Sains Malaysiana 53(8)(2024): 1749-1765
http://doi.org/10.17576/jsm-2024-5308-03

Proteomic Profiling of Serum of Women with BI-RADS 1 to 5: Identification of Potential Complementary Biomarkers for Early Detection of Breast Cancer

(Pemprofilan Proteomik Serum Wanita dengan BI-RADS 1 hingga 5: Pengenalpastian Penanda Bio Pelengkap Berpotensi untuk Pengesanan Awal Kanser Payudara)

Jaime Jacqueline Jayapalan[1,2,*], Christina Jane Vellan[1], Tania Islam[3], Nur Aishah Mohd Taib[3] & Karuthan Chinna[4]

[1]*Department of Molecular Medicine, Faculty of Medicine, Universiti Malaya, 50603 Kuala Lumpur, Malaysia*
[2]*Universiti Malaya Centre for Proteomics Research (UMCPR), Universiti Malaya, 50603 Kuala Lumpur, Malaysia*
[3]*Department of Surgery, Faculty of Medicine, Universiti Malaya, 50603 Kuala Lumpur, Malaysia*
[4]*Faculty of Business and Management, UCSI University, Cheras, 56100 Kuala Lumpur, Malaysia*

Received: 20 February 2024/Accepted: 26 June 2024

ABSTRACT

Mammography remains the gold standard for the screening of breast cancer (BrCa) despite its shortcomings. Cancer antigen 15-3, an FDA-approved biomarker, is most useful as a treatment response and recurrence monitoring tool rather than for early detection of BrCa. Given this, we aimed to screen for potential complementary diagnostic protein biomarkers in the serum of women with BI-RADS 1 to 5. Individual neat sera of women with BI-RADS 1 to 5 ($N = 33$) were subjected to two-dimensional electrophoresis (2-DE) for the separation of proteins. Comparative analysis of the 2-DE silver-stained gel images was performed using Progenesis SameSpots software. The identification of protein spots of interest was determined following tandem MS/MS analysis and database search using either MASCOT or X! Tandem Vengeance search engines. Data are available *via* ProteomeXchange with the identifier PXD040427. The Bioinformatics tools of The Database for Annotation, Visualization, and Integrated Discovery were used for the functional annotation of the proteins of altered abundance. A total of 8 non-redundant proteins including albumin, apolipoprotein A-I, apolipoprotein A-II, clusterin, complement C3, immunoglobulin kappa constant, kininogen-1, and leucine-rich alpha-2 glycoprotein were found significantly overexpressed in the sera of women with BI-RADS 4 and/or 5 ($p < 0.01$, FC ≥ 2). Functional annotation of the significantly differentially expressed proteins showed their possible roles in the development of BrCa. The identified protein signatures are potential biomarkers for use in complement with mammography for improved detection of BrCa at an early stage.

Keywords: BI-RADS; biomarker; breast cancer; early detection; mass spectrometry; two-dimensional electrophoresis

ABSTRAK

Mamografi tetap dianggap sebagai piawaian emas dalam saringan kanser payudara (BrCa) walaupun terdapat beberapa kelemahan. Antigen kanser 15-3 yang diluluskan oleh FDA lebih berguna sebagai alat pemantauan tindak balas rawatan dan pengesan kanser berulang daripada untuk pengesanan awal BrCa. Oleh itu, kajian ini bertujuan untuk menyaring dan mengenal pasti penanda protein diagnostik yang mungkin menjadi pelengkap dalam serum wanita yang tergolong dalam kategori BI-RADS 1 hingga 5. Serum individu wanita tersebut telah diuji menggunakan teknik elektroforesis dua dimensi (2-DE) untuk memisahkan protein ($N = 33$). Analisis imej gel 2-DE dilakukan dengan menggunakan perisian Progenesis SameSpots. Pengenalpastian tompok protein yang berkaitan dilakukan melalui analisis spektrometri jisim berganda dan pencarian dalam pangkalan data menggunakan enjin carian *MASCOT* atau *X! Tandem Vengeance*. Maklumat mengenai data kajian boleh didapati melalui repositori ProteomeXchange dengan kod pengenalan PXD040427. Alat Bioinformatik Pangkalan Data untuk Anotasi, Visualisasi dan Penemuan Bersepadu (DAVID) telah digunakan untuk anotasi fungsi protein yang mempunyai kelimpahan yang berbeza. Sejumlah 8 protein termasuk *albumin*, *apolipoprotein A-I*, *apolipoprotein A-II*, *clusterin*, *complement C3*, *immunoglobulin kappa constant*, *kininogen-1* dan *leucine-rich alpha-2 glycoprotein* didapati meningkat secara signifikan dalam serum wanita yang tergolong dalam kategori BI-RADS 4 dan/atau 5 ($p < 0.01$, FC ≥ 2). Anotasi fungsi protein ini menunjukkan keterlibatan potensi mereka dalam proses perkembangan BrCa. Penemuan protein ini menonjolkan potensi sebagai penanda bio tambahan untuk digunakan bersama mammografi bagi meningkatkan keupayaan pengesanan BrCa pada peringkat awal.

Kata kunci: BI-RADS; elektroforesis dua dimensi; kanser payudara; penanda bio; pengesanan awal; spektrometri jisim

Introduction

Breast cancer (BrCa) has surpassed lung cancer for being the most reported cancer worldwide while it remains the paramount cause of global female cancer deaths (Sung et al. 2021). Despite the high incidence, unfortunately, prevention is not an established part of BrCa management (Borgquist et al. 2018; Shah & Guraya 2017). Mammography remains the gold standard in routine BrCa screening, albeit the emergence of new imaging modalities such as breast ultrasound and magnetic resonance imaging (Harkness, Astley & Evans 2020). The American College of Radiology has inaugurated a classification system, Breast Imaging-Reporting and Data System (BI-RADS) to standardize the risk assessment and quality control of mammography, in addition to ensuring uniformity while reporting the mammographic findings (Sickles, D'Orsi & Bassett 2013). The BI-RADS scoring system includes seven assessment categories, denoted as scores 0 to 6 (Sickles, D'Orsi & Bassett 2013). BI-RADS scores 1 to 5 indicate negative, benign, probably benign, suspicious of malignancy, and highly suggestive of malignancy, respectively, representing a spectrum from essentially no chance (0%) to more than 95% chance of advocating a positive breast cancer (BrCa) diagnosis. BI-RADS score 0 indicates an incomplete assessment, providing no clinically relevant diagnostic information, while BI-RADS score 6 denotes a known BrCa diagnosis confirmed via breast tissue biopsy. Nonetheless, mammographic findings alone are not specific and reliable to be truly diagnostic without complementing evidence from tissue biopsy (Bevers et al. 2018) for confirmatory diagnosis. In addition, mammographic screening does possess several limitations in its sensitivity and specificity (Monticciolo, Helvie & Hendrick 2018; Seely & Alhassan 2018), thus causing needless anxiety and overtreatment among patients.

In addition to the shortcomings of mammographic screening, the invasiveness of breast tissue biopsy has further prompted the interest of the research community to explore other alternative yet reliable markers for the early detection of BrCa (Li et al. 2020). Among these, protein-based biomarkers are of particular interest due to their functional and effector roles in biological systems (Duffy, McDermott & Crown 2018). The proteome is found to show a more dynamic physiological state of the cell(s), particularly in part due to genetic alterations, hence reflected as aberrantly expressed proteins (Zografos et al. 2019). Dynamically evolving proteomics techniques (Kwon et al. 2021) such as two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) can identify these aberrations in the blood/urine, which are less invasive alternatives to tissue biopsy (Nunez 2019).

Even though cancer antigen (CA) 15-3 is the most promising serum biomarker for BrCa, it is used for the monitoring of therapeutic responses and detection of recurrence in metastatic BrCa patients (Yang et al. 2017). The levels of serum CA 15-3 are rarely elevated among those with localized BrCa. Hence, it is inappropriate for use as a biomarker for early detection of BrCa, despite the recent reports expounding on its enhanced detection limit using antibody-lectin sandwich assays (Choi et al. 2018).

Recognizing the limitations inherent in traditional mammographic screening, our study seeks to discover novel protein signature markers with greater specificity for the early detection of BrCa. Leveraging 2-DE, a gel-based proteomics technique, we aim to examine serum samples from women spanning across the spectrum of BI-RADS categories (1 to 5). Through this analysis, we aim to identify complementary biomarkers that can significantly improve the accuracy and reliability of mammographic screening, ultimately transforming the approach to early BrCa detection.

Materials and Methods

Subject Recruitment

The study was conducted with approval granted by the Medical Ethics Committee of Universiti Malaya Medical Centre (Ethics Approval Number: MEC No. 435.18) and following the declaration of Helsinki. Only those women aged 18 years and above, with mammography-based BI-RADS scoring of 1 to 5 (Sickles, D'Orsi & Bassett 2013) were included in the study to represent negative, benign, probably benign, suspicious of malignancy, and highly suggestive of malignancy states of BrCa, respectively. Additionally, those women who were annotated with BI-RADS 4 and 5 were required to undergo a breast tissue biopsy procedure for confirmatory diagnosis. Besides this, those subjects who were presented with other co-morbidities, undergoing hormonal replacement therapy, or receiving any forms of medication were excluded from the study. Informed written consent was obtained from all subjects, before the collection of samples.

Sample Collection and Processing

Approximately 3 mL of blood was collected into plain BD vacutainers (Becton, Dickinson & Co, Franklin Lakes, New Jersey, USA) from the subjects. The specimens were left to stand at room temperature (RT) for 30 min before centrifugation at 1200 × *g* for 15 min at 4 °C. The separated sera were then carefully collected and stored at −80 °C until further use. The concentration of serum proteins for each sample was estimated using Pierce™ bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's protocols.

2-DE AND SILVER STAINING

2-DE was performed as previously reported (Jayapalan et al. 2012). Briefly, the respective neat serum samples containing approximately 800 µg of proteins were subjected to first-dimensional isoelectric focusing (p*I*) based separation using Ettan IPGphor 3 Isoelectric Focusing System (GE Healthcare, Uppsala, Sweden) followed by a second-dimensional electrophoretic separation using SE 600 Ruby Electrophoresis System (GE Healthcare). The electrophoresed 2-DE gels were then stained using a modified silver staining protocol (Yan et al. 2000).

