
- Nik Mohamed Kamal, N.N.S.B.; Shahidan, W.N.S. Non-Exosomal and Exosomal Circulatory MicroRNAs: Which Are More Valid as Biomarkers? *Front. Pharmacol.* **2020**, *10*. [[CrossRef](#)] [[PubMed](#)]
- Goyal, T.; Schmotzer, C.L. Validation of hemolysis index thresholds optimizes detection of clinically significant hemolysis. *Am. J. Clin. Pathol.* **2015**, *143*, 579–583. [[CrossRef](#)]
- Azhar, M.; Galgalkar, S.; Chakraborty, I.; Mehta, K.; Ragavender, M.S.; Prabhu, V.; Ledden, D. Hemolysis Detection in Sub-Microliter Volumes of Blood Plasma. *IEEE Trans. Bio-Med. Eng.* **2020**, *67*, 1243–1252. [[CrossRef](#)]
- Lippi, G.; Blanckaert, N.; Bonini, P.; Green, S.; Kitchen, S.; Palicka, V.; Vassault, A.J.; Plebani, M. Haemolysis: An overview of the leading cause of unsuitable specimens in clinical laboratories. *Clin. Chem. Lab. Med.* **2008**, *46*, 764–772. [[CrossRef](#)]
- Grant, M.S. The effect of blood drawing techniques and equipment on the hemolysis of ED laboratory blood samples. *J. Emerg. Nurs.* **2003**, *29*, 116–121. [[CrossRef](#)]

18. Fang, L.; Fang, S.H.; Chung, Y.H.; Chien, S.T. Collecting factors related to the haemolysis of blood specimens. *J. Clin. Nurs.* **2008**, *17*, 2343–2351. [[CrossRef](#)]
19. Petinos, P.; Gay, S.; Badrick, T. Variation in Laboratory Reporting of Haemolysis—A Need for Harmonisation. *Clin. Biochem. Rev.* **2015**, *36*, 133–137.
20. He, X.; Park, S.; Chen, Y.; Lee, H. Extracellular Vesicle-Associated miRNAs as a Biomarker for Lung Cancer in Liquid Biopsy. *Front. Mol. Biosci.* **2021**, *8*, 630718. [[CrossRef](#)]
21. Vasconcelos, M.H.; Caires, H.R.; Ábols, A.; Xavier, C.P.R.; Linē, A. Extracellular vesicles as a novel source of biomarkers in liquid biopsies for monitoring cancer progression and drug resistance. *Drug Resist. Updates* **2019**, *47*, 100647. [[CrossRef](#)] [[PubMed](#)]
22. Challagundla, K.B.; Wise, P.M.; Neviani, P.; Chava, H.; Murtadha, M.; Xu, T.; Kennedy, R.; Ivan, C.; Zhang, X.; Vannini, I.; et al. Exosome-mediated transfer of microRNAs within the tumor microenvironment and neuroblastoma resistance to chemotherapy. *J. Natl. Cancer Inst.* **2015**, *107*, djv135. [[CrossRef](#)] [[PubMed](#)]
23. Valadi, H.; Ekstrom, K.; Bossios, A.; Sjostrand, M.; Lee, J.J.; Lotvall, J.O. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **2007**, *9*, 654–659. [[CrossRef](#)] [[PubMed](#)]
24. Jalalian, S.H.; Ramezani, M.; Jalalian, S.A.; Abnous, K.; Taghdisi, S.M. Exosomes, new biomarkers in early cancer detection. *Anal. Biochem.* **2019**, *571*, 1–13. [[CrossRef](#)] [[PubMed](#)]
25. Stoorvogel, W. Functional transfer of microRNA by exosomes. *Blood* **2012**, *119*, 646–648. [[CrossRef](#)] [[PubMed](#)]
