

RESEARCH ARTICLE

Reliability of tumor markers, chemokines, and metastasis-related molecules in serum

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ABSTRACT. There is a growing interest in the role that cancer biomarkers, metastasis-related molecules, and chemokines may play in the development and progression of various cancers. However, few studies have addressed the reliability of such biomarkers in healthy individuals over time. The objective of this study was to investigate the temporal reliability of multiple proteins in serum samples from healthy women who donated blood over successive years. Thirty five, postmenopausal women with two, repeated annual visits, and thirty, premenopausal women with three, repeated annual visits were randomly selected among eligible subjects from an existing, prospective cohort. Multiplexing Luminex xMAPTM technology was used to measure the levels of 55 serum proteins representing cancer antigens, chemokines, angiogenic and anti-angiogenic factors, proteases, adipokines, apoptotic molecules, and other markers in these women. The biomarkers with high detection rates (> 60%) and acceptable reliability (intraclass correlation coefficient, ICCs ≥ 0.55) using xMAPTM method were: cancer antigens: AFP, CA 15-3, CEA, CA-125, SCC, SAA; growth factors/related molecules: ErbB2, IGFBP-1; proteases and adhesion molecules: MMP-1, 8, 9, sE-selectin, human kallikreins (KLK) 8,10, ICAM-1, VCAM-1, chemokines: fractalkine, MCP-1,2, RANTES, MIP-1 α , MIP-1 β , Eotaxin, GRO- α , IP-10; inhibitors of angiogenesis: angiostatin and endostatin; adipokines leptin and resistin; apoptotic factor: Fas, and other proteins mesothelin, myeloperoxidase (MPO), and PAI-1. The rest of the biomarkers under investigation either had ICCs less than 0.55 or had low levels of detection (< 60%). These included cancer antigens: CA 19-9, CA 72-4, MICA, S100, TTR, ULBP1, ULBP2, ULBP3; proteases: MMP 2, 3, 7, 12, 13; chemokines: MCP-3, MIF, MIG; adipokines: leptin and resistin; apoptotic factors: FasL, DR5, Cyfra 21-1; and inhibitors of angiogenesis and other markers: thrombospondin and heat shock protein (HSP) 27. In conclusion, 34 out of the 55 biomarkers investigated were present in detectable levels in > 60% of the samples, and with an ICC ≥ 0.55 , indicating that a single serum measurement can be used in prospective epidemiological studies using the xMAPTM method.

Keywords: reliability, tumor markers, chemokines, metastasis-related molecules, prospective cohort

Biomarkers are cellular or soluble indicators of health status, making them very important for monitoring people who are both healthy, and those who have an established disease. As a newly discovered biomarker assay makes the transition from a research setting to the clinical diagnostic laboratory, it should progress through defined stages of assay evaluation [1]. Reliability is one of the key issues in biomarker validation, and several reliability studies have already been conducted by our research group [2, 3]. Biomarker validation studies can detect the various compo-

nents of variability of a biomarker and indicate directions for assay improvement, along with possible use in epidemiological or clinical settings [4].

Although a number of studies have measured the reliability of hormones and growth factors, very few studies have evaluated the temporal reliability of multiple, serum-based potential biomarkers of cancer [2-4]. In this study, we assessed the reliability of 55 biomarkers including cancer antigens (AFP, CA 125, CA 15-3, CA 19-9, CA 72-4, CEA, MICA, SAA, SCC, S100, TTR, ULBP1, 2, 3);

growth factors/related molecules: (ErbB2, IGFBP1); cell adhesion molecules, proteases, and protease inhibitors (sE-selectin, sICAM-1, sVCAM-1, PAI-1 (total and active) matrix metalloproteinases (MMP 1, 2, 3, 7, 8, 9, 12, 13), kallikreins (KLK8, 10); chemokines (eotaxin, fractalkine, GRO- α , IP-10, MCP-1, MCP-2, MCP-3, MIF, MIG, MIP-1 α , MIP-1 β , RANTES); adipokines (leptin, resistin), apoptotic factors (sFas, sFasL, DR5, Cyfra 21-1); angiogenesis inhibitors (angiostatin, endostatin, thrombospondin); and other markers (mesothelin, HSP 27, MPO). This study is one of the first evaluating the reliability of multiple biomarkers in serum samples from an existing prospective cohort, using the multiplexing Luminex technology.

