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Biomarkers of Breast Cancer Cell Lines A; Pilot Study on Human Breast Cancer Metabolomics

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Abstract

Metabolism of a cancer cell is significantly different from that of a normal cell. Therefore the metabolites of a breast cancer cell are also different from the metabolites of a normal breast epithelial cell and identification of the altered metabolites in body fluids may help us identify the presense of cancer in the body. Metabolites produced by breast cancer cells can serve as potential biomarkers of breast cancer.

In this study the breast cancer cell lines were cultured and the metabolites released into the culture media were obtained into three extracts. Liquid chromatography/mass spectrometry were then used to separate and analyze these metabolites. Data sets produced were aligned by mass lynx software and subsequently subjected to multivariate statistical analysis. Metabolites present in higher amounts in breast cancer cell lines were identified by comparison with the known masses in databases like METLIN. The fold rise of metabolites in breast cancer cell lines compared to the non tumourigenic cell line was used to identify the potential biomarkers of breast cancer cell lines. It was found that there are more than eighty metabolites which can be regarded as biomarkers of cancer cell lines and there are six metabolites which can be regarded as specific potential biomarkers of breast cancer cell lines.

Introduction

Breast cancer is the most common cancer among women in the U.K(1). Every year more than 40000 women in the UK develop breast cancer and nearly 10000 die because of this disease(2). To detect breast cancer at such a stage where medical intervention can alter mortality and morbidity statistics of this condition, we need to identify some measurable substance in the body that is specifically associated with breast cancer. Such a substance if identified would serve as a marker of breast cancer. Its presense or rise in the normal value (if present under normal conditions) would mean breast cancer is present in the body. Such substances often called biomarkers of cancer are usually products of metabolism of tumour cells(3). Although it has been known for quite a long time now that tumour cell metabolism is different from the metabolism of normal cell, the altered metabolites of breast cancer cells have not yet been utilized as metabolic biomarkers for breast cancer screening. It would be extremely useful to identify the biomarkers of breast cancer in body fluids as they would provide non biopsy tests which would be highly sensitive and specific for this condition. Identification of the altered metabolites originating from breast cancer cell lines that differ significantly from the metabolites of non tumourigenic breast epithelial cell line was the focus of this study.

Aims & Objectives

The overall aim was to identify classes of metabolites that are associated with the development of breast tumour phenotype in the cell lines. The specific aims were

a) To develop sample extraction methods in order to profile a wide range of metabolites in cultured breast epithelial cell lines.
b) To use liquid chromatography/mass spectrometry and multivariate statistical methods to determine how metabolite profiles differ between normal and tumourigenic breast cell lines.

Materials And Methods

Cell Lines: Three breast cell lines were used; MCF-7, an early stage breast cell line obtained from Cell Line Services (CLS), Germany.

MDA-MB-231, an invasive human breast cancer cell line obtained from Cell Line Services (CLS) ,Germany.

MCF-10A, a non tumourigenic breast epithelial cell line obtained from American Type Culture Collection, USA used for comparison.

Cell Culture Media: The breast cell lines were cultured in the DMEM/F12 medium (Invitrogen Cat no 21331020). It was supplemented with fetal bovine serum, pencillin, streptomycin, glut amine, epidermal growth factor, hydrocortisone, cholera toxin and

bovine insulin. After being purchased the breast cell lines were maintained in an incubator at 37° C with 5% CO2 & splited 2-3 times/week. The culture media with supplements was put in the six well culture plates. 3ml of a cell line (having 2.0×10>cells/ml) was placed in the walls of these culture plates. Thus we had many replicates of each cell line. These culture plates were kept in the incubator overnight and thus the three cell lines got cultured under similar conditions. Metabolites produced by the cell lines during culture were expected to be present in the culture media. Therefore only the supernatant from the culture media was taken in tubes that were labeled as the replicate numbers of the cell lines. Some tubes were kept as controls and these had media that was not in contact with cell cultures. All the tubes were stored at -80°C.

Solid Phase Extraction (SPE)

These method was used for concentrating metabolites from the media in the tubes. 0.5ml methanol had been added to the tubes. SPE method involved loading the sample solutionon to SPE phase, wash away undesired components and then washing off the desired analytes with another solvent into a collection tube. For all extractions we made an internal standard mixture of stable isotopes. In 100µl ethanol we added 1ong each of d4 estradiol, d4 estradiol sulfate and d9progesterone. The stock solution of 1µg/ml concentration was kept at -20°C. The tubes were taken out from the 80°C freezer and thawed. 5ml of media from the replicates were taken each time. Then the tubes were vortexed and centrifuged. The top 2ml of the supernatent were placed in labelled glass tubes. 2ml of 2% formic acid in water was added to buffer them. 100µl of internal standard was added and tubes were vortexed. The cartridges used for SPE were strata X-AW 60mg/3ml manufac tured by Phenomenex. The sorbent lot number used was S308-19. For conditioning the cartridges 3ml ethyl acetate, 3ml methanol and 3ml 2% formic acid in water was used. Then the sample was applied to SPE. 3ml deionised pure water was used for washing each tube and the tubes were then dried for 15 minutes. One set of tubes was put under SPE, eluted with 3ml ethyl acetate and labelled ETAC n. Second set of tubes was put under SPE eluted with MEOH and labelled MEOHn. Third set of tubes was put under SPE eluted with ammonium hydroxide in methanol and labelled AMMn. N indicated the replicate no.of the cellline. The samples were stored at -20C overnight. ETAC extract of the media was expected to contain neutral or lipophilic metabolites (eg- steroids, nucleosides, fatty acids, phospholipids) MEOH extract of the media was expected to contain more polar neutral molecules, prostaglandins and phospholipids. AMM fraction was expected to contain conjugated anionic 3metabolites including organic acids. Ultra performance liquid chromatography electrospray ionisation time of flight mass spectroscopy [UPLC-ESI-T OF MS] Chromatographic separation was performed using a Waters ACQUITY UPLC™ system (Waters Corp., Milford, USA), equipped with a binary solvent delivery system and an auto-sampler. A Waters 100mm × 2.1 mm ACQUITY C18 1.7 µm column was used to separate the endogenous metabolites. The mobile phase consisted of SOLVENT (A) 0.2% formic acid in water and 5% acetonitrile in water(B) 100% acetonitrile and 0.2% formic acid in water. The following gradient program was used for the MS analysis in positive mode: 0-15.0 min from 0.0 to 100% B then held in 100% B for 10 mins. In negative ESI mode the same gradient program was used. Analytes were detected with a Micromass (Waters,Manchester, UK) TO F-MS system with an ESI source operated in either negative or positive mode. Capillary voltage was set at 2.60kV in positive mode and at between -2.60V and -2.75V in negative mode. Argon was used as collision gas at TOF penning pressures of 274.83 × 10^-7 to 5×10^-7 mbar. Collision energy was set at 10 eV to avoid fragmentation of the analytes. Sulfadimethoxine (5pg/µl in methanol/water, 1:1, v/v, plus, in positive mode only, 0.1% formic acid) was used as internal lock mass infused at 40µl/min via a lockspray interface (baffling frequency;0.2 s^-1) to ensure accurate mass measurement. The internal lockmass m/z ratios were 311.0814 and 309.0658 in positive and negative mode respectively. Source temperature was 1000C and desolvation temperature was 3000C. The nebulising and desolvation nitrogen flows were maintained at 100 and 400 l/h respectively. The mass spectrometer was calibrated with sodium iodide and the spectra were collected in full scan mode from 100 to 1000 m/z. Full scan mass spectra of the range of metabolites were recorded in positive and negative modes in order to select the most abundant m/z ion. Thetheoretical parent ions for each metabolite were calculated from the atomic mass of the most abundant isotope of each element by using the Molecular Weight Calculator software (Mass Lynx 4.1 Software). The ETAC & MEOH extracts of samples were analysed in +ESI & AMM extracts were analyzed in– ESI mode. Mass Lynx Software: Mass Lynx Software was used for MS analyses. Extraction of the spectral peaks from the raw data and then chromatogram alignment were carried out automatically by using Marker lynx v 4.1 softwarepackage [waters corporation, Milford, MA,

USA] The parameters used for detecting the spectral peaks were optimised to minimize noise level of the detected signal. The parameters used were; mass accuracy of the acquired data[mass tolerance]:0.05 Da ; width of an average peak at 5% height: 5s; baseline noise between the peaks [peak to peak baseline noise]:100; number of masses per RT submitted to the collection algorithm:50 minimum intensity allowed for a spectral peak to be defined as a marker: 1% of the base peak intensity [BPI]. The BPI chromatograms were used to calculate the presense of internal standard in this study (Figure 1)

The mass lynx software provided base peak intensity (BPI) chromatograms of the samples. The following figure illustrates BPI chromatograms of the media(M)samples analyzed in +ESI mode.