IMAGE ANALYSES

Silver-stained 2-DE gels were scanned using ImageScanner III (GE Healthcare) and analyzed using Progenesis SameSpots version 4.5 (Nonlinear Dynamics, Newcastle, UK). Spots detected with an area of 200 pixels or less, presumed artefacts, were removed at the 'filtering' stage. Those spots that fulfilled the statistical filters including p-value < 0.01 and fold change (FC) ≥ 2, were manually reviewed for inclusion in the comparative analysis.

PROTEIN IDENTIFICATION *VIA* TANDEM MS (MS/MS) ANALYSIS AND DATABASE SEARCH

Protein spots of interest were carefully excised from 2-DE gels and were subjected to the in-gel tryptic digestion method as previously reported (Jayapalan et al. 2012). The tryptic-digested peptides were analyzed using either a 5800 matrix-assisted laser desorption/ionization-tandem time of flight mass analyzer (Applied Biosystems, Foster City, USA) or Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific). The resulting MS/MS spectra were searched against *Homo sapiens* entries in the Swiss-Prot database (updated on 23 September 2020; 26556 sequences) via a concatenated target/decoy strategy, with decoy sequences generated by reversing the target sequences. This was performed with the MASCOT search engine (Perkins et al. 1999) or X! Tandem Vengeance search engine version 2015.12.15.2 (Craig & Beavis 2004) incorporated in SearchGUI software version 4.0.12 (Barsnes & Vaudel 2018). For the former, Mascot peptide scores higher than 30 indicate identity or extensive homology ($p < 0.05$). For the latter, the identity of peptides and proteins was inferred from the search results using PeptideShaker version 2.0.10 (Vaudel et al. 2015). A 1% false discovery rate (FDR) was used as a threshold for validation of the peptide spectrum matches using PeptideShaker. The search parameters were set as follows: (i) Enzyme: trypsin; (ii) Specificity: specific; (iii) Maximum missed cleavage: 1; (iv) Fixed modification: carbamidomethylation of cysteine; (v) Variable modification: oxidation of methionine; (vi) MS precursor ion mass tolerance: 100 ppm; (vii) MS/MS fragment ion mass tolerance: 0.2 Da; (viii) Inclusion of monoisotopic masses only. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE (Perez-Riverol et al. 2021) partner repository with the dataset identifier PXD040427.

STATISTICAL ANALYSES

Normalized volumes of the protein spots were log-transformed prior to statistical analyses using Statistical Package for Social Sciences (SPSS) version 25.0 (IBM Corporation, Armonk, USA). Fisher's exact test was carried out to determine the association between the categorical variables. The distribution of the datasets was determined using the Shapiro-Wilk test of normality. One-way ANOVA and appropriate *post-hoc* analyses were subsequently carried out for the comparative analysis between the groups of subjects. Following an FDR analysis (Benjamini & Hochberg 1995), a corrected p-value of ≤ 0.0072 indicates significantly altered levels of proteins between the groups of subjects. The statistical outputs were appropriately reported as previously described (Lang & Altman 2015; Tomczak & Tomczak 2014).

BIOINFORMATIC ANALYSES

Functional annotation tool, The Database for Annotation, Visualization, and Integrated Discovery (DAVID 6.8) Bioinformatics Resources was used for the clustering of significantly enriched Gene Ontology (GO) terms (corrected $p < 0.05$) (Huang, Sherman & Lempicki 2009). The cluster with the highest enrichment score represents the top-ranked biological significance and *vice versa*.

RESULTS

STUDY SUBJECTS

Following the selection criteria described, only a total of 33 subjects were included in the study. The demographic characteristics of the subjects are summarized in Table 1. Age did not differ significantly between the groups of subjects [$F(4,28) = 1.861$, $p = 0.145$]. Similarly, there was no significant association between ethnicity and BI-RADS scores among the subjects ($p = 0.503$).

COMPARATIVE PROTEOMICS ANALYSIS OF SERUM OF WOMEN WITH BI-RADS 1 TO 5

2-DE was performed on individual neat serum samples ($N = 33$) obtained from women with BI-RADS 1 to 5. Figure 1 shows typical representative 2-DE profiles generated from the neat sera of a woman with BI-

RADS 4 (randomly selected). The technique was able to resolve hundreds-thousands of serum proteins that are predominantly of high abundance according to their respective p*I* and molecular mass (M_r). Image analyses resulted in the detection of a total of 39 significantly differentially expressed spots of protein (Supplemental data available upon requests). However, among these, only 20 spots of proteins (spots A - T) had retained their significant expressions post-FDR analysis (corrected $p \leq 0.0072$) (Supplemental data available upon requests). The cropped images of the differentially expressed spots of proteins (spots A - T) are shown in Figure 2.

The expression of Spot A was significantly higher among women with BI-RADS 1 [7.764 (0.216)] compared to those with BI-RADS 2 [7.295 (0.239)] [$F(4, 28) = 5.466$, $p = 0.0022$, $\eta^2 = 0.438$] (Figure 3(A)). On the other hand, spot B was significantly highly expressed in the sera of women with both BI-RADS 1 [7.484 (0.123)] and 4 [7.483 (0.122)] compared to those women with BI-RADS 3 [6.993 (0.311)] [$F(4,28) = 7.078$, $p = 0.0005$, $\eta^2 = 0.503$], respectively (Figure 3(B)). Spot C was significantly overexpressed in the sera of women with BI-RADS 4 [7.482 (0.164)] compared to those with BI-RADS 1 [6.708 (0.244)] [$F(4, 28) = 5.313$, $p = 0.0026$, $\eta^2 = 0.431$] (Figure 3(C)). Interestingly, 16 spots of serum protein, including spots D to O and spots Q to T were significantly highly expressed among women with BI-RADS 4 compared to those with BI-RADS 2 (Figures 3(D) – 3(O) & 2(Q) – 2(T)) ($p < 0.0072$). In addition, spots M, N, O, and T demonstrated higher levels of expression in the sera of women with BI-RADS 4 compared to those with BI-RADS 3 (Figures 3(M) – 3(O) & 2(T)).

Aside from this, spot T and another three spots of serum proteins including, spots Q, R and S were significantly overexpressed among women with BI-RADS 5 compared to those women with BI-RADS 3 and 2, respectively (Figure 3(T) & 3(Q) – 3(S)). Finally, the expression levels of spot P were found significantly higher in the sera of women with BI-RADS 4 [7.520 (0.230)] and 5 [7.819 (0.144)] compared to those women with BI-RADS 1 [6.936 (0.153)] [$F(4, 28) = 7.239$, $p = 0.0004$, $\eta^2 = 0.508$], respectively (Figure 3P).

MS analysis of the 20 significantly differentially expressed spots of proteins resulted in the identification of eight non-redundant proteins (Table 2; Supplemental data available upon requests). These include kininogen-1 (KNG1; spots B and M), apolipoprotein A-II (APOA2; spots C and P), complement C3 alpha chain (C3; spots D and F), leucine-rich alpha-2 glycoprotein (LRG; spot E), fragments of albumin (ALB; spots G, I, K, and L), clusterin (CLU; spots H, J, Q, and S), apolipoprotein A-I (APOA1; spots A, N, O, and R), and immunoglobulin kappa constant (IGKC; spot T). Proteins including APOA1 (spots A and O), C3 (spots D and F) and ALB (spots G, I, K, and L) demonstrated a marked shift in their mass and p*I* as denoted by their distinct locations in the 2-DE gel profiles (Figure 1) as well as the MS/MS-derived amino acid sequences of the proteins (Supplemental data available upon requests), thus suggesting the presence of proteoforms for the respective proteins (Lee, Saraygord-Afshari & Low 2020).

FUNCTIONAL ANNOTATION CLUSTERING

Functional annotation clustering was performed on all the eight serum proteins (Table 2), thus, identified a total of four clusters including extracellular region, reverse cholesterol transport (RCT), platelet degranulation, and complement activation, in that order (Figure 4). APOA1, APOA2, and CLU were found to be significantly associated with RCT. In addition to KNG1 and ALB, APOA1, and CLU were also significantly associated with platelet degranulation. While the levels of C3 and IGKC as well as CLU were significantly associated with complement activation (Figure 4).

TABLE 1. Demographic characteristics of study subjects

Characteristics	BI-RADS					*p*-values
	1 ($n = 5$)	2 ($n = 10$)	3 ($n = 10$)	4 ($n = 5$)	5 ($n = 3$)	
Age (years)[a]	54.8 (8.7)	57.0 (10.9)	48.0 (12.5)	43.2 (10.6)	56.0 (6.6)	0.145
Ethnicity (M: C: I)[b, c]	3:2:0	3:5:2	7:2:1	4:1:0	3:0:0	0.503

[a]One-way analysis of variance (ANOVA). [b] M: C: I refer to Malay: Chinese: Indian. [c] Fisher's exact test

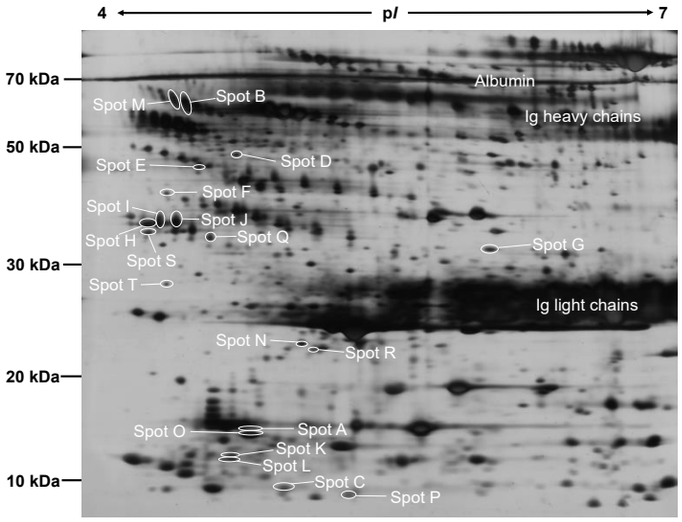


FIGURE 1. Representative silver-stained 2-DE protein profiles of neat sera of a woman with BI-RADS 4. Proteins that were differentially expressed are marked on the gels using white circles and their identities were later confirmed by MS and database search. For all panels, the acidic sides of the 2-DE gels are to the left and the relative M_r declines from the top. Representative silver-stained 2-DE protein profiles of neat sera of women with BI-RADS 1-3 and 5 are demonstrated in Supplemental Figures 1 - 4, respectively