In our previous studies, we compared the biomarker expression levels between individuals with established tumors and healthy controls. Specifically, we found differential expression of cytokines, chemokines, cancer antigens and other serum markers in patients with ovarian cancer, melanoma, head and neck cancer, endometrial cancer, and several other malignancies [5-8]. Additionally, in our previous studies we explored the differences in hormone and cytokine expression between postmenopausal and premenopausal women [3, 9, 10]. Thus, since we have already established that there are difference in biomarker expression between individuals with various diseases and healthy controls, the goal of this study was to evaluate longitudinal differences in biomarker expression in individuals free of malignancies. The null hypothesis of this study was that there is no difference in biomarker expression in healthy individuals over time.

STUDY POPULATION AND METHODS

Between March 1985 and June 1991, the New York University Women's Health Study (NYUWHS) enrolled a cohort of 14 274 women aged 34-65 years at the Guttman Breast Diagnostic Institute, a breast screening clinic based in New York City. At the time of enrollment and at annual screening visits thereafter, subjects were asked to complete questionnaires and to provide 30 ml of peripheral venous blood. Fifty one percent of cohort members donated blood on more than one occasion, usually at one-year intervals. Characteristics of the study participants have been described previously.

Blood samples were collected before breast examination between 9:00 a.m. and 3:00 p.m. Fasting was not required for study enrollment. After collection, blood specimens were kept at room temperature for approximately 1 h and at 4°C for 30 min. Samples were then centrifuged at 3 500 rpm for 15 min, and then serum was partitioned into 1ml aliquots in airtight plastic vials and frozen at - 80°C for long-term storage.

The repeat samples collected at yearly intervals from approximately half of the NYUWHS participants, were used to conduct a reliability (temporal stability) study. Subjects were selected at random among the NYUWHS participants who fulfilled the following criteria: a) large number of aliquots still in storage (> 11 at each visit), b) no diagnosis of cancer (except non-melanoma skin cancer), c) neither a case nor a control in any of the ongoing,

nested, case-control studies, d) no use of exogenous hormones (such as oral contraceptive or hormone replacement therapy) at the time of any of the blood donations. For postmenopausal women, two yearly samples were retrieved from the serum bank for 35 women, and were included on the same well-plate in a random order. The average days between collection of the two samples was 367 ($\pm$ 5). Since serum levels of some cytokines are influenced by sex hormones [11], separate groups of post- and premenopausal women were selected. Women were classified as postmenopausal if they reported: the absence of menstrual cycles in the previous six months, a total, bilateral oophorectomy or a hysterectomy without total oophorectomy if their age was 52 years or older. Women were classified as premenopausal if they reported at least one menstrual cycle during the six months prior to enrollment. For premenopausal women, three-yearly samples were retrieved from the serum bank for 30 women. The "yearly" samples were on average 442 $\pm$ 176 days apart for the 1st and 2nd samples, and 463 $\pm$ 139 days apart for the 2nd and 3rd samples. For quality control, a random sample of five premenopausal and five postmenopausal women was selected and their blinded sample duplicates were analyzed to assess intra- and inter-batch coefficients of variation (CVs). All samples were re-labeled prior to being sent to the laboratory in order to ensure blinding of the laboratory personnel.

Multiplex analysis

Never-thawed, 1mL serum samples were sent on dry ice to the University of Pittsburgh Cancer Institute, where they were stored at - 80°C until they were assayed. Serum levels of biomarkers were analyzed using xMAPTM technology. The xMAPTM technology (Luminex) combines the principle of a sandwich immunoassay with the fluorescent-bead-based technology allowing individual and multiplex analysis of up to 100 different analytes in a single microtiter well. CA 15-3, ErbB2, CEA, KLK8, hKLK10, CA 125, Cyfra 21-1, CA 19-9, ULBP1-3, MICA, SCC, SAA, TTR, thrombospondin, mesothelin, angiostatin, endostatin, AFP, CA 72-4, IGFBP1, S100 and HSP27 were measured using the assay developed and were optimized in the Luminex Core Facility of the University of Pittsburgh Cancer Institute (<http://www.upci.upmc.edu/facilities/luminex/index.html>); MMP9, resistin, fractalkine, sE-selectin, sVCAM1, MPO, tPAI-1, PAI-1 active, leptin, sFas, sFasL, MIF, sICAM-1, were measured using Linco/Millipore (St. Lois, MO, USA) kits; all MMPs other than MMP9 were measured using R&D kits, MIP-1 α , MIP-1 β , IP-10, eotaxin, RANTES, MCP-1, MCP-2, MCP-3, DR5, MIG, and GRO- α were measured using Invitrogen (Camarillo, CA, USA) kits. The xMAPTM serum assays were performed with a 96-well microplate format as previously described [7].

