DISSERTATION

Selenoproteins as predictors of prognosis after breast cancer diagnosis

Selenoproteine als Prädiktoren der Prognose nach Brustkrebsdiagnose

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List of abbreviations

Abbreviation	Full Name
AUC	Area under the curve
aAb	Autoantibodies
BI	Binding index
CV	Coefficient of variation
CI	Confidence interval
Cys	Cysteine
T2	Diiodothyronine
M	Distant metastasis
ER	Estrogen receptor
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FPKM	Fragments per kilobase per million reads
GLOBOCAN2020	Global Cancer Statistics 2020
GPx	Glutathione peroxidase
HR	Hazard ratio
HER2	Human epidermal growth factor receptor 2
IHC	Immunohistochemistry
ISH	In situ hybridization
IQR	Interquartile range
NST	Invasive carcinoma of no special type
ILC	Invasive lobular carcinoma
DIO	Iodothyronine deiodinase
L	Lymph node involvement
mRNA	Messenger ribonucleic acid
MICE	Multiple imputation by chained equations
NKBC	National Quality Registry for Breast Cancer
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NHG	Nottingham Histological Grade
NPC	Nutritional Prevention of Cancer

PBS	Phosphate buffered saline
PR	Progesterone receptor
RCT	Randomized controlled trial
ROC	Receiver operating characteristic
rT3	Reverse triiodothyronine
RNA-seq	RNA-sequencing
SEAP	Secreted embryonic alkaline phosphatase
SELECT	Selenium and Vitamin E Cancer Prevention Trial
Sec, Se-Cys	Selenocysteine
SECIS	Selenocysteine insertion sequence
SECISBP2	Selenocysteine insertion sequence binding protein 2
eEFSec	Selenocysteine specific eukaryotic elongation factor
SEPHS2	Selenophosphate synthetase 2
SELENOP	Selenoprotein P
SD	Standard deviation
SCAN-B	Sweden Cancerome Analysis Network – Breast Initiative
TDLU	Terminal duct lobular unit
TXNRD	Thioredoxin reductase
TSH	Thyroidea Stimulating Hormone
TXRF	Total reflection X-ray fluorescence
T3	Triiodothyronine
T4	Tetraiodothyronine (Thyroxine)
TME	Tumor microenvironment
T	Tumor size
US	United States

Abstract

Selenium (Se) is an essential micronutrient incorporated into selenoproteins involved in e.g., antioxidative defence, cellular redox regulation, and thyroid hormone metabolism. Se is implicated to affect cancer progression, however its association with cancer survival and related mechanisms are inconclusive. This thesis describes a collaboration with the multicenter Sweden Cancerome Analysis Network - Breast Initiative (SCAN-B) which recruits breast cancer patients. After diagnosis, baseline serum, tumor tissue, clinical data and follow-up with death and recurrence were collected. RNA-sequencing for tumor tissues were conducted. 1996 eligible patients with a follow-up of up to ~ 9 years (310 deaths, 167 recurrences) were included and RNA-seq was available for 1453. Four serum Se biomarkers, i.e., total Se (Total reflection X-ray fluorescence), selenoprotein P (SELENOP) (Sandwich ELISA), activity of glutathione peroxidase 3 (GPx3) (NADPH coupled enzyme reaction) and autoantibodies to SELENOP (SELENOP-aAb) (immunoprecipitation assay) were quantified. Multivariable Cox regression models adjusted for potential confounders estimated the association of Se parameters with prognosis. Timepoint-specific receiver operating characteristic analyses (ROCt) determined the prognostic value. Interaction between circulating Se and tumor selenotranscriptome was tested. In adjusted models, low Se, SELENOP or GPx3 at diagnosis were dose-dependently associated with higher mortality; hazard ratio (HR), 95% confidence interval (CI) for quintile 5 vs. quintile 1 was 0.42(0.28–0.63) for Se, 0.51(0.36–0.73) for SELENOP and 0.52(0.36–0.75) for GPx3. Participants in the lowest quintile of all biomarkers had a distinctly low survival of ~50%. Se biomarkers increased the prognostic value of a model containing clinically established prognostic markers (integrated area under the curve 0.754 to 0.780). SELENOP-aAb were present in serum samples of 7.65% of all patients and dose-dependently associated with prognosis, HR (95%CI) for mortality per log-increment of autoantibody titers was 1.31(1.13-1.51). This association was pronounced in Se deficiency. The association of tumor *DIO1*, *DIO3* and *SELENOM mRNAs* with mortality was dependent on circulating Se. Particularly, *DIO1* associated with a favourable outcome, while *DIO3* associated with a poor outcome, both only with high Se levels ($p_{interaction}$ <0.001 and 0.020). The three studies establish low Se status as an independent determinant for a poor prognosis with breast cancer. Quantifying Se, SELENOP, GPx3 or SELENOP-aAb in serum at time of diagnosis identifies patients with poor prognosis. The observed consistent dose-dependent findings indicate that Se-deficient patients may benefit from intensified therapy

and from a personalized substitution to correct their deficits, which merits testing in clinical intervention trials.

