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Identification of Potential Biomarkers and Metabolic Changes in the Serum of Breast Lump Patients Among Kelantanese Based on ^{1}H NMR Metabolomics

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Abstract: This case-control study, conducted at the Hospital USM BestARi unit, aimed to identify the serum metabolic fingerprint among individuals with breast lumps and healthy controls, and to discover potential biomarkers. Serum samples from healthy controls, benign breast lump patients, and malignant breast lump patients were analyzed using proton nuclear magnetic resonance spectroscopy (^{1}H NMR). A multivariate data analysis approach was employed, with the OPLS-DA and clustered heat map techniques effectively differentiating between the three groups. The study revealed significant metabolite variations across the groups and proposed D-glucose, glycerol, and glycine as potential biomarkers for breast cancer diagnosis. Metabolic pathways such as alanine, aspartate, glutamate metabolism, and glycine, serine, and threonine metabolism were implicated. The metabolomics approach coupled with multivariate analysis successfully identified key metabolites leading to group separation and suggested altered metabolic pathways. However, further research and integration with other 'omics' technologies are necessary for clinical translation.

Keywords: Metabolomics; Serum Metabolic-Fingerprint; Breast lump; ^{1}H-NMR, Biomarker; SDG 3 Good health and well-being

1. Introduction

Breast lumps are a typical presentation for patients with breast pathology, and they can be caused by benign or malignant lesions. Most breast lumps are benign. Although malignant breast lumps are less common, distinguishing the signs and symptoms of benign and malignant lesions can be difficult at times. Breast cancer is the most common type of cancer for women worldwide [1]. The characteristics of cancer cells are angiogenesis, resistance to cell-death signaling, genomic instability, unlimited proliferation, and immune surveillance evasion [2]. Breast cancer management requires investigations such as radiological imaging, invasive procedures such as histology, and blood testing for diagnosis, prognosis, and treatment [3, 4]. Although these investigations offer data to support their everyday clinical application, they are far from ideal and still have shortcomings.

Tumour markers can provide useful information for breast cancer management. Gene biomarkers, such as mutations in key tumour suppressor genes such as BRCA1, BRCA2, and TP53, play a pivotal role in characterizing various tumour types and guiding targeted therapies [5-7]. MicroRNA and DNA methylation biomarkers also contribute to diagnostic precision, with dysregulated miRNA expression patterns and epigenetic alterations serving as indicators of cancer types and prognostic factors [8-12]. Apart from the standard biomarkers, emerging evidence suggests the involvement of circular RNAs (circRNAs) in the regulation of cancer cell proliferation, invasion, angiogenesis, and metastasis [13, 14]. The integration of these molecular biomarkers into cancer diagnostics enhances early detection, subtyping, prognosis assessment, and personalized treatment strategies, ultimately improving the clinical management and outcomes for individuals affected by breast cancer and beyond.

This means that learning more about molecular genetic alterations is crucial, although they are restricted in their ability to detect early breast cancer [15-17].

To enhance our understanding of breast cancer beyond genetic alterations, the integration of metabolomic studies is crucial for validating and complementing the information derived from tumour-related genes and miRNA biomarkers [18, 19]. Metabolomics is particularly significant as it represents the most direct reflection of the phenotypic and biological functions, acting as the terminal end of the biological system [20]. Additionally, because huge metabolite concentration changes can be noticed with only minor changes in enzyme activities, metabolomics can be thought of as the biological system's amplified output, making it a more sensitive method [21]. As the search for a more sensitive biomarker for early detection and a molecular target for effective treatment of breast cancer continues, metabolomics is currently one of the areas of interest in identifying metabolic abnormalities in disease states and could potentially provide useful novel biomarkers and offer insightful information to address biological issues and breast cancer management [22, 23]. Nuclear magnetic resonance (^{1}H NMR) spectroscopy-based metabolomics method is one of the powerful analytical techniques employed to study the metabolic profile of a wide range of biological samples, such as serum, faecal and urine, which require only a minimal amount of samples [24, 25]. Several published studies have proved the utility of metabolic profiles anaysed by ^{1}H NMR in the detection of breast cancer [26, 27]. When analysing serum samples with ^{1}H NMR, Zhou et al. [28] discovered that nine metabolites were substantially altered in breast cancer compared to healthy controls. Other studies that used ^{1}H NMR with different sample types were also able to demonstrate significant differences in metabolites between breast cancer and healthy control and the possibility of being used as diagnostic biomarkers [29, 30]. Only a few research studies have looked at benign breast lesions; however, they were examined using different types of samples or instruments [31, 32]. Regarding breast lump patients, we hypothesised that different metabolic fingerprints would be found in breast lump patients compared to healthy controls.

This study was done to determine the serum metabolic fingerprinting level among breast lumps and healthy controls. This study also aims to identify potential biomarkers between breast lump and healthy control. To the best of our knowledge, there was no study in Malaysia focusing on metabolomics in breast lump patients.

2. Materials and Methods

2.1. Study Design

A case-control study was conducted at the Breast cancer awareness & Research unit (BestaRi) clinic, Hospital USM, Malaysia, from June 2020 to June 2021. The study subjects were divided into three groups: healthy control (HC), benign breast lump (BE), and malignant breast lump patients (BC). Breast lump patients were consecutively selected from those who were presented with a breast lump and had not received any treatment. HC were those without medical illness and current breast pathology after clinical assessment. A total of 177

participants were included in this study, of which 56 were BE, 53 were BC, and 68 were HC subjects. Demographic details of all study subjects were tabulated in Table S1,supplementary material. All aspects of this study complied with the Declaration of Helsinki. This study was approved by the Human Research Ethics Committee of USM, USM/JEPEM/16050172.

