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# A novel serum metabolome score for breast cancer diagnosis

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**ABSTRACT**

**Introduction:** Early detection of breast cancer is important in diagnosis and treatment, and so in enhancing patient survival and reducing death rates. Because of the low diagnostic sensitivity and specificity of widely used breast cancer biomarkers such as CA15-3, we hypothesised that a panel of new metabolic markers would provide superior sensitivity and specificity for this disease.

**Material & Methods:** We recruited 120 women with malignant breast cancer, 47 with benign breast disease and 55 females as a healthy control group. Metabolites 8-hydroxy-2′-deoxyguanosine, 1-methylguanosine, and 1-methyl adenosine were detected and quantified by gas chromatography-mass spectrometry, CA15.3 by ELISA. Cut-off values of individual and combined metabolome with CA15-3 were analysed using the receiver operating characteristics curve (ROCC) to test the efficiency of the candidate metabolome in identifying breast cancer.

**Results:** The overall linear trend of biomarkers across the groups was significant with highest levels in breast cancer (all $p < 0.05$). Using cut-off values of CA15-3, 8-hydroxy-2′-deoxyguanosine, 1-methylguanosine and 1-methyl adenosine of 30.5 U/l, 15.0 µg/l, 18.5 µg/l and 22.0 µg/l, respectively, diagnostic performance analyses of combined metabolome with CA15-3 gave a ROCC area under the curve of 0.94 (95% CI: 0.91–0.98)($p < 0.01$) with good sensitivity (88.8%), specificity (86.8%) and efficiency (90.6%). Unlike CA15.3, the highest levels of each of the metabolite were in the early stage of breast cancer.

**Conclusion:** The diagnostic combination test of candidate metabolome with CA15.3 may be a useful tool for the early detection of breast cancer and used as a metabolomics signature in this disease.

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Metabolomics; mass spectrometry; metabolome; breast cancer; CA15-3

## Introduction

Breast cancer is the most frequent cancer among women worldwide [1]. Mammography and ultrasonography, together with biopsy, are used for routine screening and staging. Limitations of these investigations are exposure to radiation, a breast tumour must be at least a few millimetres in size for detection and often produce inaccurate results. The common biomarkers of breast cancer such as human epidermal growth factor receptor-2, oestrogen and progesterone receptors, are linked to poor prognoses [2]. Serum biomarkers, such as CEA, CA15-3, and CA27-29 often respond late to cancer recurrence and associated with low sensitivity and/or low specificity and often respond late to recurrence [3].

Metabolomic studies and their application are becoming an increasingly popular tool in life science and clinical applications. It is a fast, accurate, cost-efficient and automated technique [4,5]. Mass spectrometry can often be paired with a gas or liquid chromatography (i.e. GC-MS) and is considered the gold standard to analyse many small compounds (lipids, metabolites of drugs, and environmental pollutants), volatile, thermostable and low molecular weight molecules [6–8]. The metabolome (the sum of all metabolites in an organism) is defined as all small-molecule metabolites (<1000 Da) released through cellular metabolic processes. Subtypes of the overall metabolome provide a simple functional tool for understanding specific cellular activity and physiology, such as in endocrinology or cancer. Modifications of RNA molecules, regulation of gene transcription and translations of proteins are controlled with several different types of metabolome [9].

Because of the low molecular weight of selected metabolites, they have no interaction with serum proteins, and are retained in the circulation [10], so that the serum content of a metabolome is much less than that in the urine [11]. However, serum specimens are preferred in the determination of the metabolome as serum is less affected by exogenous factors than those in the urine, and the creatinine level measurement is necessary for urinary metabolome estimation [12]. Abnormal levels of serum metabolome in breast cancer patients are associated with oxidative DNA damage or RNA's turnover and increased methyl-transferase activity [13]. Therefore various metabolomes of body fluids

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have been proposed as biomarkers for different cancers such as hepatocellular carcinoma, colorectal, breast, bladder, lymphoma, thyroid and cervical cancer [14–20].