26. Moghaddam, A.S.; Afshari, J.T.; Esmaili, S.A.; Saburi, E.; Joneidi, Z.; Momtazi-Borojeni, A.A. Cardioprotective microRNAs: Lessons from stem cell-derived exosomal microRNAs to treat cardiovascular disease. *Atherosclerosis* **2019**, *285*, 1–9. [[CrossRef](#)]
27. Aushev, V.N.; Zborovskaya, I.B.; Laktionov, K.K.; Girard, N.; Cros, M.P.; Herceg, Z.; Krutovskikh, V. Comparisons of microRNA patterns in plasma before and after tumor removal reveal new biomarkers of lung squamous cell carcinoma. *PLoS ONE* **2013**, *8*, e78649. [[CrossRef](#)]
28. Słomka, A.; Kornek, M.; Cho, W.C. Small Extracellular Vesicles and Their Involvement in Cancer Resistance: An Up-to-Date Review. *Cells* **2022**, *11*, 2913. [[CrossRef](#)]
29. Wandrey, M.; Jablonska, J.; Stauber, R.H.; Gül, D. Exosomes in Cancer Progression and Therapy Resistance: Molecular Insights and Therapeutic Opportunities. *Life* **2023**, *13*, 2033. [[CrossRef](#)]
30. Aqil, F.; Gupta, R.C. Exosomes in Cancer Therapy. *Cancers* **2022**, *14*, 500. [[CrossRef](#)]
31. Ahmad, A. Exosomes in Cancer Diagnosis and Therapy. *Int. J. Mol. Sci.* **2022**, *23*, 9930. [[CrossRef](#)] [[PubMed](#)]
32. Tan, B.; Qiu, Y.; Zou, X.; Chen, T.; Xie, G.; Cheng, Y.; Dong, T.; Zhao, L.; Feng, B.; Hu, X.; et al. Metabonomics identifies serum metabolite markers of colorectal cancer. *J. Proteome Res.* **2013**, *12*, 3000–3009. [[CrossRef](#)] [[PubMed](#)]
33. Hanash, S.M.; Ostrin, E.J.; Fahrman, J.F. Blood based biomarkers beyond genomics for lung cancer screening. *Transl. Lung Cancer Res.* **2018**, *7*, 327–335. [[CrossRef](#)] [[PubMed](#)]
34. Zhang, L.; Zheng, J.; Ahmed, R.; Huang, G.; Reid, J.; Mandal, R.; Maksymuk, A.; Sitar, D.S.; Tappia, P.S.; Ramjiawan, B.; et al. A High-Performing Plasma Metabolite Panel for Early-Stage Lung Cancer Detection. *Cancers* **2020**, *12*, 622. [[CrossRef](#)] [[PubMed](#)]
35. Park, J.H.; Pyun, W.Y.; Park, H.W. Cancer Metabolism: Phenotype, Signaling and Therapeutic Targets. *Cells* **2020**, *9*, 2308. [[CrossRef](#)] [[PubMed](#)]
36. Zhang, L.; Zhu, B.; Zeng, Y.; Shen, H.; Zhang, J.; Wang, X. Clinical lipidomics in understanding of lung cancer: Opportunity and challenge. *Cancer Lett.* **2020**, *470*, 75–83. [[CrossRef](#)]
37. Langsted, A.; Nordestgaard, B.G. Nonfasting lipids, lipoproteins, and apolipoproteins in individuals with and without diabetes: 58 434 individuals from the Copenhagen General Population Study. *Clin. Chem.* **2011**, *57*, 482–489. [[CrossRef](#)]
38. Langsted, A.; Freiberg, J.J.; Nordestgaard, B.G. Fasting and nonfasting lipid levels: Influence of normal food intake on lipids, lipoproteins, apolipoproteins, and cardiovascular risk prediction. *Circulation* **2008**, *118*, 2047–2056. [[CrossRef](#)]
39. Nordestgaard, B.G. A Test in Context: Lipid Profile, Fasting Versus Nonfasting. *J. Am. Coll. Cardiol.* **2017**, *70*, 1637–1646. [[CrossRef](#)]