See Illustration 1

Figure 1: The BPI chromatograms of these media samples show similar features as expected since none of these samples have any breast cells. Some differences can however be noted. It was expected that the chromatograms within a class (eg MCF10A) in any extract (eg methanol) should be similar. Infact the BPI chromatograms were very similar to each other as expected although some differences were also there. The differences could either be due to biological variability in the samples or due to the variability in extraction of the samples. The variation in extraction was revealed by the difference in the content of internal standards (calculated from their BPI chromatograms) in the samples of the same class.

Multivariate Analyses Of The Metabolomic Data: Data was the nexported to SIMCA-P software [Umetrics UK ltd, Winkfield,Windsor Berkshire,UK] for analysis. Before the multivariate analysis, dataa recentred pare to scaled and log transformed in order to optimise data and limit skewness. Each dataset comprised an SPE fraction of the control and samples. Initially, principal component analysis [PCA] was performed to obtain an overview of the data and to identify outliers. Data was then subjected to projections using PLS-DA to find class– separating differences [variables] in pairwise comparisons of the treatments. Finally, OPLS-DA was performed with the data to filter the information that was only due to class separation. Cross validation CV, default parameters) was used to determine the significant components of the models and thus minimise overfitting. The performance of the models was then described by the explained variation [R2X for PCA and OPLS-DA and R2Y for PLS-DA and OPLS-DA] and predictive ability [Q2] parameters of the models { WORK FLOW illustrated in Figure 2}

The following figures represent the models obtained by the SIMCA software after the mass lynx data analysis. In the models the three classes indicate the three cell lines. The blue dots represent the MDA-MB-231 cell line replicates, the red dots represent the MCF-7 cell line replicates and MCF 10A cell line replicates are represented by black dots. The model characteristics are given in the tables following the figures.

See Illustration 2

Figure 1a: shows the PCA model (labelled M21 in the study) of MEOH extracts of the three cell line replicates & it can be seen that classes are not grouped together

See Illustration 3

Figure 2b: shows three dimensional view of the same M21 model. The characteristics of this PCA- model M21 are shown in the table below.

See Illustration 4

Figure 2a: shows the PLS-DA model(M23) of the earlier shown PCA model, here class separation is much better

See Illustration 5

Figure 2b: shows the three dimensional view of the above figure The class separation is evident. This PLS-DA model M23 had the following characteristics:

See Illustration 6

Figure 3: Indicates the OPLS model of the same data set from which the preceding PCA & PLS-DA models were obtained. The cell line MCF 10A (black) replicates & cell line MCF-7 (red) replicates are distinctly separate.

See illustration 7

Figure 4: Shows the S-plot obtained from the OPLS model in the previous figure. The lower end of the S-plot has the masses of metabolites which are significantly produced in higher amounts by the cancer cell line MCF-7 replicates as compared to the MCF-10A non tumourigenic cell line replicates.

Six S-plots were obtained during this study. Three could be used to compare the amount of metabolites produced differentially by MCF-7 cell line replicates and MCF-10A cell line replicates in the three different extracts.

The other three S-plots were used to compare the amount of metabolites produced differentially by MDA-MB-231 cell line replicates and MCF-10A cell line replicates in the three different extracts.

After noting the metabolite masses at the extreme ends of the S-plots, comparisons could be made between their presense in the breast cancer cell line sample replicates against the control i.e MCF 10A cell line replicates.

Compounds that were totally absent in the normal cell line but present in significant amount in the cancer cell line were selected as potential biomarkers

Their identity could be established by comparing them with the masses of known compounds in the known data bases.

Results

After noting the metabolites (m/z i.e. mass/charge ratios) at the extreme ends of the S-plots, comparisons could be made between theirpresense in the breast cancer cell line sample replicates against the control i-eMCF 10A cell line replicates. The metabolites were identified from their m/z values by comparison with the known compounds in data bases like human metabolome database and metlin. The information obtained by the comparison has been summarized in the form of table1. The first column shows the retention time (RT) of the metabolite. The second column shows the mass charge ratio of the metabolite (m/z). The third column shows the range in which the metabolite is produced by the breast cancer cell line (MCF 7 or MDM-MB-231) the fourth column shows the range in which the metabolite is produced by the breast cell line MCF10A. The fifth column shows the fold rise (^)

The sixth column indicates the mass of the closest matched known substance found in the databases. The last column gives the common names of the known substances of similar masses. The columns up headings MCF 7, MCF10A & MDM-MB-231 within the table indicate the cell extract in which the metabolite was found as given in the table. There are also subheadings AMM, MEOH & ETAC which indicate the extract in which the following metabolites were found. The matches from the human metabolome are given first under the heading common name of known compound. The matches from the metlin database are written below in the same column shaded in blue. The metabolites analyzed in Amm extract were searched for in [M-H] mode & the metabolites in ETAC & MEOH extract were searched in [M+H] mode. {Table 1} The table shows the identified metabolites belong to all classes of compounds which means that metabolism of carbohydrates, fats, proteins and all other classes of compounds is altered in a tumourigenic cell.

See Illustration 8

This means that a metabolite found in the ETAC extract having mass/charge [m/z] ratio of 332. 326 with a retention time [Rt] 9.29 is produced by some MDM-MB-231 cancer cells upto a value of 100 & by some upto a value of 5; therefore range used is 5-100. But the same metabolite is produced by the normal cell MCF 10A upto a value of 0-4. So I infer that this is a metabolite that is normally produced by a breast epithelial cell and its production can rise upto 25 fold in breast cancer {100/4=25}. The fold rise has been calculated by the ratio as illustrated. The fold rise has been rounded to nearest non-decimal value in all rows of the column.

Thus it is clear from the table that there are more than eighty metabolites whose levels are significantly higher in breast cancer cell line extracts as compared to non cancer cell lines.

Analysis

The compounds that were found to be differentially produced in two cell groups in this study are numerous. But only those metabolites can be potential biomarkers of breast cancer which are markedly different as indicated by their fold rise in the tables. e.g. Substances with a fold rise 2 are produced in double amount by tumour cell lines than by non tumour cell lines. But this may happen in inflammatory diseases also. So I concentrated on substances whose fold rise was much higher.

The following information about the metaboites identified as potential biomarkers was obtained from the human metabolome database. The chemical formulas and the mass weight differences are also given in the website but I have not noted them here

because there are numerous possible adduct ions and I only had to list the nearest matches in the table that I made & only seven columns could be incorporated in the table.

There are more than eighty metabolites identified in the table which seem to be potential biomarkers of cancer cell lines in general as the metabolism is altered in every cancer. However I noted six metabolites which seem to be potential specific breast cancer cell line biomarkers.

