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Serum selenium, selenoprotein P and glutathione peroxidase 3 as predictors of mortality and recurrence following breast cancer diagnosis: A multicentre cohort study

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ABSTRACT

The trace element selenium is of essential importance for the synthesis of a set of redox active proteins. We investigated three complementary serum selenium status biomarkers in relation to overall survival and recurrence following diagnosis of primary invasive breast cancer in a large prospective cohort study. The Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B) is a prospective population-based study including multiple participating hospitals. Main analyses included 1996 patients with a new diagnosis of primary invasive breast cancer, with blood sampling at the time of diagnosis. In sera of the patients, total serum selenium, selenoprotein P (SELENOP), and glutathione peroxidase 3 (GPx3) activity was analysed. All three biomarkers showed a positive correlation ($p < 0.001$), supporting the high quality of samples and analytical techniques. During a total of 13,306 person years of follow-up, 310 deaths and 167 recurrent breast cancer events occurred. In fully adjusted Cox models, all three biomarkers correlated inversely with mortality ($p_{trend} < 0.001$) and compared with the lowest quintile, hazard ratios (95% confidence interval) for overall survival in the highest quintile of selenium, SELENOP and GPx3 were 0.42 (0.28–0.63), 0.51 (0.36–0.73) and 0.52 (0.36–0.75), respectively. Low GPx3 activity was associated with more recurrences (Q5 vs Q1: fully adjusted HR (95%CI); 0.57 (0.35–0.92), ($p_{trend} = 0.005$). Patients with low selenium status according to all three biomarkers (triple deficient) had the highest mortality risk with an overall survival probability of ~50% after 8 years, in particular as compared to those having at least one marker in the highest quintile; fully adjusted HR (95%CI); 0.30 (0.21–0.43). Prediction of mortality based on all three biomarkers outperformed established tumour characteristics like histologic grade, number of involved lymph nodes or tumour size. An assessment of Se status at breast cancer diagnosis identifies patients at exceptionally high risk for a poor prognosis.

1. Introduction

Over the last years, improved screening as well as optimized personalized adjuvant therapy collectively has increased survival chances following breast cancer diagnosis [1–3]. However, given that breast cancer still accounted for 685,000 deaths globally in 2020, it is highly relevant to identify those patients with poor survival odds. This may allow for intensified adjuvant therapy, which could improve prognosis. Currently, established prognostic factors include tumour characteristics and stage [4–6].

The trace element selenium (Se) is of prime importance for the biosynthesis of a limited set of selenoproteins implicated in anti-oxidative protection, thyroid hormone metabolism, tumour growth and cell proliferation [7,8]. Accordingly, Se intake and Se status have been

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discussed as potentially affecting breast cancer development. Unfortunately, the largest randomized control trials (RCT) for elucidating a role of Se in chemoprevention were mainly or exclusively enrolling male subjects, studying prostate cancer. This limitation applies to both the Nutritional Prevention of Cancer (NPC) and the Selenium and Vitamin E Cancer Prevention Trial (SELECT) [9–11]. Data from observational studies is similarly inconclusive and no strong association between Se status and breast cancer risk have been reported [12,13]. A corresponding Cochrane analysis indicated no significant association between total Se and cancer incidence [14]. Considering Se as a potential prognostic factor, despite the lack of support for a relevant role of Se for breast cancer incidence, two recent studies indicated associations between breast cancer survival and total serum Se concentration [15,16]. However, one of these studied pre-diagnostic Se concentrations and it is not known if they reflect levels at diagnosis, and, hence, the possibility to use Se status at diagnosis as a prognostic marker in newly diagnosed cases. The other study used samples taken at diagnosis but included about 500 cases only. Most importantly, both of these studies assessed Se status with a single biomarker only. Total serum Se concentration is a composite parameter comprising different Se-containing fractions [17]. The majority of circulating Se is contained in the liver-derived Se transport protein selenoprotein P (SELENOP) and the kidney-derived extracellular glutathione peroxidase GPx3 [18]. Depending on the dietary intake, certain selenocompounds with low molecular weight are present, along with proteins containing small amounts of selenomethionine [19]. Serum GPx3 activity and SELENOP concentration are positively associated with Se intake and total serum Se concentration until the thresholds for maximal expression are reached [20–23]. The relationship between nutritional Se intake and saturated selenoprotein expression, in particular full expression of SELENOP, is used to deduce recommendations on an optimal Se supply both under basal conditions and in disease or pregnancy [24,25]. The quantification of two or even three biomarkers of Se status enables a more robust assessment than from total blood Se concentrations alone, providing a more reliable and authentic insight into the nutritional supply and whether it is marginal or sufficient, as the protein biomarkers reach a saturated expression level once a replete status is given [19,26].

The aim of our study was to test the association of low Se status with poor survival and high recurrence following breast cancer diagnosis and compare the prognostic value of three different biomarkers of Se status.

2. Material and methods

2.1. SCAN-B

The Sweden Cancerome Analysis Network - Breast Initiative (SCAN-B) (ClinicalTrials.gov ID NCT02306096) is a prospective real-world, population based multicentre study enrolling patients since August 30th, 2010. It aims to identify new prognostic factors and targets for individualized therapy by genomic profiling of breast cancer [27–29]. Patients treated in the participating hospitals in Malmö, Lund, Helsingborg, Kristianstad, Växjö, Halmstad, Uppsala, Karlskrona, Varberg, and Ljungby were included in the analyses. Briefly, patients in Sweden newly diagnosed with primary invasive breast cancer without distant metastases were enrolled before surgery, representing approximately 85% of all breast cancer incidences in the catchment region within the enrolment period [29].

2.2. Assesment of clinical data and follow up

Clinical information was collected before and after surgery by the surgical department and the responsible pathologist. All data was reported to the Swedish National Quality Registry for Breast Cancer (NKBC) [30].

Age, sex, menopausal status, surgical procedures (involving both the breast and the axilla), and planned adjuvant therapy were reported. Tumour characteristics were evaluated by the local pathology department of the participating hospital, and remainder of fresh tumour specimens was preserved in RNAlater and stored frozen [29]. The histopathological type was categorized into four categories for the purpose of this study; ductal, lobular, other, ductal + lobular/other. Histological grade was evaluated according to Nottingham grading system (NHG) and categorized into I,II, and III [31]. Estrogen receptor (ER) and progesterone receptor (PGR) status was determined as positive, if >10% of cells stained positive. HER2 status was regarded as negative with an immunohistochemistry score (IHC) of 0, 1+. For samples scoring 2+ and 3, an ISH test was performed to decide whether the receptor was amplified. Registration routines differed slightly between centres, and those with no ISH test performed (i.e. likely HER2 negative tumours) were in some centres denoted as “missing” for amplification status, i.e. HER2 status. As evaluation of HER2 has been part of the routine during the entire period, and for the purpose of the current analysis, those with missing for amplification were regarded as HER2-negative. Ki67 was dichotomized into low with less than 20% staining in hotspot regions and positive otherwise. A patient was considered to have an axillary metastasis if there was a micrometastasis (0.2 mm–2 mm) or a macrometastasis (>2 mm). A “submicrometastasis”/isolate tumour cells (ITC) (<0.2 mm) was described separately. Tumour size in mm was provided following the pathological examination.

All patients received a reference date for diagnosis and were followed until recurrence (in the analysis of disease-free survival), death or end of follow-up time. End-of follow up was a date between April 1, 2019 and June 30, 2019, but in order to conserve patient privacy, and not to reveal the exact date of diagnosis, only number of days between date of diagnosis and end of follow up was provided to the authors by the SCAN-B steering committee. NKBC extracts vital status from the Swedish Population Registry. Recurrent disease was reported by the treating centre, when diagnosed. As NKBC covers the entire country, death and recurrent disease were also recorded if the patient had moved to another area.

