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# Live single cell mass spectrometry reveals cancer-specific metabolic profiles of circulating tumor cells

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Recently, there has been increased attention on the analysis of circulating tumor cells (CTCs), also known as liquid biopsy, owing to its potential benefits in cancer diagnosis and treatment. Circulating tumor cells are released from primary tumor lesions into the blood stream and eventually metastasize to distant body organs. However, a major hurdle with CTC analysis is their natural scarcity. Existing methods lack sensitivity, specificity, or reproducibility required in CTC characterization and detection. Here, we report untargeted molecular profiling of single CTCs obtained from gastric cancer and colorectal cancer patients, using live single cell mass spectrometry integrated with microfluidics-based cell enrichment techniques. Using this approach, we showed the difference in the metabolomic profile between CTCs originating from different cancer groups. Moreover, potential biomarkers were putatively annotated to be specific to each cancer type.

**KEYWORDS**
cancer biomarker, circulating tumor cells, liquid biopsy, mass spectrometry, single cell analysis

**Abbreviations:** %RSD, percent relative standard deviation; CRC, colorectal cancer; CTC, circulating tumor cell; DA, discriminant analysis; FA, fatty acyl; GC, gastric cancer; GPL, glycerophospholipid; LSC-MS, live single-cell mass spectrometry; MS, mass spectrometry; PC, phosphatidylcholine; PCA, principle component analysis; PE, phosphatidylethanolamine; PS, phosphatidylserine; SIM, selective ion monitoring; SL, sterol lipid.

Yasmine Abouleila and Kaoru Onidani contributed equally to this work.

## 1 | INTRODUCTION

Recent studies have shown that cancer cells are highly heterogeneous on the single cell level, which might be one of the factors contributing to tumor relapse and increased incidence of metastasis.[1-3] However, studying cancer on the single cell level poses several challenges, such as the difficulty of getting tumor samples in large-scale clinical studies. Aside from imaging techniques for diagnostic purposes, the only reliable way of studying cancer cells in a clinical setting is to carry out a surgical biopsy on the tumor site, a process that is inconvenient for the patient because of its invasive nature. Moreover, such invasive examination is unsuitable for large-scale studies to monitor the biological behavior in real time, which can be altered under the pressure of various therapeutic agents.

An alternative procedure termed "liquid biopsy" has been introduced as a possible method that can allow access to molecular information of cancer cells without the need for invasive procedures.[4-8] Liquid biopsy is a simple, non-invasive technique that targets circulating free nucleic acids (cfDNA and cfRNA) or CTCs that are often found in the peripheral blood of cancer patients. Circulating tumor cells, in particular, present wide-ranging features of phenotypic and genotypic variation depending on their primary tumor source.[9] Circulating tumor cells are cells that shed from the primary tumor into the vasculature after undergoing epithelial-mesenchymal transition,[10] which is a phenotypic conversion of epithelial cells to gain more mesenchymal features, allowing them to circulate through the blood stream and potentially metastasize to various body organs. Accordingly, they ought to have insightful and essential information on the primary tumor, which will be of great importance for differential cancer diagnoses.

Previous CTC studies have mainly focused on enumerating their presence in peripheral blood, which can act as a predictive biomarker for early detection of tumor metastasis as well as monitoring the therapeutic efficacy and response of anticancer drugs.[11] Despite the prognostic value of CTC enumeration, it is still not enough to gain a comprehensive understanding of the tumor nature and its characteristics. Recent studies have reported genome and transcriptome analyses of single CTCs using a next generation sequencer.[12] Circulating tumor cell sequencing proved to be an efficient liquid biopsy tool that can monitor the variation in gene expression among different cancer stages, which could be used to investigate tumor origin, evolution, and tumor progression during treatment. However, there is an untapped potential that lies in CTC metabolomic profiling that can shed light on the heterogeneity of cancer cells as well as the possible role played by CTCs in cancer progression and metastasis. In addition, analysis of CTCs provides the capability of obtaining a snapshot on the functional state of endogenous metabolites, thus, playing an essential role in filling the "genotype-phenotype gap". Furthermore, the metabolic profile as a whole is considered as a complex biomarker that can be of great value in the field of oncology.[9,13-18]

However, CTC metabolomic profiling is often a difficult task. The number of CTCs obtained per patient could greatly vary depending on the cancer type and the clinical stage. In general, the number of CTCs usually varies between zero to a few hundred or even more in approximately 7.5 mL peripheral blood.[18,19] Therefore, obtaining sufficient numbers of CTCs suitable for carrying out analysis still remains a great challenge. On this background, achieving efficient CTC enrichment with minimal sample loss is an issue for CTC metabolic profiling. Among the methods used for CTC enrichment are size-based filtration, immune-mediated capturing,[20] and FACS.[21] However, multistep processes with harsh conditions could result in low throughput, CTC recovery, and cell viability. To overcome such problems, a microfluidics device that relies on differences in size and deformability of CTCs and blood cells for CTC separation was developed, which ensures an accurate and effective label-free approach that maintains cell viability for further downstream analysis.[22]

Another issue is selection of suitable analytical techniques. Conventional metabolomic techniques such as liquid/gas chromatography are unsuitable due to their insufficient sensitivity and inapplicability to small volumes associated with single cells. Live single-cell mass spectrometry[23] has been developed as a promising technique that has enough sensitivity for single cell metabolic profiling. In LSC-MS, a single cell is collected into a tapered glass microcapillary under videomicroscopy, which is then ionized and directly introduced to a mass spectrometer. This technique was successfully applied to plant cells,[24] mammalian cells,[25] and CTCs,[21] albeit with focus on targeted analysis for a limited number of compounds. To achieve molecular characterization of CTCs for future diagnostics, untargeted analysis must be achieved to gain comprehensive metabolomic information about the primary tumor.

In this study, by integrating LSC-MS and a microfluidics-based CTC enrichment technique, untargeted analysis was undertaken for CTCs obtained from 2 cancer types, GC and CRC (Figure 1). We explored the possibility of discriminating between CTCs and lymphocytes obtained from the same patients, as well as discriminating between CTCs obtained from different cancer types and patients on the single cell level.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and peripheral blood samples

Participants comprised 10 patients with advanced GC and CRC. The patients' information is summarized in Table 1. Once informed consent was secured from these patients, their blood samples were processed for CTC analysis. Peripheral blood was collected in 5 mL EDTA vacutainers or Streck tubes (TERUMO, Tokyo, Japan) and processed within 24 hours. This study was approved by the ethics committee of the National Cancer Center (2013-001; the term is during 2013-2022) and RIKEN (Kobe1 2017-07; the term is during 2017-2022).