PE(14:1(9Z)/14:1(9Z)) a phosphatidylethanolamine (PE) was found to be upto 14 fold more in the ETAC extract of the MCF 7 breast cancer cell extracts than in non cancer MCF 10A cell line extracts in our experiment. Previous studies using NMR spectro scopy(25) have shown high amounts of PE in breast cancer cells. Our experiment confirms that and we also find that the amount produced by breast cancer cell can be upto 14 times more than that produced by non cancer breast epithelial cell. Although glycerophosphoethanolamines can have many different com binations of fatty acids, but PE(14:1(9Z)/14:1(9Z)), in particular, consists of two chains of myristoleic acid at the C-1 and C-2 posi tions. The myristoleic acid moieties are derived from milk fats. Milk is produced only in the breast tissue. So I think that this metabolite can be a specific breast cancer biomarker. Its increased production also means that phospholipid degradation is much more in breast cancer cells than in normal breast epithelial cell. This was illustrated further by the increased amounts of other phospholipids found in the present study.

Another glycerophosphoethanolamine in the ETAC extract of MCF-7 breast cancer cell lines was PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/14:1(9Z)), which consists of one chain of docosahexaenoic acid at the C-1 position and one chain of myristoleic acid at the C-2 position. The myristoleic acid moiety is derived from milk fats which makes this also a specific breast cancer marker. Its levels were double in the breast cancer cell line extracts. PE(15:0/18:4(6Z, 9Z, 12Z,15Z)) a phosphatidylethanolamine(PE) consisting of one chain of pentadecanoic acid at the C-1 position and one chain of stearidonic acid at the C-2 position. The pentadecanoic acid moiety is derive ed from milk fat. Thus this is also specific to the breast tissue. It was found in the ETAC extract of MCF 7 CA cells. It was found to be upto 37 fold more in CA cell extracts. PE(18:4(6Z,9Z, 12 Z,15Z)/15:0) consists of one chain of stearidonic acid at the C-1 position and one chain of pentadecanoic acid at the C-2 position. The pentadecanoic acid moiety is derived from milk fat making this PE specific to breast tissue. It was found to be three fold more in breast cancer ETAC extracts of MCF 7 cell line.

DG(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0) a diacylglycerol which usually can have many different combinations of fatty acids but DG(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0), in particular, consis ts of one chain of myristoleic acid at the C-1 position and one chain of eicosapentaenoic acid at the C-2 position. The myristoleic acid moiety is derived from milk fats making it a specific breast tissue metabolite. It was found elevated in MCF-7 breast cancer cell line ammonium extracts as compared to the MCF10A non tumour igenic cell line.

DG(20:5(5Z,8Z,11Z,14Z,17Z)/14:1(9Z)/0:0) a glyceride consists of one chain of eicosapentaenoic acid at the C-1 position and one chain of myristoleic acid at the C-2 position. The myristoleic acid moiety is derived from milk fat making this a specific metabolite of breast tissue. It was found in the ammonium extract of MDA-MB-231 breast cancer cell lines. The amount produced was upto five fold more in the breast cancer cell lines as compared to the MCF 10A non cancer cell lines.

LysoPE(0:0/20:3(11Z,14Z,17Z)) a lysophospholipid(LPL) that was found to be produced upto 14 fold more by breast cancer cells. LPLs are breakdown products of phosphatidylethanol amine found in all the cells. But the amount produced is much smaller. That indicates that it might be useful as a general tumour marker but not as a specific breast cancer marker.

LysoPC(20:2(11Z,14Z)) another lysophospholipid (LyP) is found in normal conditions in the blood plasma. An enzyme lecithin: cholesterol acyltransferase (LCAT) secreted from the liver is invoved in determining its plasma level under normal conditions. In this study it was found that a breast cancer cell produces it upto 7 fold more of this metabolite than a normal breast epithelial cell. This I feel can also be a suitable marker as it is measured in the plasma at present also and no new tests need to be devized. Although it is not specific to breast cancer, but if we do know the normal range in plasma, we can evaluate its levels in breast cancer to see how useful its measurement can be.

Sphingomyelin SM(d17:1/24:1(15Z)) or SM(d17:1/24:1(15Z)) and Sphingomyelin (d18:0/14:0)

or SM(d18:0/14:0) are both sphingolipids found in cell membranes especially in the myelin sheath of nerve cells. Sphingomelins besides having a ceramide core (sphingosine bonded to a fatty acid via an amide linkage) additionally contain either phosphocholine or phosphoethanolamine. In breast cancer cell extract not only these two sphingomyelins but also their component molecules were found in abundance which again reflects the increased metabolism (20 fold more in our exp eriment) of sphingolipids in breast cancer cells. Ceramide was found to be produced upto 100 fold more by some cancer cells in contrast to non cancer cells. Whether this rise is associated only with breast cancer cells or it occurs in other cancers also needs further studies.

TG(20:5(5Z,8Z,11Z,14Z,17Z)/18:3(9Z,12Z,15Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))[iso6] is a monodocosahexaenoic acid triglyceride. It was found to be present 2-20 times more in breast cancer extracts as compared to normal cells.

Another metabolite found to rise upto thirty fold more was Farnesol. It is an intermediate in the isoprenoid/cholesterol bio synthetic pathway & plays a role in controlling the degradation of 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase. Studies have shown that it can activate the farnesoid receptor (FXR), a nuclear receptor that forms a functional heterodimer. The exogenous farnesol effects various physiological processes like inhibition of phosphatidylcholine biosynthesis induction of apoptosis, inhibition of cell cycle progression and actin cytoskeletal disorganization. The elevated farnesol found in breast cancer extracts in our study thus validate these findings as the cancer cell division is uncontrolled apoptosis is absent and phospholipid turnover is high. However I cant be sure if its level is so high in breast cancer only (thirty fold rise) or in other cancers also. But it can be again a general tumour marker.

A substance which is usually found in the nerve tissue was found to be particularly raised in breast cancer extracts in our study. This was Gamma Glutamylglutamic acid. It was found to be 20 fold more in cancer as compared to non cancer cell extracts. It is made of two glutamate molecules. Normally glutamate plays a role in synaptic plasticity. In brain injury it accumulates out side the cells which causes calcium ions to enter the cells via NMDA receptor channels. That causes neuronal damage and cell death. Excessively high intracellular Ca2+ is believed to bring about the fatal changes by damaging mitochondria and inducing pro-apoptotic genes. I think that this could be a mechanism of the normal breast epithelial cell death under the effect of metabolites produced by breast cancer cells nearby.

S-(PGJ2)-glutathione a glutathione conjugate of prostaglandin J2 was found to be two to five fold more in cancer cell extracts. It is one of the PGD2 dehydration product 9-deoxy- Δ9-PGD2 (also called prostaglandin J2). It has known cytotoxic activity and its elevated levels indicate that glutathione conjugation is increased in breast cancer cells. It is two to five fold increased in breast cancer cell extracts but is absent/negligible in normal cells. So it may be a potential marker of this tumour.

Similarly
2-N-[5-(4-bromophenyl)-1,3,4-thiadiazol-2-yl]-1-N-(3,4-difluoro phenyl) pyrrolidine-1,2-dicarboxamide is present in cancer cell extracts but absent in normal cell extracts. This can be a potential biomarker specific for breast cancer.

3 alpha,7alpha-Dihydroxy-5beta-cholestan-26-al is an intermediate in bile acid biosynthesis, specifically in the synthesis of chenodeoxyglycocholate and litho cholate. It was found to be 20 fold more in breast cancer cell extracts than in non cancerous cells. Bile acids are believed to regulate all key enzymes involved in cholesterol homeostasis. Bile acids have potent membrane disrupting potential. These facts indicate that in breast cancer cells there are possibly enzymes which direct bile acid synthesis although normally this only occurs in liver. If we consider the other way round it could be that there are enzymes which degrade cholesterol and lipids to compounds which are similar to bile synthesis intermediates.

