Chitotriosidase, a marker of innate immunity, is elevated in patients with primary breast cancer.

Mya Sanda Thein[1,2], Anita Kohli[1,3], Rohit Ram[1,4], Maria Clara Ingaramo[1,5],

Alka Jain[1], Neal S. Fedarko[1]; The Johns Hopkins University, Baltimore, MD

[1]Department of Medicine, Johns Hopkins University, Baltimore, MD

[2]Current address: University of Maryland Community Oncology Group, Glen Burnie, MD

[3]Current address: Scheie Eye Institute at the University of Pennsylvania, Philadelphia, PA

[4]Current address: Department of Radiology, University of Pittsburgh Medical Center, Pittsburgh, PA

[5]Current address: Instituto de Agrobiotecnología del Litoral, Santa Fe, Argentina

Running Title: Serum chitotriosidase activity in cancer

Key Words: Neopterin, chitotriosidase, macrophages, cancer

Corresponding Author: Neal Fedarko, PhD, Room: 1A-12 JHACC, 5501 Hopkins Bayview Circle, Baltimore, MD 21224, Email: ndarko@jhmi.edu

Conflicts of Interest: None.

Word Count: 2706

Total number of Figures: 2 Tables: 2

Abstract:

Background: Cancer progression has been associated with altered immune cell function and activation. Neopterin, which is secreted by interferon-γ stimulated macrophages, exhibits an association with multiple cancer types and metastatic disease. Chitotriosidase, which is secreted by chronically activated macrophages and granulocyte-macrophage colony-stimulating factor stimulated neutrophils has not been studied in the setting of cancer.

Objective: The goal of this discovery study was to screen chitotriosidase for diagnostic capacity in detecting cancer and compare its operating characteristics with those of neopterin.

Methods: Serum from subjects with breast (n = 66) or prostate (n = 70) cancer, and from 204 subjects free of malignant disease were studied. Chitotriosidase was measured by enzyme activity assay, while neopterin was measured by a competitive enzyme immunoassay. Statistical analyses included group comparisons by Mann Whitney U test, diagnostic capacity by receiver operating characteristics (ROC) curve analysis and biomarker associations with physiologic and clinical measures by Spearman correlation.

Results: Chitotriosidase activity was significantly higher in both cancer types compared with gender matched controls, though only in breast cancer was the diagnostic capacity significant (area under the ROC curve of 0.97 ± 0.01). In contrast, neopterin was significantly elevated in prostate cancer and exhibited discriminatory capacity (area under the ROC curve of 0.76 ± 0.05). Age, BMI, % body fat and metastasis were variables that correlated with neopterin, but not chitotriosidase levels.

Conclusions: The operating characteristics of serum chitotriosidase were different from neopterin and further analysis of chitotriosidase as a biomarker for breast cancer is warranted.

Introduction

Tumor growth and progression is associated with inflammation, altered immune function and activation [1]. The tumor microenvironment is characterized by the presence of host leucocytes in the tumor and its surrounding stroma [2]. Tumor-associated macrophages are a significant component of inflammatory infiltrates in malignant tumor tissues. They play an important role in cancer progression by stimulating tumor-cell proliferation, promoting angiogenesis, tumor invasion and metastasis [2,3] and are associated with poor prognosis in a variety of human cancers [4]. Neutrophils are normally relatively short-lived rapid responders to inflammation. Tumor associated neutrophils have also been associated with tumor progression and poor prognosis [5-7].

Neopterin, a GTP metabolite, is a product of human monocyte-derived macrophages stimulated by activated T cell release of interferon-γ [8]. Therefore, elevated neopterin can reflect systemic immune activation and the effect of cytokines on macrophages [9]. Although the biological function of neopterin is still not well understood, it may be an indicator of oxidative stress and reactive oxygen species formation [10]. Reactive oxygen species are known to play a significant role in initiation, proliferation and survival of cancer cells [11]. A positive correlation between high levels of neopterin and advanced tumor stage has been observed for a number of malignancies including leukemia, lymphoma, multiple myeloma, malignant melanoma and cancer of the breast, cervix, colon, liver, lung, ovaries, pancreas, prostate, and stomach [9].

Chitotriosidase, a member of the glycosyl hydrolase family, is a secreted enzyme that catalyzes the hydrolysis of both chitin and chitin-like substrates [12]. Chitotriosidase serves as a diagnostic marker of Gaucher disease where tissue resident macrophages are chronically activated

and secrete high levels [13]. Chitotriosidase production was also found to be associated with pathogen-driven diseases, particularly with chronic fungal infection and with reticulo-endothelial activations, indicating its role as an innate immunity component of the macrophage-driven inflammatory process [14,15]. Neutrophils can also be a potential source of serum chitotriosidase activity, releasing it from granules when stimulated with granulocyte-macrophage colony-stimulating factor [15]. The serum distribution of chitotriosidase has not been described in the setting of cancer.

We hypothesized that serum chitotriosidase activity would reflect a tumor extrinsic (host) contribution to modifying disease progression and, given its association with chronic inflammation, that chitotriosidase would exhibit greater sensitivity and specificity than neopterin for cancer detection. Hence, the current discovery study characterized a novel cancer biomarker, chitotriosidase, and compared it with the extensively studied innate immune biomarker neopterin. The biomarkers were characterized in terms of distribution and diagnostic capacity using serum from normal subjects and subjects with breast or prostate cancer. Breast and prostate cancer share a number of similarities including steroid hormone modulation, stromal cell/tumor microenvironment promotion of disease progression, and immune system changes that facilitate cellular transition through different oncogenic steps [16]. Biomarker associations with physiologic covariates (age, BMI, % body fat) and clinical measures were also determined.

Materials and Methods

Sample selection.