2.3. Assesment of selenium status

The infrastructure underlying SCAN-B is fully integrated in the clinical routine and has been previously described in detail [29]. Briefly, blood sampling was conducted at time of diagnosis, before surgery. Within 2 h, serum samples were allocated in 200 μL aliquots and stored on dry ice when transported to the biobank at the Department of Clinical Chemistry, Skåne University Hospital, where they were subsequently stored at −80 °C. Analysis of Se biomarkers took place in a laboratory in Berlin, Germany. Samples were randomized with regard to date of diagnosis (i.e. storage time) and clinical data was completely blinded for the recipient of the samples as well as the technicians and scientists conducting the laboratory measurements. Laboratory results were linked with clinical information when all laboratory analyses were completed.

Total reflection X-ray fluorescence (TXRF) method was used to analyse total serum Se in the samples using a TXRF analyser (T-Star, Bruker Nano GmbH, Berlin, Germany), as outlined before [32]. In brief, patient serum was diluted 1:2 with a buffer containing gallium (1000 μg/l), to serve as standard. Eight μl of the dilution was applied to polished quartz glass slides (Bruker Nano GmbH, Berlin, Germany) and dried overnight in a 37 °C incubator. Serum standard Seronorm (Sero AS, Billingstad, Norway) served as control. The inter- and intra-assay coefficient of variation (CV) were below 8%.SELENOP in serum was analysed using a validated commercial ELISA (selenOtest™, selenOmed GmbH, Berlin, Germany), as described recently [33]. In brief, 5 μL serum was used in a sandwich ELISA procedure with human SELENOP specific monoclonal antibodies. According to manufacturer’s instructions, three controls resembling the working range of the assay served as control. The inter- and intra-assay CV were below 10% for low and medium concentrations and below 20% for the high concentration control.GPx3

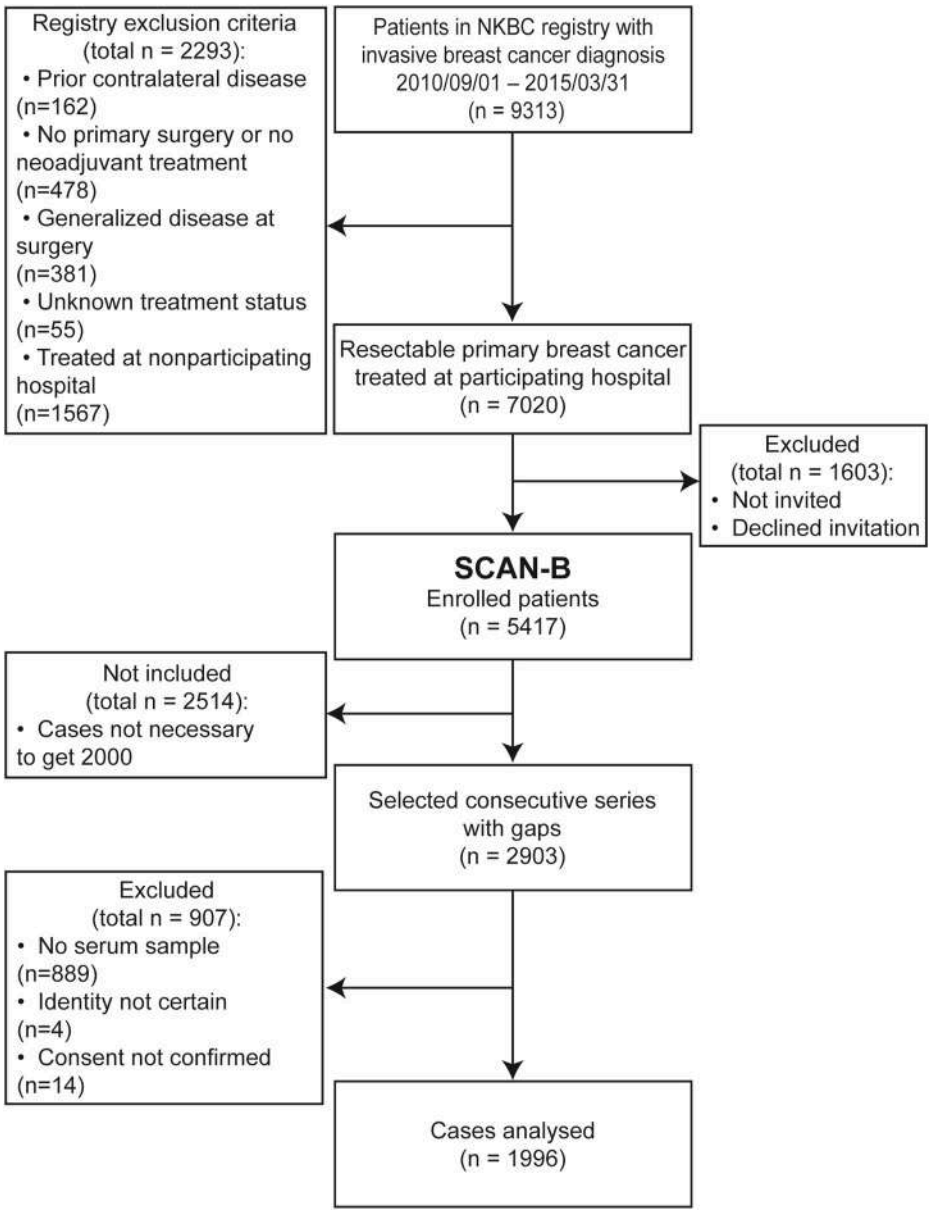

Fig. 1. Flow chart explaining inclusion and exclusion criteria.

enzyme activity was determined with a coupled-enzyme reaction by measuring the consumption of NADPH, as described earlier [34,35]. The consumption is proportional to the reduction of UV absorption at 340 nm, which in turn is proportional to the activity measured in 5 μL serum. The measurement was done in triplicates and the activity is listed as the mean activity. A standard serum was measured in triplicates to serve as control. The inter-assay CV was below 15% at all times and intra-assay CV was below 10%.

2.4. Statistical analysis

The Shapiro-Wilk-Test as well as visual inspection of quantile-quantile and histogram plots were used to evaluate normality of data. GPx3 was found to be normally distributed, while Se and SELENOP were non-normal. Median (interquartile range) or mean (standard deviation) were used when summarizing non-normal or normal continuous data, respectively.

All biomarkers were subsequently categorized into quintiles. Different quintiles of each biomarker, vital and recurrence status were compared to prognostic factors and treatment methods. Patients assigned to the first quintile regarding all biomarkers at the same time (triple deficient) and patients in the fifth quintile regarding all biomarkers at the same time were identified.

Correlation between biomarkers was assessed via Spearman's rank correlation coefficient among the whole cohort, and in the triple deficient group as well as in patients in the fifth quintile regarding all biomarkers at once.

For all survival analyses, start for both overall survival (OS) and recurrence free survival (RFS) was defined as the time of diagnosis. Event for OS was death from any cause. Event for RFS was defined as any recurrence including local, regional, and distant metastases, while death was censored. Kaplan Meier estimate curves were used to visually assess survival probability, differences in groups were detected using a log-rank-test. Hazard ratios (HR) along with 95% confidence intervals (CI) were calculated using Cox regression models, crude and multivariable adjusted for potential confounders of mortality or recurrence following breast cancer. The first model included the respective biomarker of Se status only, the second model was adjusted for age at diagnosis (year).

Table 1
Baseline patient and tumour characteristics in relation to vital and recurrence status.

