# Early-stage cancer results in a multiplicative increase in cell-free DNA originating from healthy tissue

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**Abstract**

Cell-free DNA is a promising biomarker for cancer detection. However, both the sources of elevated cell-free DNA (cfDNA) in patients with early-stage cancer, and the mechanisms by which cfDNA is shed into, and subsequently cleared from, the circulation are still poorly understood. Leveraging a rich dataset of cfDNA in healthy individuals and early-stage cancer patients, we find that early-stage cancer results in a multiplicative increase in the concentration of cfDNA. Our results demonstrate that this increase in cfDNA does not originate from the tumor, but from healthy-tissue, implying that the presence of cancer either increases cell turnover of healthy cfDNA-shedding cells, or slows down clearance of cfDNA by a cancer-type specific factor. This finding is further corroborated by the fact that, for each cancer type, we observe similar multiplicative increases in the concentration of mutant DNA fragments in plasma that contain mutations originating from non-tumor sources. The magnitude of the multiplicative increase in cfDNA concentration varies greatly between cancer types, ranging from a $\sim$1.3-fold increase in lung cancer, to a $\sim$12-fold increase in patients with liver cancer. As cfDNA is cleared in the liver, the large increase in patients with liver cancer may imply that the systemic increase in cfDNA levels in the presence of cancer is due to slower clearance rate rather than higher cell turnover. Our findings quantify cfDNA dynamics in patients with cancer, with implications for improving the accuracy of liquid biopsies for early cancer detection.

## Introduction

Successful detection of cancer in early stages results in more effective treatment, and significantly improved survival prognosis. Identifying biomarkers that can reliably indicate the presence of early-stage cancers is one of the major challenges in oncology [1]. One such promising biomarker is cell-free DNA (cfDNA), composed of the DNA fragments not bound to cells, that can be detected in the circulation. The presence of cfDNA in the blood was first discovered by Mandel and Metais [2] in the 40's; since then, increased levels of cfDNA concentrations have been reported in cancer patients [3-6]. especially in advanced and metastatic stages [7]. Furthermore, Stroun et al. [8] uncovered in the 80's that cfDNA from cancer patients harbored mutations that were also present in patients' tumors.

These discoveries motivated further research on detecting cancer by performing "liquid biopsies" on cfDNA, as a less intrusive alternative to the biopsy on the tumor itself. However, the mechanisms by which DNA is shed into, and subsequently cleared from, the circulation are not fully understood [9-11]. Recent work demonstrates that the majority of cfDNA in cancer patients with highly elevated cfDNA originates from leukocytes, and that the presence of cancer likely results in a systemic

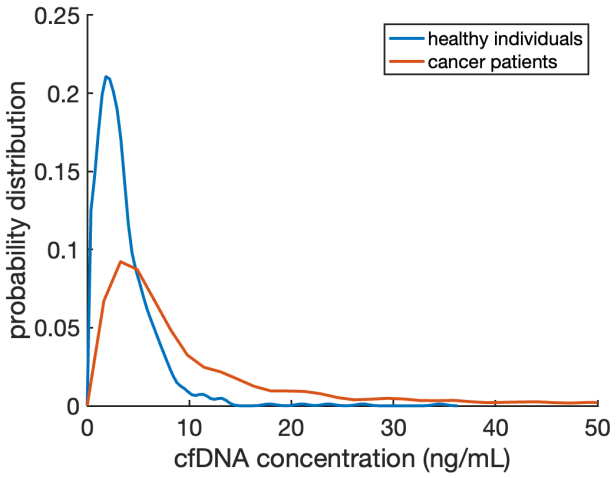


**Figure 1. Probability distribution of cfDNA concentrations in healthy individuals and patients with stage I-III cancer.** Probability density functions for the cfDNA concentration in healthy individuals with mutations detected in plasma (N=572) and patients with lung, breast, colorectal, pancreatic, ovarian, esophageal, stomach and liver cancer in stages I-III (N=952), as reported in Cohen et al. (2018) [13].

increase in cfDNA concentration in these patients [12]. Whether a similar systemic increase due to cancer is present in all cancer patients is currently not known, as many patients, in particular those with early-stage cancer, do not exhibit elevated cfDNA levels.