### 2.2 | Circulating tumor cell enrichment

The ClearCell FX system (ClearBridge Biomedics, Singapore) was used to capture and enrich CTCs from peripheral blood samples according to the manufacturer's protocol. Five milliliters of blood

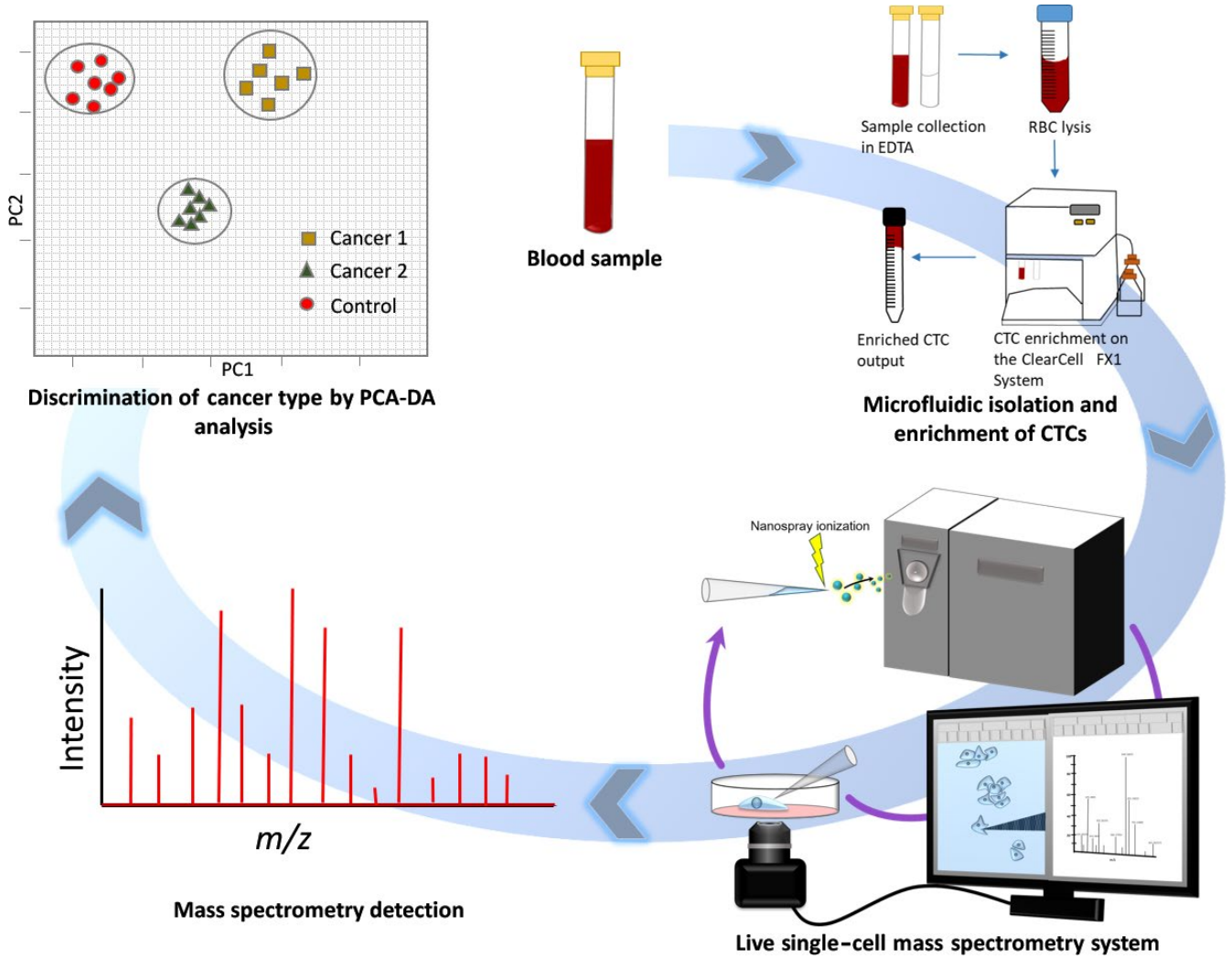


**FIGURE 1** Schematic of single-cell analysis of circulating tumor cells (CTCs) using live single-cell mass spectrometry. Blood samples were collected from gastric cancer and colorectal cancer patients. CTCs were isolated and enriched using a microfluidics technique. Single CTCs were sampled and analyzed using the live single-cell mass spectrometry system. Data processing and statistical analysis (*t* test and principle component analysis) was done. RBC, red blood cell

was mixed with 15 mL red blood cell lysis buffer (G-Biosciences, St. Louis, MO, USA) at room temperature for 10 minutes. After incubation, the samples were centrifuged at 500 *g* for 10 minutes followed by aspiration of supernatant, and finally resuspended in 4.3 mL suspension reagent supplied by the manufacturer. The samples were then processed through the ClearCell FX system. The ClearCell FX system is an automated CTC enrichment system driven by the CTChip FR1, a microfluidic biochip to isolate CTCs based on size, deformability, and inertia. The isolation principle takes advantage of the inherent Dean vortex flows present in curvilinear channels for CTC enrichment, termed Dean flow fractionation.[22]

## 2.3 | Single-cell sampling

The enriched CTC samples were centrifuged at 500 *g* for 10 minutes followed by aspiration of supernatant and resuspended in 300 μL PBS. After reconstitution, CTC sample solution was transferred to a Cell Imaging Dish (145 μm, 35 mm × 10 mm; Eppendorf, Hamburg, Germany) for microscopic visualization. The samples were stained with fluorescent Ab, mouse anti-human CD45-FITC (130-080-202; Miltenyi Biotec, Bergisch Gladbach, Germany) (Figure S1). A single CD45-negative CTC was chosen under the microscope and sucked into a Cellomics tip (CT-2; Humanix, Hiroshima, Japan) using a micromanipulator and piston syringe. Similarly, a single CD45-positive lymphocyte was also sucked into a tip as a control. Single CTCs and lymphocytes were selected by morphology. Afterwards, the samples were frozen at −80°C until subsequent MS analysis.

## 2.4 | Sample preparation

The collected samples were thawed, and the organic solvent was introduced from the rear end of the Cellomics tip. The organic solvent consisted of 80% methanol, 10% DMSO, and 0.1% formic acid. All the

**TABLE 1** Clinical characteristics of study subjects with advanced gastric cancer or colorectal cancer

| Patient no. | Gender | Age, years | Primary site | Histology | Histological differentiation | Disease status | Distant metastatic site |
|---|---|---|---|---|---|---|---|
| 1 | Male | 60 | Stomach | Adenocarcinoma | Poorly | Recurrence | Liver, bone |
| 2 | Female | 71 | Colon | Adenocarcinoma | Well to moderately | Recurrence | Small intestinal, uterus |
| 3 | Male | 63 | Stomach | Adenocarcinoma | Moderately to poorly | Stage IV | Liver, adrenal gland, para-aortic lymph nodes |
| 4 | Male | 80 | Stomach | Adenocarcinoma | Moderately to poorly | Stage IV | Liver, peritoneum |
| 5 | Male | 51 | Colon | Adenocarcinoma | Poorly | Stage IV | Liver |
| 6 | Male | 70 | Colon | Adenocarcinoma | Unknown | Recurrence | Lung |
| 7 | Male | 70 | Colon | Adenocarcinoma | Moderately | Recurrence | Liver, lung |
| 8 | Male | 68 | Colon | Adenocarcinoma | Poorly | Recurrence | Nonregional lymph nodes |
| 9 | Male | 56 | Colon | Adenocarcinoma | Moderately | Recurrence | Liver, lung |
| 10 | Male | 72 | Colon | Adenocarcinoma | Well | Recurrence | Liver, lung |

reagents used in the organic solvent were of LC-MS grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrasonication was then applied to the Cellomics tips containing the cells using a homogenizer rod (UR-20P; TOMY Seiko, Tokyo, Japan) outfitted with an in-house attachment for the tips. Sonicating the tips before MS measurement enhances the extraction of metabolites and lipids in addition to improving robustness by reducing tip plugging.[21]