Statistical analysis

All analyses were performed on the natural logarithm-transformed values as previously described [11]. The temporal reliability was estimated by the intraclass correlation coefficient (ICC) [12, 13]. The variance components were estimated with a random effects, one-way

analysis of variance model, using the SAS procedure MIXED. Exact 95% confidence intervals (CIs) for the ICCs were calculated as described by McGraw & Wong [14]. We determined *a priori* that serum markers worthy of future consideration should be detectable in at least 60% of the samples and should have an ICC of at least 0.55 based on our previous experience [3], and recommendations in the literature. The bootstrap method was used in calculating the Spearman correlation coefficient (*r*) between continuous variables as previously described [10]. Differences in the median biomarker expression level between premenopausal and postmenopausal women were evaluated with the Wilcoxon-Mann-Whitney test. All analyses were performed using SAS 9.1 (SAS Institute, Cary, NC). All *p* values are two-sided.

RESULTS

ICC and its 95% CI for the Biomarkers

Table 1 lists biomarkers for which more than 60% of the analyzed samples had values above the lower limit of detection (LLD); the ICC was ≥ 0.55 . The highest ICC was observed for AFP, which was 0.97. The results demonstrate that 34 of the 55 markers under investigation had ICCs ≥ 0.55 , indicating that a single measurement of these biomarkers can represent the long-term average level, for up to two or three years. The 34 biomarkers that were found to be stable include; cancer antigens AFP, CA 15-3, CEA, CA-125, SCC, SAA; growth factors/related molecules: ErbB2, IGFBP-1; proteases and adhesion molecules: MMP-1, 8, 9, sE-selectin, KLK8,10, sICAM-1, sVCAM-1; **chemokines**: fractalkine, MCP-2,

Table 1
Percentage of samples above detection limit, intra batch CVs, intraclass correlations (95% CIs), and medians (25th and 75th percentiles) of serum biomarkers measured by the Luminex xMap™ method*

Biomarker (unit)	% of samples above detection limit	CV (%)	ICC (95%CI)	Median (25 th -75 th percentiles)
Cancer Antigens AFP (ng/mL)	100%	0.9	0.97 (0.95-0.98)	1.7 (1.3-2.1)
CA 15-3 (pg/mL)	99%	0.7	0.95 (0.92-0.97)	3.5 (2.6-4.7)
ErbB2 (ng/mL)	99%	0.7	0.85 (0.77-0.90)	2.1 (1.9-2.4)
IGFBP-1 (ng/mL)	100%	0.8	0.82 (0.73-0.88)	2.8 (1.5-4.9)
CEA (ng/mL)	86%	16.3	0.68 (0.54-0.79)	1.3 (1.0-1.8)
CA 125 (pg/mL)	84%	5.8	0.68 (0.55-0.79)	4.5 (1.7-9.1)
SCC (pg/mL)	99%	2.0	0.67 (0.54-0.78)	272 (191-428)
SAA (ug/mL)	98%	5.2	0.67 (0.54-0.78)	6.4 (3.0-13)
Adhesion molecules				
MMP1 (ng/mL)	99%	1.5	0.88 (0.82-0.92)	1.8 (1.0-3.2)
sE-selectin (ng/mL)	98%	11.0	0.86 (0.80-0.91)	21 (13-27)
KLK10 (ng/mL)	99%	2.6	0.81 (0.72-0.87)	2.5 (2.2-3.1)
KLK8 (ng/mL)	99%	3.2	0.80 (0.70-0.87)	2.9 (2.4-3.5)
Mesothelin (ng/mL)	100%	1.5	0.78 (0.68-0.85)	8.4 (7.1-11)
sICAM-1 (ng/ml)	100%	0.8	0.64 (0.50-0.75)	177 (134-204)
MMP9 (ng/mL)	100%	0.6	0.63 (0.48-0.74)	185 (137-261)
sVCAM-1 (ug/mL)	100%	0.8	0.62 (0.48-0.74)	1.2 (0.99-1.37)
PAI-1(active) (ng/mL)	100%	4.1	0.58 (0.43-0.71)	9.7 (7.6-14)
MMP8 (ng/mL)	100%	1.7	0.56 (0.40-0.69)	9.9 (6.1-15)
tPAI-1 (ng/mL)	100%	1.1	0.69 (0.57-0.79)	35 (29-43)
Chemokines				
Fractalkine (pg/mL)	71%	4.8	0.85 (0.76-0.91)	78 (< LLD -746)
MCP-2 (pg/mL)	94%	2.9	0.78 (0.68-0.86)	23 (14-29)
RANTES (ng/mL)	91%	0.3	0.76 (0.66-0.84)	4.6 (3.0-7.0)
MCP-1 (pg/mL)	100%	3.4	0.75 (0.65-0.84)	224 (177-271)
MIP-1 α (pg/mL)	100%	3.0	0.74 (0.63-0.83)	97 (68-166.)
Eotaxin (pg/mL)	100%	1.8	0.70 (0.58-0.80)	73 (58-94)
GRO- α (pg/mL)	63%	7.4	0.70 (0.54-0.82)	18 (< LLD-112)
MIP-1 β (pg/mL)	96%	11.4	0.62 (0.47-0.74)	97 (59-153)
IP-10 (pg/mL)	100%	2.0	0.60 (0.46-0.73)	15 (11-19)
Angiogenic inhibitors				
Endostatin (ng/mL)	100%	3.1	0.79 (0.70-0.86)	57 (47-68)
Angiostatin (ug/mL)	100%	11.3	0.66 (0.52-0.76)	21 (18-25)
Adipokines				
Leptin (ng/mL)	100%	2.2	0.82 (0.74-0.88)	8.8 (5.1-16)
Resistin (ng/mL)	100%	5.1	0.63 (0.49-0.75)	12 (8.3-16)
Apoptotic Factors				
sFas (ng/mL)	100%	3.5	0.92 (0.88-0.95)	4.4 (3.8-5.2)