2.2. Chemicals

The chemicals used for NMR analysis, such as deuterated oxide (D_2O, 99%) was purchased from Sigma-Aldrich (St. Louis, USA), while trimethylsilanepropionic acid sodium salt (TSP), KH_2PO_4, imidazole, and NaOD were acquired from Merck (Darmstadt, Germany).

2.3. Specimen Collection and Analysis

A total of 3 mL fasting venous blood samples were collected from the participants in a plain tube without gel for metabolite fingerprinting using NMR analysis. The serum was taken after separation from the blood clot after centrifugation and stored at -80 °C in the freezer until further analysis. The ^{1}H NMR analysis of serum was conducted based on the procedure described by Maulidiani et al. [33] with minor modifications. 200 µL of the supernatant was taken and mixed with 400 µL of D_2O (pH 7.4) containing 0.1 % of TSP. The sample solution was transferred into a 5 mm NMR tube prior to ^{1}H NMR analysis. The mixed serum sample solution was then sonicated for 5 min at room temperature to remove air bubbles. The ^{1}H NMR spectrum of the serum sample was acquired from a Bruker Ultrashield™ 400 NMR operating at 400.1 MHz and temperature 23 °C with 1024 scans, pulse width (PW) 21.0 µs (90°), and relaxation delay 2s to obtain 8012.821 data points over the set spectral width ranged from 1.00 to 14.00 ppm and total acquisition time of 30 min. Water signals and broad protein resonances of the serum samples were suppressed using the combination of PRESAT and the Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence. D_2O and TSP were used as the internal lock and standard calibration, respectively. All spectra were manually phased and baseline corrected.

2.4. Data processing and statistical analysis

^{1}H NMR spectral data preprocessing and binning were conducted using online NMRProcFlow software version 1.4 (https://www.nmrprocflow.org/). Spectral preprocessing included calibration, baseline correction, and alignment. Following this, a spectral region from 0.50-10.00 ppm was binned into 0.04 ppm, and normalisation was applied before data extraction. For multivariate data analysis (MVDA) purposes, water signals at 4.70–5.02 ppm were excluded. The MVDA was performed using SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden) and MetaboAnalyst online software (https://www.metaboanalyst.ca/). MVDA model, such as orthogonal projection to latent structure analysis (OPLS-DA) was used to discriminate the BC, BE, and HC groups as well as to identify the biomarkers. The statistical differences in metabolites between groups were analysed using an independent t-test. The p values ≤ 0.05 were considered statistically

significant (Table S2, supplementary material). Multivariate analyses for OPLS-DA models were carried out in accordance with the provided design in Figure 1.

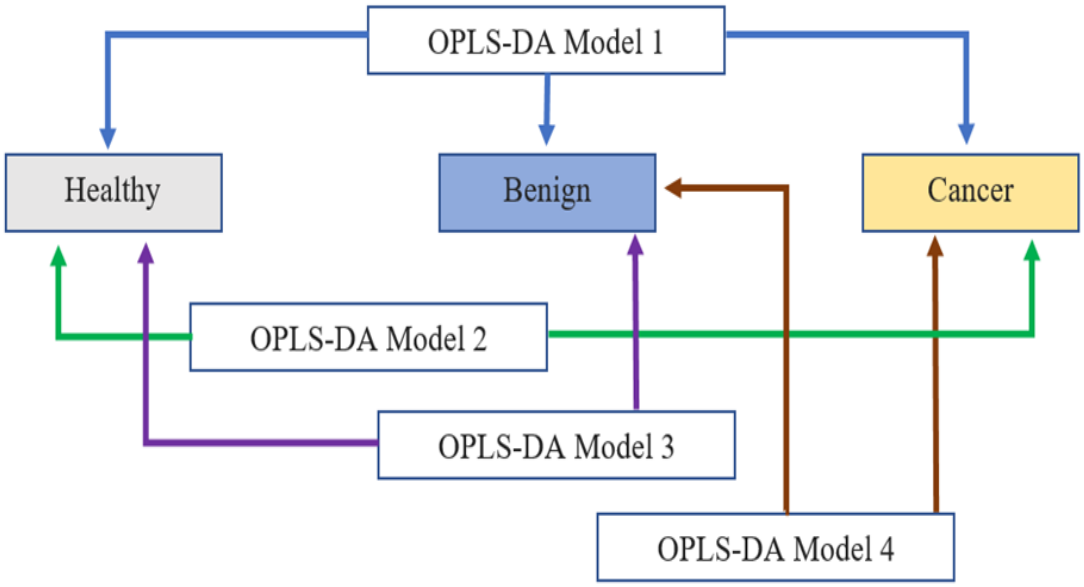


Figure 1. The design for multivariate models.

3. Results

3.1. Visual inspection of ^{1}H NMR spectra of the serum of BC, BE, and HC subjects

Figure 2 shows the representative ^{1}H NMR spectra for serum from the 3 groups. Each of the spectra was individually inspected, and the metabolites were visually identified by comparing their chemical features to those of standard reference compounds that were available in Chenomx library. A total of 44 metabolites were assigned and tabulated in Table S3, supplementary material together with their characteristic's proton signals. The 3 groups could not be separated by comparing the spectra peaks alone, and thus, multivariate data analysis approaches were needed to further analyse the data.