40. de Vries, M.; Klop, B.; Castro Cabezas, M. The use of the non-fasting lipid profile for lipid-lowering therapy in clinical practice—Point of view. *Atherosclerosis* **2014**, *234*, 473–475. [[CrossRef](#)]
41. Sun, L.; Yu, Y.; Niu, B.; Wang, D. Red Blood Cells as Potential Repositories of MicroRNAs in the Circulatory System. *Front. Genet.* **2020**, *11*, 442. [[CrossRef](#)] [[PubMed](#)]
42. Wakabayashi, I.; Sotoda, Y.; Eguchi, R. Relationships among erythrocyte-derived microRNAs in serum of healthy donors. *Clin. Chim. Acta* **2020**, *507*, 7–10. [[CrossRef](#)] [[PubMed](#)]
43. Heber, S.; Laky, M.; Ansheringer, I.; Wolschner, L.; Mussbacher, M.; Krammer, T.; Haririan, H.; Schrottmaier, W.C.; Volf, I.; Hackl, M.; et al. Platelet Activation Is Not Always Associated With Platelet-Related Plasma microRNA Abundance—Results From a Randomized Controlled Trial of Periodontal Patients. *Front. Physiol.* **2021**, *12*, 613515. [[CrossRef](#)] [[PubMed](#)]
44. Maués, J.; Aquino Moreira-Nunes, C.F.; Rodriguez Burbano, R.M. MicroRNAs as a Potential Quality Measurement Tool of Platelet Concentrate Stored in Blood Banks—A Review. *Cells* **2019**, *8*, 1256. [[CrossRef](#)] [[PubMed](#)]
45. Markert, L.; Holdmann, J.; Klinger, C.; Kaufmann, M.; Schork, K.; Turewicz, M.; Eisenacher, M.; Savelsbergh, A. Small RNAs as biomarkers to differentiate benign and malign prostate diseases: An alternative for transrectal punch biopsy of the prostate? *PLoS ONE* **2021**, *16*, e0247930. [[CrossRef](#)] [[PubMed](#)]

46. Slonchak, A.; Clarke, B.; Mackenzie, J.; Amarilla, A.A.; Setoh, Y.X.; Khromykh, A.A. West Nile virus infection and interferon alpha treatment alter the spectrum and the levels of coding and noncoding host RNAs secreted in extracellular vesicles. *BMC Genom.* **2019**, *20*, 474. [[CrossRef](#)]
47. DiMarco, N.M.; Beitz, D.C.; Whitehurst, G.B. Effect of fasting on free fatty acid, glycerol and cholesterol concentrations in blood plasma and lipoprotein lipase activity in adipose tissue of cattle. *J. Anim. Sci.* **1981**, *52*, 75–82. [[CrossRef](#)]
48. Burrage, L.C.; Miller, M.J.; Wong, L.J.; Kennedy, A.D.; Sutton, V.R.; Sun, Q.; Elsea, S.H.; Graham, B.H. Elevations of C14:1 and C14:2 Plasma Acylcarnitines in Fasted Children: A Diagnostic Dilemma. *J. Pediatr.* **2016**, *169*, 208–213.e2. [[CrossRef](#)]
49. Costa, C.C.; de Almeida, I.T.; Jakobs, C.; Poll-The, B.T.; Duran, M. Dynamic changes of plasma acylcarnitine levels induced by fasting and sunflower oil challenge test in children. *Pediatr. Res.* **1999**, *46*, 440–444. [[CrossRef](#)]
50. Lee, J.S.; Kim, M.; Seong, M.W.; Kim, H.S.; Lee, Y.K.; Kang, H.J. Plasma vs. serum in circulating tumor DNA measurement: Characterization by DNA fragment sizing and digital droplet polymerase chain reaction. *Clin. Chem. Lab. Med.* **2020**, *58*, 527–532. [[CrossRef](#)]