27-Norcholestanehexol is a bile alcohol present in minute amounts in the bile and urine in healthy subjects. Bile alcohols are end products for cholesterol elimination. Presense of elevated amounts of this bile alcohol in breast cancer cell extracts indicates the increased metabolism of cholesterol in cancer cells.

Another metabolite found to be 20 fold more in breast cancer cell extracts. This is either a,24R,25-Trihydroxyvitamin D3 or 7alpha,26-Dihydroxy-4-cholesten-3-one. A precursor of chenodeoxycholic acid, 7 alpha, 26-dihydroxy-4-cholesten-3-one, found in elevated amounts again establishes the presense of bile compounds in breast tissue. As regards alpha 24R, 25-trihydroxy vitamin D3 it is known that prostate cells

can produce 1a,25-dihydroxyvitamin D3 (1a,25(OH)2D3) from 25-hydroxyvitamin D3 (25(OH)D3) to regulate their own growth. Whether the same can occur in breast cancer cells needs further studies.

Demethylphylloquinone a form of vitamin K was found to be raised 5 fold to 70 fold in breast cancer cell extracts. This is therefore a potential marker for breast cancer.

Mesaconic acid and itaconic acid were found to be five fold more in cancer cells than normal cells. Increased amounts of these metabolites establishes the fact that anaerobic carbohydrate metabolism is carried out excessively in breast cancer cells. Deuteroporphyrin IX is a non-natural dicarboxylic porphyrin usually described as a fecal porphyrin in patients with endemic chronicar senic poisoning. Deuteroporphyrin IX was found more than thirty fold more in cancer cells than in non cancer cells. Protoporphyrinogen IX was a similar metabolite found to be 4 fold more from cancer cells. Gamma-delta-Dioxovaleric acid is produced by enzymes acting in porphyrin metablosm. Its levels in breast cancer extracts were four fold more than in non cancer cell extracts. Thus we can conclude that even porphyrin metabolism products are produced in increased amounts by breast cancer cells.

Nicotinate D-ribonucleoside was found to be 2-6 fold more in breast cancer cell extracts than in non cancer cell extracts. This validates the finding in previous studies that ribonucleoside levels are altered in breast cancer cells as compared to non cancer breast epithelial cells. Normally it is involved in the nicotinate and nicotinamide metabolism pathways. Its altered levels indicate that energy production pathways are also altered as expected in tumour.

Uridine 2',3'-cyclic phosphate is a cyclic nucleotide. Cyclic phosphates are commonly found at the 3' end of mRNAs and other small RNAs. Uridine 2',3'-cyclic phosphate is a substrate for the enzyme 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNPase, EC 3.1.4.37) which hydrolyses it to Uridine 2'-phosphate. CNPase is a unique RNase in that it only cleaves nucleoside 2',3'-cyclic phosphates and not the RNA internucleotide linkage, like other RNases such as RNase A and RNase T1.

1,7-dimethylxanthine (paraxanthine) is a metabolite belonging to Super Class- Nucleosides and Nucleoside conjugates. It is the preferential path of caffeine metabolism in humans. It was found to be produced more than 20 fold by breast cancer cells than by non cancer cells indicating a massive alteration of nucleoside metabolism in cancer cells (33).

Cervonyl carnitine is an acylcarnitine. It was found to be three fold more in breast cancer cell extracts than in non cancer cell extracts. It could well be a marker of tumour.

4-Nitrocatechol a by-product of the hydroxylation of 4-Nitrophenol by the human cytochrome P450 (CYP) 2E1 is a useful metabolic marker for the presence of functional cytochrome P450 2E1 in microsomes of the cells.

Enterostatin VPGPR (Val-Pro-Gly-Pro-Arg) is a pentapeptide that is released from procolipase during fat digestion. Enterostatin levels are elevated in the plasma of obese women but were found to be raised in breast cancer cell lines also.

There are also metabolites which are significantly higher in the normal breast epithelial cells and almost produced negligibly by the breast cancer cell lines. These are located in the S-plot on the end opposite (i.e. the higher extreme) to the end where the cancer cell line marker metabolites are located (i.e.lower extreme).

Discussion and Conclusion

Historical background:

The field of cancer metabolomics is relatively new. The few studies done on breast cancer metabolism so far haverevealed high phosphocholine levels in breast cancer cells as compared to normal breast cells. 31 P spectroscopy showed more phosphomonoesters in breast cancer cells than in normal cells and the phosphocholine and phosphoethanolamine were found to contribute to the high PME sig nal in breast cancer cells in vivo in a multinuclear Nuclearmagnetic resonance spectroscopy study (4). In a proteomics study, proteome analysis of different breast cancer cell lines identified differentially expressed proteins. O16 /O18 peptide labelling was done so as tocompare peptides in one sample [labelled with O18] with peptides in another sample [label led with O16] by mass spectrometry. Heirarchial clustering showed that various proteins were differentially expressed in the cancer cell lines (5). Another study focussed on the lipid and carbohydrate metabolites of breast cancer

cells. Lipid biosynthesis in tumour cells was found to be altered as compared to normal cells.Levels of enzymes of lipid biosynthesis pathway like fattyacid synthase & 2,4-dienoyl coenzyme. A reductase were also found to be different as compared to normal cells. The altered carbohydrate metabolism in tumour cells was found to be characterized by increased glucose uptake and elevated glycolysis. Expression of lactate dehydrogenase and otherglycolytic control enzymes was also found to be altered (6) Studies on nucleic acid metabolites in breast cancer cells showed that there occur DNA/RNA modifications and there is an elevation in the amount of excreted modified nucleosides in breast cancer cells as compared to normal cells. 26 of 36 metabolites identified with breast cancer cells, were found to be modified ribonucleosides. Ribonucleosides and those compounds which have cis-diol structure were detected. Marked differences were found in 5-methyluridine, N2,N2,7-trimethylgua nosine,N6-methyl-N6-threonylcarbamoyl adenosine and 3-(3-amino carbox ypropyl)-uridine. 1-ribosyl-4-carboxamido-5-amino imidazole andS-adenosyl methionine occurredonly in supernatants of MCF-7 cells(7). Systematic methods were proposed for identifying metabolic markers inurine samples of breast cancer patients and comparison was done between 50 breast cancer patients and 50 normal persons in one study. Nine metabolic pathways were found to be altered in the breast cancer patients. Four metabolic substances (Homovanillate, 4-hydroxyphenylacetate, 5-hydroxy indoleacetate and urea) were identified to be significantly different in cancer & normal subjects (8). In another study urine from breast cancer patients was analysed for metabolites by using multivariate methods and five potential urinary markers forbreast cancer could be identified with high accuracy(9).