Serum from 66 subjects with breast cancer (n = 8 for stage I, n = 22 for stage II, n = 22 stage II and n = 14 for stage IV), 70 subjects with prostate cancer (n = 22 for T2, n = 26 for T3, and n = 22 for T4), and from a group of 204 normal healthy women and men were obtained from an existing Johns Hopkins Bayview Medical Center repository [17,18]. Inclusion criteria as a normal healthy serum donor included measures within the normal range for fasting glucose (< 100 mg/dl) and TSH (0.5 - 2.1 mIU/mL), as well as a clinical assessment by a physician. Serum samples from cancer subjects were collected at time of diagnosis, before initiation of treatment. Exclusionary criteria included a history of diabetes mellitus, congestive heart failure, stroke, pulmonary disease, renal or hepatic dysfunction, dementia, cancer of any other organ system, any chronic inflammatory condition (e.g., rheumatoid arthritis), or the use of anti-inflammatory agents (e.g., steroids).

Clinical Measures.

Anthropometric measures included height and weight. Foot-to-foot bio-impedance analysis was conducted to estimate percentage of body fat (% fat) using a Tanita scale (Tanita Corporation of America; Arlington Heights, IL). The physiological covariates associated with each specimen included age, gender, BMI and % body fat. Clinical findings associated with the serum samples included Union for International Cancer Control (UICC) staging, estrogen receptor (ER), progesterone receptor (PR) and HER-2 neu expression status for subjects with breast cancer and prostate-specific antigen levels (PSA) for subjects with prostate cancer. The hormone sensitivity of the prostate tumors was not determined. The study was approved by Johns Hopkins Bayview Medical Center institutional review board.

Serum Assays:

Serum specimens were obtained in a resting and fasting state in the morning. All venous samples were placed at 4^{o} C prior to serum separation. After centrifugation at 3000 rpm for 20 minutes, the serum was aliquoted and stored at -80^{o}C. Chitotriosidase enzyme activity was measured by kinetic assay using the substrate 4-methylumbelliferyl-β-D-N', N', N'' triacetylchitotrioside as previously described [18]. Briefly, enzyme activity was measured by incubating 5µL of serum with substrate (22 µmol/L 4-methylumbelliferyl-β-D-N', N', N'' triacetylchitotrioside) in McIlvain's phosphate citrate buffer, pH = 5.2. The evolution of the fluorescent product 4-methylumbelliferone was measured at excitation 360 nm and emission 450 nm for a total reaction time of one hour. Standard curves for the reaction product, 4-methylumbelliferone, spanning 1 and 100 nM were included to convert fluorescent values to nmols of product and the slope over 6 minutes was then used to calculate enzyme activity expressed as pmol/min/ml. Neopterin was measured as previously described using a commercially available competitive ELISA following the manufacturer's protocol [19]. For both assays, serum samples were analyzed in duplicate and if the replicate coefficient of variance was > 15%, the sample was reanalyzed.

The lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were determined for the chitotriosidase assay following methodology proposed by the International Council for Harmonization [20]. LLOD was calculated using the formula LLOD = 3.3σ/*S*, where σ is the standard deviation of the y-intercept of the regression line of the calibration curve (n = 15) and *S* is average slope of the calibration curve. LLOQ was calculated using the formula LLOQ = 10σ/*S*. The enzyme activity assay had a lower limit of detection of 10 pmol/min/ml and a lower limit of quantitation of 30 pmol/min/ml. The chitotriosidase assay had an inter-assay coefficient of variance of 11 % for a control serum sample with an average activity of 70 pmol/min/ml. The competitive

ELISA had a sensitivity of 0.8 nM and and a control sample with an average value of 8.0 nM had a coefficient of variance of 6.0%.

Statistical Analysis

All statistical calculations were carried out using Prism and InStat software (GraphPad, Inc.). The comparison of variables or serum markers between groups were performed by using a Mann-Whitney U-test. The relationship between specificity and sensitivity for each marker was profiled by receiver operating characteristic (ROC) curves. Optimal cut off values for biomarkers were identified using Youden's J statistic. Correlations between serum markers and individual physiologic covariates or clinical measures were assessed using Spearman rank correlations. There were limited numbers of subjects in some clinical stage, hence cancer samples were divided into two groups for comparison: those with localized disease and those with evidence of distant metastasis.

Results

The characteristics of normal and cancer subjects are listed in Table I. Age was significantly higher in breast cancer subjects when compared to the normal female serum donors (unpaired t-test $p < 0.005$). BMI and % body fat were not significantly different between the normal and breast cancer groups. Approximately two thirds of breast cancer subjects were ER positive (ER+) or PR positive (PR+), while one tenth were both ER and PR negative. One fifth of the breast cancer subjects were Her-2 positive (Her2+). Age was significantly higher for prostate cancer when compared to gender-matched normal subjects, $p < 0.0001$. PSA levels were also significantly higher in subjects with prostate cancer compared to disease-free male subjects ($p < 0.0001$). No difference in age, BMI % or body fat was found between subjects with localized or metastatic breast or prostate cancer.

An enzymatic assay was employed to quantify the amount of chitotriosidase present in serum. An age-dependent difference in the rate of substrate conversion into product was apparent in serum from normal female and male subjects (Figure 1A and B). Serum from subjects with breast cancer exhibited elevated enzyme activity rates independent of age (Figure 1C). Prostate cancer serum also exhibited no age-associated change in enzyme activity and their rates of substrate conversion were less then in breast cancer serum (Figure 1D). The median chitotriosidase activity for serum from normal female donors was 67.3 (range 14.8-340.8) pmol/min/ml which was not significantly different from the median value of 70.2 (range 20.0 to 267.1) pmol/min/ml for serum from normal males (Figure 2A). Serum from subjects with breast cancer had a median chitotriosidase activity of 396.4 (range 119.7 to 978.4) pmol/min/ml, $p < 0.0001$ compared with normal female serum. Serum from prostate cancer subjects had a median chitotriosidase activity of 114 (range 11 to 706) pmol/min/ml, $p < 0.05$ compared with normal male serum.