We focused on the quantification of three candidate serum metabolites derived from nucleosides: 8-hydroxy-2′-deoxyguanosine (8-OHdG), 1-methylguanosine (1-MG) and 1-methyl adenosine (1-MA). Through oxidative DNA damage, which increases in cancer, the rate of 8-OHdG production increases [21]. Metabolites 1-MA, and 1-MG are related to RNA turnover and methyl-transferase activity which increase in the cancer cell process. This small metabolome may be quantified as a mixture utilizing gas GC-MS [22,23]. We hypothesised that individual and combined levels of serum 8-OHdG, 1-MG, and 1-MA would provide discrimination of breast cancer patients, and so develop a new diagnostic tool as a potential biomarker for the early diagnosis of breast cancer. We tempered our hypothesis by including one of the leading breast cancer marker – serum CA15-3.

## Materials and methods

We tested out hypothesis with 120 women with malignant breast cancer, 47 with benign breast diseases and 55 healthy females as a negative control group. Based on the American Joint Committee on Cancer (AJCC), patients with malignant breast diseases were classified into their clinicopathological characteristics and their TNM staging into early breast cancer, advanced cancer and metastatic disease. Diagnoses in the benign breast diseases were 35 with fibroadenoma and 12 with a papilloma. Patients with benign breast disease and healthy controls had no history of any malignancy. All subjects had normal laboratory data (e.g. albumin, total bilirubin, AST, ALT, creatinine, fasting blood sugar, cholesterol, HDL-C, LDL-C, and triglycerides) with a normal estimated glomerular filtration rate (eGFR). CA15-3 levels in serum were measured by ELISA (DRG International, Inc., Springfield Township, NJ, USA).

Three ml venous blood was withdrawn in a blank tube in the morning after overnight fasting. Serum was obtained by centrifuging the sample at 4000 rpm for 5 minutes which were kept in – 20° C until analysis. Up to 2 days after breast cancer diagnosis and before any cancer treatment started, blood specimens were collected from patients. This study was carried out in accordance with the ethical guidelines of the 1975 declaration of Helsinki and was approved by the medical ethics committee of the Ismailia Teaching Oncology Hospital, Ismailia, Egypt. Written informed consent was obtained from each subject. Standard 8-OHdG, 1-MG, 1-MA compounds, internal standards, and sources of human serum purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol and n-hexane purchased from Merck (Darmstadt, Germany). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), the derivatizing agent, was purchased from Pierce Chemical Co (Rockford, Ill., USA).

The serum specimens were thawed for 30–60 minutes in a water bath at 4 °C. To 200 µ/L serum, 0.5 ml of methanol, including 1 mg/L of internal standard was added then vortexed for 15 seconds and centrifuged for 15 minutes at 5000 rpm at 4° C. A quantity of 150 µ/L of the supernatant was transferred to a separate new tube and dried by nitrogen gas followed by adding 100 µ/L MSTFA and mixing for 15 seconds, then incubated for 1 hour (65°C) after that kept at room temperature 15 minutes followed by adding of 1 mL n-hexane in clear analytical vial [24]. Quality control, calibration curves and assay performance indices (repeatability, precision, accuracy reproducibility limit of detection, etc.) by standard techniques [25,26]. Analyte recoveries and limit of quantitation (LOQ) are calculated with excellent relative standard deviations (RSDs). Recoveries of 8-OHdG, 1-MG, and 1-MA were 108%, 96% and 95% with RSDs 5.7%, 5.1%, and 6.2%, respectively. Intra-assay precision of the method was tested by injecting (n = 6) individual solutions of standards and samples within the mid-range of the calibration curves. Inter-assay method precision was tested in the same manner, but in two separate days and results from both days (n = 12). RSD of intra-assay was lower than 5% and inter-assay was lower than 11%. For all participants, 1-µ/L of each prepared sample was injected and quantified. Thus, the concentrations of candidate metabolome in serum were determined.

Nonparametric analyses were performed to assess differences of candidate biomarker measurements across different groups. Data are shown as median values (interquartile range) or mean [standard deviation]. Differences in disease severity (healthy controls < benign disease < breast cancer) were performed using linear trend analysis. The area under the curve (AUC) and cut-off values were derived from running the ROC analysis on SPSS 22.0 (SPSS, Chicago, IL, USA) software. A multiple linear regression stepwise model was run to fit the combinations between each candidate metabolite and CA15-3 and between each metabolite with CA15-3, which we name a Serum Metabolome Score (SMS). For individual and combined variables, a performance model including accuracy (efficiency) sensitivity, specificity, positive and negative predictive values (PPV and NPV) were derived using a $2 \times 2$ cross-tabulation. P-values for all tests were significant at $<0.05$ and the confidence level was 95%.