In this setting, several questions must be answered in order to better understand the mechanics of DNA circulating in plasma, which could lead to improving the accuracy of liquid biopsy screening tests. In what way, and how much does cancer presence affect the total cfDNA concentration? Is this effect cancer type-specific, and does it differ between early cancer stages? In the present study, we address these questions through a novel analysis of a large cfDNA dataset from from Cohen et al. [13], and quantify the differences in cfDNA dynamics between healthy individuals and patients with stage I-III ovarian, liver, stomach, pancreatic, esophageal, colorectal, lung, and breast cancer.

## Results

Cohen et al. [13] report the cfDNA concentrations in plasma of healthy individuals and cancer patients, as well as the concentration and mutant allele frequency (MAF) of DNA fragments in plasma harboring mutations in cancer driver genes (Materials and Methods); we refer to this portion of cfDNA as circulating mutant DNA (cmDNA). We first observe that the 631 healthy individuals (Materials and Methods) can be divided into two groups: 572 (90.6%) that have cmDNA detected and have median cfDNA concentration 2.83ng/mL, and 59 (9.4%) that have no mutations detected in plasma and exhibit significantly (54 times) lower cfDNA concentrations (median 0.05ng/mL) (Supplementary Fig. S3). Since we aim to quantify and compare the effect of cancer on both cfDNA and cmDNA concentrations, we focus on the 90.6% of healthy individuals with cmDNA detected in plasma.

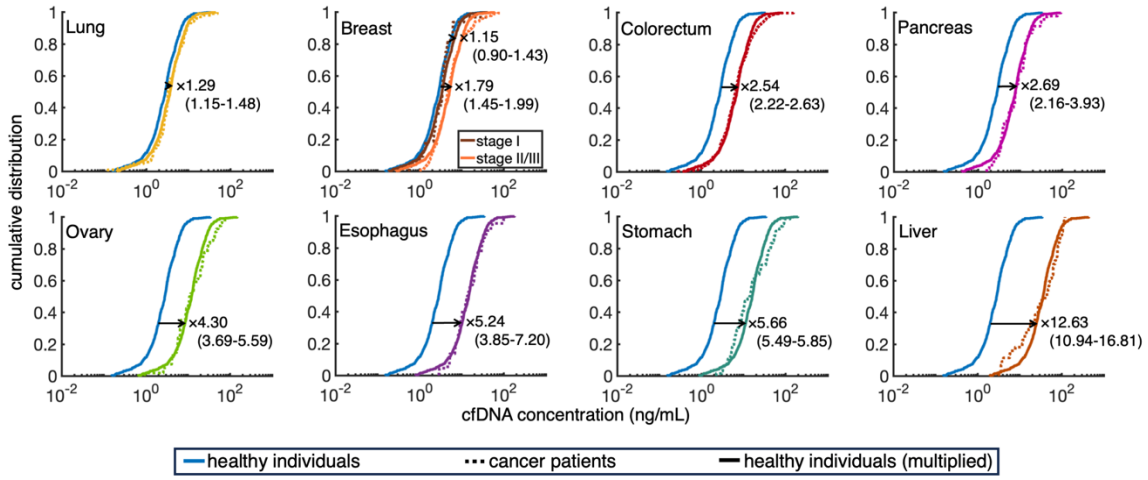


**Figure 2. Quantifying the multiplicative increase in cfDNA concentration in the presence of cancer.** Cumulative distribution functions for the cfDNA concentration in healthy individuals with mutations detected in plasma (*N*=572) and patients with lung (*N*=103), stage I breast (*N*=32), stage II/III breast (*N*=177), colorectal (*N*=385), pancreatic (*N*=47), ovarian (*N*=54), esophageal (*N*=44), stomach (*N*=66) and liver (*N*=44) cancer. Median empirical cfDNA concentrations (corresponding to 0.5 value of cumulative distribution) are 2.83 ng/mL for healthy individuals, and 3.31, 3.42, 4.60, 6.62, 7.76, 11.18, 13.97, 14.14, and 42.27 ng/mL for patients with lung, stage I breast, stage II/III breast, colorectal, pancreatic, ovarian, esophageal, stomach and liver cancer, respectively.

Patients with late-stage cancer typically have cfDNA concentrations that are significantly higher than those in healthy individuals [7, 11]. However, cfDNA concentrations in patients with early-stage cancer often overlap with those found in healthy individuals (Fig. 1). Performing a mathematical analysis of probability distributions of cfDNA concentration in healthy individuals and cancer patients, we find that the presence of stage I-III lung, breast, colorectal, pancreatic, ovarian, esophageal, stomach and liver cancer leads to a multiplicative increase in cfDNA concentration compared to healthy individuals (Fig. 2). In other words, we find that the entire dataset of cfDNA concentrations is multiplied by a positive factor in patients with cancer compared to healthy individuals (Kolmogorov-Smirnov test at 5% significance level, Materials and Methods). This effect can be seen as a horizontal "shift" or translation when the cumulative distribution function is plotted on a logarithmic x-scale (Fig. 2).