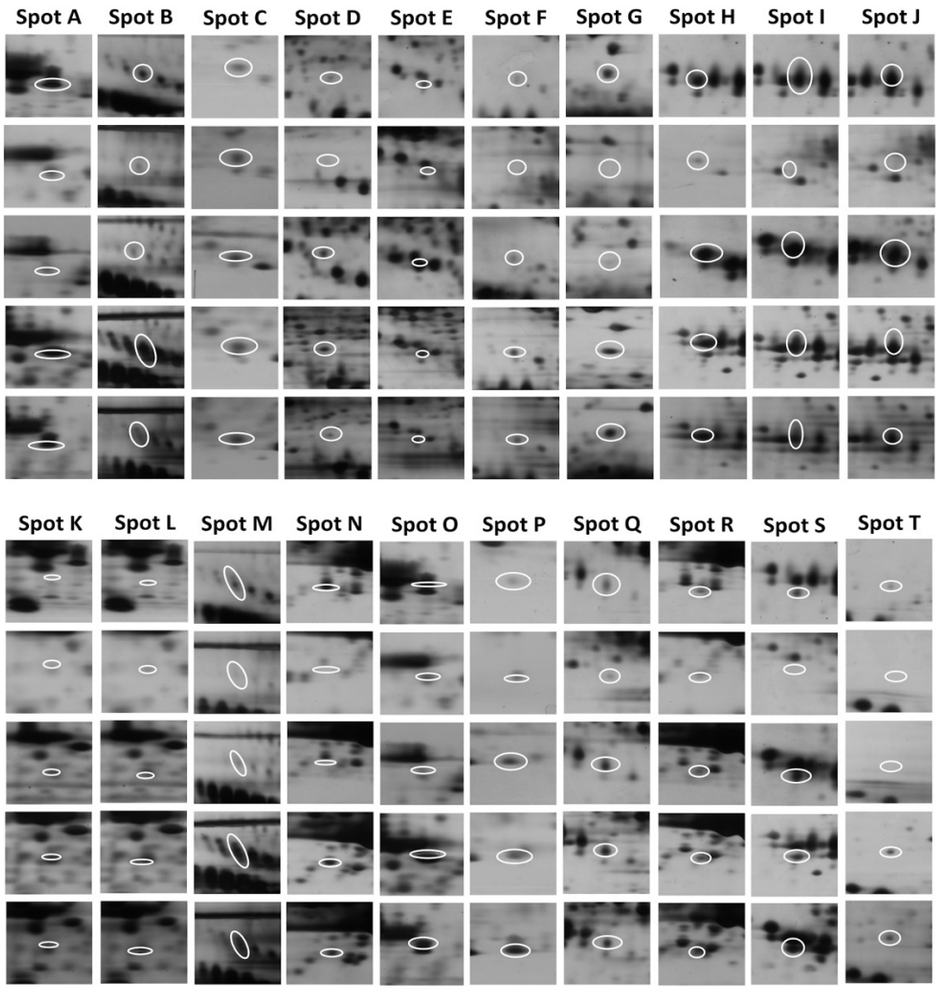
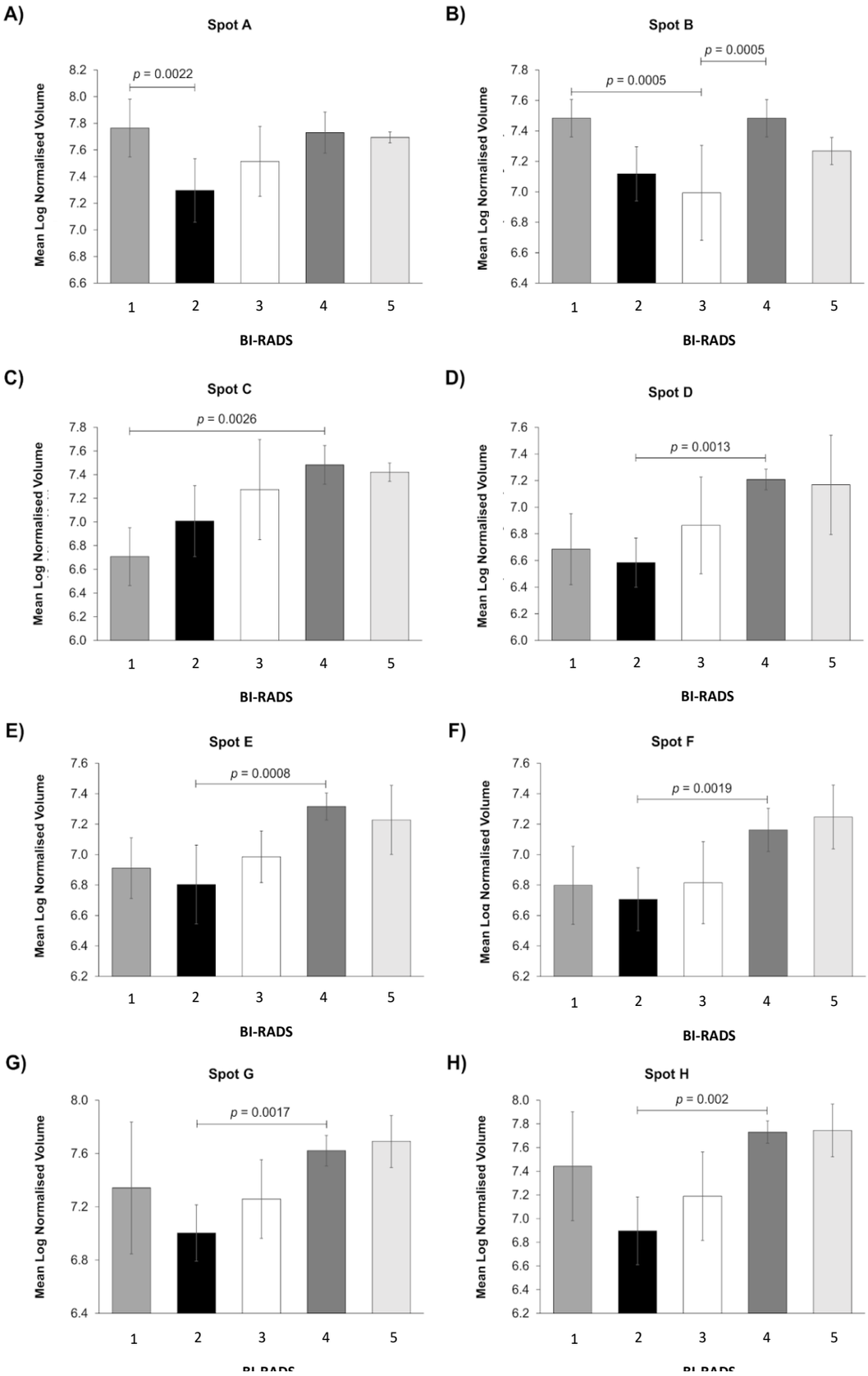
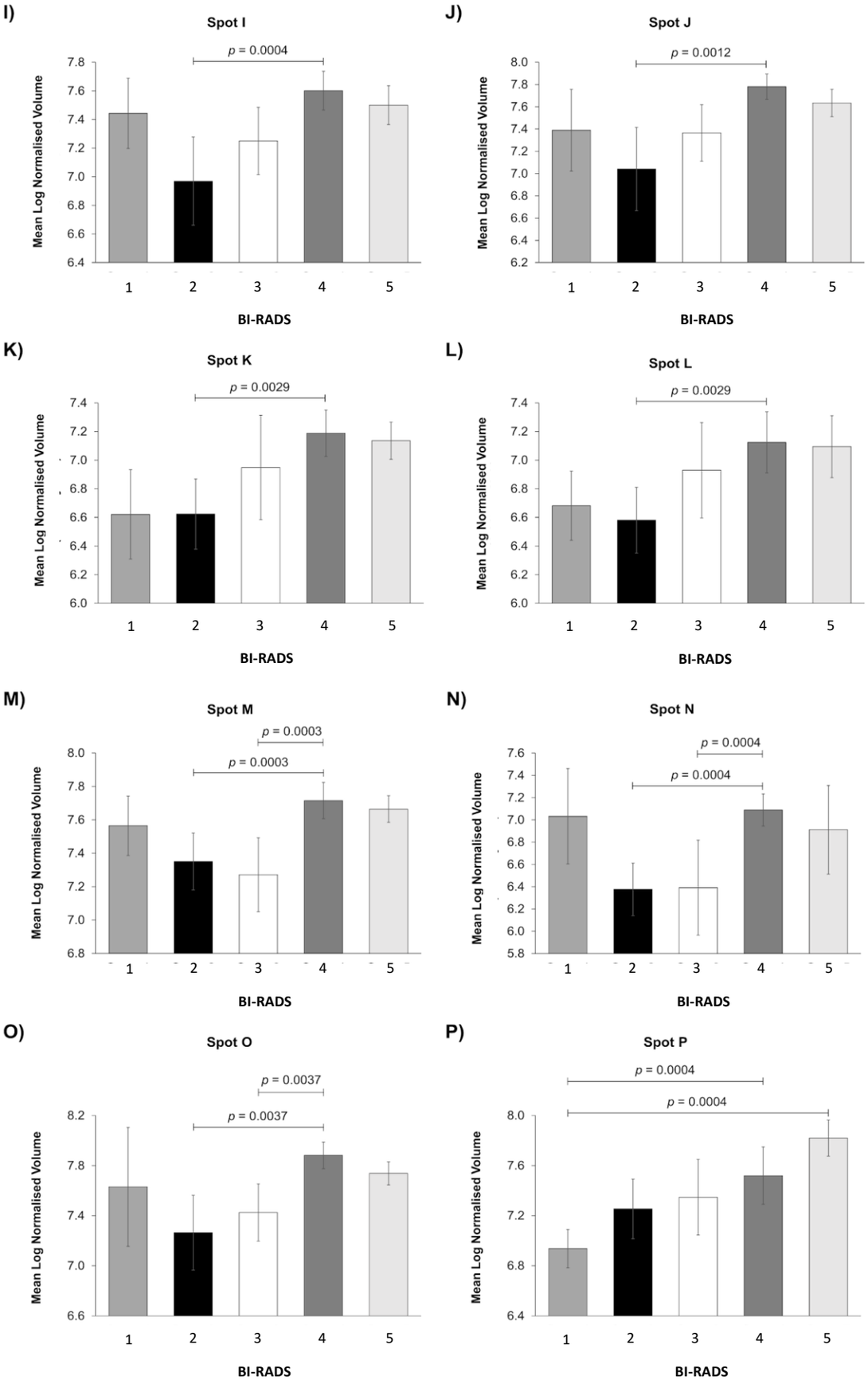