Characteristic	Vital Status		Recurrence Status	
	Alive N = 1686	Dead N = 310	Recurrence Free N = 1829	Recurrence N = 167
Age (years)	*63 (52–69)*	*72 (65–82)*	*64 (54–70)*	*65 (54–74)*
Menopausal Status				
Pre-menopausal	342 (21)	23 (7.5)	335 (19)	30 (18)
Post-menopausal	1246 (75)	278 (91)	1396 (77)	128 (77)
Uncertain	79 (4.7)	4 (1.3)	75 (4.2)	8 (4.8)
Laterality				
Left	861 (51)	177 (57)	946 (52)	92 (55)
Right	825 (49)	133 (43)	883 (48)	75 (45)
Size (mm)	*15 (11–21)*	*22 (14–30)*	*15 (11–22)*	*21 (14–30)*
Lymph Nodes				
≥4	122 (7.5)	53 (18)	139 (7.9)	36 (22)
1-3	401 (25)	60 (20)	429 (24)	32 (20)
No Involvement	1066 (66)	174 (59)	1149 (65)	91 (57)
Submicrometastasis	35 (2.2)	7 (2.4)	40 (2.3)	2 (1.2)
(Missing)	62	16	72	6
NHG				
I	351 (21)	32 (11)	372 (21)	11 (7.1)
II	790 (48)	128 (43)	856 (48)	62 (40)
III	502 (31)	136 (46)	555 (31)	83 (53)
(Missing)	43	14	46	11
Ki67 Expression				
Low	208 (50)	18 (31)	219 (50)	7 (19)
High	212 (50)	40 (69)	223 (50)	29 (81)
(Missing)	1266	252	1387	131
Histological Type				
Ductal	1356 (81)	241 (78)	1461 (80)	136 (81)
Lobular	221 (13)	39 (13)	241 (13)	19 (11)
Other	79 (4.7)	26 (8.4)	98 (5.4)	7 (4.2)
Ductal + Lobular/Other	28 (1.7)	4 (1.3)	27 (1.5)	5 (3.0)
HER2 Expression				
Negative	1462 (88)	259 (86)	1587 (88)	134 (83)
Positive	206 (12)	42 (14)	220 (12)	28 (17)
ER Expression				
Negative	201 (12)	80 (26)	232 (13)	49 (30)
Positive	1481 (88)	229 (74)	1593 (87)	117 (70)
PGR Expression				
Negative	423 (25)	134 (43)	488 (27)	69 (42)
Positive	1258 (75)	176 (57)	1337 (73)	97 (58)
Selenium (μg/l)	*72 (62–82)*	*63 (52–74)*	*71 (60–81)*	*69 (57–81)*
Selenoprotein P (mg/l)	*4.13 (3.36–4.93)*	*3.70 (2.72–4.51)*	*4.10 (3.29–4.89)*	*3.80 (3.16–4.58)*
GPx3 Activity (U/l)	*209 (47)*	*187 (56)*	*206 (48)*	*197 (54)*

Median (IQR); *Mean (SD)*; n (%).
Missing not shown if <2%.
NHG = Nottingham histological grade, HER2 = human epidermal growth factor receptor 2, ER = estrogen receptor, PGR = progesterone receptor, GPx3 = glutathione peroxidase 3, Lymph Nodes = number of lymph nodes involved.

The third model was additionally adjusted for menopausal status (pre-, post-menopausal or uncertain), mode of breast cancer detection (clinical or screening), tumour size (mm), lymph node involvement (≥4, 1–3, submicrometastasis (<0.2 mm) or none), Nottingham Histologic Grade (I, II or III), histological type (ductal, lobular, ductal + lobular/other, other), expression status of HER2 receptor (positive or negative), estrogen receptor (positive or negative), and progesterone receptor (positive or negative). Proportional hazards assumption for the models was met, as checked visually, as well as by Schoenfeld residuals and plots. Biomarkers were categorized into quintiles when using regression modelling, and also evaluated as continuous parameters. Trend among quintiles reported were calculated modelling the ordinal quintile variable as continuous. Further, survival in the triple deficient group was compared to patients with at least one biomarker in the highest quintile and rest. The group with lowest Se, i.e. first quintile or triple deficient group, was always set as reference. In a further sensitivity analysis, adjuvant therapy method and surgical procedure regarding breast and axilla were added to the fully adjusted models one by one for each biomarker.

Predictive value of Se status for death was compared with established tumour characteristics and age. For that purpose, time dependent receiver operating characteristic (ROCt) analyses were conducted using the incident/dynamic approach by Heagerty P.J. et al. [36]. For visualization, areas under the curves (AUCt) were extracted from ROCt analyses computed at time point of each death event using the risksetROC [37] package and compared in a line chart. In order to compare the models based on a global estimation parameter, the integrated area under the curve was computed [36].

Data on Se, SELENOP, GPx3, and age at diagnosis were complete for all included patients, therefore all crude and age adjusted Cox regression analyses comprise complete cases. Missing data among covariates included in the fully adjusted models made up 0.7% (Supplementary Fig. 1) of all values in covariates and were imputed when applying fully adjusted Cox models. Multiple imputation by chained equations was performed for that purpose [38]. Ten imputations and 10 iterations were performed. All variables in the multivariable Cox model, including the endpoints OS, RFS and time to event, were considered in the prediction matrix of the model. Proportional odds model was used for ordered categorical variables, polytomous logistic regression was used for unordered categorical variables, logistic regression was performed for

Table 2
Diagnosis and therapy options in relation to vital and recurrence status.

Characteristic	Vital Status		Recurrence Status	
	Alive N = 1686	Dead N = 310	Recurrence Free N = 1829	Recurrence N = 167
Diagnosis				
Clinical	722 (43)	205 (66)	829 (46)	98 (59)
Screening	942 (57)	104 (34)	978 (54)	68 (41)
Surgical Procedure Breast				
Mastectomy	612 (36)	211 (68)	720 (39)	103 (62)
Partial Mastectomy	1074 (64)	99 (32)	1109 (61)	64 (38)
Surgical Procedure Axilla				
Sentinel Node	1094 (65)	176 (57)	1181 (65)	89 (53)
Sentinel Node + Clearence	390 (23)	56 (18)	419 (23)	27 (16)
Clearence Only	179 (11)	67 (22)	198 (11)	48 (29)
Sampling	18 (1.1)	5 (1.6)	21 (1.1)	2 (1.2)
No Axillary Surgery	4 (0.2)	5 (1.6)	8 (0.4)	1 (0.6)
Endocrine Therapy				
No	417 (25)	101 (33)	450 (25)	68 (41)
Yes	1264 (75)	207 (67)	1373 (75)	98 (59)
Chemotherapy				
No	1088 (65)	226 (73)	1215 (67)	99 (60)
Yes	593 (35)	82 (27)	608 (33)	67 (40)
Immunotherapy				
No	1496 (89)	285 (93)	1633 (90)	148 (89)
Yes	185 (11)	23 (7.5)	190 (10)	18 (11)
Radiotherapy				
No	512 (30)	165 (54)	614 (34)	63 (38)
Yes	1169 (70)	143 (46)	1209 (66)	103 (62)

n (%).
Missing not shown if <2%.

binary categorical variables, predictive mean matching method was used for the continuous size variable [39]. Due to missing values above 50%, information on Ki67 expression was not imputed and not included in the analyses. Alongside convergence check, robustness of the imputation was evaluated in sensitivity analyses by comparing the regression results to complete case analyses, which yielded similar outcomes. Fifty-four patients were missing information on RFS in NKBC. With respect to the validated completeness of NKBC otherwise [30], those were considered recurrence free and information on overall survival was used instead. All analyses were also conducted excluding this group, providing constant results (data not shown).