## Results

Table 1 shows age, metabolites and CA15-3 in the three groups. There was no difference in age, or in any index between the healthy controls and benign disease controls. However, there was a significant linear trend in the three groups with increasing disease severity. Compared with cancer-free women, in those with cancer, CA15-3 was approximately 61% higher, 8-OHdG was some 124% higher, 1-MG was around 107% higher, whilst 1-MA levels were 194% higher.

Table 2 shows age and the serum markers in the three stages of breast cancer. Although age increased and both CA15-3 and 1-MG decreased with disease severity, these were not significant. In contrast, the fall in both 1-MA and 8-OHdG was significant, the latter being markedly more significant.

The optimal cut-off levels of the metabolome and CA15-3 between non-cancer (n = 102) and cancer (n = 120) were determined by ROCC analysis. Table 3 shows the cut-off points that gave the highest AUCs, and their respective assay performance indices. When combining each metabolite with CA15.3, AUCs were improved (from an average of 0.80 to 0.89), and were further improved (to 0.91) when two metabolites were combined with CA15.3. However, for best discrimination models and to achieve patterns and relationships between variables, multivariate analyses were performed by combinations between CA15-3 (as the most significant biomarker used in the diagnosis of breast cancer) and candidate metabolites using linear regression analysis and a stepwise model. The sensitivity, specificity, and efficiency (accuracy) of combined biomarkers increased compared to single biomarkers. This analysis formed a Serum Metabolome Score (SMS) for defining breast cancer, generated from a formula where SMS = 1.045 + (0.005 x CA15-3) + (0.017 x 8-OHdG) + (0.011 x 1-MG) + (0.005 x 1-MA). The AUC of the SMS was 0.94 (Figure 1) with the highest sensitivity, specificity and efficiency.

## Discussion

As in most malignant diseases, early breast cancer detection is crucial in effective diagnosis, treatment, enhanced patient survival and in reducing the rate of death [3]. Although CA15-3 is the most widely used biomarker for the diagnosis of breast cancer, its use is limited because of the low diagnostic sensitivity and specificity. Therefore, it is necessary to develop novel biomarkers to accurately differentiate patients with and without cancer. Many studies have applied the metabolomics approach to demonstrate the importance of the metabolome for diagnoses and prognoses in breast and other cancers [14–20]. Many recent studies focused on the determination and identification of the metabolome of malignant breast disease in serum and body fluid to emerge its medical significance [16,27,28]. Specific assignments of biochemical metabolite compounds in breast tumours have used a combination of the different platforms of mass spectrometry and nuclear magnetic resonance analysis techniques [6,29].

Our study tested the hypothesis of an improvement in the diagnostic sensitivity of breast cancer diagnosis using individual candidate metabolites and their combination into a metabolome. This we achieved by quantifying the concentration of serum 8-OHdG, 1-MG, and 1-MA in healthy individuals, benign and malignant breast disease patients using GC-MS, and then studied the associations between serum levels of CA15-3 and the combined metabolome with the different groups. The findings support the major set of changes in the metabolism of cancer cells resulting in a turn to increased nucleoside metabolism levels through accelerated methylation, as in 1-MA and 1-MG, and dihydroxylation, as in 8-OHdG. Within breast cancer patient serum, we found increased levels of candidate metabolome

**Table 1.** Characteristics of participants.

| Parameters | Control group n = 55 | Benign group n = 47 | *P*-value | Malignant group n = 120 | $P_{trend}$ |
|---|---|---|---|---|---|
| Age (years) | 44 [8.2] | 45 [9.3] | 0.479 | 44 [10.0] | 0.521 |
| CA15-3 (U/l) | 31.0 (21.5–34.5) | 30.0 (12.8–36.2) | 0.253 | 49.0 (28.0–73.0) | 0.004 |
| 8-OHdG (µg/l) | 15.0 (11.2–26.5) | 18.0 (12.0–25.0) | 0.278 | 37.0 (17.0–49.0) | 0.009 |
| 1-MG (µg/l) | 11.0 (7.5–19.2) | 15.5 (11.0–19.8) | 0.140 | 27.5 (15.5–39.0) | 0.031 |
| 1-MA (µg/l) | 12.5 (8.0–19.5) | 14.0 (10.0–17.2) | 0.067 | 39.0 (20.5–60.5) | 0.001 |

Data are presented as mean [SD] or median (interquartile range), P-value from comparing the benign to control groups, P trend from linear trend analysis by comparing control to benign to malignant groups.