* LLD: lower limit of detection.

RANTES, MCP-1, MIP-1 α , Eotaxin, GRO- α , MIP-1 β , IP-10; angiogenesis inhibitors: angiostatin and endostatin; adipokines: leptin and resistin; apoptotic factor: sFas; and other molecules: mesothelin, MPO, and PAI-1 (total and active). A detailed description is provided in *table 1*. The rest of the biomarkers under investigation either had ICCs less than 0.55 or had low levels of detection (< 60%). These included cancer antigens: CA 19-9, CA 72-4, MICA, S100, TTR, ULBP1, ULBP2, ULBP3; cell adhesion molecules: MMP 2, 3, 7, 12, 13; chemokines: MCP-3, MIF, MIG; adipokines: leptin and resistin; apoptotic factors: sFasL, DR5, Cyfra 21-1; angiogenic inhibitors and other markers: thrombospondin and HSP 27. Eight of the 55 biomarkers had ICCs less than 0.55, including MMP7, thrombospondin, MMP3, MIG, HSP 27, MIF, TTR, and MMP2 (listed in the order of decreasing reliability). The remaining biomarkers were detectable in less than 60% of the samples. ICCs for these 21 markers have not been calculated because of the very small percentage of samples above the detection limit and are not included in the table.

Differences in the median biomarker expression level between premenopausal and postmenopausal women. The Wilcoxon-Mann-Whitney test was used to evaluate the differences in the medians between the pre- and postmenopausal women selected for this study. Based on this test, 15 markers out of 55 were differentially expressed between the two groups using $p < 0.05$ as the significance level. These biomarkers included TTR, eotaxin, GRO- α , PAI-1(active), fractalkine, sICAM-1, sE-Selectin, tPAI-1, SCC, thrombospondin, MMP-2, MCP-1, CA-125, SAA, and resistin.

DISCUSSION

To date, few studies have addressed the temporal reliability of chemokines, cancer antigens, growth factors, apoptotic factors, and adipokines in healthy subjects. Our results are consistent with previous studies of CA 15-3 [15], MCP-1 [16], and RANTES [17]. To our knowledge, our group is the first to evaluate the temporal reliability of the majority of these biomarkers in healthy individuals, as, other than CA 15-3, CA 125, MCP-1, and RANTES, few markers have been explored in previous research of temporal reliability.

CA 125 has been used extensively for the diagnosis and follow-up of ovarian cancer patients [18]. A previous study evaluating CA 125 in healthy, menopausal women, using radioimmunoassay suggested that single, low CA 125 values are reliable indicators of a woman's true CA 125 value [19]. Using the multiplexing method, we confirmed that CA 125 is a reliable marker.

Resistin, a recently discovered adipokine, is purportedly involved in metabolic and inflammatory processes in humans and may be an important marker with which to assess disease risk in large-scale epidemiological studies. In our study, resistin was one of the most reliable markers, which confirmed the results of a recent study using ELISA. In that study, individual blood resistin concentra-

tions did not significantly change over a period of one year, and showed a high degree of reliability [20].