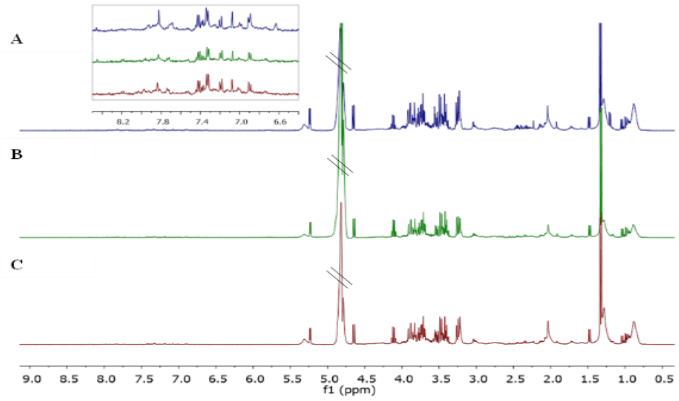


Figure 2. Representative ^{1}H NMR spectra of serum of BC (A), BE (B), and HC (C).

3.2. Metabolite fingerprint of BC, BE, and HC subjects

OPLS-DA was conducted using the data from quantification results of the identified metabolites to show the maximal class separation between the 3 groups. Figure 3 shows the scores plot produced from the OPLS-DA of the entire dataset. The BC group was on the left side of component 1 of the score plot, while the other two groups, BE and HC, were on the right. The second component was able to distinguish the BE and HC groups, indicating that there were differences between the two groups. Variable important to the projection (VIP) was utilised to determine the variable's contribution to the class discrimination of OPLS-DA model. There were 24 metabolites out of 44 metabolites that had VIP values of > 0.7 [34] with CV-SE which is less than VIP (Table S4). The issue with creating an OPLS-DA model based on all three classes at the same time will yield a reference point that may complicate the model interpretation [35]. As a result, two-class models were also constructed to further verify those metabolites that can be used as putative biomarkers.

Heat map analysis, as shown in Figure 4, revealed an apparent separation between the three groups whereby most of the metabolites were upregulated in the BC compared to BE and HC group.

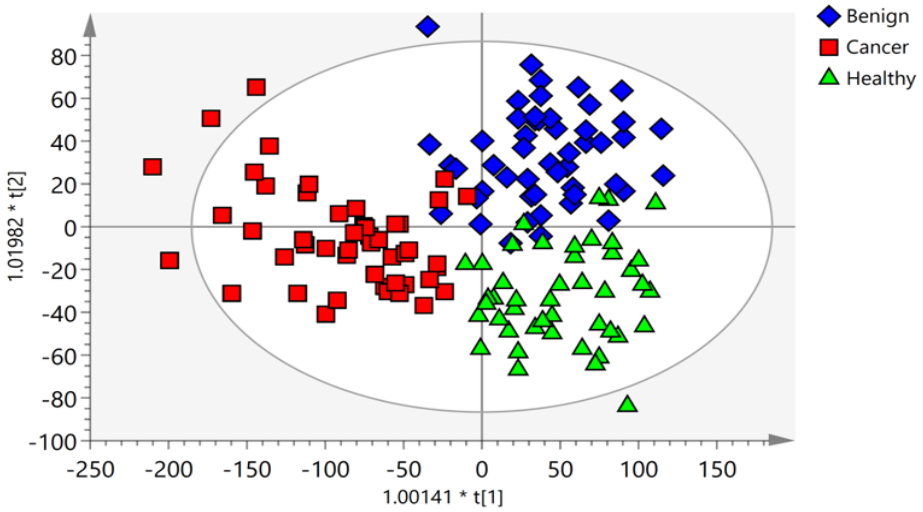


Figure 3. OPLS-DA scores plot of BC, BE, and HC groups. Validation: see Supplementary Material Table S5, Table S6, and Figure S1.