The multiplicative increase in cfDNA concentration is specific to each cancer type: 1.29 (value range not rejected by KS test at 5% significance level: 1.15-1.48) for lung cancer, 1.15 (0.9-1.43) for stage I breast cancer, 1.79 (1.45-1.99) for stage II/III breast cancer, 2.54 (2.22-2.63) for colorectal, 2.69 (2.16-3.93) for pancreatic, 4.3 (3.69-5.59) for ovarian, 5.24 (3.85-7.2) for esophageal, 5.66 (5.49-5.85) for stomach, and 12.63 (10.94-16.81) for liver cancer (Fig. 1, Supplementary Fig. S1, Supplementary Table S1, Materials and Methods). There are no statistically significant differences in cfDNA concentration between stages I to III, for all cancer types except for breast and liver cancer; however, the data sample for stage I liver cancer patients is small (5 patients). We found no significant differences in cfDNA concentrations between sexes.

We next asked if the source of the increased cfDNA concentration in early-stage cancer patients is the cancer itself or if the increase is originating from the healthy tissue. We find that the mutant allele frequency (MAF) for the most prevalent mutation detected in plasma of cancer patients of all

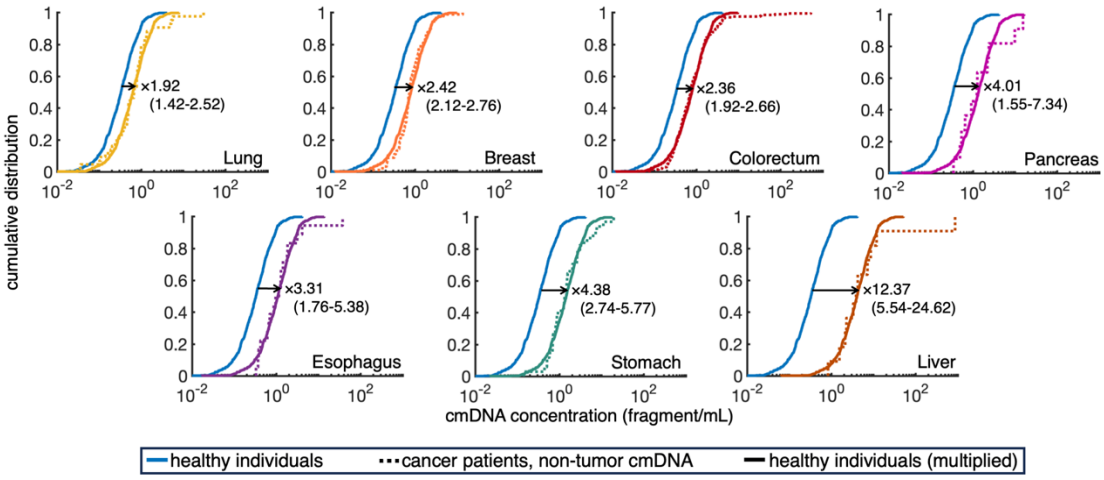


**Figure 3. Mutations detected in plasma of cancer patients but not detected in tumor exhibit a multiplicative increase in concentration compared to mutations detected in plasma of healthy individuals.** Cumulative distribution functions for the concentration of the most prevalent mutation found in plasma of healthy individuals (*N*=572) and patients with lung (*N*=43), breast (*N*=90), colorectal (*N*=205), pancreatic (*N*=11), esophageal (*N*=18), stomach (*N*=34) and liver (*N*=11) cancer in which the most prevalent mutation in plasma was not detected in tumor. Median empirical cmDNA concentrations (corresponding to 0.5 value of cumulative distribution) are 0.33 fragment/mL for healthy individuals, and 0.67, 0.73, 0.72, 1.23, 1.05, 1.39, and 3.85 fragment/mL for patients with cancer of the lung, breast, colorectum, pancreas, esophagus, stomach and liver, respectively.

types is low - median MAF is 0.06%, and 88.2% of mutations detected in plasma have MAF below 1%. Combined with the fact that 82% of cancer patients' tumors harbor mutations (at high frequency) that can be detected by the liquid biopsy panel [13], this indicates that early-stage tumors in all cancer types analyzed do not contribute significantly to cfDNA concentration; most of cfDNA originates from non-tumor (healthy) tissue.