51. Wang, K.; Yuan, Y.; Cho, J.H.; McClarty, S.; Baxter, D.; Galas, D.J. Comparing the MicroRNA spectrum between serum and plasma. *PLoS ONE* **2012**, *7*, e41561. [[CrossRef](#)] [[PubMed](#)]
52. Foye, C.; Yan, I.K.; David, W.; Shukla, N.; Habboush, Y.; Chase, L.; Ryland, K.; Kesari, V.; Patel, T. Comparison of miRNA quantitation by Nanostring in serum and plasma samples. *PLoS ONE* **2017**, *12*, e0189165. [[CrossRef](#)] [[PubMed](#)]
53. Zhang, X.; Takeuchi, T.; Takeda, A.; Mochizuki, H.; Nagai, Y. Comparison of serum and plasma as a source of blood extracellular vesicles: Increased levels of platelet-derived particles in serum extracellular vesicle fractions alter content profiles from plasma extracellular vesicle fractions. *PLoS ONE* **2022**, *17*, e0270634. [[CrossRef](#)] [[PubMed](#)]
54. Ho, J.E.; Larson, M.G.; Vasani, R.S.; Ghorbani, A.; Cheng, S.; Rhee, E.P.; Florez, J.C.; Clish, C.B.; Gerszten, R.E.; Wang, T.J. Metabolite profiles during oral glucose challenge. *Diabetes* **2013**, *62*, 2689–2698. [[CrossRef](#)] [[PubMed](#)]
55. Robertson, D.G.; Ruepp, S.U.; Stryker, S.A.; Hnatyshyn, S.Y.; Shipkova, P.A.; Aranibar, N.; McNaney, C.A.; Fiehn, O.; Reilly, M.D. Metabolomic and transcriptomic changes induced by overnight (16 h) fasting in male and female Sprague-Dawley rats. *Chem. Res. Toxicol.* **2011**, *24*, 481–487. [[CrossRef](#)] [[PubMed](#)]
56. Sampson, J.N.; Boca, S.M.; Shu, X.O.; Stolzenberg-Solomon, R.Z.; Matthews, C.E.; Hsing, A.W.; Tan, Y.T.; Ji, B.T.; Chow, W.H.; Cai, Q.; et al. Metabolomics in epidemiology: Sources of variability in metabolite measurements and implications. *Cancer Epidemiol. Biomark. Prev.* **2013**, *22*, 631–640. [[CrossRef](#)]
57. Cross, A.J.; Moore, S.C.; Boca, S.; Huang, W.Y.; Xiong, X.; Stolzenberg-Solomon, R.; Sinha, R.; Sampson, J.N. A prospective study of serum metabolites and colorectal cancer risk. *Cancer* **2014**, *120*, 3049–3057. [[CrossRef](#)]
58. Pascual, G.; Domínguez, D.; Elosúa-Bayes, M.; Beckedorff, F.; Laudanna, C.; Bigas, C.; Douillet, D.; Greco, C.; Symeonidi, A.; Hernández, I.; et al. Dietary palmitic acid promotes a prometastatic memory via Schwann cells. *Nature* **2021**, *599*, 485–490. [[CrossRef](#)]
59. Townsend, M.K.; Bao, Y.; Poole, E.M.; Bertrand, K.A.; Kraft, P.; Wolpin, B.M.; Clish, C.B.; Tworoger, S.S. Impact of Pre-analytic Blood Sample Collection Factors on Metabolomics. *Cancer Epidemiol. Biomark. Prev.* **2016**, *25*, 823–829. [[CrossRef](#)]
60. Di Ciaula, A.; Wang, D.Q.; Molina-Molina, E.; Lunardi Baccetto, R.; Calamita, G.; Palmieri, V.O.; Portincasa, P. Bile Acids and Cancer: Direct and Environmental-Dependent Effects. *Ann. Hepatol.* **2017**, *16* (Suppl. 1), S87–S105. [[CrossRef](#)] [[PubMed](#)]