All statistical tests were two-sided and p-values less than 0.05 were considered to be statistically significant. Statistical analyses were conducted using the software R (The R Foundation), version 4.0.4., implementing the packages tidyr [40], dplyr [41], ggplot2 [42], gtsummary [43], MICE [38], survminer [44], and risksetROC [37].

3. Results

Eligibility criteria for participation were a preoperative diagnosis or preoperative suspicion of primary invasive breast cancer. Patients with prior contralateral disease, generalized disease status at diagnosis, unknown treatment status, no planned treatment or patients treated at a nonparticipating hospital were excluded. Considering these criteria, a total of 5417 consecutive patients were successfully enrolled in SCAN-B during the time period 2010/09/01–2015/03/31 [45]. Our study planned to include 2000 cases in the analyses. Therefore, 2903 consecutive cases between 2010 and 2013 were selected, and a total of 907 were not included due to lack of available serum sample, uncertain identity or few cases of unconfirmed consent. With regards to time of recruitment, 140 of the patients were recruited in 2010, 689 in 2011, 764 in 2012, and 403 in 2013. Finally, 1996 patients were included for Se status assessment and statistical analyses (Fig. 1).

Among the 1996 primary invasive breast cancer patients, 8 were male. Median (IQR) follow-up time from baseline to censoring or event in the study cohort was 6.94 (6.28–7.63) years for OS and 6.87 (6.25–7.61) for RFS. A total of 310 deaths and 167 recurrences were recorded in 13,306 and 13,039 person years, respectively. In Table 1, patient and tumour characteristics were compared in relation to vital and recurrence status. Patients who died were older at baseline, more frequently in a post-menopausal state, had a higher number of involved lymph nodes, had more frequently PGR negative, ER negative, larger tumours, higher Nottingham Histologic Grade (NHG) and higher Ki67 expression, and displayed lower serum Se and SELENOP levels and lower serum GPx3 activity. Patients who had recurrent disease showed similar characteristics to the deceased, except that they were not older, and not more frequently in a post-menopausal state.

Table 2 compares diagnostic and treatment methods in relation to vital and relapse status. Patients who died following breast cancer were more frequently diagnosed clinically than by screening, more likely to have had mastectomy, more likely to have axillary node dissection and less likely to receive endocrine, chemo- or radiotherapy. Patients with recurrent disease had similar diagnostic and treatment characteristics, except that those with recurrence more frequently received chemotherapy. In a non-participation analysis (data not shown), we compared the patients included in this study to those without serum. Distributions among different patient/tumour or treatment/diagnosis characteristics were very similar.

Supplementary Table 1 compares distribution of patient and tumour characteristics across quintiles of each biomarker. We have observed an inverse association between age and total Se and GPx3, when comparing the quintiles. This association was not as distinct for SELENOP. Furthermore, participants in the first quintile of total Se had larger and more frequently ER negative tumours.

3.1. Correlation of biomarkers

In this study, Se status was assessed by three biomarkers, which were in strong correlation in the whole cohort as shown in Fig. 2, indicating high quality of serum samples and validity of analyses. The correlations were particularly stringent among the biomarkers in the lowest quintiles, while they do not reach statistical significance among patients

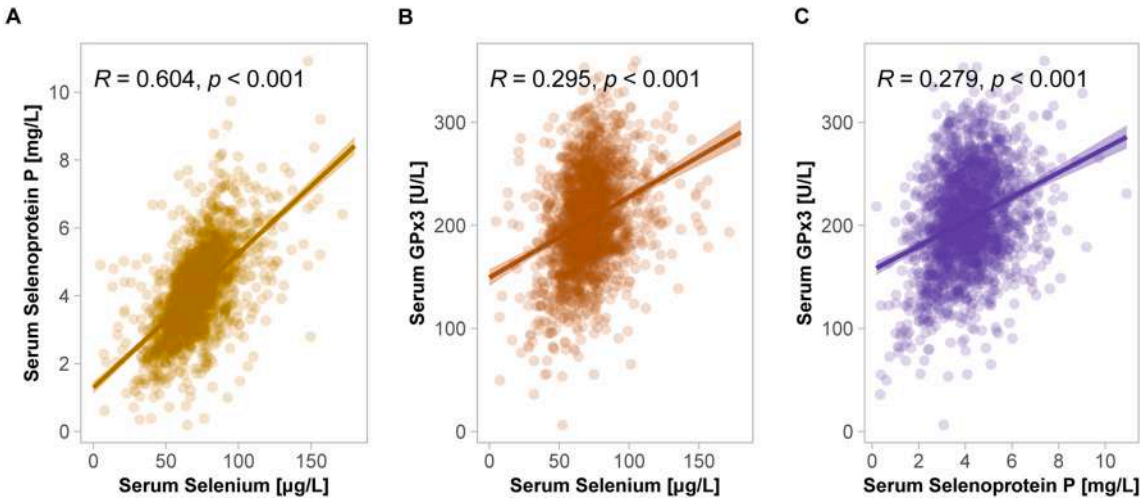

Fig. 2. Spearman's correlation analysis in patients with primary invasive breast cancer. In the total cohort, all three biomarkers of Se show significant correlations, p<0.001. (A) Total serum Se and SELENOP display a tight correlation, R = 0.604. (B) Total serum Se correlates with the GPx3 activity in sera of the patients, R = 0.294. (C) Serum SELENOP and GPx3 show a similarly strong correlation as total Se and GPx3, with R = 0.279. N (A,B) = 1993, 3 data points missing in the figures, n(C) = 1996, Spearman's R, two-tailed.