Present study:

There are more than eighty identified metabolites in Table 1 that seem to be potential bio markers of cancer cell lines. Among these are six potential specific breast cancer cell line biomarkers. What makes them specific is their origin from the milk fats as can be inferred from the presense of pentadecanoic acid moiety or myristoleic acid moiety. Milk is produced only by the breast tissue the human body. Thus anything derived from milk fat can be regarded as specific for breast. The following are the metabolites which can be regarded as the six potential specific biomarkers of breast cancer celllines

1. PE(14:1(9Z)/14:1(9Z)
2. PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/14:1(9Z
3. PE(15:0/18:4(6Z,9Z,12Z,15Z))
4. PE(18:4(6Z,9Z,12Z,15Z)/15:0)
5. DG(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)
6. DG(20:5(5Z,8Z,11Z,14Z,17Z)/14:1(9Z)/0;0)

PE(14:1(9Z)/14:1(9Z)) (having two chains of myristoleic acid at the C-1 and C-2 positions) was fourteen fold more, PE (22:6(4Z,7Z,10Z,13Z,16Z,19Z)/14:1(9Z)) (having one chain of docosahexaenoic acid at the C-1 position and one chain of myristoleic acid at the C-2 position) was double PE (15:0/18:4(6Z,9Z,12Z,15Z)) (having one chain of pentadecanoic acid at the C-1 position and one chain of stearidonic acid at the C-2 position) was thirty seven fold more PE (18:4(6Z,9Z,12Z,15Z)/15:0)) (having one chain of stearidonic acid at the C-1 position and one chain of pentadecanoic acid at the C-2 position) was three fold more in the breast cancer cellline MCF-7 than by non cancer cellline MCF-10A.

DG(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0) containing myristoleicacid moiety derived from milk fats was found to be 5 fold more in the AMM extracts of MDA-MB-231 CA cells

Suggested future work:

The findings of this study must be validated further by tandem massspectrometry. It should be established whether the identified markers are general tumour markers or not and whether the six identified metabolites are the ideal potential specific biomarkers of breast cancer cell lines. It is important to verify whether the metabolites found elevated in this study involving breast cancer cell lines are also elevated in all patients who suffer from breastcancer. If so then tests should be devised to measure these biomarkers in body fluids of patients suffering from breast cancer and find out how the levels fluctuate at different stages of breast cancer so as to use these biomarker levels in body fluids for early detection of breast cancer. That would help to initiate treatment early and thus prevent mortality due to this condition.