## 2.5 | Mass spectrometry measurement

Mass spectrometry measurement was done using an LTQ Orbitrap Velos Pro instrument (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nanospray source (Nanospray Flex; Thermo Fisher Scientific). The distance between the Cellomics tip and the inlet of the instrument was set to 2 mm and the inlet capillary temperature was set to 200°C. The spray voltage was chosen to be 1–1.5 kV, maintaining a spray current between 100–150 nA. The resolution was set to 100 000 full width half maximum. As the mass spectrometer used was an LTQ Orbitrap Velos Pro, which requires 90 minutes of stabilization time after switching the polarity between positive and negative modes, positive mode was selected in this study to ensure wider metabolite and lipid coverage. Generally, positive ion mode exhibits overall more exhaustiveness than negative mode due to the higher efficiency of protonation compared to the deprotonation process.[26,27] Despite using positive mode, several lipids that are usually detected in negative mode (ie, FAs), could still be detected by our method as cationic ion-conjugates (potassium and sodium adducts).[28,29] These adducts are readily formed and observed in electrospray ionization analyses.[30] For untargeted analysis, the instrument was set to SIM mode and it scanned from 100 *m/z* to 2000 *m/z* in 50 *m/z* increments. This method of "SIM stitching" allows for higher dynamic range as well as lower overall signal to noise ratios without compromising mass accuracy, thus improving the number of metabolites and lipids detected.[31]

## 2.6 | Data handling

The data generated from the mass spectrometer was converted from Thermo Fisher Scientific's raw proprietary format to text files using an in-house script, peak alignment was done using MarkerView software (AB Sciex, Framingham, MA, USA). Afterwards, text files were imported to R statistical software (R Foundation, Vienna, Austria) for further processing. Peaks with signal to noise ratio of <3 were eliminated. In addition, peaks appearing in less than 4 samples (10% of the total number of samples) have been removed as a preprocessing step to eliminate any noise or insignificant peaks. Furthermore, log transformation was applied to the spectra, followed by total ion count normalization by using the MALDI-quant package.[32]

## 2.7 | Statistical analysis

To visualize the metabolomic differences across multiple samples in a reduced dimensional space, a supervised approach utilizing PCA-DA was carried out using MarkerView software. The loading plots for all PCA-DA are shown in Figures S2-S4. Furthermore, to discern the possible unique peaks to the different groups, Welch's *t* test was applied between CTCs and lymphocytes (control) and between GC CTCs and CRC CTCs; the test was done on R statistical software. Peaks with more than 1 log2 fold change or less than −1 log2 fold change and with *P* value <.05 were selected (Figure S5). For peak identification, we followed a 2-pronged approach depending on the accurate mass (*m/z* <5 ppm) and isotopic pattern following the metabolomics standard initiative.[33] The significant peaks were run through an in-house script that matches possible peaks against the Kyoto Encyclopedia of Genes and

Genomics,[34] Human Metabolome Database,[35] and LIPID MAPS structure database.[36] All the annotations of putatively identified lipids was done by using the shorthand lipid notation system suggested by Liebisch et al,[37] except for eicosanoids lipids, where common names were used. Lipid candidates were annotated on the FA/alkyl position level, as exact mass measurements are incapable of determining the stereochemistry, and double bond geometry.

## 3 | RESULTS

### 3.1 | Identification of unique metabolic profile between CTCs and lymphocytes

We enriched the CTCs using a microfluidics-based enrichment technique and cytosolic metabolites were harvested from single-cell CTCs according to the processes described in the Materials and Methods (Figures 1 and S1). To examine whether LSC-MS can detect the metabolomic profile difference between a single CTC and other control cells, we analyzed CTCs and lymphocytes collected from the same patient. After peak alignment and normalization, we undertook supervised PCA followed by DA to visualize the samples in a reduced dimensional space (Figures 2 and S6). The supervised PCA-DA showed clear clustering behavior between 3 groups: CTCs, lymphocytes, and the organic solvent blank per cancer (Figure 2) and per patient (Figure S6). Due to the limited number of cells obtained per patient, supervised PCA-DA was only carried out on patients' samples, in which the number of CTCs and lymphocytes analyzed is more than 2. Detailed information about the patients and obtained samples is shown in Table 1.

### 3.2 | Possible biomarkers for CTCs

It is known that there are aberrant profiles with the presence of unique metabolites or lipids in malignant cells compared with normal cells.[38-40] Accordingly, we investigated the biomarkers found in all CTCs compared to the control lymphocyte cells. As the sample numbers have unequal size and variance, Welch's *t* test was used to determine which metabolites are present only in CTCs across all patients. The comparison was undertaken between CTCs (n = 22) and lymphocytes collected from all patients regardless of a cancer type (n = 16), after subtracting solvent peaks from both groups. The resulting peaks were filtered by selecting statistically significant peaks ($P < .05$).

In total, 119 peaks were putatively identified that were specific to CTCs by using their respective exact mass values with mean error of <5 ppm (Figure 3). Interestingly, out of the 119 peaks found, 75 were identified as GPLs. Considering their function in the cell as a structural component in biological membranes, the possible unique membrane profile common to CTCs can be shown. A table with the putatively identified metabolites and lipids unique to CTCs with *P* value, %RSD, chemical formula, and class for each compound is shown in Table S1.

### 3.3 | Single-cell profiling of GC and CRC CTCs

Several studies have suggested that unique metabolomic profiles were observed in the primary site of different cancer types.[41-43] However, to the best of our knowledge, the metabolomic profiles of single CTCs in several cancer types have never been reported. In addition, CTCs are easily accessible from peripheral blood and their molecular characterization could have significant prognostic and diagnostic value,[44-46] especially if metabolic differences can be