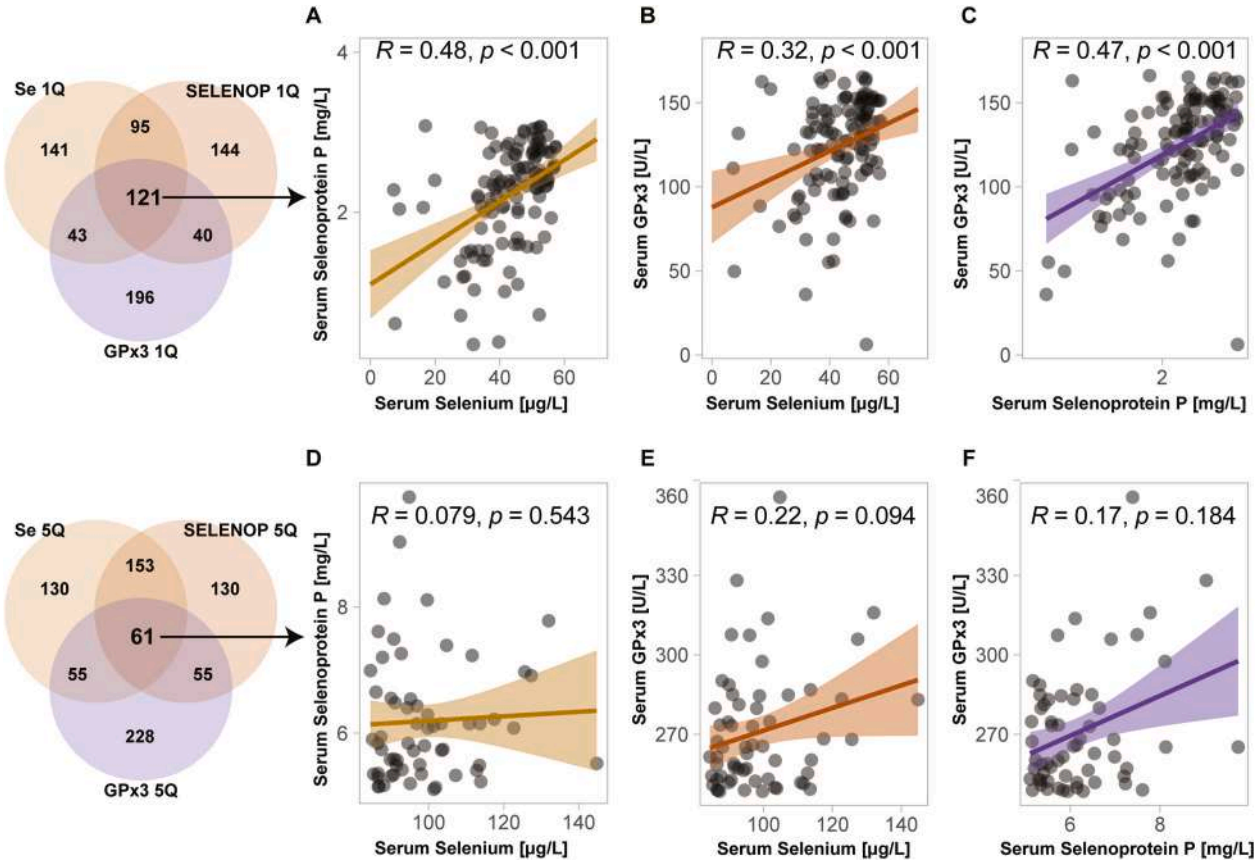

Fig. 3. Spearman's correlation of biomarkers in patients who display all biomarkers in the first or last quintile. The Venn diagram and the plots in the top section of figure (A,B,C) highlight the significant correlations between the biomarkers in samples of the 121 patients who are assigned to the first quintile regarding each of the three biomarkers. The correlations are non-significant for patients in the highest quintile regarding all three biomarkers, as shown in the lower Venn diagram and the plots in the lower panel of figure (D,E,F). This comparison underlines the saturation of selenoprotein expression under high Se status.

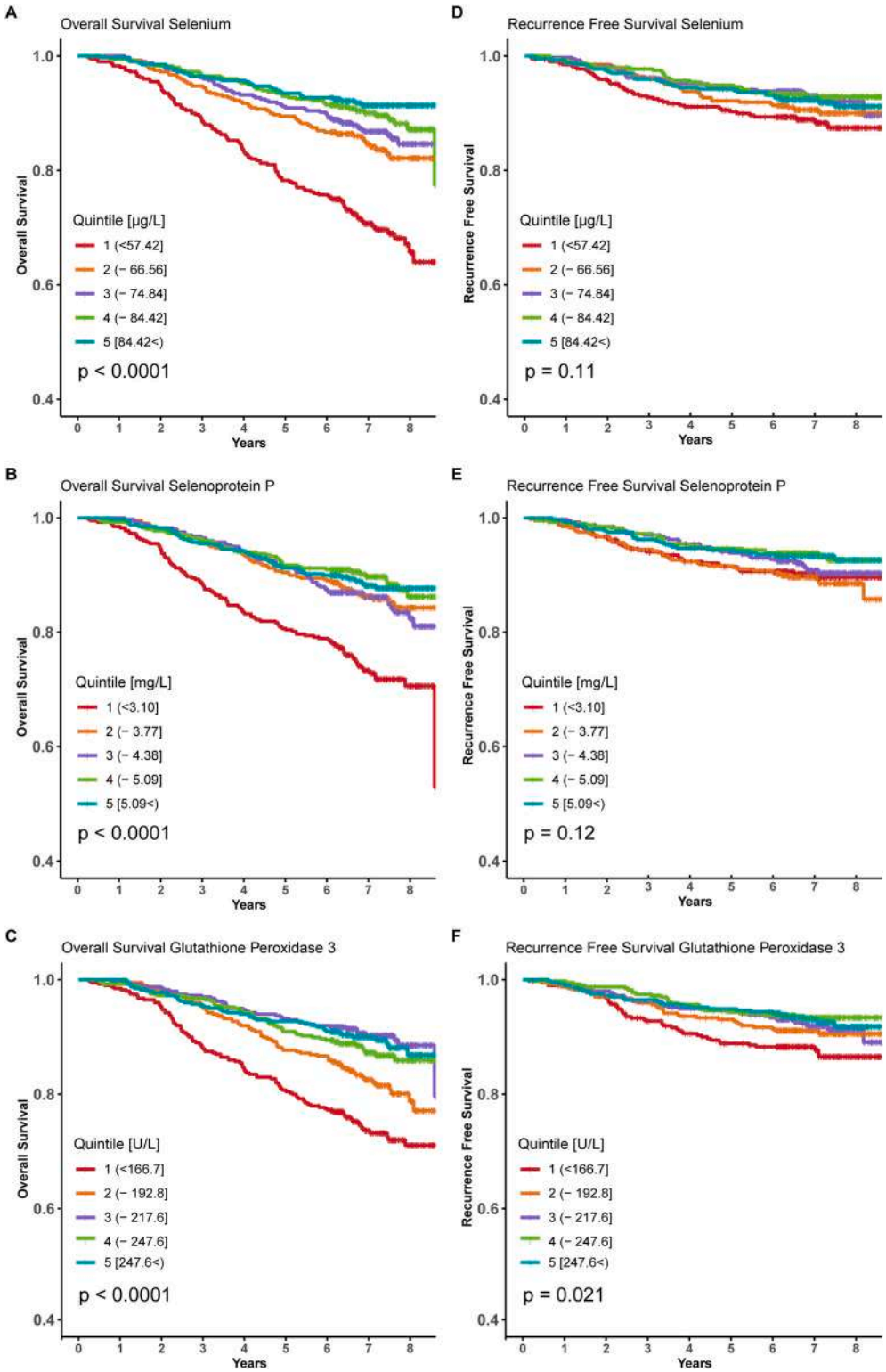

Fig. 4. Kaplan Meier curves for overall survival (A,B,C), and for recurrence free survival (D,E,F), by quintiles of the three biomarkers. Log-Rank-Test was used to evaluate differences. Y-axis-limits were set between 0.4 and 1.0 for better visualization, no points are missing. P-value was calculated with log-rank test.

assigned to the highest quintile, as serum Se is closer to be saturated (Fig. 3).

3.2. Overall and recurrence free survival

OS and RFS were compared in relation to quintiles of the three biomarkers of Se status individually with Kaplan Meier plots (Fig. 4). Results from univariate, age adjusted and fully adjusted Cox regression models are presented for OS (Table 3) and RFS (Table 4). Lowest quintile regarding each individual biomarker (first) was set as reference point. All three biomarkers display a significant inverse correlation with OS, with a $p_{trend} < 0.001$. GPx3 activity is inversely correlated with RFS, $p_{trend} = 0.005$. In sensitivity analyses, treatment methods were added one by one to the fully adjusted models for each biomarker, without

Table 3
Cox Regression models for overall survival.