FIGURE 2. Cropped 2-DE images of spots of significantly differentially expressed serum proteins. Differentially expressed serum proteins are marked using white circles


A)
Spot A
p = 0.0022
Mean Log Normalised Volume
BI-RADS
B)
Spot B
p = 0.0005
p = 0.0005
Mean Log Normalised Volume
BI-RADS
C)
Spot C
p = 0.0026
Mean Log Normalised Volume
BI-RADS
D)
Spot D
p = 0.0013
Mean Log Normalised Volume
BI-RADS
E)
Spot E
p = 0.0008
Mean Log Normalised Volume
BI-RADS
F)
Spot F
p = 0.0019
Mean Log Normalised Volume
BI-RADS
G)
Spot G
p = 0.0017
Mean Log Normalised Volume
H)
Spot H
p = 0.002
Mean Log Normalised Volume


I)
Spot I
p = 0.0004
Mean Log Normalised Volume
BI-RADS
J)
Spot J
p = 0.0012
Mean Log Normalised Volume
BI-RADS
K)
Spot K
p = 0.0029
Mean Log Normalised Volume
BI-RADS
L)
Spot L
p = 0.0029
Mean Log Normalised Volume
BI-RADS
M)
Spot M
p = 0.0003
p = 0.0003
Mean Log Normalised Volume
BI-RADS
N)
Spot N
p = 0.0004
p = 0.0004
Mean Log Normalised Volume
BI-RADS
O)
Spot O
p = 0.0037
p = 0.0037
Mean Log Normalised Volume
BI-RADS
P)
Spot P
p = 0.0004
p = 0.0004
Mean Log Normalised Volume
BI-RADS

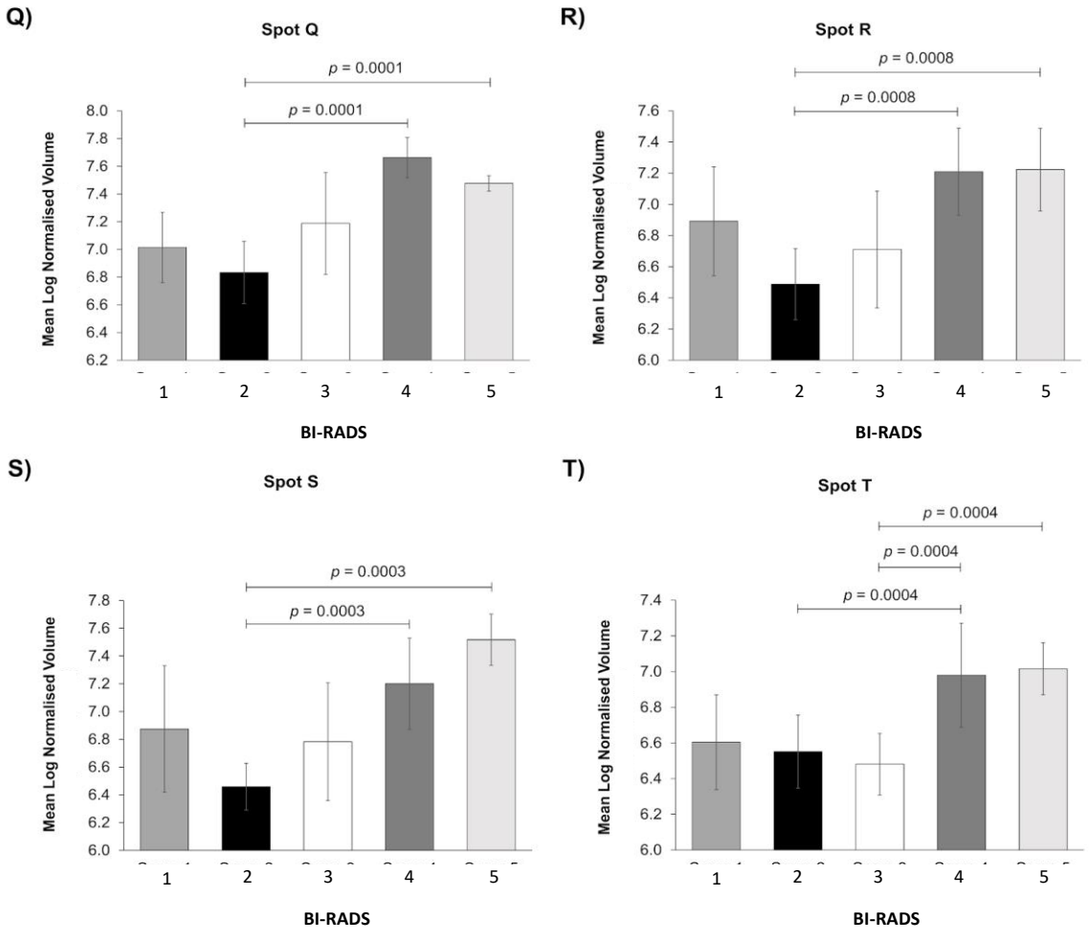


FIGURE 3. Proteins with altered expression in the serum of women with varying BI-RADS categories. Data are presented in mean log normalized volume (standard deviations (SD)) for the respective BI-RADS (1 to 5). Error bars represent the SD of the mean log normalized volume for each BI-RADS (1 to 5). Raw data are available upon requests

DISCUSSION

Proteomics profiling of individual neat serum samples obtained from 33 women with varying BI-RADS (1 to 5) resulted in the identification of 8 non-redundant serum proteins (spots A – T) that were significantly differentially expressed between the groups of subjects (Figure 3 & Table 2). The serum proteins include ALB, APOA1, APOA2, CLU, C3, IGKC, KNG1, and LRG. The expression signatures of these proteins in relevance to BI-RADS scoring are summarized in Figure 5. Many spots of proteins were found unanimously significantly overexpressed in the sera of women with either BI-RADS 4 (a category which indicates suspicious for malignancy) or 5 (a category which is highly suggestive of malignancy) compared to other groups of subjects, thus suggesting their possible roles in the development of BrCa. Except for IGKC, all other identified proteins are acute phase proteins (APPs). Interestingly, the association between cancer (including BrCa), and the aberrant expression of APPs expression had long been established, especially in terms of its potential as biomarkers for cancer (Pang et al. 2010).

APOA1 and APOA2 are the two major protein compositions of high-density lipoprotein (HDL) (van der Vorst 2020). HDL is attributed to multiple protective roles such as antioxidant and anti-inflammatory properties, especially against cardiovascular diseases (Kosmas et al. 2018). However, in cancer including BrCa (Yuan et al. 2016), the modulation of HDL on RCT is being exploited by the actively proliferating cancer cells to satisfy their increased cholesterol consumption through the upregulated expression of scavenger receptor class B type 1 (Sharma & Agnihotri 2019). This is indeed consistent with the present findings of overexpressed levels of APOA1 in the sera of women with BI-RADS 4 and 5 as well as others (Ben Hassen et al. 2020). Nevertheless, there are also contradictory reports, in which the levels are usually inversely associated with risks of BrCa

(Cedó et al. 2019), suggesting apolipoprotein-mediated cholesterol metabolism may be BrCa subtype-specific (Sun et al. 2023). This is not the first time, elevated levels of APOA2 have been associated with BrCa (Lobo et al. 2017). Unfortunately, overexpression of APOA2 was also reported in other cancers (Ren et al. 2019), hence may not be specific for the detection of BrCa, if used alone. However, when used in combination with other protein markers, APOA2 may stand a chance for use as a biomarker for the early detection of BrCa, as it did for pancreatic cancer (Honda et al. 2019).

Aside from promoting tumor angiogenesis and offering protection against apoptosis (Tellez, Garcia-Aranda & Redondo 2016), reports on the metastatic role of CLU in cancer are numerous (Peng et al. 2019). Given this, although an APP, CLU is present at high levels throughout the cancer days (Jin et al. 2012). In line with this, Chen et al. (2021) demonstrated elevated levels of CLU in BrCa patients as compared to healthy controls, but in higher-grade tumors (Chen et al. 2021). Thus, highlighting its potential as a diagnostic biomarker to discriminate patients with BrCa from those without, in general.