**Table 2.** Characteristics of breast cancer patients.

| Parameters | Early stage, n = 75 (62.5%) | Advanced stage, n = 30 (25.0%) | Metastasis stage, n = 15 (12.5%) | $P_{trend}$ |
|---|---|---|---|---|
| Age (years) | 44.0 [11.1] | 45.0 [8.2] | 46.0 [7.3] | 0.192 |
| CA15-3 (U/l) | 44.0 (27.0–65.5) | 41.0 (35.0–58.0) | 37.5 (21.0–73.0) | 0.558 |
| 8-OHdG (µg/l) | 45.0 (27.5–53.0) | 31.0 (26.0–38.5) | 29.0 (24.0–35.5) | 0.001 |
| 1-MG (µg/l) | 38.0 (22.0–53.0) | 34.0 (13.5–46.0) | 27.0 (19.0–43.5) | 0.223 |
| 1-MA (µg/l) | 47.0 (24.0–65.0) | 38.0 (28.0–61.0) | 35.0 (24.0–46.5) | 0.041 |

Data are presented as mean [SD] or median (interquartile range). $P_{trend}$ from linear trend analysis across the malignant categories.

**Table 3.** The diagnostic performance analysis of each candidate metabolome and combined metabolomes together with CA15-3 to discriminate between non-cancer (n = 102) and cancer patients (n = 120).

| Test result variable(s) | AUC | 95% CI | Cut-off | Sn. (%) | Sp. (%) | PPV (%) | NPV (%) | Efficiency (%) |
|---|---|---|---|---|---|---|---|---|
| CA15-3 (U/l) | 0.74 | 0.67–0.81 | 31.5 | 65.4 | 59.3 | 61.4 | 55.1 | 60.5 |
| 8-OHdG (µg/l) | 0.80 | 0.76–0.87 | 23.5 | 67.3 | 67.4 | 69.2 | 65.4 | 65.4 |
| 1-MG (µg/l) | 0.77 | 0.71–0.84 | 18.5 | 66.4 | 75.5 | 74.4 | 67.3 | 70.7 |
| 1-MA (µg/l) | 0.82 | 0.75–0.86 | 18.0 | 74.7 | 68.3 | 72.1 | 71.3 | 71.1 |
| CA15-3 + 8-OHdG | 0.89 | 0.83–0.93 | 2.08 | 81.3 | 80.6 | 82.1 | 79.8 | 81.0 |
| CA15-3 + 1-MG | 0.87 | 0.83–0.92 | 2.13 | 80.4 | 80.6 | 81.9 | 79.0 | 80.5 |
| CA15-3 + 1-MA | 0.91 | 0.87–0.95 | 2.05 | 84.1 | 83.7 | 84.9 | 82.8 | 83.9 |
| CA15-3 + 8-OHdG + 1-MG | 0.91 | 0.88–0.94 | 1.95 | 82.6 | 78.5 | 80.2 | 84.0 | 83.5 |
| CA15-3 + 8-OHdG + 1-MA | 0.92 | 0.88–0.96 | 1.85 | 85.2 | 81.2 | 77.9 | 80.6 | 85.0 |
| 8-OHdG + 1-MG + 1-MA | 0.89 | 0.84–0.93 | 2.21 | 84.0 | 76.0 | 80.5 | 85.0 | 84.2 |
| SMS | 0.94 | 0.91–0.98 | 2.06 | 88.8 | 86.8 | 89.6 | 87.8 | 90.6 |

AUC; the area under curve, CI; confidence interval, Sn.; Sensitivity, Sp.; specificity, PPV; positive predictive values, NPV; negative predictive values, SMS; Serum metabolomes score of linear combination of different variables (CA15-3 + 8-OHdG + 1-MG +1- MA)