The median value of neopterin was 6.77 (range 2.92 to 13.43) nM in serum from normal female donors and 6.56 (range 3.37 to 10.50) nM in serum from normal male donors, not significantly different (Figure 2B). The median value of serum neopterin was 6.58 (range 3.60 to 15.69) nM in breast cancer subjects, no different from that in gender matched normal serum. A median neopterin value of 8.28 (range 3.88 to 60.74) nM in prostate cancer subjects was significantly higher from that in normal male serum, $p < 0.0001$.

The discriminatory capacity of serum levels of chitotriosidase to detect cancers was profiled by ROC curve analysis (Figure 2C). For distinguishing between breast cancer subjects and disease-

free females, the area under the curve (AUC) was significant for chitotriosidase with an AUC of 0.97 (95% confidence interval 0.95 - 0.99), ($p < 0.0001$). Based on the ROC curve analysis, on average, a breast cancer patient will have a more abnormal chitotriosidase test result than 97% of the normal subjects. At a serum level cut off of 230 pmol/min/ml, chitotriosidase had a sensitivity of 79% (95% confidence interval 67 - 88) and a specificity of 96 % (95% confidence interval 91 - 99) with a likelihood ratio of 22. A subject with a chitotriosidase activity of greater than 230 pmol/min/ml will be 22 times more likely to have breast cancer than someone with lower activity. Serum levels of chitotriosidase exhibited diminished ability to discriminate between serum from normal and prostate cancer (AUC of 0.64, 95% confidence interval 0.53 to 0.75, $p < 0.05$). At the serum level cut off of 230 pmol/min/ml, chitotriosidase had a sensitivity of 19% (95% confidence interval 8 to 35) and a specificity of 97 % (95% confidence interval 92 to 99) with a likelihood ratio of 6.5 for prostate cancer.

The capacity of serum levels of neopterin to detect cancer was also profiled by ROC curve analysis (Figure 2D). The AUCs were 0.68 (95% confidence interval 0.42 to 0.59, $p = 0.88$) and 0.76 (95% confidence interval 0.68 to 0.84, $p < 0.0001$), for detecting breast or prostate cancer, respectively from gender-matched normal sample donors. Neopterin had no discriminatory power to detect breast cancer while on average, a prostate cancer patient would have a more abnormal neopterin test result than 76 % of the controls. At a serum level cut off of 7.6 nM, neopterin had a sensitivity of 70 % (95% confidence interval 58 to 80) and a specificity of 85 % (95% confidence interval 76 to 92) with a likelihood ratio of 4.7 for prostate cancer.

Associations between the two biomarkers and physiologic covariates were investigated next. Significant correlations were observed in normal serum between age and chitotriosidase with a Spearman r of 0.46 (95% confidence interval 0.29 - 0.60), $p < 0.0001$ for serum from females and a Spearman r of 0.30 (95% confidence interval 0.09 to 0.48), $p < 0.005$ for serum from males. The association of age with chitotriosidase activity lost significance in serum samples from cancer. BMI and % body fat did not significantly correlate with chitotriosidase activity in serum from normal donors as well as from subjects with breast or prostate cancer.

In contrast, neopterin was significantly correlated with age in serum from normal females (Spearman r of 0.30, 95% confidence interval 0.11 to 0.46, $p < 0.005$), normal males (Spearman r of 0.54, 95% confidence interval 0.37 to 0.68, $P < 0.0001$), and from subjects with breast cancer (Spearman r of 0.27, 95% confidence interval 0.02 to 0.48, $p< 0.05$). Significant associations between BMI and and neopterin were observed observed in serum from normal females (Spearman r of 0.40, 95% confidence interval 0.23 to 0.55, $p < 0.001$) and males (Spearman r of 0.40, 95% confidence interval 0.23 to 0.55, $p < 0.001$), but not in serum from subjects with cancer. Percent body fat significantly correlated with neopterin activity only in samples from normal females (Spearman r = 0.39, 95% confidence interval 0.21 to 0.54, $p < 0.0001$).

The relationship between the two biomarkers and clinical measures were assessed. For chitotriosidase in breast cancer, there were no significant associations with receptor status. The median chitotriosidase activity in localized breast cancer was 387.4 (range 119.7 to 978.4) pmol/min/ml versus 443.1 (range 191.8 to 786.1) pmol/min/ml for metastatic breast cancer. The values were not significantly different. In prostate cancer, serum chitotriosidase did not correlate with

PSA levels. The median chitotriosidase activity in localized prostate cancer was 93.8 (range 11.2 to 583.8) pmol/min/ml while metastatic prostate cancer had a median chitotriosidase activity of 130.6 (range 37.1 to 606.9) pmol/min/ml, not significantly different.

Neopterin also had no significant associations with receptor status in breast cancer. In contrast to chitotriosidase, neopterin exhibited a number of associations with other clinical measures. The median value of neopterin in localized breast cancer was 6.34 (range 3.60 to 15.69) nM versus 10.02 (range 5.54 - 15.69) nM for metastatic breast cancer, $p < 0.005$. Neopterin was significantly correlated with PSA levels in prostate cancer with a Spearman r of 0.58 (95% confidence interval of 0.32 to 0.76), $p < 0.0001$. Localized prostate cancer had a median of 8.26 (range 2.88 to 14.17) nM while metastatic prostate cancer had a median of 21.7 (range 4.18 to 60.74) nM, $p < 0.0005$. A summary table reporting study design and results following the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines [21] adapted to an earlier phase discovery study are given in Table 2.