61. Nordestgaard, B.G.; Langsted, A.; Mora, S.; Kolovou, G.; Baum, H.; Bruckert, E.; Watts, G.F.; Sypniewska, G.; Wiklund, O.; Borén, J.; et al. Fasting is not routinely required for determination of a lipid profile: Clinical and laboratory implications including flagging at desirable concentration cut-points—a joint consensus statement from the European Atherosclerosis Society and European Federation of Clinical Chemistry and Laboratory Medicine. *Eur. Heart J.* **2016**, *37*, 1944–1958. [[PubMed](#)]
62. Simundic, A.M.; Cornes, M.; Grankvist, K.; Lippi, G.; Nybo, M. Standardization of collection requirements for fasting samples: For the Working Group on Preanalytical Phase (WG-PA) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM). *Clin. Chim. Acta* **2014**, *432*, 33–37. [[CrossRef](#)] [[PubMed](#)]
63. Singh, P.K.; Patel, A.; Kaffenes, A.; Hord, C.; Kesterson, D.; Prakash, S. Microfluidic Approaches and Methods Enabling Extracellular Vesicle Isolation for Cancer Diagnostics. *Micromachines* **2022**, *13*, 139. [[CrossRef](#)] [[PubMed](#)]
64. Chen, J.; Li, P.; Zhang, T.; Xu, Z.; Huang, X.; Wang, R.; Du, L. Review on Strategies and Technologies for Exosome Isolation and Purification. *Front. Bioeng. Biotechnol.* **2021**, *9*, 811971. [[CrossRef](#)] [[PubMed](#)]
65. Brennan, K.; Martin, K.; FitzGerald, S.P.; O’Sullivan, J.; Wu, Y.; Blanco, A.; Richardson, C.; Mc Gee, M.M. A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. *Sci. Rep.* **2020**, *10*, 1039. [[CrossRef](#)]
66. Taylor, C.; Chacko, S.; Davey, M.; Lacroix, J.; MacPherson, A.; Finn, N.; Wajnberg, G.; Ghosh, A.; Crapoulet, N.; Lewis, S.M.; et al. Peptide-Affinity Precipitation of Extracellular Vesicles and Cell-Free DNA Improves Sequencing Performance for the Detection of Pathogenic Mutations in Lung Cancer Patient Plasma. *Int. J. Mol. Sci.* **2020**, *21*, 9083. [[CrossRef](#)]
67. Williams, C.; Royo, F.; Aizpurua-Olaizola, O.; Pazos, R.; Boons, G.J.; Reichardt, N.C.; Falcon-Perez, J.M. Glycosylation of extracellular vesicles: Current knowledge, tools and clinical perspectives. *J. Extracell. Vesicles* **2018**, *7*, 1442985. [[CrossRef](#)]
68. Wang, X.; Tian, L.; Lu, J.; Ng, I.O. Exosomes and cancer—Diagnostic and prognostic biomarkers and therapeutic vehicle. *Oncogenesis* **2022**, *11*, 54. [[CrossRef](#)]

69. Li, X.; Corbett, A.L.; Taatizadeh, E.; Tasnim, N.; Little, J.P.; Garnis, C.; Daugaard, M.; Guns, E.; Hoorfar, M.; Li, I.T.S. Challenges and opportunities in exosome research-Perspectives from biology, engineering, and cancer therapy. *APL Bioeng.* **2019**, *3*, 011503. [[CrossRef](#)]
70. Evangelista, E.B.; Kwee, S.A.; Sato, M.M.; Wang, L.; Rettenmeier, C.; Xie, G.; Jia, W.; Wong, L.L. Phospholipids are A Potentially Important Source of Tissue Biomarkers for Hepatocellular Carcinoma: Results of a Pilot Study Involving Targeted Metabolomics. *Diagnostics* **2019**, *9*, 167. [[CrossRef](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.