Characteristic	At Risk	Death	Univariate[a]		Age Adjusted[b]		Fully Adjusted[c]		p – Trend[d]
	N	N	HR	95% CI	HR	95% CI	HR	95% CI	
Selenium Quintiles									<0.001
1	400	119	–	–	–	–	–	–	
2	399	62	0.48	0.35 to 0.65	0.59	0.44 to 0.81	0.63	0.46 to 0.87	
3	399	53	0.40	0.29 to 0.55	0.53	0.38 to 0.74	0.53	0.38 to 0.74	
4	399	43	0.32	0.22 to 0.45	0.46	0.32 to 0.66	0.47	0.33 to 0.67	
5	399	33	0.24	0.17 to 0.36	0.37	0.25 to 0.55	0.42	0.28 to 0.63	
Total	1996	310							
Selenium per SD increase			0.59	0.52 to 0.66	0.71	0.63 to 0.81	0.72	0.63 to 0.82	
SELENOP Quintiles									<0.001
1	400	106	–	–	–	–	–	–	
2	399	55	0.47	0.34 to 0.65	0.53	0.38 to 0.73	0.54	0.39 to 0.76	
3	399	60	0.51	0.37 to 0.70	0.61	0.44 to 0.83	0.60	0.43 to 0.83	
4	399	43	0.36	0.26 to 0.52	0.42	0.30 to 0.61	0.46	0.32 to 0.66	
5	399	46	0.39	0.27 to 0.55	0.46	0.32 to 0.65	0.51	0.36 to 0.73	
Total	1996	310							
SELENOP per SD increase			0.65	0.58 to 0.73	0.71	0.63 to 0.80	0.74	0.65 to 0.83	
GPx3 Quintiles									
1	400	105	–	–	–	–	–	–	<0.001
2	399	72	0.64	0.48 to 0.87	0.80	0.59 to 1.08	0.76	0.56 to 1.03	
3	399	40	0.34	0.24 to 0.49	0.45	0.31 to 0.65	0.43	0.30 to 0.63	
4	399	50	0.44	0.31 to 0.61	0.60	0.43 to 0.85	0.59	0.42 to 0.84	
5	399	43	0.37	0.26 to 0.53	0.53	0.37 to 0.76	0.52	0.36 to 0.75	
Total	1996	310							
GPx3 per SD increase			0.65	0.58 to 0.73	0.76	0.68 to 0.85	0.75	0.66 to 0.84	

HR = Hazard Ratio, CI = Confidence Interval.
[a] Crude model. No missing values.
[b] Age adjusted model. No missing values. Adjusted for age at diagnosis of breast cancer.
[c] Fully Adjusted Model. Missing values in adjustment factors were imputed via multiple imputation. Model includes Age, Menopausal Status, ER Expression, PGR Expression, HER2 Expression, Nottingham Histologic Grade, Histologic Type, Number of Lymph Nodes involved, Mode of Diagnosis, and Tumour Size [mm].
[d] p – Trend calculated in fully adjusted models by entering quintile variable as continuous.

Table 4
Cox Regression models for recurrence free survival.

Characteristic	At Risk	Recurrence	Univariate[a]		Age Adjusted[b]		Fully Adjusted[c]		p – Trend[d]
	N	N	HR	95% CI	HR	95% CI	HR	95% CI	
Selenium Quintiles									0.2
1	400	43	–	–	–	–	–	–	
2	399	36	0.78	0.50 to 1.22	0.80	0.52 to 1.25	0.86	0.54 to 1.35	
3	399	29	0.61	0.38 to 0.98	0.64	0.40 to 1.02	0.67	0.41 to 1.09	
4	399	27	0.56	0.35 to 0.91	0.59	0.36 to 0.96	0.63	0.38 to 1.03	
5	399	32	0.67	0.42 to 1.05	0.70	0.44 to 1.11	0.85	0.53 to 1.37	
Total	1996	167							
Selenium per SD increase			0.90	0.77 to 1.06	0.92	0.78 to 1.08	0.96	0.82 to 1.13	
SELENOP Quintiles									0.10
1	400	37	–	–	–	–	–	–	
2	399	43	1.10	0.71 to 1.71	1.13	0.73 to 1.75	1.15	0.73 to 1.81	
3	399	34	0.84	0.53 to 1.35	0.87	0.54 to 1.38	0.93	0.58 to 1.49	
4	399	26	0.65	0.39 to 1.07	0.66	0.40 to 1.08	0.72	0.43 to 1.19	
5	399	27	0.67	0.41 to 1.10	0.67	0.41 to 1.11	0.79	0.47 to 1.32	
Total	1996	167							
SELENOP per SD increase			0.84	0.71 to 0.99	0.84	0.71 to 1.00	0.85	0.72 to 1.00	
GPx3 Quintiles									0.005
1	400	47	–	–	–	–	–	–	
2	399	35	0.71	0.46 to 1.09	0.73	0.47 to 1.13	0.71	0.46 to 1.12	
3	399	32	0.62	0.40 to 0.98	0.64	0.41 to 1.01	0.65	0.41 to 1.03	
4	399	25	0.49	0.30 to 0.80	0.51	0.31 to 0.83	0.48	0.29 to 0.79	
5	399	28	0.55	0.34 to 0.88	0.57	0.35 to 0.91	0.57	0.35 to 0.92	
Total	1996	167							
GPx3 per SD increase			0.81	0.68 to 0.95	0.83	0.70 to 0.98	0.82	0.70 to 0.96	

HR = Hazard Ratio, CI = Confidence Interval.
[a] Crude model. No missing values.
[b] Age adjusted model. No missing values. Adjusted for age at diagnosis of breast cancer.
[c] Fully Adjusted Model. Missing values in adjustment factors were imputed via multiple imputation. Model includes Age, Menopausal Status, ER Expression, PGR Expression, HER2 Expression, Nottingham Histologic Grade, Histologic Type, Number of Lymph Nodes involved, Mode of Diagnosis, and Tumour Size [mm].
[d] p – Trend calculated in fully adjusted models by entering quintile variable as continuous.

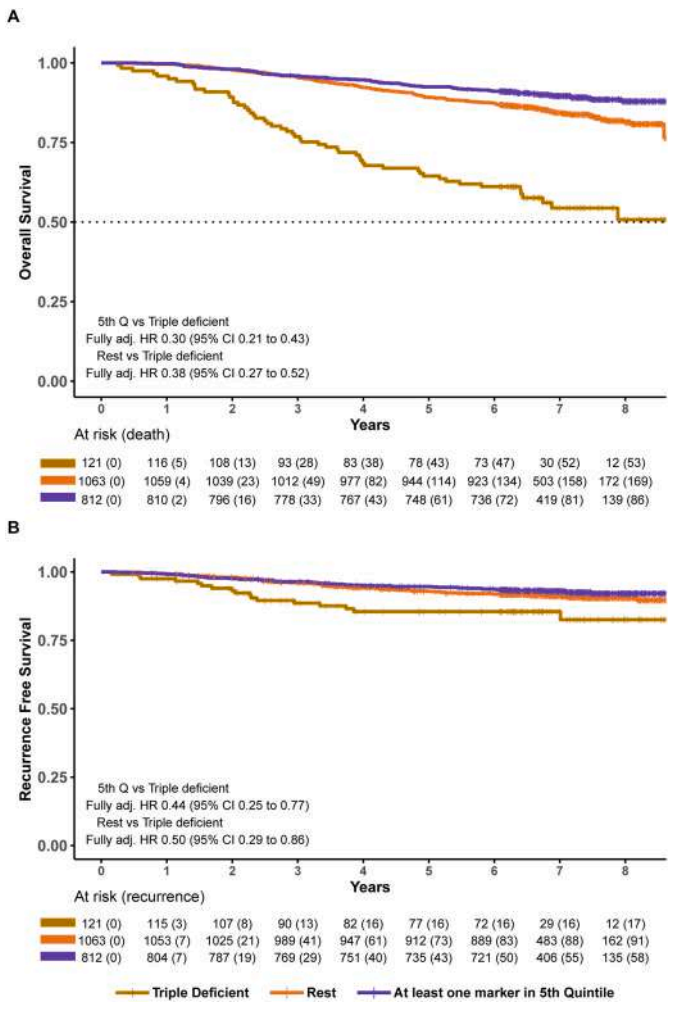

Fig. 5. Overall survival (A) and recurrence free survival (B) assessed for the triple deficient group. Triple deficient stands for patients who are in the first quintile regarding all three biomarkers; ~ (Se < 57 μg/l and SELENOP <3 mg/l and GPx3 <167 U/l). Purple curve stands for patients who are in the fifth quintile regarding at least one biomarker ~ (Se > 84 μg/l or SELENOP >5 mg/l or GPx3 >248 U/l). Q = Quintile, HR=Hazard ratio, CI=Confidence interval, Fully adj. = Fully adjusted for confounders of breast cancer mortality as listed in Tables 3 and 4 (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

significant adjustment effects (Supplementary Tables 2 and 3). Further sensitivity analyses using a complete case analysis comprising 1798 patients showed very similar results (Supplementary Tables 4 and 5). Lastly, OS and RFS were evaluated in fully adjusted models excluding the 8 male breast cancer cases with almost equal results (Supplementary Table 6).