Discussion

Based on 2010-2012 data, at some point during their lifetime approximately 12 % of women will be diagnosed with breast cancer and 14 % of men will be diagnosed with prostate cancer [22]. Age-adjusted annual death rates per 100,000 for 2012 were 21.9 for breast cancer and 21.4 for prostate cancer [23]. Cancer progression and metastases are the cause of 90% of human cancer deaths [24]. Chronic immune activation is believed to contribute to tumor growth and metastasis [25,26]. Validated tumor markers in breast cancer include tissue based ER, PR, and HER-2, status [27]. In the

current study, there was no association between ER, PR and HER-2 status with neopterin or chitotriosidase levels. This is consistent with hormone and growth factor receptors being tumor intrinsic markers. whereas chitotriosidase and neopterin reflect the tumor extrinsic host immune response. Serum tumor markers that are clinically used for breast cancer are CA 15-3, CA 27-29, and CEA (carcinoembryonic antigen). However, due to their low sensitivity and specificity, the American Society of Clinical Oncology expert panel in breast cancer recommends using those serum tumor markers in metastatic disease only [28]. For prostate cancer, PSA has been used for screening purposes though it does not have ideal specificity as it can be elevated in certain benign conditions such as acute prostatitis and benign prostatic hyperplasia and approximately 15% of PCA cases occur in men with very low serum PSA values [29,30]. The identification of novel candidate diagnostic biomarkers for both cancer types remains a priority.

During cancer progression, activated tumor associated macrophages and neutrophils can can promote genetic instability, tumor growth, angiogenesis and invasive behavior [31]. In the current study, markers associated with macrophage and neutrophil activation were correlated with physiological and clinical correlates in breast and prostate cancer. The earliest stage of breast cancer was associated with significant elevation of chitotriosidase, while neopterin was significantly elevated in late stage metastatic disease. The observation of elevated neopterin levels in subjects with metastatic disease are in agreement with previous studies demonstrating that higher serum levels of neopterin were associated with poor prognosis and the presence of distant metastasis in breast and prostate cancer [32,33]. The age- and BMI-associated contributions to biomarker levels observed in the current study are consistent with observations in other populations. Chitotriosidase was reported to exhibit an age-associated increase in serum levels in normal subjects [18]. The biomarker

neopterin has been reported to be influenced by both age and BMI in serum from normal and obese subjects and subjects with sleep apnea [34,19].

Breast and prostate cancer share similar biological (sex hormone sensitive and insensitive states) and epidemiological characteristics (incidence and mortality). For both breast and prostate cancer, a high neutrophil to lymphocyte ratio (a marker of inflammation) was prognostic for worse overall survival and disease-free survival [35,36]. Despite these similarities, differences in underlying pathophysiology have been observed. Chronic inflammation was higher in biopsies from men with a benign prostate versus those with prostate cancer and inversely correlated with tumor volume in a two year repeat biopsy in men who had a negative baseline biopsy [37,38]. In the current study, elevated chitotriosidase activity associated with breast but not prostate cancer may be indicative of a further difference between the two cancer types. Chitotriosidase is a marker of the chronic activation of the dedicated phagocytic cells - macrophages and neutrophils, hence there may be an organ-specific difference in how these immune cells interact with the tumor microenvironment.

Limitations to this study include that it involved relatively small discovery groups, the serum samples were cross-sectional and considerable overlap between normal and cancer values were evident in some cases. Measuring circulating levels of a biomarker does not necessarily identify the secreting cells, so it is unclear whether macrophages or neutrophils or some combination may be contributing to altered levels of chitotriosidase in breast cancer. Additionally, as potential biomarkers for cancer, chitotriosidase and neopterin may lack disease specificity in that they are induced in a number of other pathological states. Neopterin serum levels are increased in viral infections, various malignant disorders, autoimmune diseases, liver, kidney and heart disease [39,40]. Serum levels of

chitotriosidase are elevated in Gaucher disease [41] and in other disorders associated with chronic macrophage activation including Wegener's granulomatosis [42], and sarcoidosis [43]. A large case - control validation study of chitotriosidase as a marker of early stage breast cancer would address a number of these limitations and is being planned.

CONCLUSION:

Chitotriosidase exhibited a significant ability to distinguish between disease-free subjects and subjects with diagnosed breast cancer. Neopterin, though elevated in metastatic disease, did not exhibit diagnostic capacity in breast or prostate cancer. Further studies should be done to validate chitotriosidase as a biomarker and to investigate the biology of macrophage and neutrophil activation in breast cancer progression.

Acknowledgements:

This research was supported by Department of Defense, CDMRP grant BC010478 (N.S.F.), T35 AG026758 (R.R. and A.K.).and NIH T32 training grant 5T32AG000120 (M.S.T.). The authors declare that they have no conflict of interest.

Drs. Thein, Kohli, and Fedarko participated in the conception and design of the study. Drs. Ram and Jain and Ms. Ingaramo developed the methodology to measure chitotriosidase in serum. Data was acquired by Ms. Ingaramo and Drs. Thein, Kohli, and Ram. Drs. Thein, Kohli, Jain and Fedarko performed the analysis and interpretation of data. The study was supervised by Drs. Thein and Fedarko. Writing and reviewing of the manuscript was carried al by all authors.

Human Subjects: This research protocol performed in this study was reviewed and approved by the Johns Hopkins Medicine Institutional Review Board.

Informed consent: For this discovery study, formal consent was not required.

References:

1. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L (1992) The origin and function of tumor-associated macrophages. *Immunology today* 13 (7):265-270.

2. Balkwill F, Mantovani A (2001) Inflammation and cancer: back to Virchow? *Lancet* 357 (9255):539-545.

3. Balkwill F, Coussens LM (2004) Cancer: an inflammatory link. *Nature* 431 (7007):405-406.

4. Siveen KS, Kuttan G (2009) Role of macrophages in tumour progression. *Immunol Lett* 123 (2):97-102.

5. Jiang C, Hu WM, Liao FX, Yang Q, Chen P, Rong YM, Guo GF, Yin CX, Zhang B, He WZ, Xia LP (2016) Elevated preoperative neutrophil-to-lymphocyte ratio is associated with poor prognosis in gastrointestinal stromal tumor patients. *Onco Targets Ther* 9:877-883.