The uniform increase in cfDNA concentration observed in all evaluated cancer types could stem from two sources: either cfDNA shedding from non-tumor sources is systemically increased by a multiplicative factor in patients with cancer, or the presence of cancer results in a similar-fold lower clearance of cfDNA from circulation. There is evidence that an excess of dying cells can lead to slower cfDNA clearance, e.g. during vigorous exercise; the presence of cancer may also lead to an increase in dying cells, potentially slowing down the clearance of cfDNA. Cell-free DNA is typically cleared in spleen, kidney, and the liver [9]; it is of note that cfDNA concentration is highest in patients with liver cancer (~12 times increase in cfDNA concentration compared to healthy individuals), indicating the slower cfDNA clearance rate as a potential mechanism of how tumor presence results in the multiplicative increase of cfDNA levels.

We next hypothesized that, since cfDNA is mainly shed from non-tumor sources and exhibits a multiplicative increase in the presence of cancer, then a similar increase should also be observed in circulating mutant DNA (cmDNA) that does not originate from tumor. In the dataset we use [13], both the most prevalent mutation in plasma and the mutations detected in tumor are reported for 645 of the 959 cancer patients (Materials and Methods). Comparing the cmDNA concentration in healthy individuals and in cancer patients in which cmDNA detected in plasma is not detected in

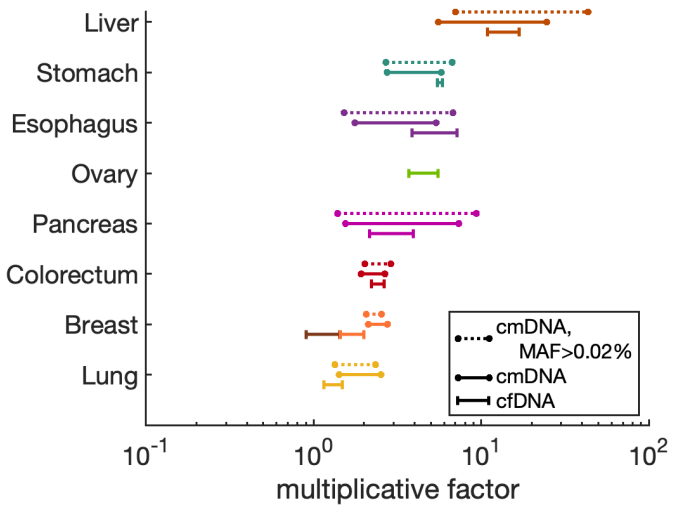


**Figure 4. Multiplicative factor ranges for each cancer type, quantifying the increase in concentrations of total cfDNA in plasma, and of cmDNA not shed from cancer tissue, in the presence of cancer.** For each cancer type, we depict the ranges of multiplicative factor not rejected by KS test at 5% significance level for cfDNA and cmDNA concentrations.

tumor, we find uniform increases by a multiplicative factor for every cancer type: 1.92 (1.42-2.52) for lung, 2.42 (2.12-2.76) for breast, 2.36 (1.92-2.66) for colorectal, 4.01 (1.55-7.34) for pancreatic, 3.31 (1.76-5.38) for esophageal, 4.38 (2.74-5.77) for stomach, and 12.37 (5.54-24.62) for liver cancer (Fig. 3, Supplementary Fig. S2, Supplementary Table S2, Materials and Methods). We do not calculate the multiplicative factor for ovarian cancer, since the dataset reported only one ovarian cancer patient with cmDNA not originating from the tumor. We observe that the ranges of multiplicative factor values for cfDNA and cmDNA from healthy tissue are overlapping for each of the cancer types (Fig. 4), except for breast cancer where ranges are comparable yet non-overlapping. We found no significant differences in cmDNA concentrations between cancer stages or sexes.

Mutant DNA in plasma may originate from healthy or tumor tissue; however, it may also be detected artificially due to sequencing errors. In a more recent work [14], Cohen et al. found that the artefactual mutations in plasma appeared with a MAF lower than 0.013%. Hence, we perform the cmDNA analysis again, disregarding the mutations with low MAFs; the results remain similar to the results obtained for all mutations (Fig. 4, Supplementary Table S2, Materials and Methods).