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Illustrations

Illustration 1

Figure 1

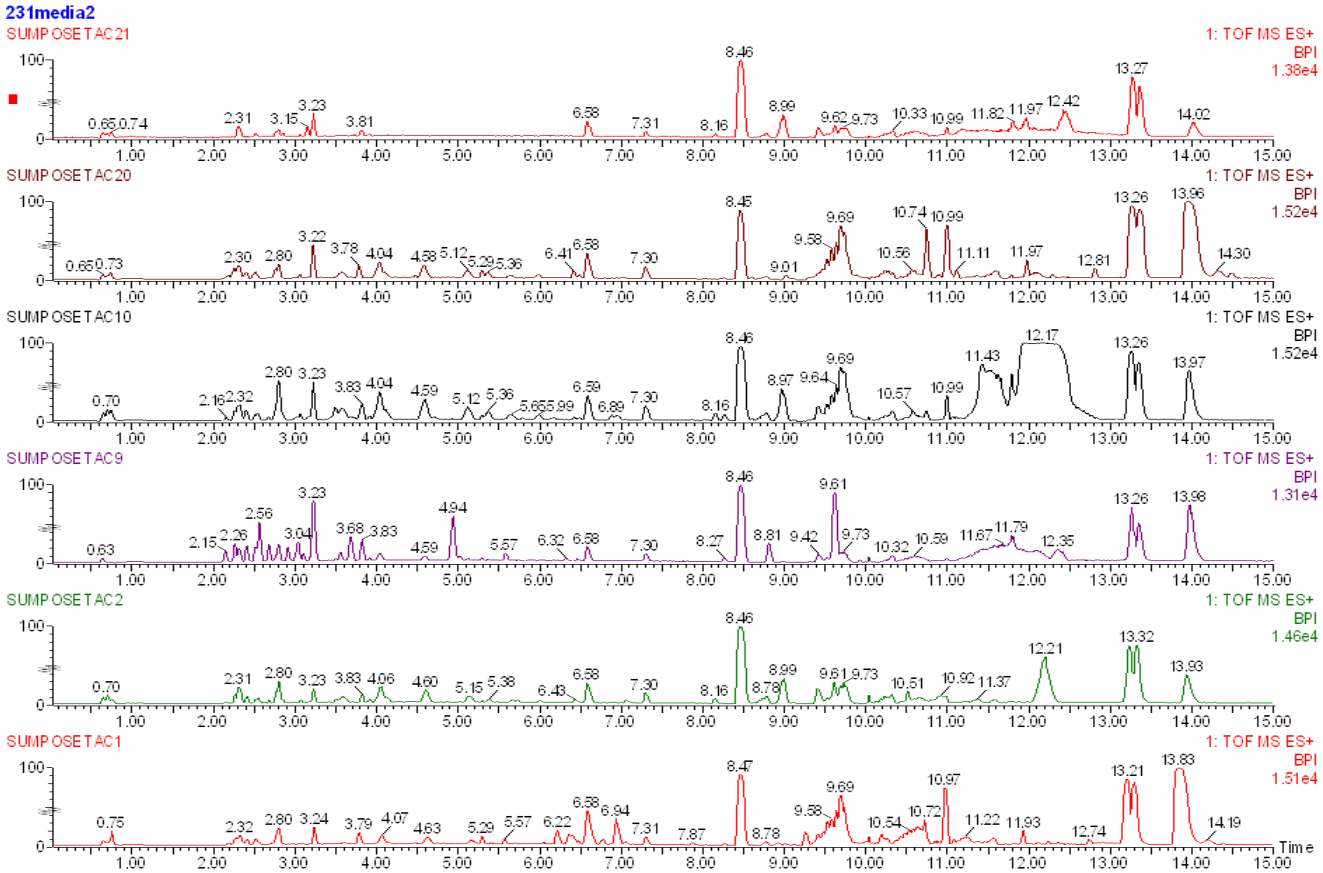

Illustration 2

Figure 2

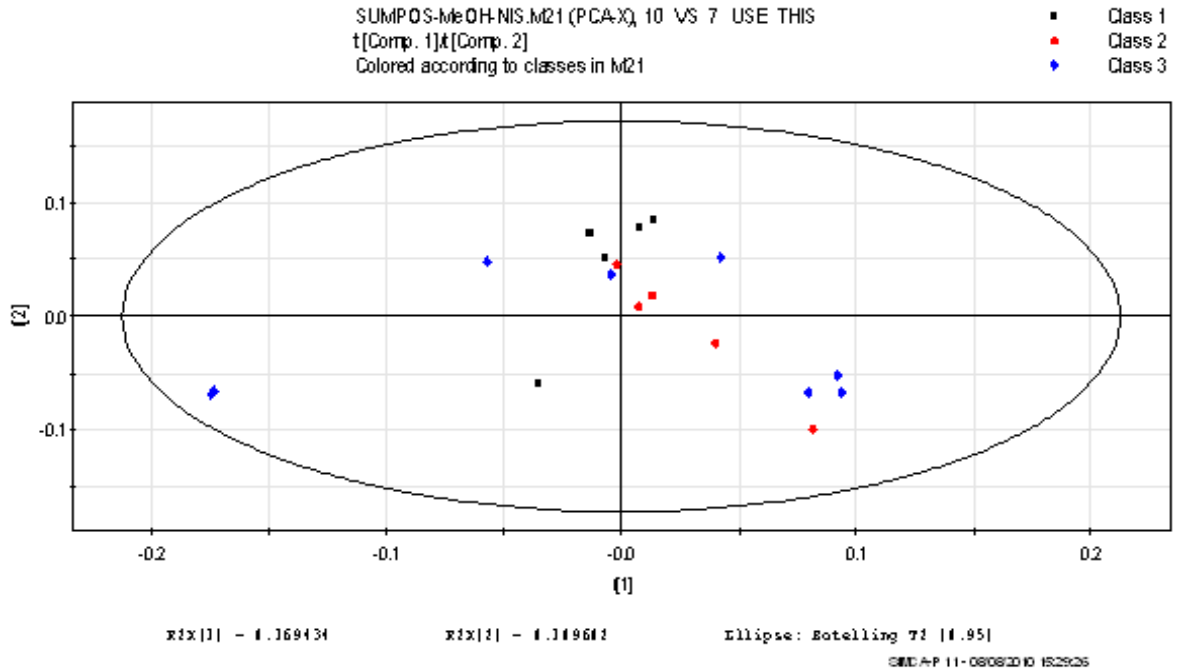

Illustration 3

Figure 1a

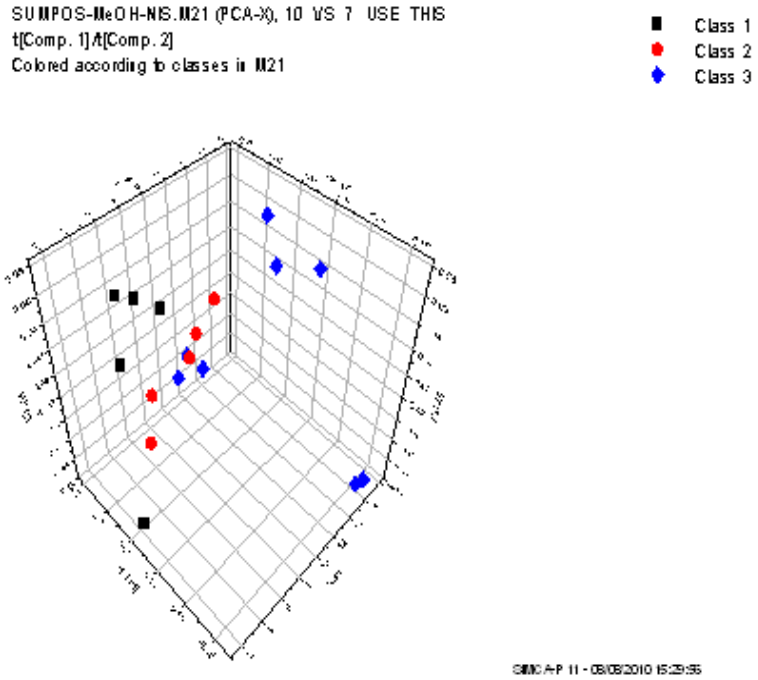

Illustration 4

Figure 1b

MODEL		A	R2	Q2
M21	PCA-X	2	0.279	0.0348

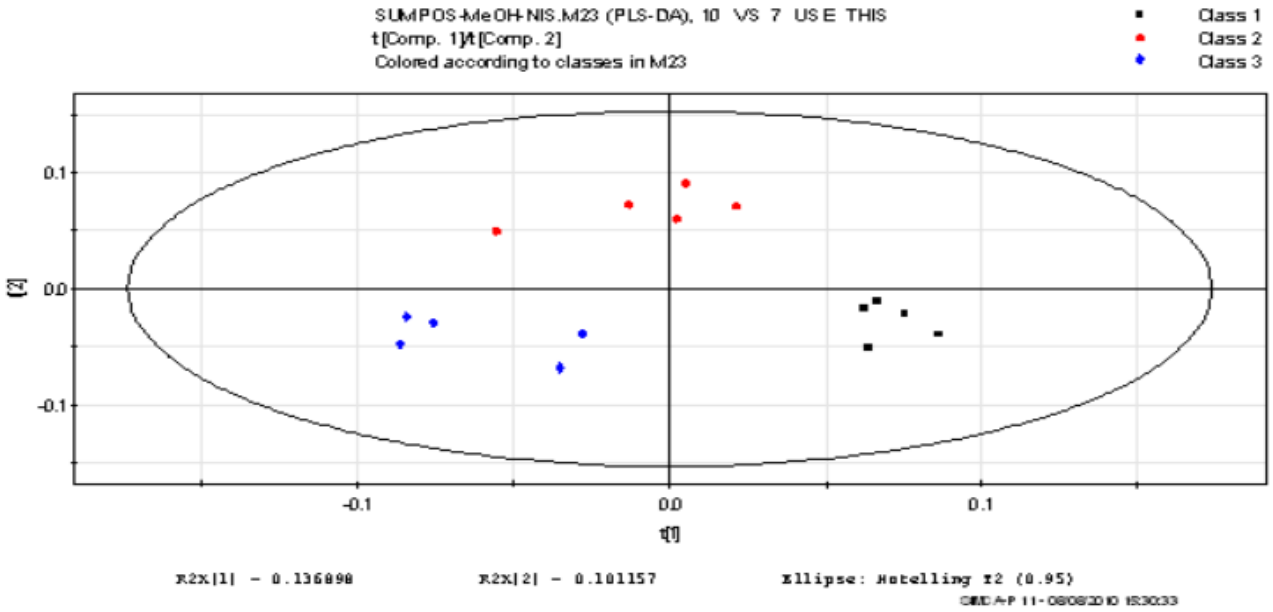

Illustration 5

Figure 2a

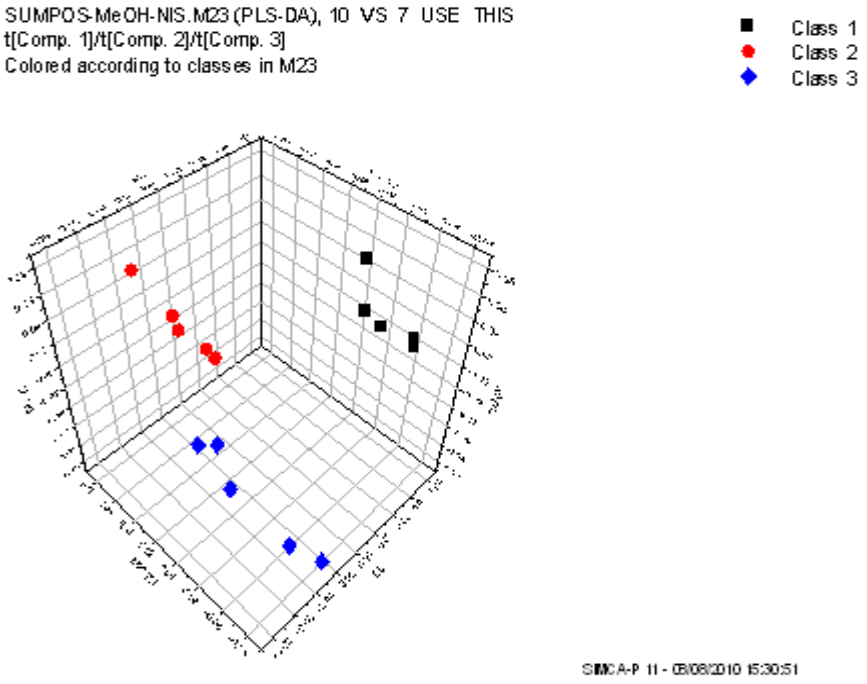

Illustration 6

Figure 2b

MODEL	A	R2	R2	Q2
M23	4	0.416	0.993	0.736

These values show that this PLS-DA model is a very good model

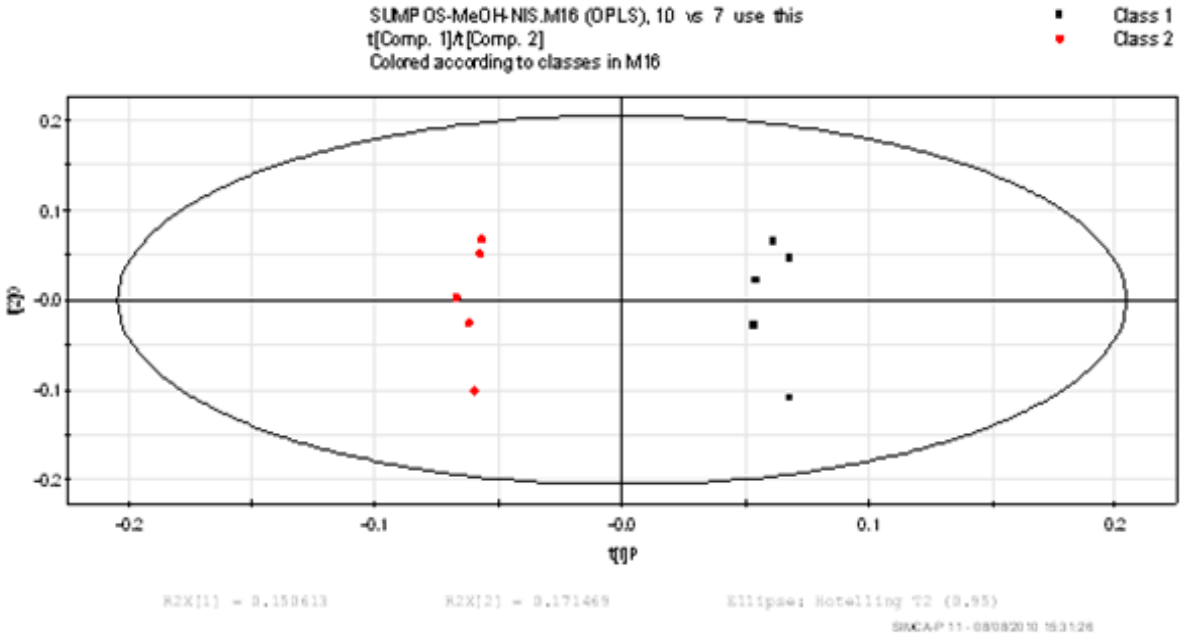

Illustration 7

Figure 3

Model		R2X	R2Y	Q2
M16	OPLS 1+1	0.322	0.993	0.536

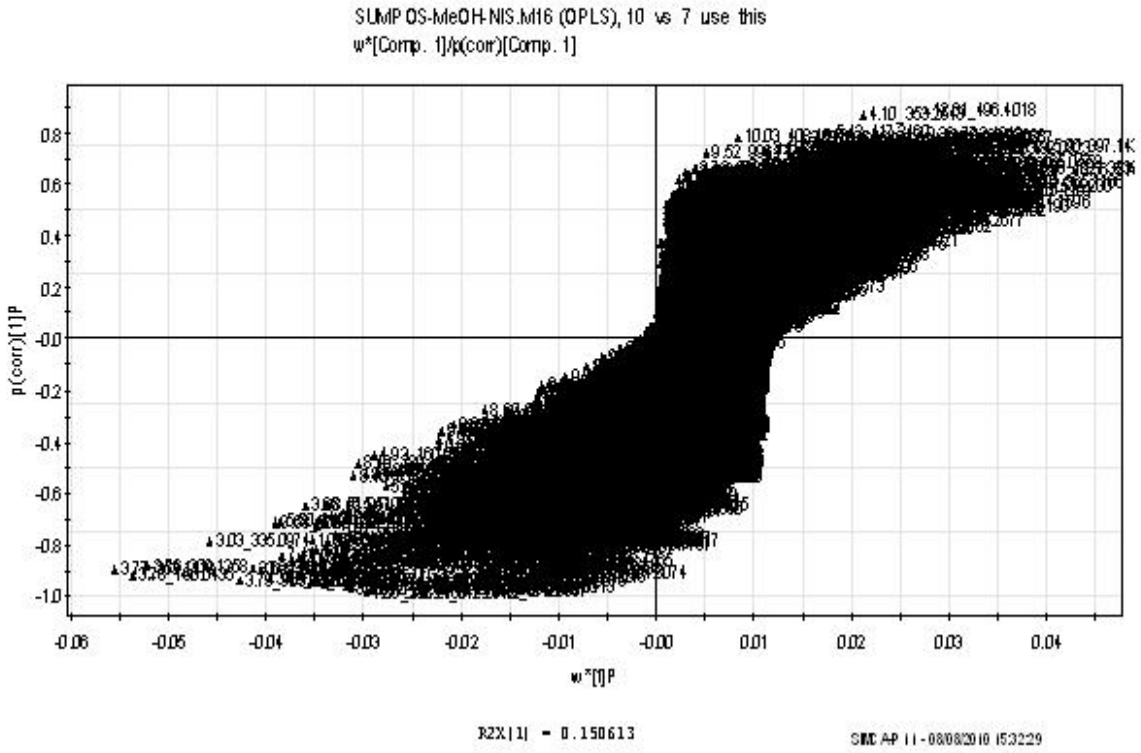

Illustration 8

Table

AMM	*extract*					
RT	**m/z**	**MDA-MB 231**	**MCF10A**	**^**	**MATCH**	**COMMON NAME of known compound**
2.99	129.0529	25-90	0-28	3	129.055710	2-Methyl-3-ketovaleric acid Ketoleucine, Mevalonolactone 2-Ketohexanoic acid Adipate semialdehyde 3-Oxohexanoic acid
					130.0630	**3-oxo-4-methyl-pentanoic acid**
3.73	226.9785	0.6-6	0-2.6	2	227.0326 **228.0399**	Mevalonic acid-5P **(R)-5-Phosphomevalonate**