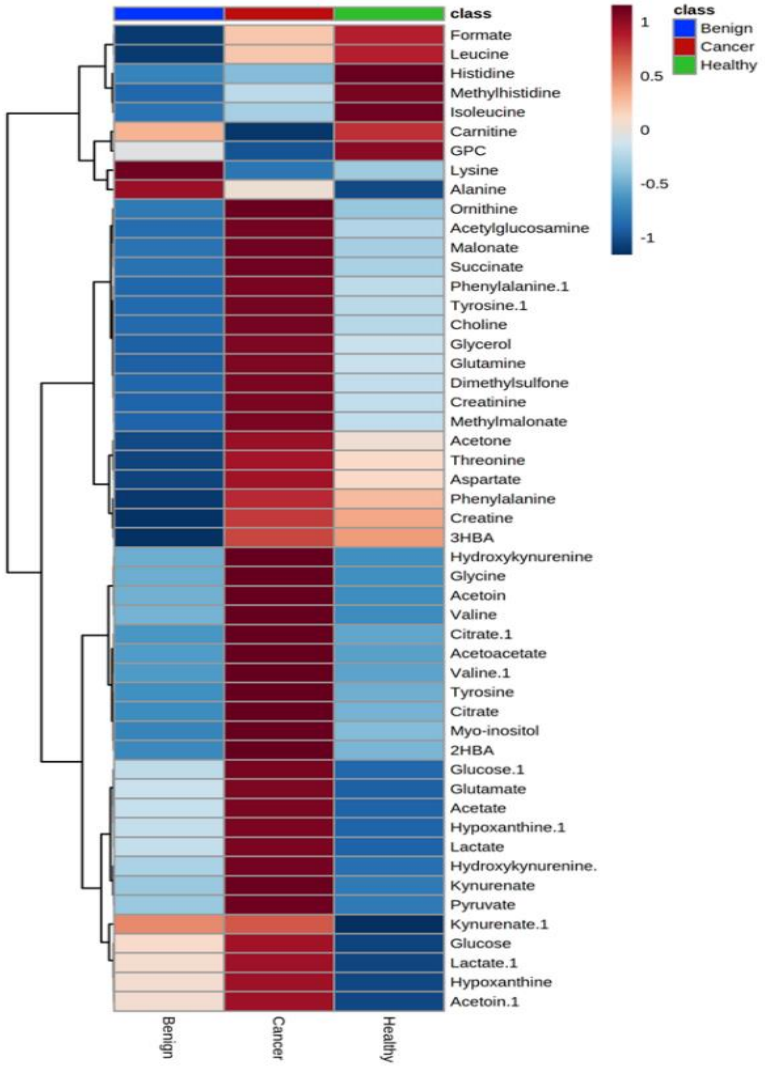


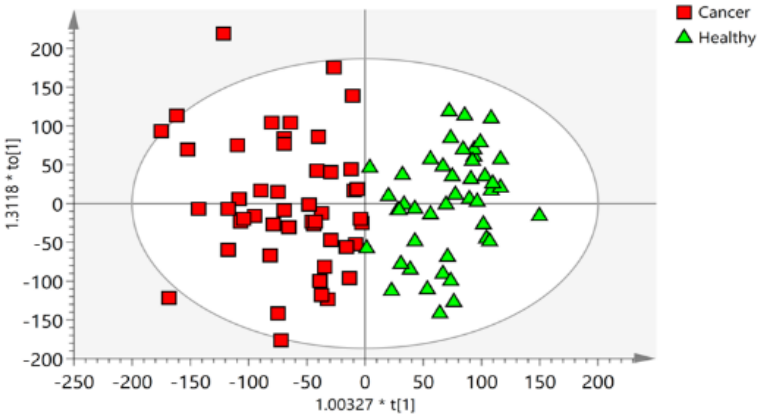
Figure 4. Heat map of the hierarchical clustering. The colours on top of the heat map represent the class: BE, BC, and HC. The colours in the heat map represent metabolites expression intensities.

3.3. Determination of Biomarkers

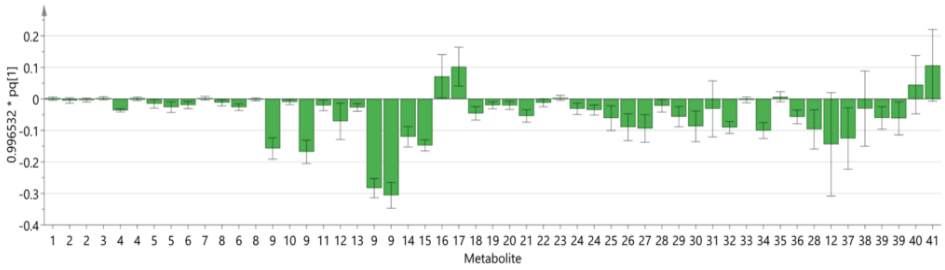
Three other OPLS-DA models were constructed for identifying putative biomarkers between two classes, which were model 2 (HC versus BC), model 3 (HC versus BE), and model 4 (BC versus BE). The combination of loading with the jack-knifed confidence intervals and S plots helped to identify the potential biomarkers [35]. In model 2 (HC versus BC), the separation between the two groups is clearly seen (Figure 5A). Most of the metabolites, for instance, D-glucose, L-lactate, glycerol, glycine, pyruvate, acetoacetate, L-glutamine, acetate, 2-HBA, acetoin, and methylmalonate were increased in BC. On the contrary, decrease in L-carnitine, GPC, L-leucine, and L-isoleucine (Figure 5B). The potential markers in the BC group are D-glucose, glycerol, and glycine, as shown by their high magnitude and high reliability in the S plot (Figure 5C) as well as low confidence interval (Figure 5B).

HC versus BC

A



B



C

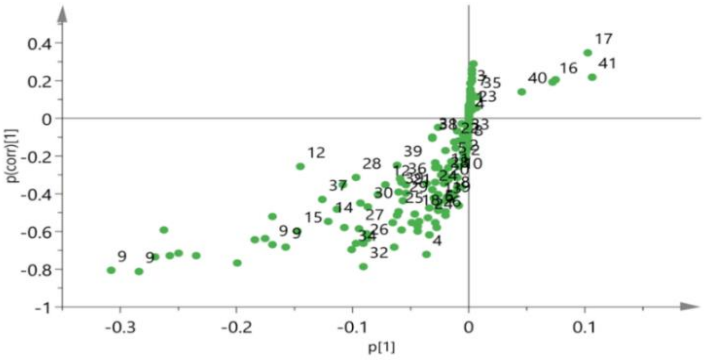


Figure 5. OPLS-DA scores plot (A), loading column plot (B), and S plot (C) of HC versus BC groups. Validation: see Supplementary Material Table S7, Table S8, Figure S2, and Figure S3.