In Fig. 5, we further evaluated OS and RFS of patients in the lowest quintile regarding all biomarkers at the same time, i.e. evaluating a triple deficient group, and compared this group to those who are in the highest quintile regarding at least one biomarker, and to the rest of the cohort. Triple deficient patients showed an even poorer prognosis than patients in the first quintile of one biomarker only, as shown in Fig. 4.

Finally, we assessed the time dependent predictive value of the Se biomarkers. Among individual predictors for death, except age at diagnosis, composite Se status including quintiles of Se, SELENOP and GPx3 had the highest incident/dynamic AUC throughout the follow up time (Fig. 6). The addition of composite Se status to combined tumour characteristics model with respect to patients' age improved the predictive value throughout the total follow up time.

4. Discussion

The present prospective cohort study provides strong evidence for a direct association of Se status at diagnosis with low mortality and low recurrence of invasive breast cancer. Notably, using all three parameters of Se status, a triple deficient patient group was identified with highest mortality risk of close to 50% after 8 years of follow up. The composite biomarker of Se status outperformed three of the most important tumour characteristics, i.e., Nottingham histologic grade, tumour size and number of lymph nodes involved, in predicting mortality. We conclude that the Se status constitutes an important prognostic parameter in breast cancer.

4.1. Strengths and limitations

The SCAN-B trial constitutes a sufficiently large and well characterized observational study with a low drop-out rate and a comprehensive data base. It is among the largest prospective studies including population-based consecutive series of newly diagnosed breast cancer patients in the world. Its high quality is reflected in the low rate of missing information in the prospectively captured covariable overview (Tables 1 and 2). The samples collected at breast cancer diagnosis have been preserved in a dedicated biobank under high quality standards, which is supported by the stringent linear correlations observed for different Se status biomarkers (Figs. 2 and 3). In view that the laboratory analyses have been conducted by scientists blinded to the clinical information at a remote site from the clinics and biobank (Berlin, Germany, versus Lund, Sweden), the reliability of the techniques used is similarly supported by the linear correlations observed and congruent results obtained. In retrospect, the comprehensive analysis of three biomarkers proved as a meaningful approach on a methodological aspect, as the correlation is well indicative of high validity of measurements. Hence, there is a low risk of misclassification in regard to the main exposure, Se status. On a contextual aspect, using three biomarkers also allowed for identifying a triple deficient group with a particularly poor overall survival. Besides correct classification of the main exposure, there is low risk of a misclassification bias regarding potential confounders and information on vital status deriving from Swedish National Register for Breast Cancer (NKBC). Completeness of NKBC was reported to be 99.9% and validity of reported information had a very high exact agreement of >90% [30]. The sufficient size and comprehensive clinical database was crucial for enabling a thorough adjustment for potential confounders, allowing the investigation of an independent effect of Se status for survival and recurrence following diagnosis of breast cancer. However, like in all observational studies, residual confounding cannot be ruled out entirely. Information on BMI, smoking, alcohol intake or socioeconomic status, which were shown to possibly associate to Se status and mortality, were not accessible [46]. Yet, we believe it is unlikely that our results would be affected significantly by adjusting for these potential confounders. While higher BMI is associated with higher Se levels, obesity on the other hand is also associated with higher mortality [47]. Further, serum Se and SELENOP were found to be positively associated with alcohol intake, including high intake of ≥30 g/day [48]. Another relevant limitation of this study is the sampling at baseline only, and with one time point only. Hereby, circumstantial factors affecting the acute Se status, e.g. a Se-enriched meal or supplement intake, cannot be identified and corrected for. However, the assessment of three biomarkers with different endogenous half-lives (total selenium | transport protein | enzyme) appears suitable to limit and balance this risk [17]. While statistical power is very high for OS with a low risk for type II error, limited number of recurrent events precludes a detailed analysis of the importance of a replete Se status for recurrence risk. Multiple comparisons were performed, which may lead to a type I error, however, strong dose-response associations and the same direction of results regarding all biomarkers argue against a chance finding. Information on Ki67-expression is incomplete, as this

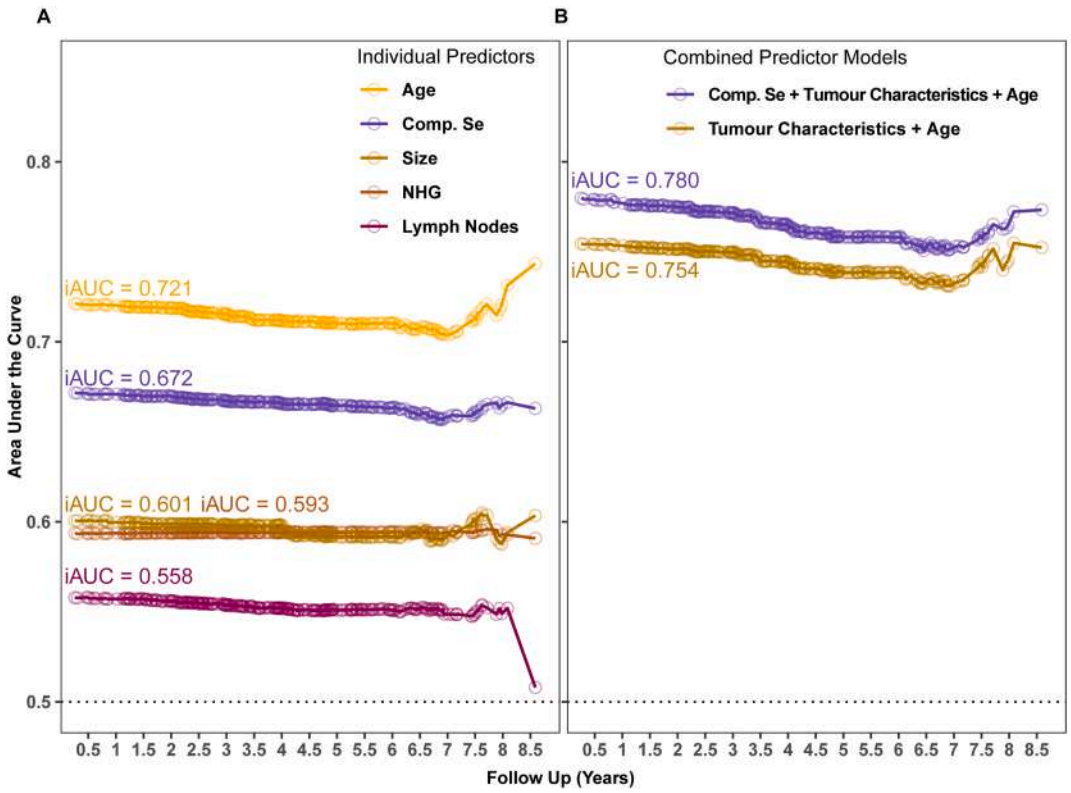

Fig. 6. Predictive value of Se status for mortality. The first panel (A) compares the predictors individually, (B) compares the predictors in combined models. AUCt (y-axis) was computed at each time of death, marked with the symbol ○. An AUC of 0.5, depicted by the dotted line, represents a random predictor without any value, while an AUC of 1.0 is a prediction model with 100% specificity and 100% sensitivity. iAUC = Integrated area under the curve, Comp. Se = Composite Se status.

analysis was not a part of clinicopathological routine in Sweden in early 2010, when SCAN-B started. For this reason, it was not possible to include Ki67-expression in the fully adjusted models. Nevertheless, the assessment of NHG was more complete, and earlier shown to be of similar importance as Ki67 for prognosis following breast cancer [49].