TABLE 2. Identification of differentially expressed serum proteins by MS/MS

Spot/ Cluster ID	Matched protein ID	Swiss-Prot accession number[a]	Theoretical mass (Da) / p*I*	Peptide scores	Matched peptides	Sequence coverage (%)
A[†]	Apolipoprotein A-I	P02647	30,759 / 5.56	100	11	37
B	Kininogen-1	P01042	72,996 / 6.34	98	5	8
C	Apolipoprotein A-II	P02652	11,167 / 6.27	100	3	21
D[†]	Complement C3 alpha chain	P01024	188,569 / 6.02	133	5	3
E	Leucine-rich alpha-2 glycoprotein	P02750	38,382 / 6.45	40	3	10
F[†]	Complement C3 alpha chain	P01024	188,569 / 6.02	100	9	5
G[†]	Albumin	P02768	71,317 / 5.92	60	2	3
H	Clusterin	P10909	52,461 / 5.89	100	10	19
I[†]	Albumin	P02768	71,317 / 5.92	105	3	3
J	Clusterin	P10909	52,461 / 5.89	105	2	4
K[†]	Albumin	P02768	71,317 / 5.92	140	2	3
L[†]	Albumin	P02768	71,317 / 5.92	100	6	9
M	Kininogen-1	P01042	72,996 / 6.34	73	3	7
N	Apolipoprotein A-I	P02647	30,759 / 5.56	37	4	14
O[†]	Apolipoprotein A-I	P02647	30,759 / 5.56	100	6	19
P	Apolipoprotein A-II	P02652	11,167 / 6.27	100	2	11
Q	Clusterin	P10909	52,461 / 5.89	100	10	19
R	Apolipoprotein A-I	P02647	30,759 / 5.56	40	4	14
S	Clusterin	P10909	52,461 / 5.89	100	7	14
T	Immunoglobulin kappa constant	P01834	11,757 / 6.11	100	4	65

[a]Accession number was retrieved from the Swiss-Prot database (https://www.uniprot.org/)

[†]Protein was identified as a fragment as indicated by its position in 2-DE gel profiles

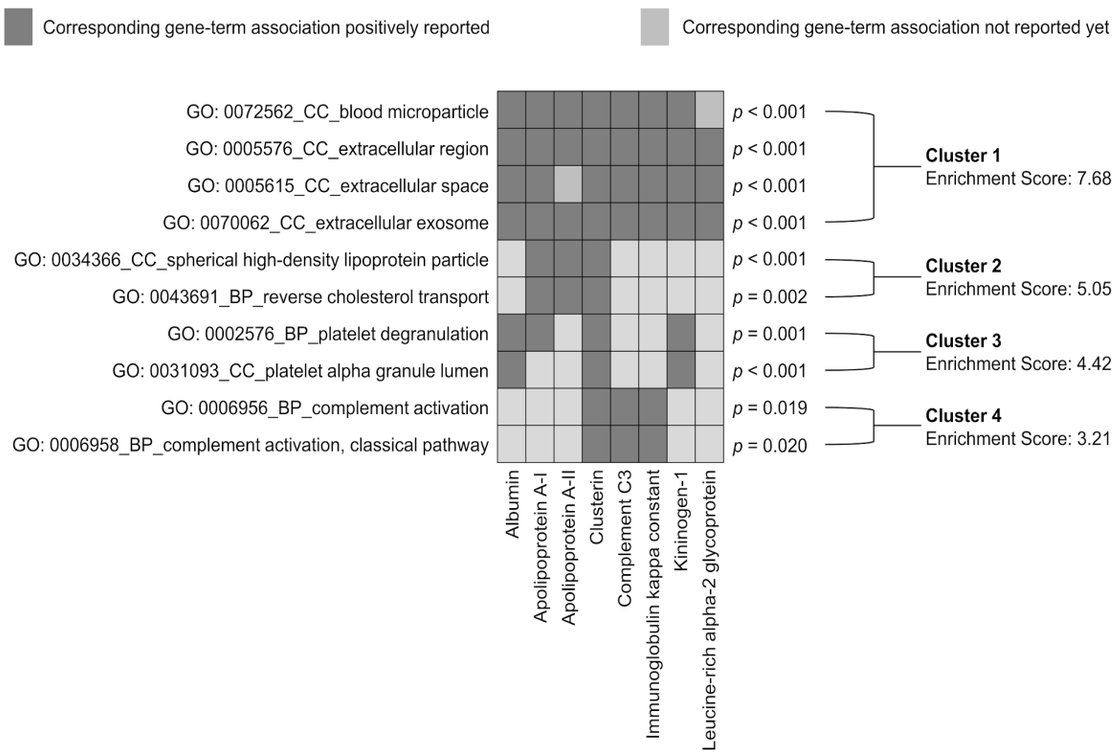


FIGURE 4. Functional annotation clustering of significantly highly expressed serum proteins among women with BI-RADS 4 and/or 5 women. Corrected *p*-values for the significantly enriched GO terms are presented. Significantly enriched GO terms were clustered into 4 main categories; the cluster with the highest enrichment score represents the top-ranked biological significance and *vice versa*

Varying levels of KNG-1 have been previously reported in various types of cancer (Abdul-Rahman et al. 2007; Wang et al. 2020) and biological fluids (2016). While the specificity and the varying altered levels of KNG-1 remain a concern as a diagnostic marker candidate, one reason behind its overexpression in the sera of women with BI-RADS 4 and 5 could be that KNG-1, together with other proteins such as alpha-1B-glycoprotein, fibrinogen gamma chain and plasminogen involved in the exocytosis of platelet alpha granule contents (platelet degranulation) (Palacios-Acedo et al. 2019), may have released various factors that provoked angiogenesis and tumor-associated inflammation in these subjects (George, Shaheed & Sutton 2021). Another possible reason is that the increased levels of KNG-1 may have likewise elevated the levels of its active peptide, bradykinin (BK), which in turn, induced tumor angiogenesis *via* the interaction with BK B2 receptors (Zhou et al. 2019). Given the involvement of KNG-1 in the angiogenesis of tumor, it could potentially serve as a biomarker for the early detection of BrCa, possibly when used in combination with other markers (Gajbhiye et al. 2016).

LRG, a glycosylated APP had been varyingly implicated both in the development (Xie et al. 2019) and the progression (Zhang et al. 2016) of many cancers (e.g., pancreatic ductal adenocarcinoma, colorectal cancer). Nevertheless, LRG indeed retained its status as a good potential diagnostic biomarker with higher sensitivity (85%) for colorectal cancer, when used in combination with other candidate markers (Fouda et al. 2021). In view of this, albeit showing varying levels of expression at different stages of BrCa (Zhang et al. 2021; Zou et al. 2022), LRG may still be considered as a panel for diagnostic biomarkers for BrCa.

Increasing evidence on the activation of the complement system in the tumor microenvironment (TME) that promotes tumorigenesis through the interactions of complement components and stromal cells is being reported (Revel et al. 2020; Senent et al. 2022), thus, suggesting the pro-tumoral properties of the complement system (Jackson et al. 2021). In the present study, the regions of alpha chain subunits of the complement C3 were highly expressed in the sera of women with BI-RADS 4 compared to those with BI-RADS 2. C3a, which is derived from the alpha chain of C3,

BI-RADS / Protein ID	1	1 & 4	4													4 & 5				
	APOA1†	KNG1	APOA2	C3†	LRG	C3†	ALB†	CLU	ALB†	CLU	ALB†	ALB†	KNG1	APOA1	APOA1†	APOA2	CLU	APOA1	CLU	IGKC
1																				
2																				
3																				
4																				
5																				

†Protein was identified as a fragment indicated by its position in the 2-DE gel profile compared to the parent protein. ■, Significant differences in the levels of protein expression were observed between the groups. ■, No significant differences in the levels of protein expression were observed between the groups.

FIGURE 5. Stratification of the identified signature serum proteins according to their respective BI-RADS

is an anaphylatoxin with pro-inflammatory properties, thus commonly found in elevated levels during tumor progression (Pio, Corrales & Lambris 2014). In line with this, the excessive C3 produced by the cancerous tumors may have diffused into the circulatory system due to their 'leaky' behavior of vasculatures in TME, hence, reflected in the serum of women with BI-RADS 4. Interestingly, a previous study has shown that the overexpression of C3 could also enhance metastasis in BrCa by disrupting the tight cellular junctions (Boire et al. 2017), modulating vascularization and endothelial cell functions (Zhang et al. 2019) as well as promoting the survival of cancer cells by increasing their interactions with platelets (Palacios-Acedo et al. 2022). Given this, the complement system has very likely activated as part of innate immunity during the initial stages of cancer in the blood of the subjects in the present study.

In addition to mature B lymphocytes and plasma cells, studies had shown that epithelium-derived carcinoma including BrCa could produce significantly high amounts of IGKC (Yang et al. 2013). Qiu et al. (2012) demonstrated the production of Ig gamma heavy chain and kappa light chain by the papillary thyroid cancer cells together with the co-localized expression of complement proteins including C1q, C3c, and C4c, thus, advocating the activation of classical complement pathway through the formation of immune complexes (Roumenina et al. 2019). These complexes prohibit the binding of the host's antibodies to tumor antigens, hence, protect the cancer cells from antibody-dependent cell-mediated cytotoxicity. On this basis, the upregulated expression of IGKC in the sera of women with BI-RADS 4 and 5 did demonstrate its potential use as a biomarker for the early detection of BrCa.

Albumin, the most abundant serum protein is a negative APP (Schrödl et al. 2016). Hence, hypoalbuminemia is often reported in many diseases including BrCa (Moujaess et al. 2017). The lower levels of ALB observed in cancer patients could very likely be due to the inhibition of ALB synthesis caused by cancer-related systemic inflammation and an increase in the turnover of ALB as utilized by tumors and/or malnutrition (Gupta & Lis 2010; Kühn et al. 2017). However, in the present study, contrastingly higher levels of fragments of ALB (spots G, I, K, and L) were observed in the sera of women with BI-RADS 4 compared to those having benign breast lesions (BI-RADS 2). Likewise, previously a positive association was also found between increased ALB levels and the risk of ovarian cancer in a population-based study (Schwartz et al. 2017). Although the reason behind these contrasting findings is unclear at present, elucidation of the involvement of ALB in BrCa oncogenesis is necessary for their potential application as biomarkers for the early detection of BrCa.

Given the heterogeneity and complexity of cancer, a 'single' marker is deemed insufficient for achieving the highest sensitivity and specificity for its detection (Borrebaeck 2017). Accordingly, many recent biomarker studies have now shifted their focus to the development of multi-marker panels for cancer. In line with this, APOA1, KNG-1, and IGKC (spots B, M, N, O, and T; Figures 3 and 5) were found highly expressed in the sera of women with BI-RADS 4 or 5 compared to those with BI-RADS 3 (probably benign) (Figure 5), thus, suggesting its potential role in distinguishing patients with malignant BrCa at an early stage from those with benign conditions, more accurately. This is because the interpretation of BI-RADS 3 has always resulted in confusion among the treating physicians and has caused needless anxiety among patients who have undergone mammographic screening (Lee et al. 2018).