Despite their high reliability, metabolites such as GPC, methylmalonate, L-lactate, and L-isoleucine were deemed ambiguous biomarkers since their magnitude was medium and their confidence intervals were vast, with several metabolites having confidence intervals that crossed zero. Formate, 3-HBA, kynurenate, ornithine, creatine, hypoxanthine, and L-aspartate, all of which have low magnitude and low reliability, were shown to have identical levels in both BC and HC. The separation between the groups (Figure 6A) from model 3 (HC

versus BE) was mainly caused by the elevation of D-glucose, L-lactate, acetoin, and L-alanine, while the reduction of glycerol, acetone, L-glutamine, methylmalonate, 3-HBA, and L-isoleucine in BE groups (Figure 6B). D-glucose, L-alanine, 3-HBA, and methylmalonate are the most likely putative biomarkers for distinguishing between the two groups. However, even though these metabolites exhibited high reliability, their magnitude in the S-plot (Figure 6C) was only medium, and their role as prospective biomarkers was hampered by a significantly large confidence interval in the loading plot.

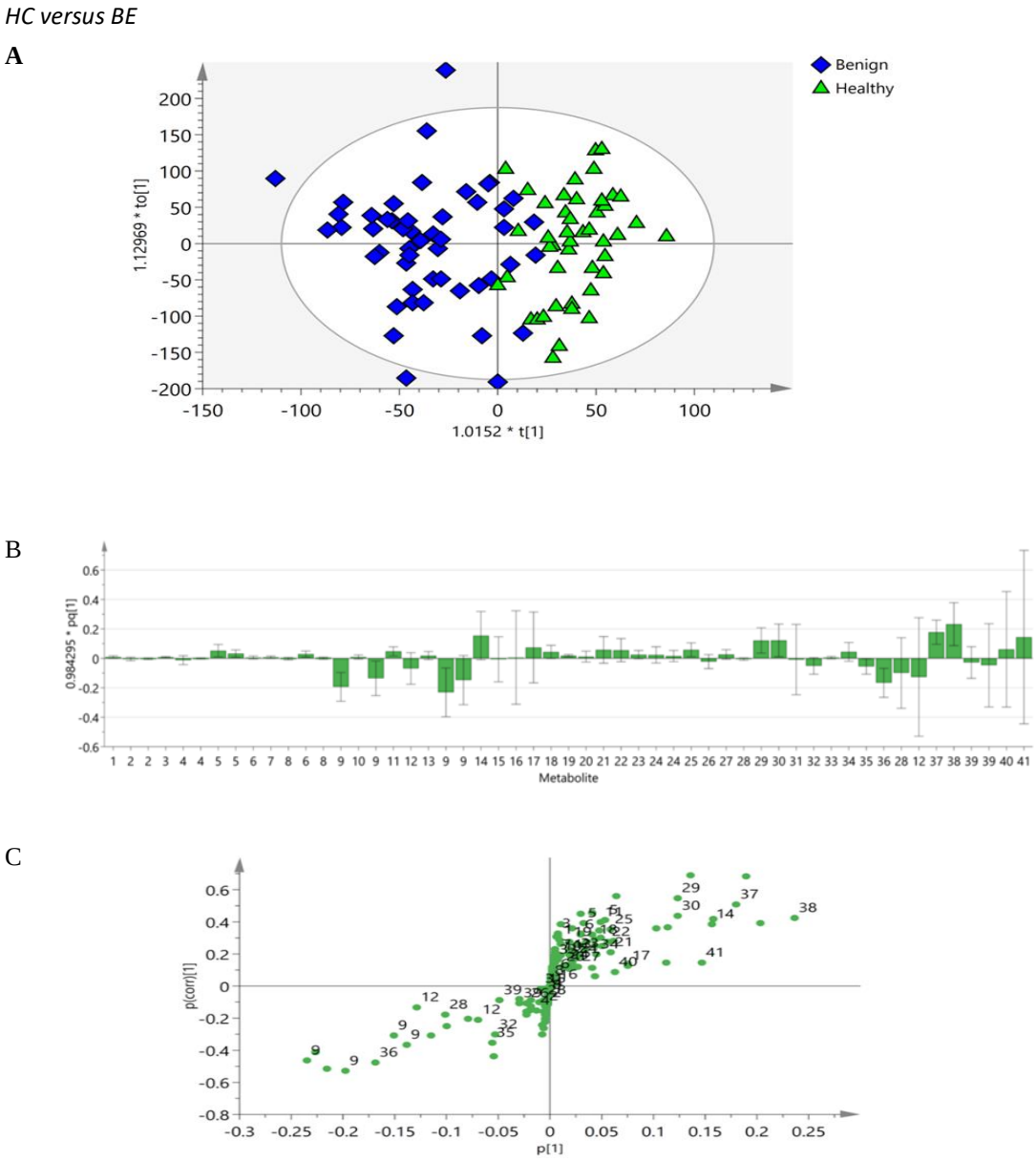
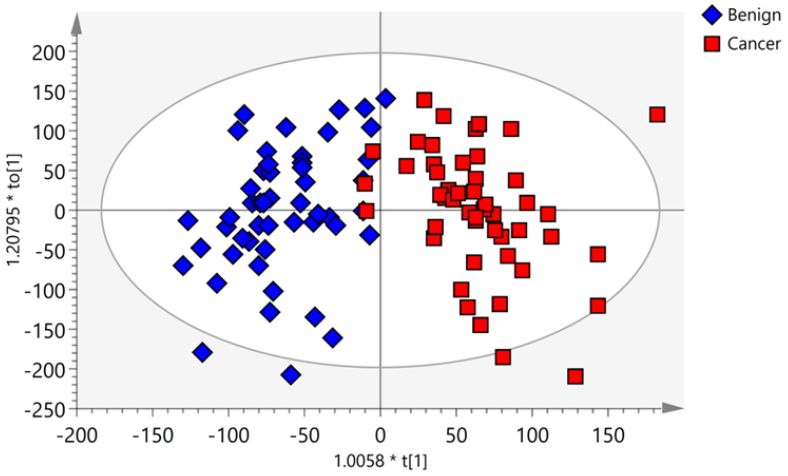