In general, very limited number of studies have evaluated longitudinal changes of these biological markers in healthy individuals [9, 10]. The majority of existing studies relied on correlations and did not report the variance components or ICCs, which provide superior assessment of reliability. Additionally, most of the existing studies on biomarker reliability to date, do not assess markers in healthy participants, evaluating only biomarker changes in patients with various benign and malignant conditions. In this study, serum levels of most of the biomarkers were similar to those measured by the same xMAPTM method in other studies [5], and those measured by ELISA in healthy populations. The differences in population characteristics (age, gender, etc.), assay sensitivity and specificity, standards used in the assays [21], or sample collection, processing, storage and assay performance [22], may contribute to the observed differences in biomarker concentrations in healthy subjects. Therefore, standardization of procedures needs to be done before there can be any direct comparison between studies.

Previous studies have suggested that the circulating levels of serum biomarkers can be affected by a wide range of factors, including age, gender, race, blood pressure, serum cholesterol, BMI, percentage body fat, visceral fat, cigarette smoking, the use of hormone replacement therapy, menopausal status, and physical exercise [23]. Reliability studies in the area of biomarkers are complicated by the fact that "normal" levels of biomarkers may differ with age. One of the previous studies found elevated cancer antigen levels in elderly individuals without any confirmed malignancies [24], suggesting that biomarkers may change over time due to the aging process rather than to occult pathology. Despite these factors affecting biomarker reliability, our study has demonstrated that a substantial number of biomarkers were stable over a one-two year period.

Our study had some limitations. The samples were not assayed in duplicate. However, this is common in assays using the Luminex method, which provides an average value based on 100 bead measurements. Our study population included women only, so the results may not be extrapolated to males. Future studies need to look into the reliability of biomarkers in premenopausal *versus* postmenopausal women in more detail, and compare the reliability of biomarkers in males and females. Despite these limitations, this is one of the first and the largest studies assessing the reliability of multiple serum markers using Luminex methodology.

In addition to addressing these limitations, in our future studies it would be important to evaluate more carefully the presence of various biological markers implicated in cancer development in the serum of healthy individuals. The presence of these biomarkers in the serum of healthy individuals is still not well understood. A good example of this concept is ErbB2, a member of the epidermal growth factor receptor family, implicated in the development of many human cancers. At this point, the presence of ErbB2 in serum samples from healthy individuals has not been explored by large epidemiological studies. Since the presence of overexpression of ErbB2 in serum of

healthy individuals could be symptomatic of the development of breast cancer or cancer in general, the detection of high and reliable-over-time levels of ErbB2 in the blood of healthy individuals could be an indication for the continuation of more frequent tests to unveil the cause.

Additionally, this study resulted in novel data on the differences between biomarker expression levels in healthy, premenopausal and healthy, postmenopausal women over time. Due to a relatively small size of this cohort, only very large differences between pre- and postmenopausal women would be detectable in our study. This study detected differences between premenopausal versus postmenopausal women in 15 out of 55 markers, including TTR, eotaxin, GRO- α , PAI-1(active), fractalkine, sICAM-1, sE-Selectin, tPAI-1, SCC, thrombospondin, MMP-2, MCP-1, CA-125, SAA, and resistin. These results were consistent with previous research of healthy women followed up through menopausal transition, which suggested that SAA, tPAI, and MCP-1 differ between premenopausal and postmenopausal status [25]. Additionally, previous evidence suggests that there are differences in PAI-1, MMP-1, and MMP-2 levels between healthy, premenopausal versus postmenopausal women [26, 27]. Consistently with our study, Grover *et al.* found that both hysterectomy and menopausal status have a clear effect on serum CA 125 levels and must be considered if serum CA 125 is to be used as a screening test [28]. Other than SAA, MCP-1, PAI-1, MMP-1, and MMP-2, the rest of the markers in our study that were differentially expressed between postmenopausal and premenopausal women have been only rarely investigated in healthy women in relation to menopausal status. In conclusion, using the xMAP™ method we found that serum concentrations of cancer antigens: AFP, CA 15-3, CEA, CA-125, SCC, SAA; growth factors/related molecules: ErbB2, IGFBP-1; proteases and adhesion molecules: MMP-1,8,9, sE-selectin, KLK8,10, sICAM-1, sVCAM-1; chemokines: fractalkine, MCP-1,2, RANTES, MIP-1 α , MIP-1 β , eotaxin, GRO- α , IP-10; angiogenesis inhibitors: angiostatin and endostatin; adipokines: leptin and resistin; apoptotic factor: sFas; and other proteins: mesothelin, MPO, and PAI-1, are detectable and remain stable for up to two years in stored serum samples, suggesting that a single measurement of this markers may be sufficient for utilization in clinical and epidemiological studies.

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