0.6	401.8844	2.5-7	0-2.5	2	401.917999	Ganglioside GM3 (d18:1/20:0)
3.12	401.0771	2-6.8	0-2.5	2	401.078918 **402.0862**	4-Phosphopantothenoylcysteine **4-Phospho-N-pantothenoylcysteine**
3.27	442.0908	0-5	0-1.5	3	442.01705442.14807 443.1553	Guanosine diphosphate Dihydrofolic acid Dihydrofolic acid

3.79	384.9347	0-18	0-3	6	384.944183	Uridine 2',3'-cyclic phosphate
3.27	464.0737	2-5.2	0-2.5	2	464.058289	4-Nitrocatechol 2,4-Dihydroxy-nitrophenol
0.88	193.8453	0-12.5	0-2.5	5	193.807419 193.76593 193.74722	DG(20:5(5Z,8Z,11Z,14Z,17Z)/14:1(9Z)/0:0) DG(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z)/0:0) Cholic acid glucuronide Bilirubin
0.75	221.8033	0-3.5	0-0.5	7	221.838715	DG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:0/0:0)
					221.83871	DG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/18:0/0:0)
AMM	**EXTRACT**					
RT	**m/z**	**MCF7**	**MCF10**	^		
2.72	384.9361	0-4	0	4	384.94418 384.948395	Uridine 2',3'-cyclic phosphate Pelargonidin
4.44	498.9129	0.5-9	0-2.5	3	498.92144 **499.9287**	[Myo-inositol 1,3,4,6-tetrakisphosphate Myo-inositol 1,3,4,6-tetrakisphosphate
ETAC	**EXTRACT**					
RT	**m/z**	**MDM--MB231**	**MCF10**	^		
2.54		1-_8	0	8	205.09715 204.0899	Tryptophan Tryptophan