6. Rahbar A, Cederarv M, Wolmer-Solberg N, Tammik C, Stragliotto G, Peredo I, Fornara O, Xu X, Dzabic M, Taher C, Skarman P, Soderberg-Naucler C (2016) Enhanced neutrophil activity is associated with shorter time to tumor progression in glioblastoma patients. *Oncoimmunology* 5 (2):e1075693.

7. Tazzyman S, Barry ST, Ashton S, Wood P, Blakey D, Lewis CE, Murdoch C (2011) Inhibition of neutrophil infiltration into A549 lung tumors in vitro and in vivo using a CXCR2-specific antagonist is associated with reduced tumor growth. *Int J Cancer* 129 (4):847-858.

8. Niederwieser D, Huber C, Gratwohl A, Bannert P, Fuchs D, Hausen A, Reibnegger G, Speck B, Wachter H (1984) Neopterin as a new biochemical marker in the clinical monitoring of bone marrow transplant recipients. *Transplantation* 38 (5):497-500.

9. Sucher R, Schroecksnadel K, Weiss G, Margreiter R, Fuchs D, Brandacher G (2010) Neopterin, a prognostic marker in human malignancies. *Cancer Lett* 287 (1):13-22.

10. Murr C, Bergant A, Widschwendter M, Heim K, Schrocksnadel H, Fuchs D (1999) Neopterin is an independent prognostic variable in females with breast cancer. *Clin Chem* 45 (11):1998-2004.

11. Tafani M, Sansone L, Limana F, Arcangeli T, De Santis E, Polese M, Fini M, Russo MA (2016) The interplay of reactive oxygen species, hypoxia, inflammation, and sirtuins in cancer initiation and progression. *Oxid Med Cell Longev* 2016:3907147.

12. Renkema GH, Boot RG, Muijsers AO, Donker-Koopman WE, Aerts JM (1995) Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. *J Biol Chem* 270 (5):2198-2202.

13. Hollak CE, van Weely S, van Oers MH, Aerts JM (1994) Marked elevation of plasma chitotriosidase activity. A novel hallmark of Gaucher disease. *The Journal of clinical investigation* 93 (3):1288-1292.

14. Kzhyshkowska J, Gratchev A, Goerdt S (2007) Human chitinases and chitinase-like proteins as indicators for inflammation and cancer. *Biomarker insights* 2:128-146.

15. van Eijk M, van Roomen CP, Renkema GH, Bussink AP, Andrews L, Blommaart EF, Sugar A, Verhoeven AJ, Boot RG, Aerts JM (2005) Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. *Int Immunol* 17 (11):1505-1512.

16. Risbridger GP, Davis ID, Birrell SN, Tilley WD (2010) Breast and prostate cancer: more similar than different. *Nat Rev Cancer* 10 (3):205-212.

17. Kavathia N, Jain A, Walston J, Beamer BA, Fedarko NS (2009) Serum markers of apoptosis decrease with age and cancer stage. *Aging* 1 (7):652-663.

18. Ramanathan R, Kohli A, Ingaramo MC, Jain A, Leng SX, Punjabi NM, Walston JD, Fedarko NS (2013) Serum chitotriosidase, a putative marker of chronically activated macrophages, increases with normal aging. *J Gerontol A Biol Sci Med Sci* 68 (10):1303-1309.

19. Spencer ME, Jain A, Matteini A, Beamer BA, Wang NY, Leng SX, Punjabi NM, Walston JD, Fedarko NS (2010) Serum levels of the immune activation marker neopterin change with age and gender and are modified by race, BMI, and percentage of body fat. *J Gerontol A Biol Sci Med Sci* 65 (8):858-865.

20. Shrivastava A, Gupta VB (2011) Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chron Young Sci* 2:21-25.

21. Altman DG, McShane LM, Sauerbrei W, Taube SE (2012) Reporting recommendations for tumor marker prognostic studies (REMARK): explanation and elaboration. *BMC Med* 10:51.

22. Howlader N, Noone AM, Krapcho M, Garshell J, Miller D, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, KA C SEER Cancer Statistics Review, 1975-2012, National Cancer Institute. Bethesda, MD, based on November 2014 SEER data submission, posted to the SEER web site, April 2015. Table 1 https://seer.cancer.gov/archive/csr/1975_2012/results_merged/topic_lifetime_risk_diagnosis.pdf. Accessed January 21, 2016

23. Howlader N, Noone AM, Krapcho M, Garshell J, Miller D, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, KA C SEER Cancer Statistics Review, 1975-2012, National Cancer Institute. Bethesda, MD, based on November 2014 SEER data submission, posted to the SEER web site, April 2015. Table 1.5.

https://seer.cancer.gov/archive/csr/1975_2012/results_single/sect_01_table.05_2pgs.pdf. Accessed January 21, 2016

24. Mehlen P, Puisieux A (2006) Metastasis: a question of life or death. *Nature reviews Cancer* 6 (6):449-458.

25. Shacter E, Weitzman SA (2002) Chronic inflammation and cancer. *Oncology (Williston Park)* 16 (2):217-226, 229; discussion 230-212.

26. Chai EZ, Siveen KS, Shanmugam MK, Arfuso F, Sethi G (2015) Analysis of the intricate relationship between chronic inflammation and cancer. *Biochem J* 468 (1):1-15.