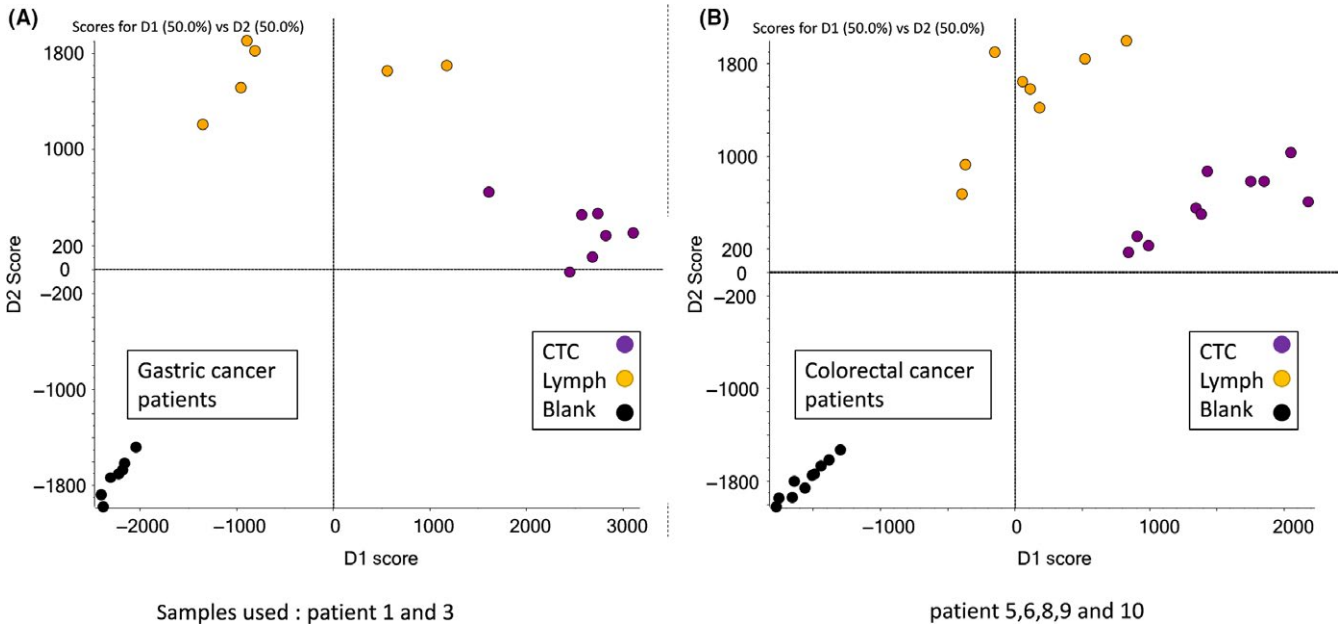


**FIGURE 2** Principle component analysis of circulating tumor cells (CTCs) and lymphocytes. A, The difference in the metabolic profile between CTCs and lymphocytes collected from gastric cancer patients. B, Metabolic profile of CTCs and lymphocytes from colorectal cancer patients. Each dot corresponds to a single cell

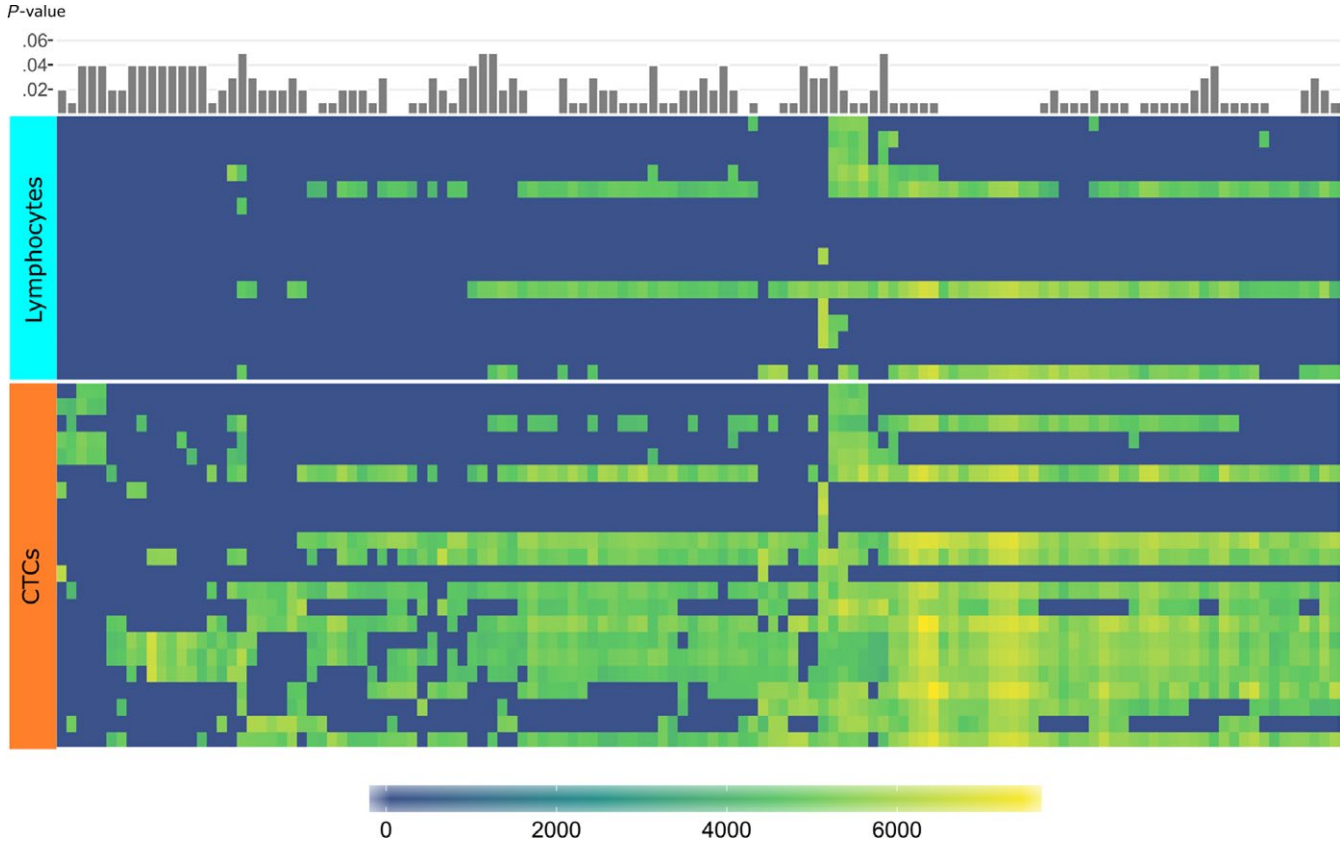


**FIGURE 3** Heatmap of significant peaks found in all collected circulating tumor cells (CTCs) in comparison with lymphocytes. The *P* value of each annotated peak is shown above the figure

discerned between CTCs originating from different cancer types. To investigate this further, we compared the CTCs obtained from GC patients to those obtained from CRC patients to visualize the unique cellular profiles of each group.

To this end, supervised PCA-DA was carried out on GC and CRC CTCs as well as the blank. Despite the heterogeneity caused by samples obtained from different patients, significant clustering could still be observed where CTCs appeared to cluster into 2 distinct groups corresponding to their cancer type, as shown in Figure 4A, suggesting the potential of CTC metabolome characterization as a future tool for cancer diagnosis.

On further inspection of the data, a trend was noticed in the frequency of peaks distributed along the *m/z* scale, especially in the case of GC, in which a higher incidence of high *m/z* peaks was detected. This was indicated by comparing the histograms of the average spectra of GC CTCs vs those of CRC, as shown in Figure 4B. As most metabolites have relatively low molecular weight, the increased incidence of relatively high molecular weight peaks in GC CTCs suggest a distinctive metabolic "fingerprint" of this cancer that most likely involves a higher distribution of lipids, which can be used in the future as a biomarker for GC.