Figure 6. OPLS-DA scores plot (A), loading column plot (B) and S plot (C) of HC versus BE groups. Validation: see Supplementary Material Table S9, Table S10, Figure S4, and Figure S5.

BC versus BE

A



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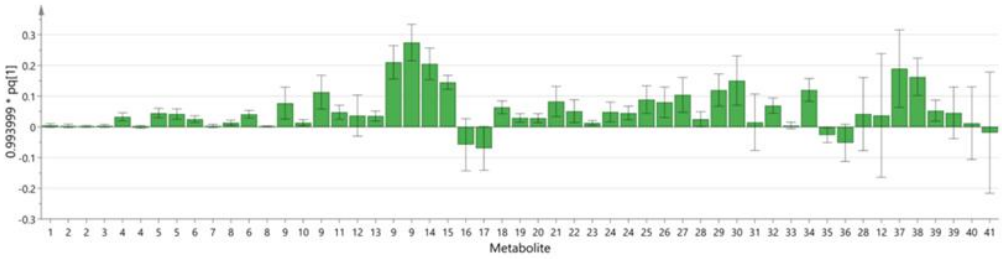

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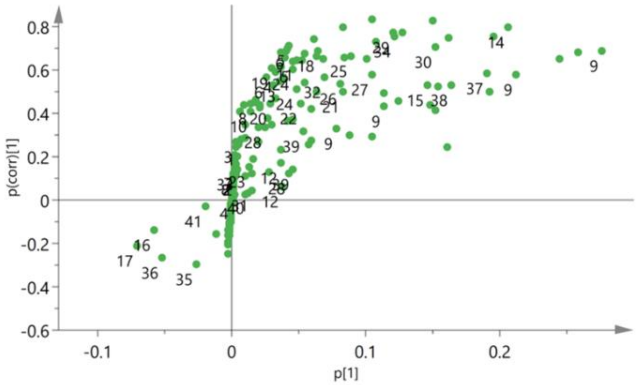

Figure 7. OPLS-DA scores plot (A), loading column plot (B) and S plot (C) of BC versus BE groups. Loading & S plots keys : (1) Formate; (2) Hypoxanthine; (3) 1-Methylhistidine; (4) Kynurenate; (5) L-Phenylalanine; (6) L-Tyrosine; (7) L-Histidine; (8) 3-Hydroxykynurenine; (9) D-Glucose; (10) N-Acetylglucosamine; (11) L-Threonine; (12) L-Lactate; (13) Myo-inositol; (14) Glycerol; (15) Glycine; (16) L-Carnitine; (17) sn-Glycero-3-phosphocholine (GPC); (18) Choline; (19) Dimethylsulfone; (20) Malonate; (21) Creatinine; (22) Creatine; (23) L-Aspartate; (24) Citrate; (25) Succinate; (26) Pyruvate; (27) Acetoacetate; (28) Acetoin; (29) Acetone; (30) L-Glutamine; (31) L-Glutamate; (32) Acetate; (33) Ornithine; (34) 2-Hydroxybutyrate (2-HBA); (35) Lysine; (36) L-Alanine; (37) Methylmalonate; (38) 3-Hydroxybutyrate (3-HBA); (39) L-Valine; (40) L-Leucine; (41) L-Isoleucine.
Validation: see Supplementary Material Table S11, Table S12, Figure S6, and Figure S7.

The metabolic differences between malignant and benign lesions were also well visualised (Figure 7A) in model 4 (BC versus BE). The loading plot revealed most of the metabolites were raised, for example, D-glucose, glycerol, glycine, acetone, L-glutamine, 2-HBA, methylmalonate, creatinine, succinate, pyruvate, acetoacetate, and 3-HBA in BC group. Meanwhile, L-carnitine, GPC, and L-alanine were reduced in the malignancy group (Figure 7B). Even though numerous metabolites were higher in the BC, the number of appropriate biomarkers was restricted to D-glucose, glycine, and glycerol, which showed excellent reliability and magnitude in the S-plot (Figure 7C) with a narrow confidence interval in the loading plot.

3.4. Analysis of the perturbed metabolic pathways

The 44 metabolites listed in Table S3 were subjected to the pathway analysis in taboAnalyst 5.0 software. The metabolic pathways generated (Table S13, supplementary materials) had p-value of ≤0.05 (statistically significant). Particularly, two pathways- 1) Alanine, aspartate, and glutamate metabolism, as well as 2) Glycine, serine, and threonine metabolism were seen to be more affected (Figure 8 & S8).