27. Sturgeon CM, Duffy MJ, Stenman UH, Lilja H, Brunner N, Chan DW, Babaian R, Bast RC, Jr., Dowell B, Esteva FJ, Haglund C, Harbeck N, Hayes DF, Holten-Andersen M, Klee GG, Lamerz R, Looijenga LH, Molina R, Nielsen HJ, Rittenhouse H, Semjonow A, Shih Ie M, Sibley P, Soletormos G, Stephan C, Sokoll L, Hoffman BR, Diamandis EP (2008) National Academy of Clinical Biochemistry laboratory medicine practice guidelines for use of tumor markers in testicular, prostate, colorectal, breast, and ovarian cancers. *Clin Chem* 54 (12):e11-79.

28. Harris L, Fritsche H, Mennel R, Norton L, Ravdin P, Taube S, Somerfield MR, Hayes DF, Bast RC, Jr. (2007) American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J Clin Oncol* 25 (33):5287-5312.

29. Balk SP, Ko YJ, Bubley GJ (2003) Biology of prostate-specific antigen. *J Clin Oncol* 21 (2):383-391.

30. Thompson IM, Pauler DK, Goodman PJ, Tangen CM, Lucia MS, Parnes HL, Minasian LM, Ford LG, Lippman SM, Crawford ED, Crowley JJ, Coltman CA, Jr. (2004) Prevalence of prostate cancer

among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *N Engl J Med* 350 (22):2239-2246.

31. Galdiero MR, Garlanda C, Jaillon S, Marone G, Mantovani A (2013) Tumor associated macrophages and neutrophils in tumor progression. *Journal of cellular physiology* 228 (7):1404-1412.

32. Yildirim Y, Gunel N, Coskun U, Pasaoglu H, Aslan S, Cetin A (2008) Serum neopterin levels in patients with breast cancer. *Med Oncol*.

33. El-Akawi ZJ, Abu-Awad AM, Sharara AM, Khader Y (2010) The importance of alpha-1 antitrypsin (alpha1-AT) and neopterin serum levels in the evaluation of non-small cell lung and prostate cancer patients. *Neuro Endocrinol Lett* 31 (1):113-116.

34. Punjabi NM, Beamer BA, Jain A, Spencer ME, Fedarko N (2007) Elevated levels of neopterin in sleep-disordered breathing. *Chest* 132 (4):1124-1130.

35. Cao J, Zhu X, Zhao X, Li XF, Xu R (2016) Neutrophil-to-Lymphocyte Ratio Predicts PSA Response and Prognosis in Prostate Cancer: A Systematic Review and Meta-Analysis. *PLoS One* 11 (7):e0158770.

36. Ethier JL, Desautels D, Templeton A, Shah PS, Amir E (2017) Prognostic role of neutrophil-to-lymphocyte ratio in breast cancer: a systematic review and meta-analysis. *Breast Cancer Res* 19 (1):2.

37. Karakiewicz PI, Benayoun S, Begin LR, Duclos A, Valiquette L, McCormack M, Benard F, Saad F, Perrotte P (2007) Chronic inflammation is negatively associated with prostate cancer and high-grade prostatic intraepithelial neoplasia on needle biopsy. *Int J Clin Pract* 61 (3):425-430.

38. Moreira DM, Nickel JC, Andriole GL, Castro-Santamaria R, Freedland SJ (2015) Chronic baseline prostate inflammation is associated with lower tumor volume in men with prostate cancer on repeat biopsy: Results from the REDUCE study. *Prostate* 75 (13):1492-1498.

39. Berdowska A, Zwirska-Korczala K (2001) Neopterin measurement in clinical diagnosis. *J Clin Pharm Ther* 26 (5):319-329.

40. Pacileo M, Cirillo P, De Rosa S, Ucci G, Petrillo G, Musto D'Amore S, Sasso L, Maietta P, Spagnuolo R, Chiariello M (2007) The role of neopterin in cardiovascular disease. *Monaldi Arch Chest Dis* 68 (2):68-73.

41. Bussink AP, van Eijk M, Renkema GH, Aerts JM, Boot RG (2006) The biology of the Gaucher cell: the cradle of human chitinases. *Int Rev Cytol* 252:71-128.

42. Koening CL, Gota CE, Langford CA, Hoffman GS, Natowicz MR (2010) Serum chitotriosidase activity and Wegener's granulomatosis. *Clin Biochem* 43 (4-5):512-514.

43. Tercelj M, Salobir B, Simcic S, Wraber B, Zupancic M, Rylander R (2009) Chitotriosidase activity in sarcoidosis and some other pulmonary diseases. *Scand J Clin Lab Invest* 69 (5):575-578.

Table 1. Study subject characteristics.

	n	Age, years	BMI kg/m^2	% body fat	ER$^+$	PR$^+$	ER$^+$PR$^+$	Her2$^+$
female NL	111	53 ± 18	27.9 ± 8.5	36.3 ± 6.6	-	-	-	-
Breast Ca	66	62 ± 14	25.2 ± 3.9	34.9 ± 2.9	41 (71)	41 (62)	29 (44)	14 (21)
localized	52	60 ± 15	24.9 ± 4.0	34.7 ± 3.1	38 (73)	33 (63)	23 (44)	9 (17)
metastatic	14	66 ± 8	26.4 ± 3.0	35.6 ± 1.8	9 (64)	8 (57)	6 (43)	5 (36)
	n	Age, years	BMI kg/m^2	% body fat	PSA ng/ml			
male NL	93	52 ± 17	24.2 ± 3.6	18.4 ± 2.7	0.85 (0.36 to 2.43)			
Prostate Ca	70	66 ± 9	24.0 ± 4.5	18.1 ± 3.4	27 (1.9 to 69)			
localized	48	66 ± 9	25.1 ± 5.7	20.2 ± 7.5	8.3 (1.9 to 67)			
metastatic	22	67 ± 8	24.8 ± 7.6	19.8 ± 10.0	53 (4.8 to 69)			

Values for age, BMI and % body fat are mean ± standard deviation. Values for prostate specific antigen (PSA) were median with range in parenthesis. Values for negative estrogen receptor (ER-), negative progesterone receptor (PR-), and positive Her-2 Neu receptor (Her2+) are number subjects (%).