### 3.4 | Possible biomarkers for CRC and GC CTCs

To identify possible metabolites or lipids that are unique to GC or CRC CTCs, Welch's *t* test was applied to the 2 groups (n = 9 and n = 13, respectively). In total, 155 significant peaks were extracted according to their log2-based fold change (more than 1 or less than −1 log2 fold change) and $P < .05$ (Figures 5A and S5). Among those peaks, 69 were present in GC CTCs, whereas 86 peaks were suggested to be specific to CRC CTCs. A histogram of the *m/z* distribution of potential biomarkers to each cancer type is shown in Figure 5B. A summary with the putatively identified metabolites and lipids unique to GC CTCs and CRC CTCs with *P* value, %RSD, chemical formula, and class for each compound is shown in Tables S2 and S3, respectively.

Among the statistically significant peaks found, acyl carnitine metabolites as well as sterol lipids were elevated in CRC. Furthermore, eicosanoids were also observed to be more abundant in CRC CTCs, which is further corroborated by other studies done on this cancer type.[47] Glycerophospholipids were noticed to be elevated in GC CTCs, which also concurs with recent reports.[48]

## 4 | DISCUSSION

Previous studies in genomics,[12] transcriptomics,[49] and proteomics[50] succeeded in depicting the effective role of CTC analyses in monitoring tumor progression and prognosis as well as its clinical impact. As metabolomics is the final frontier of omics, it is essential

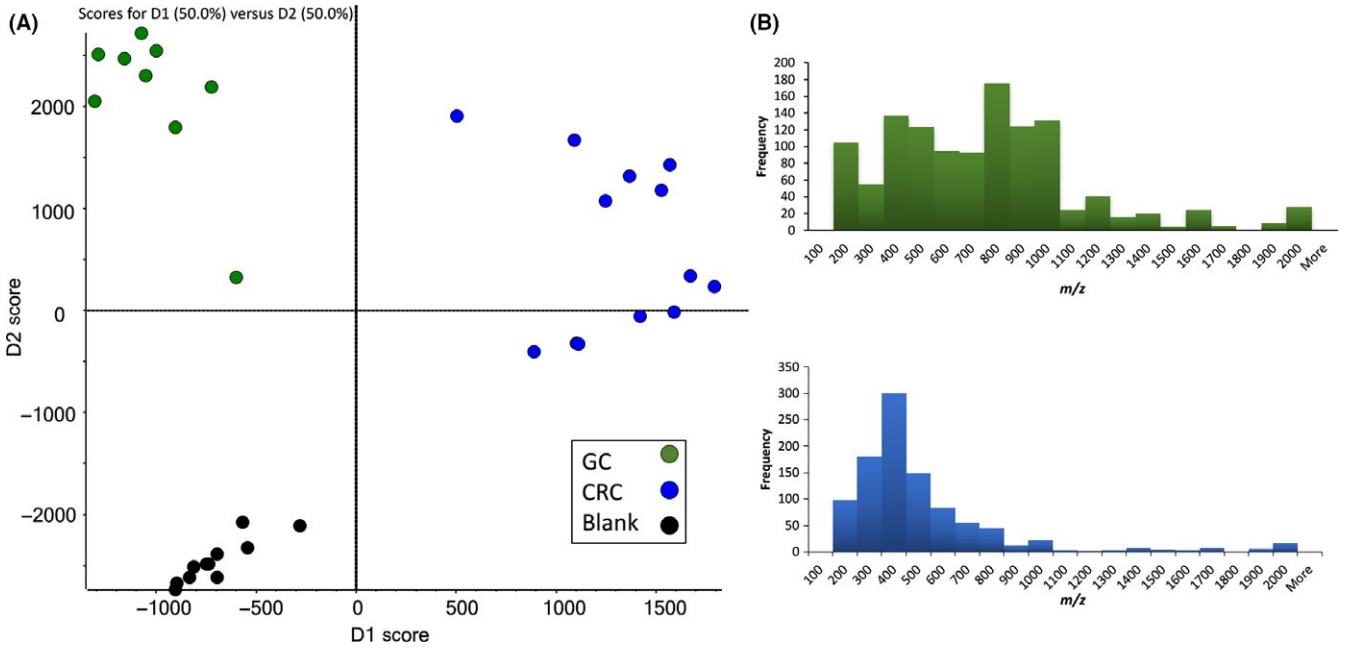


**FIGURE 4** Single cell profiling of gastric cancer (GC) and colorectal cancer (CRC) circulating tumor cells (CTCs). A, Principle component analysis-discriminant analysis discriminating between GC CTCs, CRC CTCs, and blank. Each dot corresponds to a single cell. B, Histogram of the frequency of peak distribution across the *m/z* scale of different cancer types

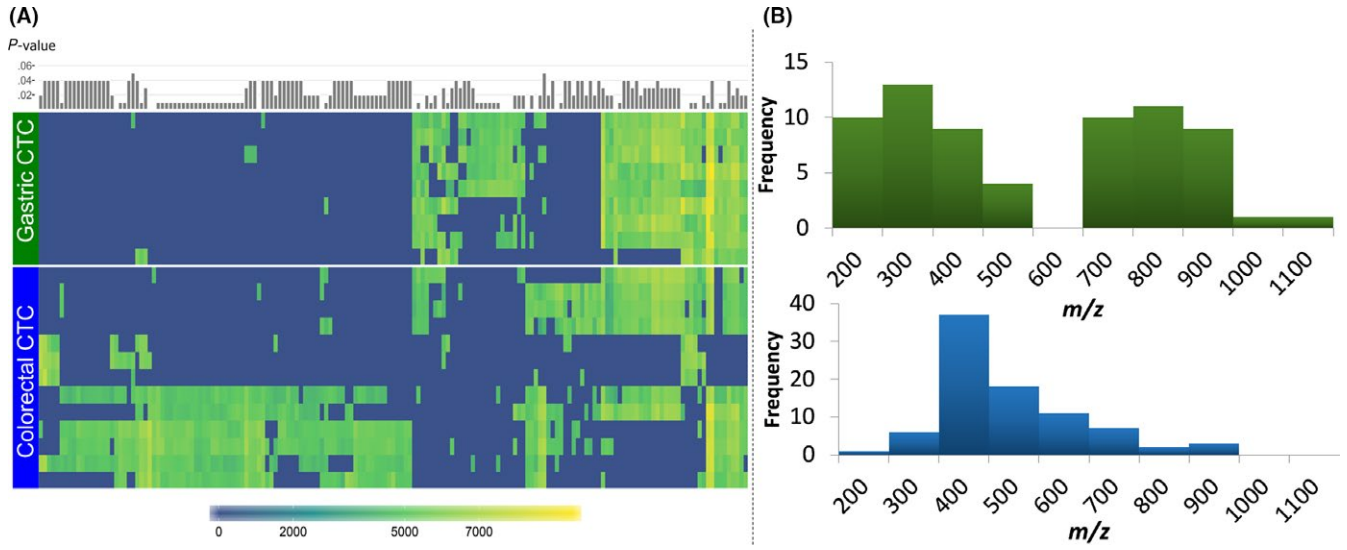


**FIGURE 5** Characterization of significant peaks found in both gastric cancer and colorectal cancer circulating tumor cells (CTCs). A, Heatmap of significant peaks found in both gastric cancer and colorectal cancer CTCs. The *P* value of each annotated peak is shown above the figure. B, Histogram showing the frequency of the unique peaks (potential biomarkers) to each cancer type distribution across the *m/z* scale of the two cancer types

to study the metabolic phenotypes of CTCs to gain a comprehensive understanding on the primary and metastatic tumor biology. However, metabolomics still faces several challenges arising from its inherent complexity and variability, especially on the single cell level. As a result, dimensional reduction techniques followed by DA are required to simplify obtained data and reveal significant biological meaning. This could possibly be achieved by using PCA followed by DA, a process we term PCA-DA. Principle component analysis provides unsupervised dimensionality reduction that simplifies the data and enhances its visualization,[51] whereas DA highlights the metabolomic differences (ie, metabolic fingerprint) among different cell types originating from different cancer phenotypes.[52] Nonetheless, there are limitations associated with DA as it can provide overly optimistic results, mainly due to the lack of suitable statistical validation and use by nonexperts without considering its potential pitfalls.[53]