## Discussion

Our results indicate that cfDNA shed from healthy tissue exhibits a multiplicative shift towards higher concentrations in the presence of early-stage cancer; this increase is quantifiable and ranges from ~30% in lung to ~1100% in liver cancer. The agreement between the cancer-type specific multiplicative factors for total cfDNA concentration, and the concentration of circulating mutant DNA not originating from tumor, is a further validation of our finding. At this point, we cannot answer definitively whether this increase is the result of excessive cfDNA shedding due to increased cell turnover or slower cfDNA clearance; it is also possible that these two causes are related.

The quantifiable increase in cfDNA in early-stage cancer patients points to the following strategy for cancer screening that could potentially be of merit: Patients with hereditary predisposition to cancer may have their cfDNA levels tested regularly; despite fluctuations due to reasons other than cancer (e.g. inflammation, physical exercise, or measurement errors), observation of a persistent multiplicative increase in cfDNA concentration could indicate the presence of early-stage cancer.

We note that the reported cfDNA concentrations may differ significantly depending on the method used for the analysis of the samples. Employing a different methodology, Cohen et al. (2017) [15] report a median cfDNA concentration of 4.25ng/mL in healthy individuals, which is significantly different than the 2.83ng/mL reported in Cohen et al. (2018) [13]. However, we find that the multiplicative factor effect is also present between cfDNA concentrations from samples of healthy individuals from the two studies (multiplicative factor value 1.57 (1.34-1.79), Supplementary Fig. S4); this finding can be proven useful when cfDNA data from different studies are combined.

Finally, the cancer-specific multiplicative factor may be affected by the effects of therapy or anesthesia. Anesthesia has been reported to reduce the concentration of cfDNA ~2.5-fold [16]. Cohen et al. (2017) [15] report cfDNA concentrations from 182 healthy individuals and 221 patients with stage I-III pancreatic cancer. As many pancreatic cancer patients in this study had their blood sample taken after administration of anesthesia, we expect that the multiplicative factor for pancreatic cancer is up to 2.5-fold lower compared to Cohen et al. (2018) [13], which excluded patients where anesthesia was known to be administered prior to sample collection. Indeed, we find that the multiplicative factor for pancreatic cancer from Cohen et al. (2017) [15] is 1.45 (1.30-1.59) (Supplementary Fig. S5). This factor is ~2 times lower that the multiplicative factor for pancreatic cancer in Cohen et al. (2018) [13] data, as expected.

## Materials and Methods

### *Patient data*

We perform a statistical analysis of the concentration (ng/mL) of cfDNA detected in plasma for 812 healthy individuals and for: 104 patients with lung cancer, 209 with breast cancer, 388 with colorectal cancer, 93 with pancreatic cancer, 54 with ovarian cancer, 45 with esophageal cancer, 68 with stomach cancer, and 44 with liver cancer reported by Cohen et al. [13]. All cancer cases reported are in stages I to III. The dataset includes samples from 181 healthy individuals and 46 pancreatic cancer evaluated in a previous study [15]. In order to ensure that all samples were analyzed following the same process, we exclude these samples from the present work.

Cohen et al. [13] also report the concentration (fragment/mL) of the circulating mutant DNA (cmDNA) of the most prevalent DNA mutation detected in plasma for the same population of healthy individuals and cancer patients. The panel for mutation detection from plasma includes 61 amplicons, each querying an average of 33 base pairs within one of the 16 cancer driver genes. Genes included in the panel and the number of amplicons for each gene are: *NRAS* (2), *CTNNB1* (2), *PIK3CA* (4), *FBXW7* (4), *APC* (2), *EGFR* (1), *BRAF* (1), *CDKN2* (2), *PTEN* (4), *FGFR2* (1), *HRAS* (1), *KRAS* (3), *AKT1* (1), *TP53* (31), *PPP2R1A* (1), *GNAS* (1). Apart from the most prevalent mutation in plasma, mutations found in tumor are also reported for 336 of the patients with colorectal, 70 with lung, 116 with breast, 15 with pancreatic, 7 with ovarian, 36 with esophageal, 47 with stomach, and 24 with liver cancer.

### *Kolmogorov-Smirnov test*

Kolmogorov-Smirnov (KS) test is a nonparametric test for continuous one-dimensional probability distributions that we employ to check whether two datasets may come from the same distribution

[17]. More specifically, in the case of two datasets, we use the Kolmogorov-Smirnov test to check whether the multiplication of all elements of one dataset by the same deterministic factor may result in the two datasets originating from the same distribution.