LIMITATIONS OF THE STUDY

Like any other research, the present study is not without its limitations. Firstly, this study employed a very small sample size. Given this, a larger cohort of samples is deemed necessary to validate the data of the present study to translate the identified biomarkers for use in

the clinic settings. Secondly, albeit versatile and widely used for various applications including biomarker discovery (Meleady 2023), the 2-DE technique used in the present study is often associated with issues related to reproducibility. Given this, opting for a gel-free targeted approach such as multiple reaction monitoring/MS for the subsequent validation study may prove beneficial for translating the present outcome for clinic use as well as towards personalized medicine. Thirdly, although the present study's design does not directly correlate serum protein levels with biopsy-confirmed malignancies, it concentrates on BIRADS categories 1-5 to pinpoint biomarkers that can overcome the limitations of mammographic screening. While the current data may not immediately influence existing diagnostic methods, it serves as a roadmap for future research endeavors. To enhance its significance, forthcoming studies should incorporate correlations with confirmed malignancies, perhaps through longitudinal investigations. Furthermore, exploring how these novel biomarkers synergize with established diagnostic tools such as mammography could fortify their role within the BrCa diagnostic landscape.

Conclusion

This study identifies promising protein signatures (e.g., APOA1, APOA2, ALB, CLU, C3, IGKC, KNG1, and LRG) as complementary biomarkers to improve mammographic screening and early BrCa detection. Aiming beyond refining existing diagnostic processes, this study seeks to reduce the psychological and physical strain on women by minimizing diagnostic ambiguities and unnecessary biopsies associated with mammographic-based false-positive results. These biomarkers, upon validation in a larger, clinically diverse population, could revolutionize BrCa screening, especially for women with dense breast tissues, making detection more precise, non-invasive, and patient-friendly. This approach promises to bridge the gap in BrCa diagnostics, enhancing patient management and marking a significant advancement in early BrCa detection strategies.

Acknowledgements

This research was funded by the Fundamental Research Grant Scheme, Ministry of Higher Education, Malaysia (Grant No.: FRGS/1/2019/SKK08/UM/02/16) and Geran Penyelidikan Fakulti by the Faculty of Medicine, Universiti Malaya (Grant No.: GPF001C-2019). The authors declare no conflict of interests. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of University Malaya Medical Centre (435.18). Informed consent was obtained from all subjects involved in the study. We are grateful for the technical contribution provided by Tan Aik Han at the initial stage of the study. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD040427.

References

Abdul-Rahman, P.S., Lim, B.K. & Hashim, O.H. 2007. Expression of high-abundance proteins in sera of patients with endometrial and cervical cancers: Analysis using 2-DE with silver staining and lectin detection methods. *Electrophoresis* 28(12): 1989-1996. https://doi.org/10.1002/elps.200600629

Barsnes, H. & Vaudel, M. 2018. SearchGUI: A highly adaptable common interface for proteomics search and de novo engines. *Journal of Proteome Research* 17(7): 2552-2555. https://doi.org/10.1021/acs.jproteome.8b00175

Ben Hassen, C., Gutierrez-Pajares, J.L., Guimaraes, C., Guibon, R., Pinault, M., Fromont, G., & Frank, P.G. 2020. Apolipoprotein-mediated regulation of lipid metabolism induces distinctive effects in different types of breast cancer cells. *Breast Cancer Research* 22(1): 38. https://doi.org/10.1186/s13058-020-01276-9

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Methodological)* 57(1): 289-300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bevers, T.B., Helvie, M., Bonaccio, E., Calhoun, K.E., Daly, M.B., Farrar, W.B., Garber, J.E., Gray, R., Greenberg, C.C., Greenup, R., Hansen, N.M., Harris, R.E., Heerdt, A.S., Helsten, T., Hodgkiss, L., Hoyt, T.L., Huff, J.G., Jacobs, L., Lehman, C.D., Monsees, B., Niell, B.L., Parker, C.C., Pearlman, M., Philpotts, L., Shepardson, L.B., Smith, M.L., Stein, M., Tumyan, L., Williams, C., Bergman, M.A. & Kumar, R. 2018. Breast cancer screening and diagnosis, version 3.2018, NCCN clinical practice guidelines in oncology. *Journal of National Comprehensive Cancer Network* 16(11): 1362-1389. https://doi.org/10.6004/jnccn.2018.0083

Boire, A., Zou, Y., Shieh, J., Macalinao, D.G., Pentsova, E. & Massagué, J. 2017. Complement component 3 adapts the cerebrospinal fluid for leptomeningeal metastasis. *Cell* 168(6): 1101-1113.e13. https://doi.org/10.1016/j.cell.2017.02.025

Borgquist, S., Hall, P., Lipkus, I. & Garber, J.E. 2018. Towards prevention of breast cancer: What are the clinical challenges? *Cancer Prevention Research* 11(5): 255-264. https://doi.org/10.1158/1940-6207.capr-16-0254

Borrebaeck, C.A.K. 2017. Precision diagnostics: Moving towards protein biomarker signatures of clinical utility in cancer. *Nature Reviews Cancer* 17(3): 199-204. https://doi.org/10.1038/nrc.2016.153

Cedó, L., Reddy, S.T., Mato, E., Blanco-Vaca, F. & Escolà-Gil, J.C. 2019. HDL and LDL: Potential new players in breast cancer development. *Journal of Clinical Medicine* 8(6): 853. https://doi.org/10.3390/jcm8060853

Chen, Q.F., Chang, L., Su, Q., Zhao, Y. & Kong, B. 2021. Clinical importance of serum secreted clusterin in predicting invasive breast cancer and treatment responses. *Bioengineered* 12(1): 278-285. https://doi.org/10.1080/21655979.2020.1868732

Choi, J.W., Moon, B.I., Lee, J.W., Kim, H.J., Jin, Y. & Kim, H.J. 2018. Use of CA153 for screening breast cancer: An antibodylectin sandwich assay for detecting glycosylation of CA153 in sera. *Oncology Reports* 40(1): 145-154. https://doi.org/10.3892/or.2018.6433

Craig, R. & Beavis, R.C. 2004. TANDEM: Matching proteins with tandem mass spectra. *Bioinformatics* 20(9): 1466-1467. https://doi.org/10.1093/bioinformatics/bth092

Duffy, M.J., McDermott, E.W. & Crown, J. 2018. Blood-based biomarkers in breast cancer: From proteins to circulating tumor cells to circulating tumor DNA. *Tumour Biology* 40(5): 1010428318776169. https://doi.org/10.1177/1010428318776169

Fouda, M.S., Aljarwani, R.M., Aboul-Enein, K. & Omran, M.M. 2021. Diagnostic performances of leucine-rich α-2-glycoprotein 1 and stem cell factor for diagnosis and follow-up of colorectal cancer. *Journal of Genetic Engineering and Biotechnology* 19(1): 17. https://doi.org/10.1186/s43141-021-00116-3

Gajbhiye, A., Dabhi, R., Taunk, K., Vannuruswamy, G., RoyChoudhury, S., Adhav, R., Seal, S., Mane, A., Bayatigeri, S., Santra, M.K., Chaudhury, K. & Rapole, S. 2016. Urinary proteome alterations in HER2 enriched breast cancer revealed by multipronged quantitative proteomics. *Proteomics* 16(17): 2403-2418. https://doi.org/10.1002/pmic.201600015

George, A.L., Shaheed, S.U. & Sutton, C.W. 2021. High-throughput proteomic profiling of nipple aspirate fluid from breast cancer patients compared with non-cancer controls: A step closer to clinical feasibility. *Journal of Clinical Medicine* 10(11): 2243. https://doi.org/10.3390/jcm10112243

Gupta, D. & Lis, C.G. 2010. Pretreatment serum albumin as a predictor of cancer survival: A systematic review of the epidemiological literature. *Nutrition Journal* 9: 69. https://doi.org/10.1186/1475-2891-9-69

Harkness, E.F., Astley, S.M. & Evans, D.G. 2020. Risk-based breast cancer screening strategies in women. *Best Practice & Research Clinical Obstetrics & Gynaecology* 65: 3-17. https://doi.org/10.1016/j.bpobgyn.2019.11.005

Honda, K., Katzke, V.A., Hüsing, A., Okaya, S., Shoji, H., Onidani, K., Olsen, A., Tjønneland, A., Overvad, K., Weiderpass, E., Vineis, P., Muller, D., Tsilidis, K., Palli, D., Pala, V., Tumino, R., Naccarati, A., Panico, S., Aleksandrova, K., Boeing, H., Bueno-de-Mesquita, H.B., Peeters, P.H., Trichopoulou, A., Lagiou, P., Khaw, K.T., Wareham, N., Travis, R.C., Merino, S., Duell, E.J., Rodríguez-Barranco, M., Chirlaque, M.D., Barricarte, A., Rebours, V., Boutron-Ruault, M.C., Romana Mancini, F., Brennan, P., Scelo, G., Manjer, J., Sund, M., Öhlund, D., Canzian, F. & Kaaks, R. 2019. CA19-9 and apolipoprotein-A2 isoforms as detection markers for pancreatic cancer: A prospective evaluation. *International Journal of Cancer* 144(8): 1877-1887. https://doi.org/10.1002/ijc.31900

Huang, D.W., Sherman, B.T. & Lempicki, R.A. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols* 4(1): 44-57. https://doi.org/10.1038/nprot.2008.211

Jackson, W.D., Gulino, A., Fossati-Jimack, L., Castro Seoane, R., Tian, K., Best, K., Köhl, J., Belmonte, B., Strid, J. & Botto, M. 2021. C3 drives inflammatory skin carcinogenesis independently of C5. *The Journal of Investigative Dermatology* 141(2): 404-414.e406. https://doi.org/10.1016/j.jid.2020.06.025

Jayapalan, J.J., Ng, K.L., Razack, A.H. & Hashim, O.H. 2012. Identification of potential complementary serum biomarkers to differentiate prostate cancer from benign prostatic hyperplasia using gel- and lectin-based proteomics analyses. *Electrophoresis* 33(12): 1855-1862. https://doi.org/10.1002/elps.201100608