Table 2. REMARK[a] Summary Table

a). Patient Samples, treatment and variables				
Study and marker	**Remarks**			
Markers	M1 = chitotriosidase, continuous enzyme activity (pmol/min/ml) M2 = neopterin, continuous nM values			
Further variables	Continuous: v1 = age, v2 = BMI, v3 = % body fat, v4 = PSA Categorical: v5 = ER, v6 = PR, v7 = HER2, v8 = metastasis			
Samples	**n**	**Remarks**		
No cancer	111 ♀ 93 ♂	Archived samples, source: Johns Hopkins Bayview Medical Center; inclusions: normal serum chemistry, anthropometrics and clinical evaluation		
Breast cancer Prostate cancer	66 70	Archived samples, source: Johns Hopkins Bayview Medical Center, inclusions: serum draw at time of diagnosis, before treatment.		
b) Statistical analysis				
Analysis	Cases	Controls	Variables	Results/Remarks
A1: Univariable	66 70	111 93	v1 to v6 v1 to v6	v1 significant v1 and v8 significant
A2: Univariable	66 70	111 93	DX: M1 DX: M2	ROC curve, Fig. 2C, AUC=0.97±0.01 ROC curve, Fig. 2D, AUC=0.76±0.05
A3: Multivariable	66 70	111 93	M1: v1 to v6 M2: v1 to v6 M1: v1 to v6 M2: v1 to v6	v1 significant (controls) v1 and v2 significant (cases & controls) v3 significant (controls) v1 significant (controls) v4 and v8 significant (cases)

[a] Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) summary table adapted to an earlier phase diagnostic study. Abbreviations: ER, estrogen receptor; PR, progesterone

receptor; HER2, HER2 neu receptor; PSA, prostate specific antigen; DX, diagnostic capacity.

Figure Legends

Figure 1. Chitotriosidase activity in human serum. Representative profiles of the evolution of fluorescent enzymatic product as a function of time are shown for serum obtained from normal female subjects (A), normal male subjects (B), subjects with breast cancer (C), and subjects with prostate cancer cancer (D). Age and cancer stage are indicated.

Figure 2. Distribution and discriminatory capacity serum markers cancer. Levels of chitotriosidase (A) and neopterin (B) were compared between normal females ($NL_{females}$), normal males (NL_{males}), breast cancer (BCA), and prostate cancer (PCA). Bar represent median values in panels A and B. Comparisons between stages were by Mann Whitney U-test and brackets mark the group that was significantly different from all other groups. Serum levels of chitotriosidase (C) and neopterin (D) were subjected to analysis by ROC curve analysis for breast (circle) and prostate (square) cancer detection. The area under the curve (AUC) and its standard deviation are shown.

Figure 1.