*Multiplicative factors for increased cfDNA/cmDNA in cancer patients*

We determine the value of multiplicative factor a for which we obtain the minimum Kolmogorov distance between the empirical CDF for a times the sample of cfDNA concentration in healthy patients with mutations detected in plasma, and the empirical CDF for the sample of cfDNA concentration in patients of each cancer type. We report the value of the multiplicative factor $a$, and the *p*-value of KS test between the sample of cfDNA concentration in healthy patients with mutations detected in plasma multiplied by $a$, and the cfDNA concentration in patients of each cancer type (Supplementary Table S1).

We also report the value range of the multiplicative factor $a$, for which the KS test does not reject, at 5% significance level, the hypothesis that cfDNA data for cancer patients and the cfDNA data for healthy patients multiplied by the respective factor come from the same distribution. We also observe the agreement between the datasets in probability-probability (P-P) plots (Supplementary Fig. S1), which stay close to the diagonal for all types of cancer.

We calculate the multiplicative factors a between the cmDNA concentrations reported in healthy individuals, and the concentrations of cmDNA not shed from tumor in patients of the various cancer types. The most prevalent DNA mutation in plasma was not detected in the tumor for 43 patients with lung, 90 with breast, 205 with colorectal, 11 with pancreatic, 1 with ovarian, 18 with esophageal, 34 with stomach and 11 with liver cancer. The value of a resulting in the least Kolmogorov distance between the empirical CDFs, along with the 5% significance level ranges, are shown in Supplementary Table S2; the respective P-P plots, which also stay close to the diagonal, are shown in Supplementary Fig. S2. We exclude ovarian cancer from cmDNA concentration analysis, due to insufficient data. We also calculate the multiplicative factors for cmDNA concentrations, excluding the samples with MAF less than i) 0.01%, ii) 0.015%, and iii) 0.02% (Supplementary Table S2), since mutations detected in such low MAFs may be artefactual [14].

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**Author Contributions:** K.M. and I.B. designed and performed research and wrote the manuscript.

**Competing Interest Statement:** The authors declare no competing interests.

**Data Availability Statement**

The data analyzed in this study were obtained from Supplementary Materials of Cohen et al. (2018) [13] https://doi.org/10.1126/science.aar3247 and Cohen et al. (2017) [15] https://doi.org/10.1073/pnas.1704961114

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**Supplementary Information**

| Cancer | multiplicative factor | KS *p*-value |
|---|---|---|
| Lung | 1.29 (1.15-1.48) | 0.62 |
| Stage I Breast | 1.15 (0.90-1.43) | 0.78 |
| Stage II/III Breast | 1.79 (1.45-1.99) | 0.48 |
| Colorectum | 2.54 (2.22-2.63) | 0.30 |
| Pancreas | 2.69 (2.16-3.93) | 0.82 |
| Ovary | 4.30 (3.69, 5.59) | 0.44 |
| Esophagus | 5.24 (3.85-7.20) | 0.94 |
| Stomach | 5.66 (5.49-5.85) | 0.10 |
| Liver | 12.63 (10.94-16.81) | 0.28 |

**Table S1: Multiplicative factors for cfDNA concentration, for each cancer type.** We report the *p*-value of the KS test between cfDNA samples from patients of each cancer and the cfDNA samples from healthy individuals with cmDNA detected multiplied by the corresponding multiplicative factor. We also report the range of values for the multiplicative factor with KS test *p*-values >0.05.

| Cancer | multiplicative factor | KS *p*-value | multiplicative factor MAF >0.01% | KS *p*-value | multiplicative factor MAF >0.015% | KS *p*-value | multiplicative factor MAF >0.02% | KS *p*-value |
|---|---|---|---|---|---|---|---|---|
| Lung | 1.92 (1.42-2.52) | 0.89 | 1.93 (1.45-2.52) | 0.88 | 1.89 (1.41-2.45) | 0.88 | 1.75 (1.34-2.34) | 0.89 |
| Breast | 2.42 (2.12-2.76) | 0.41 | 2.50 (2.22-2.79) | 0.27 | 2.44 (2.16-2.66) | 0.31 | 2.35 (2.06-2.54) | 0.31 |
| Colorectum | 2.36 (1.92-2.66) | 0.51 | 2.49 (1.98-2.70) | 0.42 | 2.51 (2.00-2.74) | 0.45 | 2.59 (2.02-2.89) | 0.44 |
| Pancreas | 4.01 (1.55-7.34) | 0.86 | 4.01 (1.43-8.53) | 0.82 | 4.01 (1.40-8.47) | 0.82 | 4.72 (1.39-9.35) | 0.78 |
| Esophagus | 3.31 (1.76-5.38) | 0.99 | 3.36 (1.77-6.07) | 0.99 | 3.64 (1.74-6.57) | 0.99 | 3.47 (1.52-6.78) | 0.99 |
| Stomach | 4.38 (2.74-5.77) | 0.86 | 4.56 (2.99-6.25) | 0.87 | 5.19 (2.98-7.70) | 0.83 | 5.01 (2.70-6.71) | 0.62 |
| Liver | 12.37 (5.54-24.62) | 0.99 | 14.56 (6.88-37.14) | 0.99 | 17.78 (7.19-46.06) | 0.99 | 16.96 (7.03-43.43) | 0.99 |