Jin, J., Kim, J-M., Hur, Y-S., Cho, W.P., Lee, K-Y., Ahn, S-I., Hong, K.C. & Park, I-S. 2012. Clinical significance of clusterin expression in pancreatic adenocarcinoma. *World Journal of Surgical Oncology* 10(1): 146. https://doi.org/10.1186/1477-7819-10-146

Kosmas, C.E., Martinez, I., Sourlas, A., Bouza, K.V., Campos, F.N., Torres, V., Montan, P.D. & Guzman, E. 2018. High-density lipoprotein (HDL) functionality and its relevance to atherosclerotic cardiovascular disease. *Drugs in Context* 7: 212525. https://doi.org/10.7573/dic.212525

Kühn, T., Sookthai, D., Graf, M.E., Schübel, R., Freisling, H., Johnson, T., Katzke, V. & Kaaks, R. 2017. Albumin, bilirubin, uric acid and cancer risk: Results from a prospective population-based study. *British Journal of Cancer* 117(10): 1572-1579. https://doi.org/10.1038/bjc.2017.313

Kwon, Y.W., Jo, H.S., Bae, S., Seo, Y., Song, P., Song, M. & Yoon, J.H. 2021. Application of proteomics in cancer: Recent trends and approaches for biomarkers discovery. *Frontiers in Medicine* 8: 747333. https://doi.org/10.3389/fmed.2021.747333

Lang, T.A. & Altman, D.G. 2015. Basic statistical reporting for articles published in biomedical journals: The "Statistical analyses and methods in the published literature" or the SAMPL guidelines. *International Journal of Nursing Studies* 52(1): 5-9. https://doi.org/10.1016/j.ijnurstu.2014.09.006

Lee, K.A., Talati, N., Oudsema, R., Steinberger, S. & Margolies, L.R. 2018. BI-RADS 3: Current and future use of probably benign. *Current Radiology Reports* 6(2): 5. https://doi.org/10.1007/s40134-018-0266-8

Lee, P.Y., Saraygord-Afshari, N. & Low, T.Y. 2020. The evolution of two-dimensional gel electrophoresis - from proteomics to emerging alternative applications. *Journal of Chromatography A* 1615: 460763. https://doi.org/10.1016/j.chroma.2019.460763

Li, J., Guan, X., Fan, Z., Ching, L-M., Li, Y., Wang, X., Cao, W-M. & Liu, D-X. 2020. Non-invasive biomarkers for early detection of breast cancer. *Cancers* 12(10): 2767. https://doi.org/10.3390/cancers12102767

Lobo, M.D., Moreno, F.B., Souza, G.H., Verde, S.M., Moreira, R.A. & Monteiro-Moreira, A.C. 2017. Label-free proteome analysis of plasma from patients with breast cancer: Stage-specific protein expression. *Frontiers in Oncology* 7: 14. https://doi.org/10.3389/fonc.2017.00014

Meleady, P. 2023. Two-dimensional gel electrophoresis and 2D-DIGE. *Methods in Molecular Biology* 2596: 3-15. https://doi.org/10.1007/978-1-0716-2831-7_1

Monticciolo, D.L., Helvie, M.A. & Hendrick, R.E. 2018. Current issues in the overdiagnosis and overtreatment of breast cancer. *AJR American Journal of Roentgenology* 210(2): 285-291. https://doi.org/10.2214/ajr.17.18629

Moujaess, E., Fakhoury, M., Assi, T., Elias, H., El Karak, F., Ghosn, M. & Kattan, J. 2017. The therapeutic use of human albumin in cancer patients' management. *Critical Reviews in Oncology/Hematology* 120: 203-209. https://doi.org/10.1016/j.critrevonc.2017.11.008

Nunez, C. 2019. Blood-based protein biomarkers in breast cancer. *Clinica Chimica Acta* 490*:* 113-127. https://doi.org/10.1016/j.cca.2018.12.028

Palacios-Acedo, A-L., Langiu, M., Crescence, L., Mège, D., Dubois, C. & Panicot-Dubois, L. 2022. Platelet and cancer-cell interactions modulate cancer-associated thrombosis risk in different cancer types. *Cancers* 14(3): 730. https://www.mdpi.com/2072-6694/14/3/730

Palacios-Acedo, A.L., Mège, D., Crescence, L., Dignat-George, F., Dubois, C. & Panicot-Dubois, L. 2019. Platelets, thrombo-inflammation, and cancer: Collaborating with the enemy. *Frontiers in Immunology* 10: 1805. https://doi.org/10.3389/fimmu.2019.01805

Pang, W.W., Abdul-Rahman, P.S., Wan-Ibrahim, W.I. & Hashim, O.H. 2010. Can the acute-phase reactant proteins be used as cancer biomarkers? *The International Journal of Biological Markers* 25(1): 1-11.

Peng, M., Deng, J., Zhou, S., Tao, T., Su, Q., Yang, X. & Yang, X. 2019. The role of clusterin in cancer metastasis. *Cancer Management & Research* 11: 2405-2414. https://doi.org/10.2147/cmar.S196273

Perez-Riverol, Y., Bai, J., Bandla, C., García-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., Kundu, Deepti J., Prakash, A., Frericks-Zipper, A., Eisenacher, M., Walzer, M., Wang, S., Brazma, A. & Vizcaíno, J.A. 2021. The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. *Nucleic Acids Research* 50(D1): D543-D552. https://doi.org/10.1093/nar/gkab1038

Perkins, D.N., Pappin, D.J., Creasy, D.M. & Cottrell, J.S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20(18): 3551-3567. https://doi.org/10.1002/(sici)1522-2683(19991201)20:18<3551::aid-elps3551>3.0.co;2-2

Pio, R., Corrales, L. & Lambris, J.D. 2014. The role of complement in tumor growth. *Advances in Experimental Medicine and Biology* 772: 229-262. https://doi.org/10.1007/978-1-4614-5915-6_11

Qiu, Y., Korteweg, C., Chen, Z., Li, J., Luo, J., Huang, G. & Gu, J. 2012. Immunoglobulin G expression and its colocalization with complement proteins in papillary thyroid cancer. *Mod. Pathol.* 25(1): 36-45. doi:10.1038/modpathol.2011.139

Ren, L., Yi, J., Li, W., Zheng, X., Liu, J., Wang, J. & Du, G. 2019. Apolipoproteins and cancer. *Cancer Medicine* 8(16): 7032-7043. https://doi.org/10.1002/cam4.2587

Revel, M., Daugan, M.V., Sautés-Fridman, C., Fridman, W.H. & Roumenina, L.T. 2020. Complement system: Promoter or suppressor of cancer progression? *Antibodies (Basel)* 9(4): 57. https://doi.org/10.3390/antib9040057

Roumenina, L.T., Daugan, M.V., Noé, R., Petitprez, F., Vano, Y.A., Sanchez-Salas, R., Becht, E., Meilleroux, J., Clec'h, B. L., Giraldo, N. A., Merle, N.S., Sun, C.M., Verkarre, V., Validire, P., Selves, J., Lacroix, L., Delfour, O., Vandenberghe, I., Thuilliez, C., Keddani, S., Sakhi, I.B., Barret, E., Ferré, P., Corvaïa, N., Passioukov, A., Chetaille, E., Botto, M., de Reynies, A., Oudard, S.M., Mejean, A., Cathelineau, X., Sautès-Fridman, C. & Fridman, W.H. 2019. Tumor cells hijack macrophage-produced complement C1q to promote tumor growth. *Cancer Immunology Research* 7(7): 1091-1105. https://doi.org/10.1158/2326-6066.cir-18-0891

Schrödl, W., Büchler, R., Wendler, S., Reinhold, P., Muckova, P., Reindl, J. & Rhode, H. 2016. Acute phase proteins as promising biomarkers: Perspectives and limitations for human and veterinary medicine. *Proteomics Clinical Applications* 10(11): 1077-1092. https://doi.org/10.1002/prca.201600028

Schwartz, G.G., Tretli, S., Vos, L. & Robsahm, T.E. 2017. Prediagnostic serum calcium and albumin and ovarian cancer: A nested case-control study in the Norwegian Janus serum bank cohort. *Cancer Epidemiology* 49: 225-230. https://doi.org/10.1016/j.canep.2017.07.004

Seely, J.M. & Alhassan, T. 2018. Screening for breast cancer in 2018 - what should we be doing today? *Current Oncology* 25(Suppl 1): S115-S124. https://doi.org/10.3747/co.25.3770

Senent, Y., Tavira, B., Pio, R. & Ajona, D. 2022. The complement system as a regulator of tumor-promoting activities mediated by myeloid-derived suppressor cells. *Cancer Letters* 549: 215900. https://doi.org/https://doi.org/10.1016/j.canlet.2022.215900

Shah, T.A. & Guraya, S.S. 2017. Breast cancer screening programs: Review of merits, demerits, and recent recommendations practiced across the world. *Journal of Microscopy and Ultrastructure* 5(2): 59-69. https://doi.org/10.1016/j.jmau.2016.10.002

Sharma, B. & Agnihotri, N. 2019. Role of cholesterol homeostasis and its efflux pathways in cancer progression. *The Journal of Steroid Biochemistry and Molecular Biology* 191: 105377. https://doi.org/10.1016/j.jsbmb.2019.105377

Sickles, E.A., D'Orsi, C.J. & Bassett, L.W. 2013. ACR BI-RADS® mammography. In *ACR BI-RADS® Atlas, Breast Imaging Reporting and Data System*. 5th ed., edited by D'Orsi, C.J., Sickles, E.A., Mendelson, E.B. & Morris, E.A. American College of Radiology. pp. 121-140.