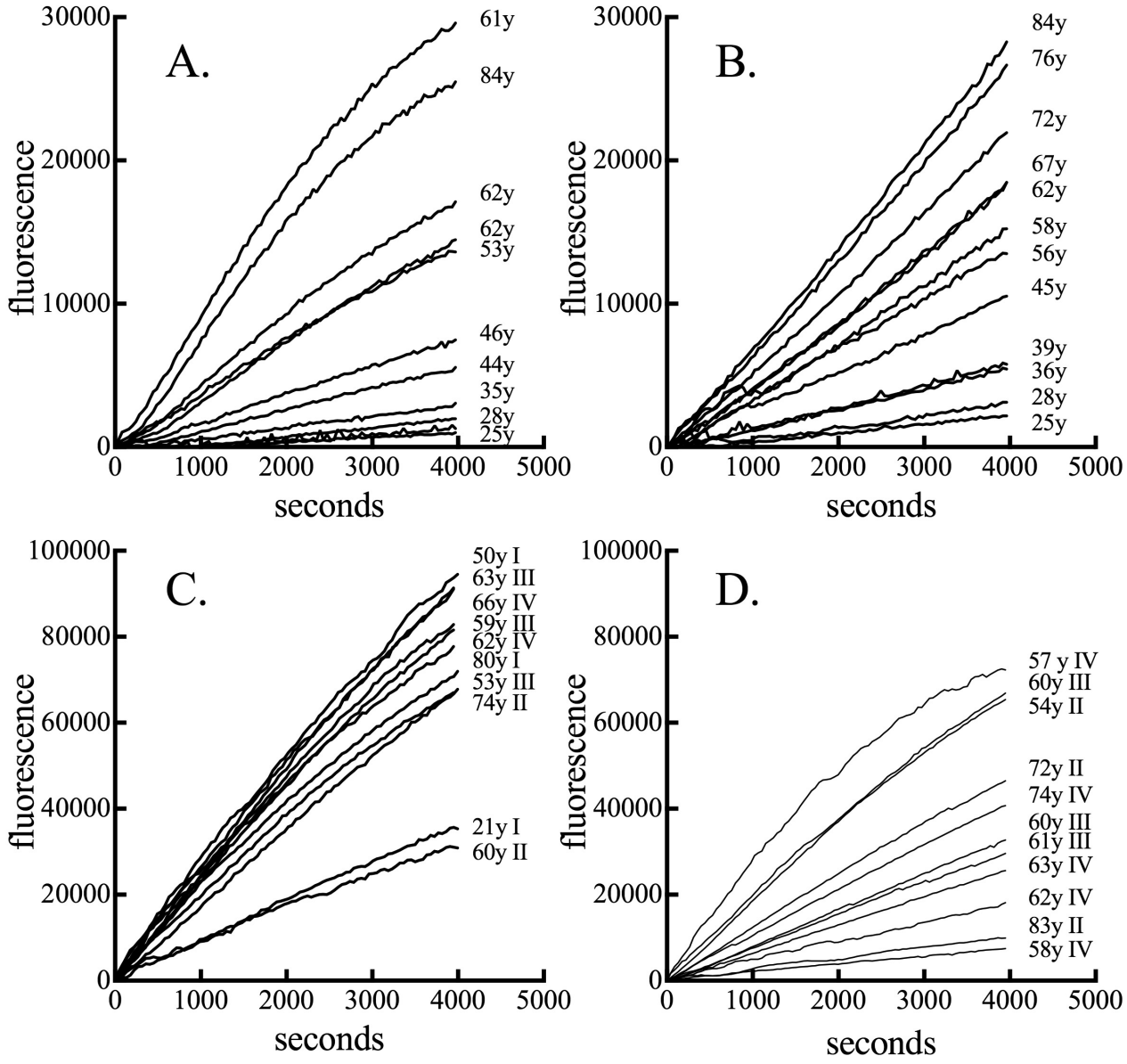
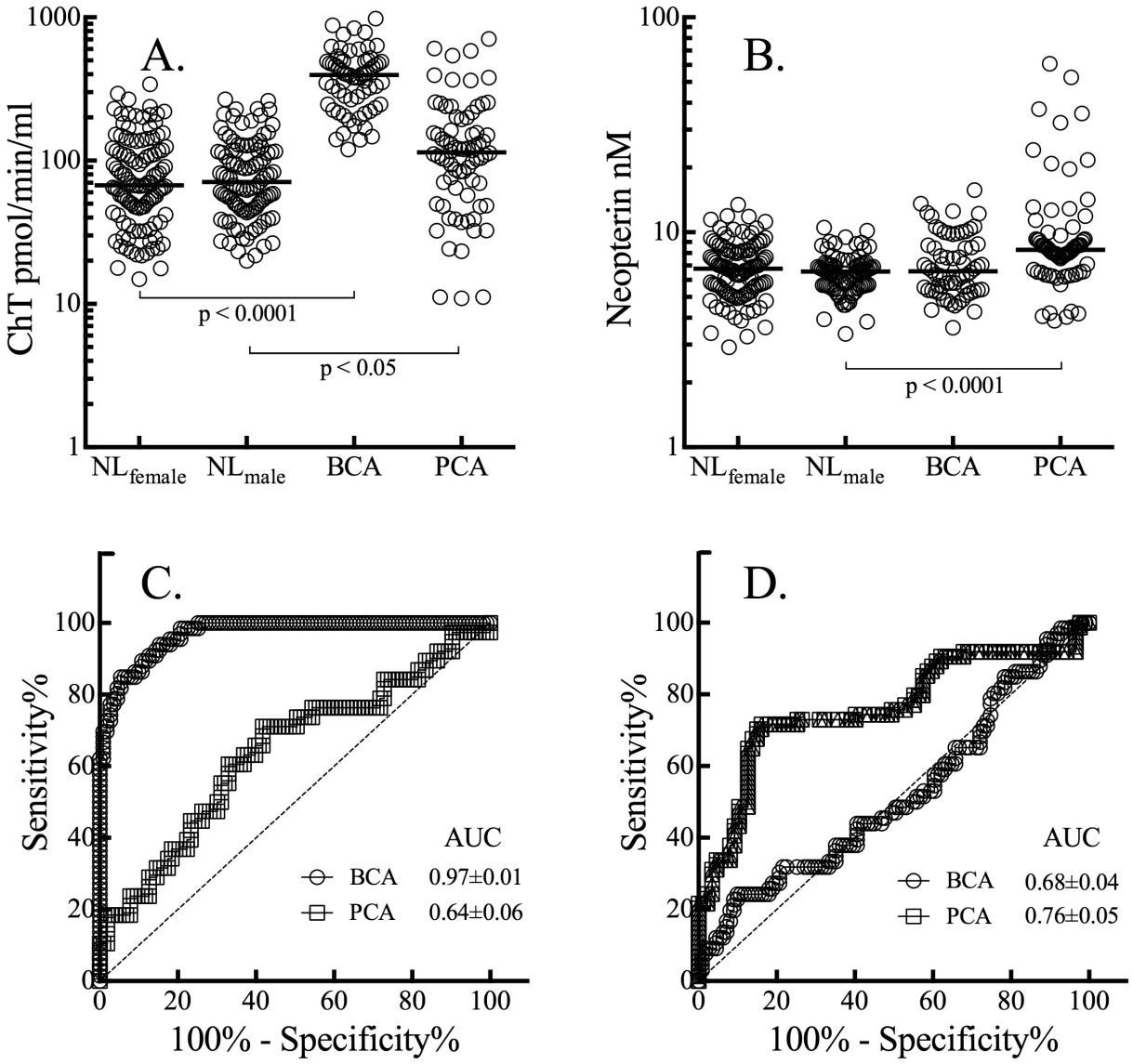

A.
B.
C.
D.
fluorescence
seconds
0
10000
20000
30000
40000
60000
80000
100000
1000
2000
3000
4000
5000
61y
84y
62y
62y
53y
46y
44y
35y
28y
25y
84y
76y
72y
67y
62y
58y
56y
45y
39y
36y
28y
25y
50y I
63y III
66y IV
59y III
62y IV
80y I
53y III
74y II
21y I
60y II
57 y IV
60y III
54y II
72y II
74y IV
60y III
61y III
63y IV
62y IV
83y II
58y IV

Figure 2.


A.
ChT pmol/min/ml
1000
100
10
1
p < 0.0001
p < 0.05
NL female
NL male
BCA
PCA
B.
Neopterin nM
100
10
1
p < 0.0001
NL female
NL male
BCA
PCA
C.
Sensitivity%
100% - Specificity%
AUC
BCA 0.97±0.01
PCA 0.64±0.06
D.
Sensitivity%
100% - Specificity%
AUC
BCA 0.68±0.04
PCA 0.76±0.05