9.29	332.3268	4-100	0-5	20	332.323547 **331.2875**	Ceramide (d18:1/22:0) N,N-dimethyl arachidonoyl amine
10.72	487.3611	0-32	0-15	2	487.28189 487.35947 486.3709	PA(20:4(5Z,8Z,11Z,14Z)e/2:0) PE(24:1(15Z)/20:4(5Z,8Z,11Z,14Z) 26,27-diethyl-1alpha,25-dihydroxy-20,21-methano-23-oxavitamin D3 / 26,27-diethyl-1alpha,25-dihydroxy
ETAC	**EXTRACT**					
RT	**m/z**	**MCF-7**	**MCF-10**	^		
11.81	695.438	5-_35	0-2.5	14	695.437073 694.4784	PE(14:1(9Z)/14:1(9Z)) GPGro(15:0/15:0)[U]
9.29	332.326	5-_52	0-10	5	332.323547	Ceramide (d18:1/22:0)
2.9	481.2634	5-_15	0-5	3	481.24319 481.273071	11-Oxo-androsterone glucuronide Leukotriene E4
3.1	564.3606	2-_7	0-2	3	564.40234 564.35601 564.366516	LysoPE(24:1(15Z)/0:0) Ganglioside GM3 (d18:0/14:0) LysoPE(0:0/20:3(11Z,14Z,17Z))
3.1	569.3163	2.8-13	0-3	4	569.312195 569.33203 569.33203	Protoporphyrinogen IX Deoxycholic acid 3-glucuronide (3a,5b,7a)-23-Carboxy-7-hydroxy-24-norcholan-3-yl-b-D-Glucopyranosiduronic acid
2.9	476.3072	3.5-8.5	0-2.8	3	476.27716 476.3040	LysoPE(18:3(9Z,12Z,15Z)/0:0) PGD2 ethanolamide

3	525.289	3.2-13.3	0-3.2	4	525.31433 525.295 **524.2725**	VPGPR Enterostatin PIP(20:3(8Z,11Z,14Z)/18:1(11Z)) 26,26,26,27,27,27-hexafluoro-1alpha,25-dihydroxyvitamin D3 / 26,26,26,27,27,27-hexafluoro-1alpha,25-
2.68	388.2551	1-_6	1-2.7	2	388.30572 388.25466 388.25466	2-Hydroxymyristoylcarnitine PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/14:1(9Z)) PE(14:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
3.33	696.4405	1.4-4	1-1.4	3	696.49627 696.442	PE(18:4(6Z,9Z,12Z,15Z)/P-16:0) Cholestane-3,7,12,25-tetrol-3-glucuronide
10.78	753.4717	1.5-19	0	19	753.4709	SM(d18:0/14:0)
13.05	707.4937	1-_7	0-0.7	7	707.496	PA(16:0e/18:0)
6.49	299.0203	4-_5	0	5	299.07614 299.1125	Benzoyl glucuronide 2-Phenylethanol glucuronide
14.71	698.5009	0-37	0	37	698.47552 697.5046 698.5094	PE(15:0/18:4(6Z,9Z,12Z,15Z)) 1-tetrahexanoyl-2-(8-[3]-ladderane-octanyl)-sn-glycerophosphoethanolamine PE(16:0/P-16:0)

MEOH	EXTRACT				MASS	COMMON NAME
RT	m/z	MDM-MB-231	MCF-10	^		
7.66	526.2931	0.5-2.1	0-0.5	4	526.293	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)
10.84	531.3871	6-_7	0-1	7	531.398560	Ganglioside GM1 (d18:0/18:0)
12.32	746.5614	1.5-3	0-1	3	746.5571	2-Hydroxylauroylcarnitine
7.44	494.3243	11-_20	0-2	10	494.324097	7 alpha-Hydroxy-3-oxo-4-cholestenoate, Cervonyl carnitine LysoPC(16:1(9Z)) MG(24:6(6Z,9Z,12Z,15Z,18Z,21Z)/0:0/0:0)
7.12	468.309	7-_10	0-1	10	468.3084	LysoPC(14:0)
7.66	482.309	2.5-7	0-0.3	2	482.309	Palmitoyl glucuronide
8.01	372.3116	1.4-2	0-0.2	10	372.3122	N-Methylnicotinium
3.31	714.7809	2-_16	0-1	16	714.805786	Liothyronine
10.07	561.3983	3-_7	0-0.8	9	561.387 561.387146	Ganglioside GA2 (d18:1/20:0 Trihexosylceramide (d18:1/20:0)
7.66	502.2947	0.7-4.8	0-0.4	10	502.293	LysoPE(20:4(8Z,11Z,14Z,17Z)/0:0)
8.63	480.3453	2-_20	0-2	10	480.345	7 alpha,26-Dihydroxy-4-cholesten-3-one Calcitriol 24R,25-Dihydroxyvitamin D3
8.56	482.3596	2-_12	0-2	6	482.360	7-a,25-Dihydroxycholesterol,7-a, 27-dihydroxycholesterol,(24R)-Cholest-5-ene-3-beta,7-alpha,24-triol
7.83	520.3397	3.5-10.5	0-2.5	5	520.3397	LysoPC(18:2(9Z,12Z))
8.26	991.6713	1-_45	0-2	22	991.6761	TG(20:5(5Z,8Z,11Z,14Z,17Z) /18:3(9Z,12Z,15Z)/22:6(4Z,7Z,10Z ,13Z,16Z,19Z))[iso6]

10.84	531.3871	1.5-6	0-1.5	2	531.3985	Ganglioside GM1 (d18:0/18:0)
8.6	522.3531	13-_130	0-23	6	522.3554	LysoPC(18:1(9Z))
8.73	522.3551	1-_8	0-1	8	522.3554	LysoPC(18:1(11Z))
0.91	203.0538	0-12	0-0.5	24	203.0539	Paraxanthine
8.59	482.3596	1-_13	0-0.5	3	482.3604	3a,7a-Dihydroxy-5b-cholestan-26-al 17a,20a-Dihydroxycholesterol,
8.26	276.6339	1.5-12.5	0-0.3	40	276.6339	Deuteroporphyrin IX
8.27	648.2812	1-4.5	0-0.8	50	648.2828	LysoPC(22:4(7Z,10Z,13Z,16Z))
10.93	421.3510	1-_4	0-1	4	421.355	SM(d17:1/24:1(15Z))
7.84	466.3286	1-_4	0-0.5	8	466.329	LPA(18:0e/0:0)
8.63	480.3453	2-_20	0-3	7	480.3448	LysoPC(P-16:0)
MEOH		**Extract**				
RT	**m/z**	**MCF-7**	**MCF-10**	^	**MASS**	**COMMON NAME**
3.76	309.1258	10-230	0-10	23	309.1292	Gamma Glutamylglutamic acid
3.77	353.1118	>320	<50	7	353.112 353.1131 353.1109	S-Formylglutathione Genistein, Apigenin Tiglylglycine,3-Methylcrotonylglycine
3.78	160.0435	20--90	<15	6	160.0439	N-Acetylgalactosamine 4-sulphate
3.76	309.1258	200-240	<20	12	309.1251	Estrone
3.79	375.1003	6--20	<3	7	375.1006	beta-Carboline

3.78	335.106	4--12.5	<2	6	335.103	Nicotinate D-ribonucleoside
5.92	316.2492	1.7--2.2	0-0.5	4	316.2482	Decanoylcarnitine
3.68	309.117	120-300	<120	3	309.119	3-Nitrotyrosine
5.5	219.1744	5--70	0-5	14	219.1743	Demethylphylloquinone
5.5	264.2335	1--35	<1	35	264.2322	Farnesol
0.91	203.0538	0--12	0-0.5	24	203.0539	Paraxanthine
8.59	482.3596	1--13	0-0.5	24	482.3608	3a,7a-Dihydroxy-5b-cholestan-26-al
8.26	276.6339	1.5-7	0-1	7	276.6339	Deuteroporphyrin IX
8.27	648.2812	1-4.5	0-0.8	5	648.2828	LysoPC(22:4(7Z,10Z,13Z,16Z))
10.93	421.351	1--4	0--1	4	421.355	SM(d17:1/24:1(15Z))
7.84	466.3286	1--4	0-0.5	8	466.329 466.3293	LPA(18:0e/0:0) Stearoylcarnitine

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