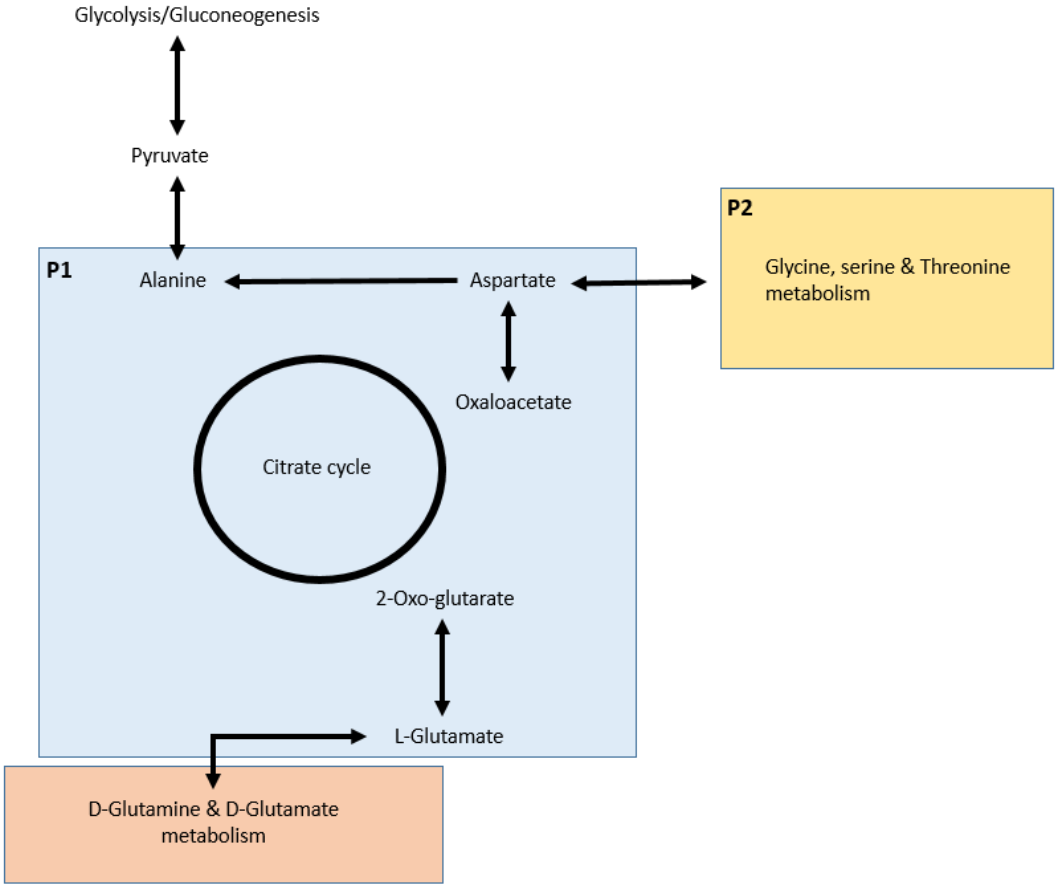

Figure 8. Involved metabolic pathways. Keys: (P1) Alanine, aspartate, and glutamate metabolism; (P2) Glycine, serine, and threonine metabolism. * The pathways were built based on results from pathway analysis in MetaboAnalyst 5.0 software and KEGG database pathway (http://www.genome.jp/kegg/).

4. Discussion

Cancer is associated with certain metabolic changes and has its own metabolic phenotyping [15]. Metabolic fingerprinting in serum samples using ^{1}H NMR spectroscopy revealed variable levels of metabolites in BC, BE, and HC in our investigation, indicating metabolic diversity. The OPLS-DA score plot of the entire data set, as depicted in , effectively distinguishes between the three groups. This clear discrimination underscores the potential of this approach for differentiating between healthy controls, benign breast lumps, and malignant breast lumps. The heat map analysis was successful in differentiating between the groups, revealing that most metabolites were upregulated in breast cancer, while branched-chain amino acids (BCAAs), with the exception of valine, were downregulated. The Variable Importance in Projection (VIP) indicated that isoleucine, lactate, and glucose were the key metabolites responsible for the separation between the three groups. This aligns with several studies that have also observed reduced levels of isoleucine in breast cancer compared to healthy controls [36, 37]. The role of BCAAs in cancer further substantiates these findings. It has been suggested that BCAA catabolism is associated with cachexia, a wasting syndrome observed in cancer patients, and the energy requirements and proliferation of the tumour. This connection between BCAA levels and cancer progression underscores the potential of these metabolites as indicators of disease state and progression [38].

We discovered that the difference in glucose and lactate concentrations in the samples between the three groups had a substantial effect on group discrimination. Our findings of increased lactate and glucose levels in breast cancer align with those of a previous study [30]. Interestingly, another researcher observed a decrease in glucose levels in breast cancer compared to the healthy control group [28]. These discrepancies can be explained by the fact that breast cancer alters carbohydrate metabolism, resulting in decreased glucose tolerance, but this is complicated by menopausal status and obesity. Luque et al. [39] likewise demonstrated that there is a direct association between breast cancer and insulin resistance in premenopausal obese patients. Meanwhile, there was also a lactate concentration disparity between earlier research [30, 37]. Lactate was once considered a product of anaerobic glycolysis in a hypoxic state [40]. Besides, lactate can also be produced under fully aerobic conditions [37] and specifically in malignancy; most of the lactate produced by the cancer cell was from aerobic glycolysis [41]. Moreover, lactate can be formed from sources other than glucose, notably glutamine catabolism [42].