**Table S2: Multiplicative factors for cmDNA concentration not shed from tumor, for each cancer type.** We consider all samples, and samples with MAF >0.01%, 0.015%, 0.02%, and report the p-value of the KS test between cmDNA samples from patients of each cancer and the cmDNA samples from healthy individuals multiplied by the corresponding multiplicative factor. We also report the range of values for the multiplicative factor with KS test *p*-values >0.05.

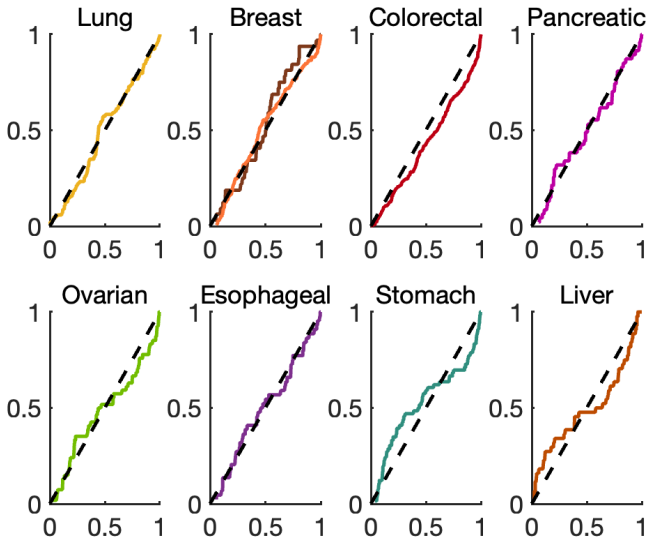


**Figure S1: P-P plots for the multiplicative factor effect in cfDNA concentration between healthy and cancer patients.** In each panel, we depict the probability pairs calculated from the empirical CDF for cfDNA concentration of cancer patients of a particular type, and the empirical CDF of cfDNA concentration in healthy patients multiplied by the respective factor from Table S1.

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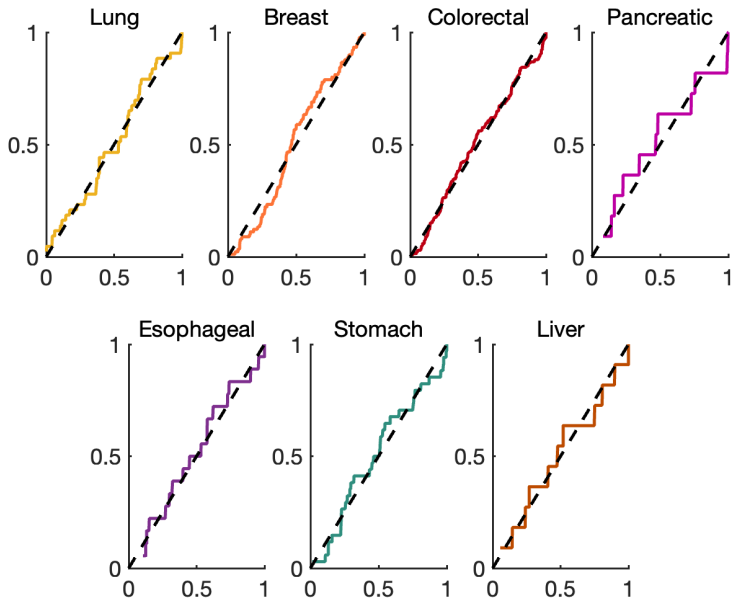


**Figure S2: P-P plots for the multiplicative factor effect in cmDNA concentration between healthy and cancer patients.** In each panel, we depict the probability pairs calculated from the empirical CDF for cmDNA concentration shed from non-tumor tissue of cancer patients of a particular type, and the empirical CDF of cmDNA concentration in healthy patients multiplied by the respective factor from Table S2 (values for all MAF). Ovarian cancer is not depicted due to insufficient data.