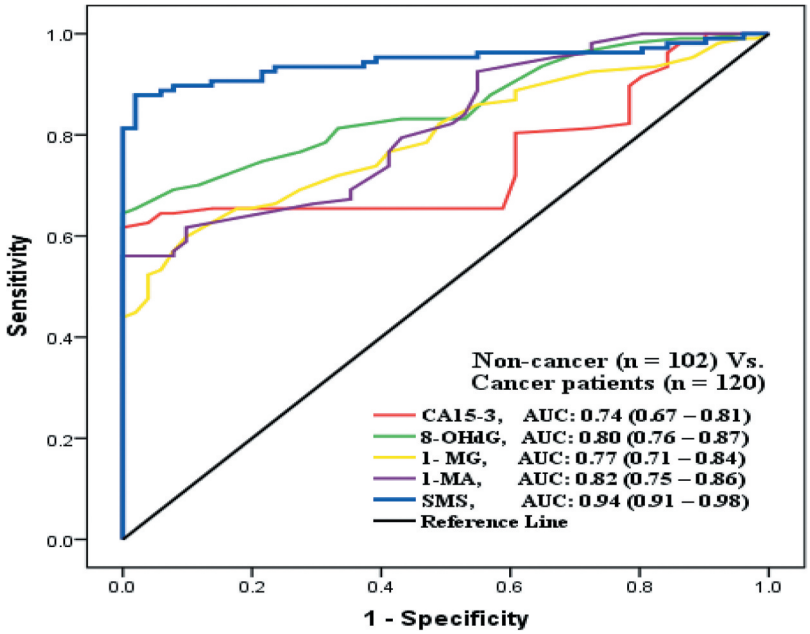


**Figure 1.** Receiver operating characteristics (ROC) curves of individual biomarkers and serum metabolome score (SMS) with AUC values and 95% confidence intervals of AUC (in brackets).

compared to normal control and benign breast disease. The most notable increase in candidate biomarker levels was in the early stage of breast cancer.

These findings are consistent with others who reported that the serum level of 8-OHdG is increased in patients with breast cancer [30,31], that serum 1-MG is increased in patients with colorectal cancer [14], and that serum 1-MA is increased in hepatocarcinoma [32]. The diagnostic score of each individual metabolite plus CA15.3, and of the metabolome combined with CA15-3 significantly improved the breast cancer diagnostic sensitivity and specificity compared to CA15-3 alone.

The elevated levels of candidate metabolome in breast cancer patients can be explained by the likelihood that nucleoside metabolome occurred in the circulation caused by oxidative DNA damage and RNA's metabolites turnover, which increases in malignant diseases [33]. 1-MG is a methylated nucleoside resulting from RNA degradation and is excreted in patients with malignant tumours at elevated levels [34]. 1-MA reflects DNA damage and modification and is expected to affect the pairing of the RNA bases. It has a reversible action in the post-transcription tRNA and mRNA and affects translation processes efficiency [35]. As a result, 1-MA has a high transcriptional rate in cancer cells and is found in increased levels in the serum of breast cancer patients.

Our findings also show a decrease in the levels of the four markers with increasing disease severity, and in two cases this is significant. These results agree with those of Kuo et al. [36]. This decrease in levels of metabolites with the severity of breast cancer can be explained by accelerated carcinogenesis that may have been associated with metabolic enzymes responsible for DNA breakdown and stimulating these metabolites' excretion in the early clinical stages [37].

This work represents an advance in biomedical science because it provides a novel diagnostic score

with high diagnostic sensitivity and selectivity that may improve breast cancer staging, diagnosis, and monitoring, and so should be a routine test in this disease.

## Summary table

*What is known about this subject*:
- Early detection of breast cancer is an important factor in initiating potential curative treatments and achieving a favourable outcome.
- Metabolomic studies and their applications are becoming increasingly popular tools in life sciences and clinical applications.
- Common breast cancer biomarkers are associated with low sensitivity and/or low specificity and often respond late to recurrence.

*What this paper adds*:
- Metabolites 8-OHdG, 1-MG and 1-MA are as accurate as CA15.3 in determining breast cancer.
- The each metabolite, but not CA15.3, is highest in early breast cancer.
- The combination of each metabolite with CA15.3 improved discriminatory power, but the most effective was a combination of all four indices.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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