Three OPLS-DA models were able to demonstrate which metabolites were upregulated and downregulated in each of the two groups. In both model 2 (HC vs BC) and model 4 (BC vs BE), D-glucose, glycine, and glycerol were shown to be elevated in the cancer group and had high reliability and magnitude to be used as possible biomarkers. According to a prediction model study that used anthropometric data and a variety of standard blood tests, glucose was the most important biomarker for predicting the existence of breast cancer by Gini coefficient and the most sensitive biomarker when ROC analysis was used [43]. Glycine was found to be one of the possible biomarkers for identifying benign from

cancer groups in a study that used serum amino acid and organic acid profiles as screening for breast cancer [44].

Amino acid metabolism was the most affected process as the pathways involved from our pathways analysis were 1) Alanine, aspartate, and glutamate metabolism & 2) Glycine, serine, and threonine metabolism. A systemic review done by Yang et al. [22] also showed a consistent finding with most of the metabolic pathways involved. Amino acids have a variety of biological and metabolic functions, and changes in their role in cancer metabolism have been observed [45]. Glutamine, in addition to the well-known primary fuel, glucose, provides an alternate energy source for cancer growth by recharging the tricarboxylic acid (TCA) cycle. Catabolism of glutamine produced glutamate, which is the source for α-ketoglutarate, a TCA cycle intermediary, and nonessential amino acids [46]. The propensity of glutamate for its product is glucose-dependent. When there is enough glucose, glutamate is converted to aspartate, alanine, glycine, and serine [47]; however, when there is not enough glucose, α-ketoglutarate is produced via glutamate dehydrogenase [47, 48].

There have been numerous metabolomics studies in breast cancer with significant separation between the case and control groups, putative biomarkers, and changed metabolic pathways. However, the results have been inconsistent. The disparity is mostly related to the heterogeneity of breast cancer, as well as differences in analytical methodologies, inter-individual biological variability, type of samples, and study protocols [49]. There are a few limitations to this study. The average age of research participants in the healthy, benign, and cancer groups could have an impact on the results, especially among healthy and benign adults, because most of the older age group will have concomitant disorders. Age can affect metabolic alterations and the metabolite level of an individual. That is why age must be considered when interpreting metabolomic research findings on cancer age-matched study participants [50, 51]. In addition, many of the metabolite changes may be potentially modifiable with drugs, diet and nutritional supplementation [52, 53]. Furthermore, metabolites in serum can originate from various sources, including the tumour itself, the host's physiological processes, and the gut microbiome [54]. Metabolic alterations in the host due to gut dysbiosis, influenced by comorbidities like obesity and diabetes [55, 56], may elevate the susceptibility to breast cancer and contribute to its advancement. Furthermore, certain metabolites originating from the gut microbiome could impact intracellular metabolism, molecular processes within breast cancer and potentially influence chemotherapy outcomes [57-59]. With that, a more well-defined case-control study with a larger sample size with multi-centre populations should be conducted in future studies.

Another limitation is the low sensitivity of ^{1}H NMR spectroscopy metabolomics in contrast to mass spectrometry-based methods. This may affect the accuracy and scope of metabolite quantification in NMR-based metabolomics [60]. A multiplatform strategy, on the other hand, would be preferable to complement the limitations of each analytical method. In conclusion, the ^{1}H NMR spectroscopy metabolomics approach was able to fingerprint the metabolites and demonstrate significant metabolites that contribute to the group separation. D-glucose, glycine, and glycerol are examples of possible biomarkers for diagnosing breast

cancer. The exact mechanism underlying the involvement of these identified metabolites in breast cancer is still elusive. Thus, a mechanistic study of several metabolites in the pathophysiology of breast cancer is required by integrating information with other upstream 'omics', such as genomics, transcriptomics, and proteomics, before any clinical translation can be attempted [61]. For instance, the machine learning approach, such as support vector machine-recursive feature elimination (SVM-RFE) [62], has been demonstrated to be invaluable in integrating the diverse 'omics' datasets, expediting the identification of robust biomarkers [63], thus potentially enhancing our understanding of the complex molecular landscape associated with breast cancer and advancing precision medicine efforts in breast cancer research.

5. Conclusions

The analysis of serum samples from three distinct groups (healthy control, benign breast lump, and malignant breast lump) revealed significant variations in metabolites across the groups. Data analysis techniques such as OPLS-DA and clustered heat maps successfully differentiated between these groups. D-glucose, glycerol, and glycine emerged as potential biomarkers for breast cancer. However, the average age of the study participants may have influenced these findings. Despite the challenges in finding age-matched study participants, it is important to acknowledge this potential bias. To enhance the precision of these findings, future studies should involve larger populations and multiple centres for validation.

Supplementary Materials: The following are available online at the journal website.

Author Contributions: Conceptualization, JO and AZ; methodology, TADAATD and WNWA; software, FA and DFS; validation, MM; resources, WFWAR and MMY; writing—original draft preparation, AZ and MSY; writing—review and editing, TADAATD and NSAM.

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Conflicts of Interest: The authors declare that they have no competing interests.

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