Sun, X-B., Liu, W-W., Wang, B., Yang, Z-P., Tang, H-Z., Lu, S., Wang, Y-Y., Qu, J-X. & Rao, B-Q. 2023. Correlations between serum lipid and Ki67 levels in different breast cancer molecular subcategories. *Oncology Letters* 25(2): 53. https://doi.org/10.3892/ol.2022.13639

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. 2021. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries *CA: A Cancer Journal for Clinicians* 71(3): 209-249. https://doi.org/https://doi.org/10.3322/caac.21660

Tellez, T., Garcia-Aranda, M. & Redondo, M. 2016. The role of clusterin in carcinogenesis and its potential utility as therapeutic target. *Current Medicinal Chemistry* 23(38): 4297-4308. https://doi.org/10.2174/0929867323666161024150540

Tomczak, M. & Tomczak, E. 2014. The need to report effect size estimates revisited. An overview of some recommended measures of effect size. *Trends in Sport Sciences* 1(21): 19-25.

van der Vorst, E.P.C. 2020. High-density lipoproteins and apolipoprotein A-I. *Subcell Biochem.* 94: 399-420. https://doi.org/10.1007/978-3-030-41769-7_16

Vaudel, M., Burkhart, J.M., Zahedi, R.P., Oveland, E., Berven, F.S., Sickmann, A., Martens, L. & Barsnes, H. 2015. PeptideShaker enables reanalysis of MS-derived proteomics data sets. *Nature Biotechnology* 33(1): 22-24. https://doi.org/10.1038/nbt.3109

Wang, W., Wang, S. & Zhang, M. 2020. Evaluation of kininogen 1, osteopontin and alpha-1-antitrypsin in plasma, bronchoalveolar lavage fluid and urine for lung squamous cell carcinoma diagnosis. *Oncology Letters* 19(4): 2785-2792. https://doi.org/10.3892/ol.2020.11376

Xie, Z-B., Zhang, Y-F., Jin, C., Mao, Y-S. & Fu, D-L. 2019. LRG-1 promotes pancreatic cancer growth and metastasis via modulation of the EGFR/p38 signaling. *Journal of Experimental & Clinical Cancer Research* 38(1): 75. https://doi.org/10.1186/s13046-019-1088-0

Yan, J.X., Wait, R., Berkelman, T., Harry, R.A., Westbrook, J.A., Wheeler, C.H. & Dunn, M.J. 2000. A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. *Electrophoresis* 21(17): 3666-3672. https://doi.org/10.1002/1522-2683(200011)21:17<3666::aid-elps3666>3.0.co;2-6

Yang, B., Ma, C., Chen, Z., Yi, W., McNutt, M.A., Wang, Y., Korteweg, C. & Gu, J. 2013. Correlation of immunoglobulin G expression and histological subtype and stage in breast cancer. *PLoS ONE* 8(3): e58706. https://doi.org/10.1371/journal.pone.0058706

Yang, Y., Zhang, H., Zhang, M., Meng, Q., Cai, L. & Zhang, Q. 2017. Elevation of serum CEA and CA15-3 levels during antitumor therapy predicts poor therapeutic response in advanced breast cancer patients. *Oncology Letters* 14(6): 7549-7556. https://doi.org/10.3892/ol.2017.7164

Yuan, B., Wu, C., Wang, X., Wang, D., Liu, H., Guo, L., Li, X.A., Han, J. & Feng, H. 2016. High scavenger receptor class B type I expression is related to tumor aggressiveness and poor prognosis in breast cancer. *Tumour Biology* 37(3): 3581-3588. https://doi.org/10.1007/s13277-015-4141-4

Zhang, J., Zhu, L., Fang, J., Ge, Z. & Li, X. 2016. LRG1 modulates epithelial-mesenchymal transition and angiogenesis in colorectal cancer via HIF-1α activation. *Journal of Experimental & Clinical Cancer Research* 35: 29. https://doi.org/10.1186/s13046-016-0306-2

Zhang, R., Liu, Q., Li, T., Liao, Q. & Zhao, Y. 2019. Role of the complement system in the tumor microenvironment. *Cancer Cell International* 19: 300. https://doi.org/10.1186/s12935-019-1027-3

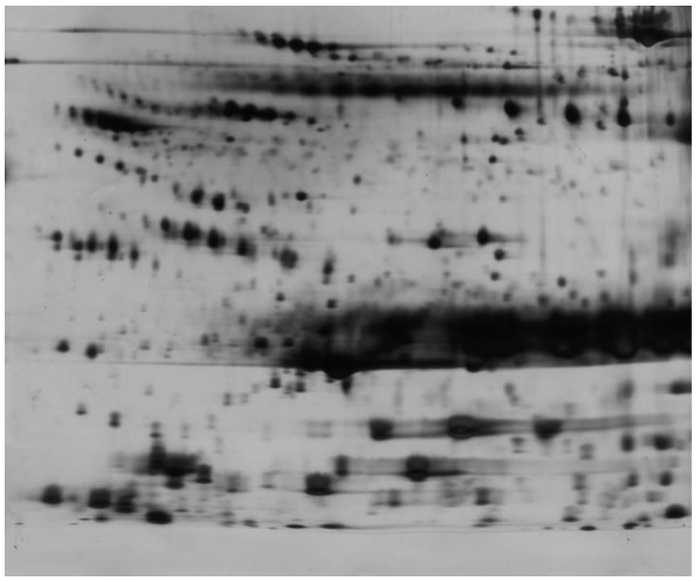
Zhang, Y.S., Han, L., Yang, C., Liu, Y.J. & Zhang, X.M. 2021. Prognostic value of LRG1 in breast cancer: A retrospective study. *Oncology Research and Treatment* 44(1-2): 36-42. https://doi.org/10.1159/000510945

Zhou, Y., Wang, W., Wei, R., Jiang, G., Li, F., Chen, X., Wang, X., Long, S., Ma, D. & Xi, L. 2019. Serum bradykinin levels as a diagnostic marker in cervical cancer with a potential mechanism to promote VEGF expression via BDKRB2. *International Journal of Oncology* 55(1): 131-141. https://doi.org/10.3892/ijo.2019.4792

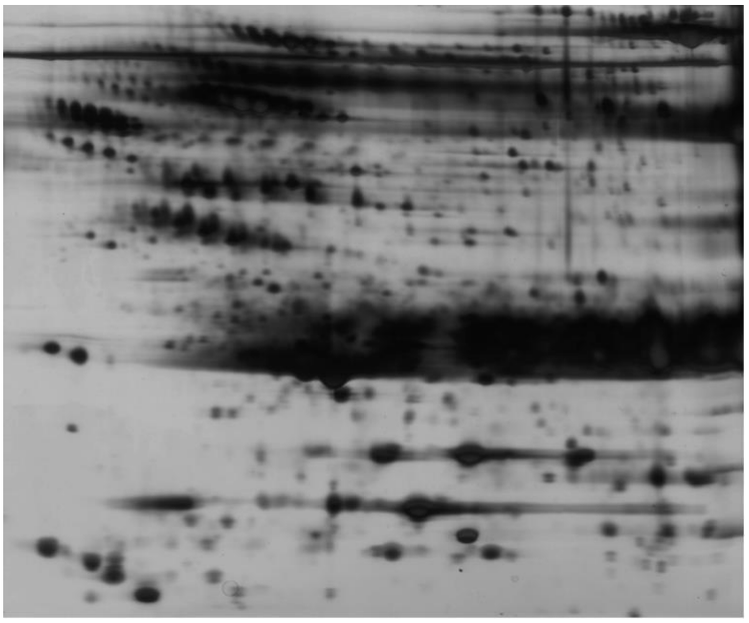
Zografos, E., Anagnostopoulos, A.K., Papadopoulou, A., Legaki, E., Zagouri, F., Marinos, E., Tsangaris, G.T. & Gazouli, M. 2019. Serum proteomic signatures of male breast cancer. *Cancer Genomics & Proteomics* 16(2): 129-137.

Zou, Y., Xu, Y., Chen, X., Wu, Y., Fu, L. & Lv, Y. 2022. Research progress on leucine-rich alpha-2 glycoprotein 1: A review. *Frontiers in Pharmacology* 12. https://doi.org/10.3389/fphar.2021.809225

*Corresponding author; email: jaime_jacklyn@um.edu.my

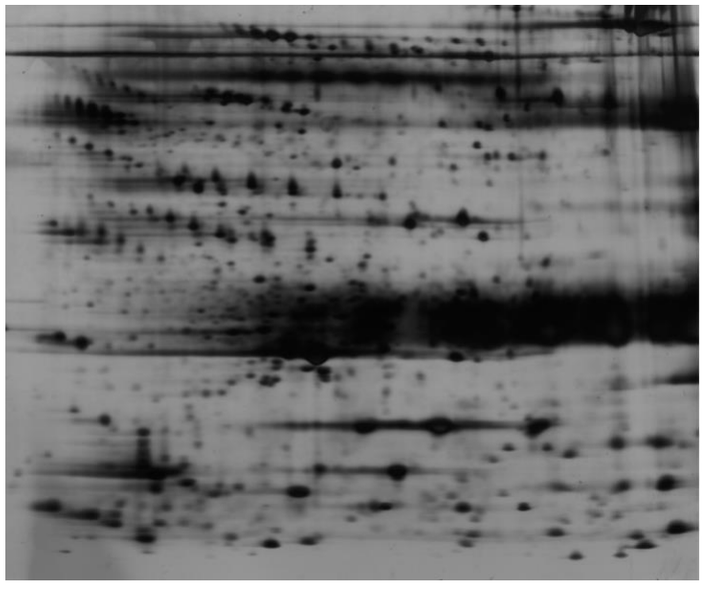


SUPPLEMENTAL FIGURE 1. Representative silver stained 2-DE protein profiles of neat sera of women with BI-RADS 1. The acidic sides of the 2-DE gels are to the left and relative Mr declines from the top



SUPPLEMENTAL FIGURE 2. Representative silver stained 2-DE protein profiles of neat sera of women with BI-RADS 2. The acidic sides of the 2-DE gels are to the left and relative Mr declines from the top

SUPPLEMENTAL FIGURE3. Representative silver stained 2-DE protein profiles of neat sera of women with BI-RADS 3. The acidic sides of the 2-DE gels are to the left and relative Mr declines from the top



SUPPLEMENTAL FIGURE 4. Representative silver stained 2-DE protein profiles of neat sera of women with BI-RADS 5. The acidic sides of the 2-DE gels are to the left and relative Mr declines from the top