In this paper, our results revealed the metabolic differences between CTCs and lymphocytes derived from the same patients (Figure 2). Additionally, PCA-DA showed clear clustering behavior between CTCs and lymphocytes per cancer, which gives credence to the theory that, despite cellular heterogeneity, overall metabolomic differences between cancer cells and control cells are evident, even on a single-cell scale. Furthermore, the metabolic difference among different cancer types (GC and CRC) was depicted in Figure 4A, where CTCs clustered into 2 distinct groups corresponding to their cancer type, suggesting the potential of CTC metabolome characterization as a future tool for cancer diagnosis. The difference in phenotype was further confirmed by a histogram, showing the discrepancy in the frequency of peak distribution across the *m/z* scale of different cancer types (Figure 4B).

The important role of lipids' synthesis and metabolism in tumorigenesis and tumor progression has been showcased in previous studies.[40,54-56] Hence, targeting specific lipids or blocking certain pathways is a promising therapeutic strategy for cancer treatment.[57] Accordingly, we focused in our study on detecting mainly lipids to explore their role in both cancer types (GC and CRC). This was done by optimizing and tuning our mass spectrometry method to focus on the higher *m/z* ranges and including DMSO in our ionization solvent. Dimethyl sulfoxide is known to improve the overall ionization performance of lipids in MS.[58] In addition, applying ultrasonication on the capillaries containing single cells proved to improve exhaustiveness and lipid coverage.[21] Consequently, most of the potential biomarkers detected in this study were mainly lipids, such as GPLs, FAs, acyl carnitines, SLs, and prenol lipids. In addition, different abundance and distribution of lipid classes was observed in each cancer type (Figure S7). In CRC CTCs, SLs were highly elevated compared to other lipid classes. Previous studies showed the correlation between high levels of SLs and distant metastasis in CRC patients,[59] which is consistent with our study subjects. Moreover, as chronic inflammation is associated with the development of CRC, we expected to detect eicosanoids in relatively high levels. Eicosanoids are believed to affect CRC development and progression by inflammation induction, regulation of cellular oxidative stress, and alteration of membrane dynamics.[60] Furthermore, low levels of FAs and GPLs were detected in CRC CTCs. This could be due to the increased catabolism of GPLs to FAs followed by the subsequent degradation of FAs during the β-oxidation process.[61,62] As β-oxidation usually takes place in the mitochondria, FAs then bind to acylcarnitines (carriers) that transport FAs to the mitochondria. Therefore, higher levels of acylcarnitines are thought to be specific to CRC (in contrast to GC),[63] which is supported by our results.

In GC CTCs, FAs and GPLs were pre-eminent. This is probably due to the increased de novo synthesis and elongation of FAs and GPLs.[64-67] The synthesis of GPLs was proved to be a key factor in cancer proliferation as it is mainly responsible for membrane and energy production.[68] Because GPL synthesis requires acetyl-CoA, and citrate is an acetyl-CoA donor for this process, higher lipid metabolism can be associated with elevated tricarboxylic acid cycle activity in GC.[69,70] As previously mentioned, GC showed overall higher levels of FAs than CRC. Specifically, saturated fatty acids were found to be relatively elevated in comparison to monounsaturated fatty acids and polyunsaturated fatty acids.[71] Recent studies have reported that saturated fatty acids can act as a source of energy in tumor cells, enhancing tumor proliferation and survival, and might also affect tumor resistance to treatment.[72,73] Numerous metabolites/lipids were detected to be common in all CTC samples vs lymphocytes. Chief among them are several GPLs that are believed to have biological significance in cancer physiology, including PC(32:1), PC(34:1), PS(38:5), PE(38:6), PC(32:3), and PC(34:2).[5,74] Detailed information about identified lipids and metabolites for each cancer group are shown in Tables S1-S3. As a result, lipid profiling of CTCs could be used for the diagnosis and prognosis of CRC and GC as well as identifying novel targets for their treatment. However, the specific impact of lipids on tumor development, progression, and metastasis is not yet fully understood,[75] which highlights the need for precise monitoring of any alterations in lipid metabolism in cancer cells. Along with our results, this presents a new avenue for the diagnostics and treatment of cancer.

Previous studies utilized human serum and plasma to undertake untargeted analysis and discriminate between different cancer types.[76-79] However, to the best of our knowledge, our study is the first to achieve this utilizing CTCs at the single-cell level. In addition to the previously mentioned advantages of CTC molecular profiling, our method can be used to investigate new potential biomarkers on the single-cell level that could not be detected using conventional methods, highlighting the novelty of this method in cancer research. Nevertheless, it is worth noting that the aim of this paper is not to provide a be all and end all definitive list of biomarkers that can be used in clinical diagnosis today. Instead, we aim to highlight the untapped potential of CTC molecular characterization in both clinical and research settings using LSC-MS. Moreover, despite using exact mass with a high degree of accuracy (<5 ppm) in this paper for annotation, additional verification techniques must be used, such as tandem MS, capillary electrophoresis, or HPLC for positive compound identification. However, performing tandem MS on signals obtained from a single cell in a robust manner is still a challenging task that needs to be addressed with improvements in the instrumentation itself or by incorporating an enrichment step before analysis.

In summary, untargeted analysis of human derived CTCs at the single-cell level was undertaken for the first time utilizing LSC-MS. Circulating tumor cells and lymphocytes obtained from the same patient could be successfully discriminated as well as CTCs of different cancer types. In addition, the possible role played by the distribution of lipids and higher molecular weight compounds at the single cell level in classifying different cancer types based on their "metabolic fingerprint" was highlighted. Several promising biomarkers were putatively annotated that are mostly specific to GC CTCs, CRC CTCs, and CTCs in general. Finally, due to the scarcity of human-derived samples, especially in inherently rare cells such as CTCs, we hope that this paper and its results spur much-needed collaborative efforts to upscale CTC characterization to established large-scale studies that will possibly aid in clinical applications in the near future.

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## CONFLICT OF INTEREST

Chwee Teck Lim is a shareholder and has a financial interest in Clearbridge Biomedics. The author authors have no conflict of interest.