4.2. Borderline selenium status of the study cohort

There are different theories for assessing Se status and defining Se deficiency. The most widely consented criterion relates Se intake to the expression level of circulating selenoproteins, assuming that both GPx3 and SELENOP reach saturated maximal levels at sufficiently high Se supply [17–19]. According to this notion, the majority of US Americans can be considered as Se replete, whereas a majority of subjects residing in e.g. Europe, Asia, or Africa would qualify as insufficiently supplied [11,50]. This interrelationship is well mirrored when correlating total serum Se concentrations with GPx3 activities and SELENOP concentrations, respectively (Fig. 3). Under replete conditions, the protein biomarkers are not closely related to total serum Se, whereas under deficient conditions, stringent correlations are observed, as the trace element then constitutes a limiting factor for selenoprotein biosynthesis. The cut-points where the stringent relation of GPx3 and SELENOP biosynthesis become saturated and independent of total serum Se correspond to 80–90 μg/l for GPx3 and 120–130 μg/l for SELENOP, respectively. According to these criteria, the SCAN-B cohort was perfectly covering both the Se-deficiency and Se-sufficiency range, with median (IQR) concentrations of 70.4 (60.1–81.3) μg/l (serum Se), and 4.08 (3.28–4.88) mg/l (serum SELENOP). The results obtained are in good agreement with a former study on subjects under supplemental Se in the UK, and our former analysis of Se deficiency as risk factor for cardiovascular disease or colorectal cancer [20,32,51,52].

The outcome observed in the present study supports the notion on the high relevance of a sufficient Se status for full expression of SELENOP and GPx3 for supporting survival in disease. SELENOP and GPx3 account for the majority of circulating Se in serum; the contribution of low molecular weight selenocompounds is estimated at around 1–3% only, depending on recent intake of organic or inorganic dietary Se sources, and amount of circulating selenosugars for Se excretion [21,23,53–56]. Antibody-mediated depletion of SELENOP and GPx3 indicated a relative contribution of these proteins accounting for 48–53% and 12–19%, respectively, to total serum Se in subjects with replete Se status, with the remainder mainly associated with selenomethionine [57–60]. However, the relative contribution of SELENOP and GPx3 to total serum Se is not constant, but depends strongly on the baseline Se status and Se supply as both selenoproteins show saturation kinetics with increasing Se intake [20,58,61]. In addition, GPx3 and SELENOP are subject to regulation by female steroid hormones and menopausal state, causing the relative contribution of SELENOP to total serum Se to vary between 48% in young and 56% in elderly women, respectively [60,62]. Notably, the Se content of SELENOP is also not constant, as other amino acids, in particular cysteine, can be inserted in place of selenocysteine in response to the UGA codons during translation, resulting in a variable range of 5–10 selenocysteine residues per SELENOP molecule [23,60,63,64]. Collectively, the dynamic interrelations between dietary Se intake, endocrine regulation and liver and kidney function in GPx3 and SELENOP biosynthesis underline the notion that an assessment of different circulating Se status biomarkers provides an improved and diagnostically more informative insight into Se status as compared to one biomarker alone.

4.3. Mechanisms and comparison with other studies

The trace element Se is essentially needed for a small set of selenoproteins, some of which catalysing redox reactions and contributing to intracellular redox status, quality control of newly synthesized proteins, control of thyroid hormone metabolism, and growth and differentiation.

A potential involvement of SELENOP in neoplasia is supported by the association of breast cancer with single nucleotide polymorphisms in the encoding *SELENOP* gene [65,66]. Furthermore, its expression is decreased in various neoplasms e.g. gastrointestinal tumours [51]. Besides kidney, GPX3 has been detected in mammary gland and is reported to be down regulated in breast cancer, where its decline was associated with a poor prognosis [67,68].

However, several observational studies and RCTs failed to indicate Se deficiency as relevant risk factor for breast cancer incidence. In our study, the association with recurrent disease was not as apparent with respect to all biomarkers, albeit the statistical power was rather limited due to the low number of recurrences following breast cancer. In view of these findings, the association observed point to a high relevance of protective selenoproteins in the aftercare of breast cancer diagnosis. The main therapeutic interventions included chemotherapy, radiotherapy, mastectomy, and others. All of these procedures are associated with an increased physical, psychological and proinflammatory stress on the breast tissue and the organism. Such measures are associated with enhanced cytokine concentrations and tissue damage, both associated with an activated immune response and elevated concentrations of reactive oxygen species and oxidative stress. The interrelations with the Se status are two-fold. Firstly, increased inflammation suppresses hepatic SELENOP biosynthesis and thereby reduced systemic Se transport and tissue Se supply, causing among other effects suppressed renal Se status and GPX3 levels [69]. Secondly, low Se status fails to control the pro-inflammatory response and may enable an overshooting activity of the immune system [70]. Collectively, both mechanisms close a feed-forward and vicious cycle, aggravating the cytotoxic therapeutic measures and hindering convalescence. In how far the declining Se status impairs regular functioning of the immune system and increases disease and mortality risks from different causes like other malignancies, CVD or infections remains to be elucidated, but the similarities observed between this study and the large prospective cancer and CVD studies argue for common mechanisms [32,52]. One common denominator of the different observational studies constitutes a strongly increased health risk when residing in the lowest percentile of Se in a given European population, defined by either an insufficient selenoprotein expression level or low total serum Se concentration or both.

5. Conclusions

The stringent and surprisingly strong association between Se deficiency and poor overall survival after breast cancer diagnosis supports the notion on the essential importance of a sufficiently high Se status for human health. As seen before, populations residing in Europe are insufficiently supplied, and a profound Se deficit is associated with worst chances of survival. Our study has identified a group of patients with breast cancer diagnosis with exceptionally high mortality risk by assessing the Se deficit via a compound biomarker consisting of three serum parameters. In contrast to genetic predisposition, Se status is amenable to correction via simple dietary or supplemental means. Hence, a solid and sufficiently powered intervention study stratified for baseline Se deficiency is needed and appears highly promising in order to test whether correcting a diagnosed Se deficit confers survival benefits in breast cancer.

Contributions

LS, JM, and KD designed the study. KD, QS, LS, ER, JM, YB, AB, LHS, LR, MM, JVC acquired the data, KD, QS, LS, ER, JM, YB, AB, LHS, LR, MM, JVC collected and cleaned the data, and KD and JM, YB, AB, ER performed the statistical analyses. KD, JM and LS wrote the first draft of the manuscript. KD, QS, LS, ER, JM, YB, AB, LHS, LR, MM, JVC interpreted the results and critically revised the manuscript. The corresponding author ensures that all listed authors have read and agreed to the final version of the manuscript, and meet authorship criteria and that no others meeting the criteria have been omitted.

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki and has been approved by the Regional Ethical Review Board of Lund (diary numbers 2007/155, 2009/658, 2009/659, 2014/8), the county governmental biobank center, and the Swedish Data Inspection group (diary number 364–2010).

Data sharing

Original data may be applied for at the SCAN-B steering committee.

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Declaration of competing interest

LS holds shares of selenOmed GmbH, a company involved in Se status assessment and supplementation; no other relationships or activities that could appear to have influenced the submitted work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102145.

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