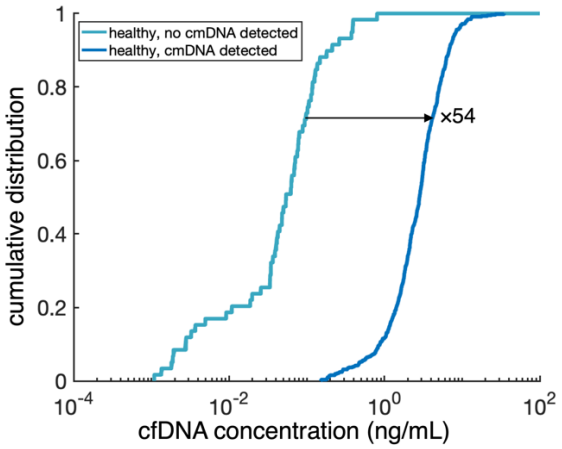


**Figure S3: Multiplicative factor effect in cfDNA concentration between healthy patients with or without cmDNA detected.** Empirical cumulative distribution functions for the cfDNA concentration in healthy individuals with no mutations detected in liquid biopsies (9.4% of healthy individuals) and healthy individuals with mutations detected in plasma (90.6% of healthy individuals). By performing the KS test, we observe that cfDNA concentrations in healthy individuals with no mutations detected are consistent with the cfDNA concentrations in healthy individuals with mutations detected in plasma, multiplied by 1/54 (*p*-value: 0.19).

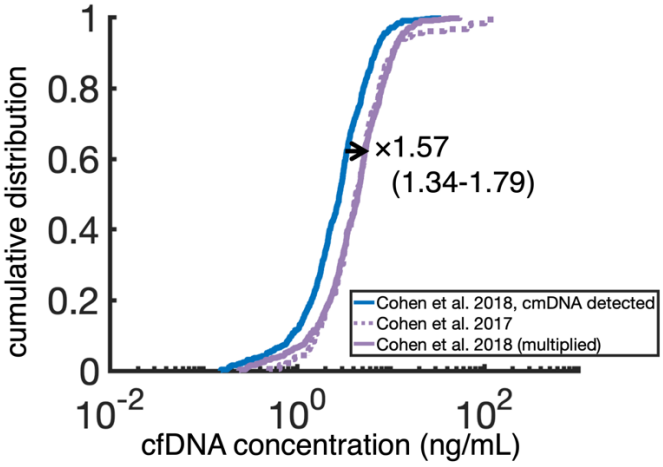


**Figure S4: Multiplicative factor effect in cfDNA concentration between healthy individuals reported between two studies by Cohen et al.** Empirical cumulative distribution functions for the cfDNA concentration in healthy individuals with mutations detected from the 2018 study by Cohen et al., and healthy individuals from the 2017 study by Cohen et al. By performing the KS test, we observe that cfDNA concentrations in healthy individuals from the 2017 study are consistent with the cfDNA concentrations in healthy individuals from the 2018 study, multiplied by 1.57 (*p*-value: 0.95).

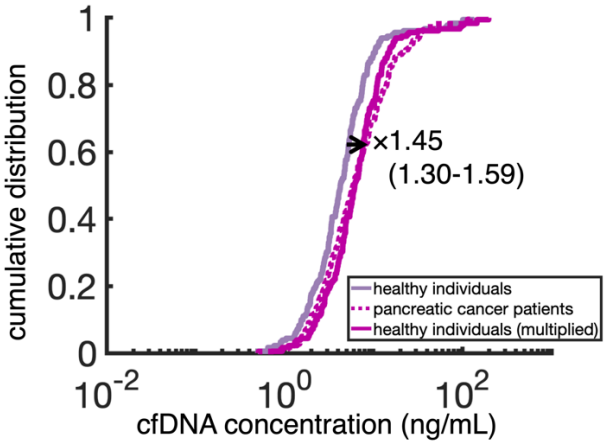


**Figure S5: Multiplicative factor effect in cfDNA concentration between healthy individuals and pancreatic cancer patients reported in Cohen et al. 2017.** Empirical cumulative distribution functions for the cfDNA concentration in 182 healthy individuals, and 221 pancreatic cancer patients from the 2017 study by Cohen et al. By performing the KS test, we observe that cfDNA concentrations in healthy individuals are consistent with the cfDNA concentrations in pancreatic cancer patients, multiplied by 1.45 (*p*-value: 0.39).