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## REFERENCES

1. Fisher R, Pusztai L, Swanton C. Cancer heterogeneity: implications for targeted therapeutics. *Br J Cancer*. 2013;108:479-485.
2. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. *Nat Rev Clin Oncol*. 2018;15:81-94.
3. Alizadeh AA, Aranda V, Bardelli A, et al. Toward understanding and exploiting tumor heterogeneity. *Nat Med*. 2015;21:846-853.
4. Karachaliou N, Mayo-de-Las-Casas C, Molina-Vila MA, et al. Real-time liquid biopsies become a reality in cancer treatment. *Ann Transl Med*. 2015;3:36.
5. Hofman P. Liquid biopsy for early detection of lung cancer. *Curr Opin Oncol*. 2017;29:73-78.
6. Pantel K. Blood-based analysis of circulating cell-free DNA and tumor cells for early cancer detection. *PLoS Med*. 2016;13:e1002205.
7. Yőrűker EE, Holdenrieder S, Gezer U. Blood-based biomarkers for diagnosis, prognosis and treatment of colorectal cancer. *Clin Chim Acta*. 2016;455:26-32.
8. Ilié M, Hofman P. Pros: Can tissue biopsy be replaced by liquid biopsy? *Transl Lung Cancer Res*. 2016;5:420-423.
9. Zhou L, Dicker DT, Matthew E, et al. Circulating tumor cells: silent predictors of metastasis. *F1000Res*. 2017;6:F1000. Faculty Rev-1445.
10. Jie XX, Zhang XY, Xu CJ. Epithelial-to-mesenchymal transition, circulating tumor cells and cancer metastasis: mechanisms and clinical applications. *Oncotarget*. 2017;8:81558-81571.
11. Toss A, Mu Z, Fernandez S, et al. CTC enumeration and characterization: moving toward personalized medicine. *Ann Transl Med*. 2014;2:108.
12. Zhu Z, Qiu S, Shao K, et al. Progress and challenges of sequencing and analyzing circulating tumor cells. *Cell Biol Toxicol*. 2018;34:405-415.
13. Krebs MG, Hou JM, Ward TH, et al. Circulating tumour cells: their utility in cancer management and predicting outcomes. *Ther Adv Med Oncol*. 2010;2:351-365.
14. Maas M, Hegemann M, Rausch S, et al. Circulating tumor cells and their role in prostate cancer. *Asian J Androl*. 2017;19:1-8.
15. Gallo M, De Luca A, Maiello MR, et al. Clinical utility of circulating tumor cells in patients with non-small-cell lung cancer. *Transl Lung Cancer Res*. 2017;6:486-498.
16. Polasik A, Tzschaschel M, Schochter F, et al. Circulating tumour cells, circulating tumour DNA and circulating microrna in metastatic breast carcinoma - what is the role of liquid biopsy in breast cancer? *Geburtshilfe Frauenheilkd*. 2017;77:1291-1298.
17. Kong SL, Liu X, Suhaimi NM, et al. Molecular characterization of circulating colorectal tumor cells defines genetic signatures for individualized cancer care. *Oncotarget*. 2017;8:68026-68037.
18. Gold B, Cankovic M, Furtado LV, et al. Do circulating tumor cells, exosomes, and circulating tumor nucleic acids have clinical utility? A report of the association for molecular pathology *J Mol Diagn*. 2015;17:209-224.
19. Galletti G, Portella L, Tagawa ST, et al. Circulating tumor cells in prostate cancer diagnosis and monitoring: an appraisal of clinical potential. *Mol Diagn Ther*. 2014;18:389-402.
20. Millner LM, Linder MW, Valdes R. Circulating tumor cells: a review of present methods and the need to identify heterogeneous phenotypes. *Ann Clin Lab Sci*. 2013;43:295-304.
21. Hiyama E, Ali A, Amer S, et al. Direct lipido-metabolomics of single floating cells for analysis of circulating tumor cells by live single-cell mass spectrometry. *Anal Sci*. 2015;31:1215-1217.
22. Hou HW, Warkiani ME, Khoo BL, et al. Isolation and retrieval of circulating tumor cells using centrifugal forces. *Sci Rep*. 2013;3:1259.
23. Masujima T. Live single-cell mass spectrometry. *Anal Sci*. 2009;25:953-960.
24. Fujii T, Matsuda S, Tejedor ML, et al. Direct metabolomics for plant cells by live single-cell mass spectrometry. *Nat Protoc*. 2015;10:1445-1456.
25. Ali A, Abouleila Y, Amer S, et al. Quantitative live single-cell mass spectrometry with spatial evaluation by three-dimensional holographic and tomographic laser microscopy. *Anal Sci*. 2016;32:125-127.
26. Zhong H, Fang C, Fan Y, et al. Lipidomic profiling reveals distinct differences in plasma lipid composition in healthy, prediabetic, and type 2 diabetic individuals. *Gigascience*. 2017;6:1-12.
27. Yanes O, Tautenhahn R, Patti GJ, et al. Expanding coverage of the metabolome for global metabolite profiling. *Anal Chem*. 2011;83:2152-2161.
28. Murphy RC, Axelsen PH. Mass spectrometric analysis of long-chain lipids. *Mass Spectrom Rev*. 2011;30:579-599.
29. Wang C, Wang M, Han X. Applications of mass spectrometry for cellular lipid analysis. *Mol BioSyst*. 2015;11:698-713.
30. Kind T, Fiehn O. Advances in structure elucidation of small molecules using mass spectrometry. *Bioanal Rev*. 2010;2:23-60.
31. Southam AD, Payne TG, Cooper HJ, et al. Dynamic range and mass accuracy of wide-scan direct infusion nanoelectrospray fourier transform ion cyclotron resonance mass spectrometry-based metabolomics increased by the spectral stitching method. *Anal Chem*. 2007;79:4595-4602.
32. Gibb S, Strimmer K. MALDIquant: a versatile r package for the analysis of mass spectrometry data. *Bioinformatics*. 2012;28:2270-2271.
33. Goodacre R, Broadhurst D, Smilde AK, et al. Proposed minimum reporting standards for data analysis in metabolomics. *Metabolomics*. 2007;3:231-241.
34. Kanehisa M, Goto S, Hattori M, et al. From genomics to chemical genomics: new developments in KEGG. *Nucleic Acids Res*. 2006;34:D354-D357.
35. Wishart DS, Feunang YD, Marcu A, et al. HMDB 4.0: the human metabolome database for 2018. *Nucleic Acids Res*. 2018;46:D608-D617.
36. Sud M, Fahy E, Cotter D, et al. LMSD: LIPID MAPS structure database. *Nucleic Acids Res*. 2007;35:D527-D532.
37. Liebisch G, Vizcaino JA, Kofeler H, et al. Shorthand notation for lipid structures derived from mass spectrometry. *J Lipid Res*. 2013;54:1523-1530.
38. Griffin JL, Shockcor JP. Metabolic profiles of cancer cells. *Nat Rev Cancer*. 2004;4:551-561.

39. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer*. 2011;11:325-337.
40. Santos CR, Schulze A. Lipid metabolism in cancer. *FEBS J*. 2012;279:2610-2623.
41. Kim HY, Lee KM, Kim SH, et al. Comparative metabolic and lipidomic profiling of human breast cancer cells with different metastatic potentials. *Oncotarget*. 2016;7:67111-67128.
42. Boroughs LK, DeBerardinis RJ. Metabolic pathways promoting cancer cell survival and growth. *Nat Cell Biol*. 2015;17:351-359.
43. Brown DG, Rao S, Weir TL, et al. Metabolomics and metabolic pathway networks from human colorectal cancers, adjacent mucosa, and stool. *Cancer Metab*. 2016;4:11.
44. Jacob K, Sollier C, Jabado N. Circulating tumor cells: detection, molecular profiling and future prospects. *Expert Rev Proteomics*. 2007;4:741-756.
45. Polzer B, Medoro G, Pasch S, et al. Molecular profiling of single circulating tumor cells with diagnostic intention. *EMBO Mol Med*. 2014;6:1371-1386.
46. Aktas B, Kasimir-Bauer S, Heubner M, et al. Molecular profiling and prognostic relevance of circulating tumor cells in the blood of ovarian cancer patients at primary diagnosis and after platinum-based chemotherapy. *Int J Gynecol Cancer*. 2011;21:822-830.
47. Xu J, Chen Y, Zhang R, et al. Global metabolomics reveals potential urinary biomarkers of esophageal squamous cell carcinoma for diagnosis and staging. *Sci Rep*. 2016;6:35010.
48. Lario S, Ramírez-Lázaro MJ, Sanjuan-Herráez D, et al. Plasma sample based analysis of gastric cancer progression using targeted metabolomics. *Sci Rep*. 2017;7:17774.
49. Welty CJ, Coleman I, Coleman R, et al. Single cell transcriptomic analysis of prostate cancer cells. *BMC Mol Biol*. 2013;14:6.
50. Sinkala E, Sollier-Christen E, Renier C, et al. Profiling protein expression in circulating tumour cells using microfluidic Western blotting. *Nat Commun*. 2017;8:14622.
51. Worley B, Powers R. Multivariate analysis in metabolomics. *Curr Metabolomics*. 2013;1:92-107.
52. Jombart T, Devillard S, Balloux F. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genet*. 2010;11:94.
53. Gromski PS, Muhamadali H, Ellis DI, et al. A tutorial review: metabolomics and partial least squares-discriminant analysis–a marriage of convenience or a shotgun wedding. *Anal Chim Acta*. 2015;879:10-23.
54. Beloribi-Djefaflia S, Vasseur S, Guillaumond F. Lipid metabolic reprogramming in cancer cells. *Oncogenesis*. 2016;5:e189.
55. Corbet C, Feron O. Emerging roles of lipid metabolism in cancer progression. *Curr Opin Clin Nutr Metab Care*. 2017;20:254-260.
56. Ray U, Roy SS. Aberrant lipid metabolism in cancer cells - the role of oncolipid-activated signaling. *FEBS J*. 2018;285:432-443.
57. Liu Q, Luo Q, Halim A, et al. Targeting lipid metabolism of cancer cells: a promising therapeutic strategy for cancer. *Cancer Lett*. 2017;401:39-45.
58. Szabó PT, Kele Z. Electrospray mass spectrometry of hydrophobic compounds using dimethyl sulfoxide and dimethylformamide as solvents. *Rapid Commun Mass Spectrom*. 2001;15:2415-2419.
59. Yan G, Li L, Zhu B, et al. Lipidome in colorectal cancer. *Oncotarget*. 2016;7:33429-33439.
60. Tuncer S, Banerjee S. Eicosanoid pathway in colorectal cancer: recent updates. *World J Gastroenterol*. 2015;21:11748-11766.
61. Halama A, Guerrouahen BS, Pasquier J, et al. Metabolic signatures differentiate ovarian from colon cancer cell lines. *J Transl Med*. 2015;13:223.
62. Schooneman MG, Vaz FM, Houten M, et al. Acylcarnitines. *Diabetes*. 2013;62:1-8.
63. Jung J, Jung Y, Bang EJ, et al. Noninvasive diagnosis and evaluation of curative surgery for gastric cancer by using NMR-based metabolomic profiling. *Ann Surg Oncol*. 2014;21:S736-S742.
64. Huang C, Freter C. Lipid metabolism, apoptosis and cancer therapy. *Int J Mol Sci*. 2015;16:924-949.
65. Xiao S, Zhou L. Gastric cancer: metabolic and metabolomics perspectives (Review). *Int J Oncol*. 2017;51:5-17.
66. Kusakabe T, Nashimoto A, Honma K, Suzuki T. Fatty acid synthase is highly expressed in carcinoma, adenoma and in regenerative epithelium and intestinal metaplasia of the stomach. *Histopathology*. 2002;40:71-79.
67. Perrotti F, Rosa C, Cicalini I, et al. Advances in lipidomics for cancer biomarkers discovery. *Int J Mol Sci*. 2016;17:E1992.
68. Dolce V, Cappello AR, Lappano R, et al. Glycerophospholipid synthesis as a novel drug target against cancer. *Curr Mol Pharmacol*. 2011;4:167-175.
69. Aa J, Yu L, Sun M, et al. Metabolic features of the tumor microenvironment of gastric cancer and the link to the systemic macroenvironment. *Metabolomics*. 2012;8:164-173.
70. Wise DR, Ward PS, Shay JES, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of α-ketoglutarate to citrate to support cell growth and viability. *Proc Natl Acad Sci USA*. 2011;108:19611-19616.
71. Song H, Wang L, Liu H-L, et al. Tissue metabolomic fingerprinting reveals metabolic disorders associated with human gastric cancer morbidity. *Oncol Rep*. 2011;26:431-438.
72. Forman MR, Mahabir S. Saturated fatty acids and cancer. In: Milner JA, Romagnolo DF, eds. *Bioactive Compounds and Cancer. Nutrition and Health*. Totowa, NJ:Humana Press;2010:213-233.
73. Ventura R, Mordec K, Waszczuk J, et al. Inhibition of de novo palmitate synthesis by fatty acid synthase induces apoptosis in tumor cells by remodeling cell membranes, inhibiting signaling pathways, and reprogramming gene expression. *EBioMedicine*. 2015;2:808-824.
74. Chen X, Chen H, Dai M, et al. Plasma lipidomics profiling identified lipid biomarkers in distinguishing early-stage breast cancer from benign lesions. *Oncotarget*. 2016;7:36622-36631.
75. Baenke F, Peck B, Miess H, et al. Hooked on fat: the role of lipid synthesis in cancer metabolism and tumour development. *Dis Model Mech*. 2013;6:1353-1363.
76. Yang L, Cui X, Zhang N, et al. Comprehensive lipid profiling of plasma in patients with benign breast tumor and breast cancer reveals novel biomarkers. *Anal Bioanal Chem*. 2015;407:5065-5077.
77. Shen S, Yang L, Li L, et al. A plasma lipidomics strategy reveals perturbed lipid metabolic pathways and potential lipid biomarkers of human colorectal cancer. *J Chromatogr B*. 2017;1068–1069:41-48.
78. Del Boccio P, Perrotti F, Rossi C, et al. Serum lipidomic study reveals potential early biomarkers for predicting response to chemoradiation therapy in advanced rectal cancer: a pilot study. *Adv Radiat Oncol*. 2017;2:118-124.
79. Jinping Gu, Xiaomin Hu, Shao Wei, et al. Metabolomic analysis reveals altered metabolic pathways in a rat model of gastric carcinogenesis. *Oncotarget*. 2016;7:60053-60073.


## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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