Zusammenfassung

Selen (Se) ist ein essentielles Spurenelement, das in Selenoproteine eingebaut wird, welche in antioxidativer Abwehr, zellulärer Redoxregulation und dem Schilddrüsenhormonstoffwechsel involviert sind. Se wird mit Brustkrebsprogression in Verbindung gebracht, jedoch ist der Zusammenhang zwischen dem Se-Status und Prognose nach Krebsdiagnose unklar. Diese Arbeit beschreibt eine Kooperation mit der multizentrischen Sweden Cancerome Analysis Network - Breast Initiative (SCAN-B), die Patient*Innen mit neuem primär invasivem Brustkrebs prospektiv einschließt und Serumproben, Tumorgewebe mit anschließender RNA-Sequenzierung, klinische Daten sowie Information zur Sterblichkeit und zum Brustkrebsrezidiv sammelt. Für die Analysen konnten 1996 Patient*Innen mit einer Nachbeobachtungszeit von etwa 9 Jahren (310 Todesfälle, 167 Rezidive) eingeschlossen werden. RNA-Seq-Daten lagen für 1453 Proben vor. Es wurden drei Se-Biomarker aus Serum quantifiziert: Gesamt-Se (Totalreflexions-Röntgenfluoreszenz), der Se-Transporter Selenoprotein P (SELENOP) (Sandwich-ELISA), Aktivität von Glutathionperoxidase 3 (GPx3) (NADPH-gekoppelte Enzymreaktion), sowie neuartige Autoantikörper gegen SELENOP (SELENOP-aAb) (Immunopräzipitations-Assay). Multivariate Cox-Regressionsmodelle mit Adjustierung für Störfaktoren, sowie Receiver-Operating-Characteristic-Analysen (ROCt) wurden durchgeführt. Die Interaktion von Gesamt-Se mit dem Tumorselenotranskriptom wurde untersucht. In adjustierten Modellen waren ein niedriges Se, SELENOP oder GPx3 beim Diagnosezeitpunkt jeweils dosisabhängig mit einer höheren Sterblichkeit assoziiert; das Hazard Ratio (HR) mit 95% Konfidenzintervall (KI) für das höchste Quintil im Vergleich zum niedrigsten betrug 0,42(0,28–0,63) für Se, 0,51(0,36–0,73) für SELENOP und 0,52(0,36–0,75) für GPx3. Teilnehmende im niedrigsten Quintil aller drei Biomarker wiesen eine deutlich geringe Überlebensrate von nur etwa 50% auf. Die Se-Biomarker erhöhten den prognostischen Wert eines Modells, das klinisch etablierte prognostische Marker enthielt (integrated area under the curve von 0,754 auf 0,780). SELENOP-aAb waren in Serumproben von 7,65 % aller Patient*Innen nachzuweisen und assoziierten dosisabhängig mit der Prognose; das Hazard Ratio (HR) (95%-KI) für die Sterblichkeit pro Log-Anstieg betrug 1,31(1,13–1,51). Diese Assoziation war bei Patient*Innen mit bereits niedrigem Serum-Se oder SELENOP-Spiegeln ausgeprägt. Serum-Se interagierte dosisabhängig mit der Assoziation zwischen Tumorexpressionen von *DIO1*, *DIO3* und *SELENOM* und der Sterblichkeit. Mit steigender Serum Se

Konzentration assoziierte *DIO1* mit einer guten Prognose, während *DIO3* mit einem ungünstigen Verlauf assoziierte ($p_{Interaktion}$ <0,001 und 0,020). Die drei Studien etablieren den Se-Status als unabhängigen prognostischen Faktor bei Brustkrebs. Patient*Innen mit niedrigem Se-Status könnten von einer Korrektur des Se-Defizits profitieren, was in einer randomisiert kontrollierten Studie getestet werden sollte.

1. Introduction

1.1. Selenium

Selenium, a member of the chalcogen group, is characterized by the atomic number 34, was first isolated by Jöns Jacob Berzelius in 1817 and derives its name from the Greek word "selēnē," which refers to its moon-like metallic shine (1). Initially, selenium was perceived as a toxic element due to its adverse effects in excess doses, leading to garlic-like halitosis, alopecia, nail fragility/sloughing or even death following the intake of extremely high and toxic amounts (2, 3). The essentiality of dietary selenium for mammalian life was first noted when it was found to hinder hepatic necrosis in a rat model of vitamin E deficiency (4). Following this discovery, it was gradually deciphered that dietary selenium carries out a number of physiological functions via integration into a specific group of proteins termed selenoproteins (5). These specialized enzymes are involved in various processes relevant for various biochemical pathways and pathologies, among others in antioxidative defence and thyroid hormone metabolism, encouraging extensive investigation of the contribution of a low selenium intake to the development of many widespread and also certain rare diseases over the last years (5, 6).

1.1.1. Dietary selenium intake and geographical variation

Unlike most trace elements, but similar to iodine, selenium intake differs vastly across the world. Hence, populations such as the majority of the United States (US) or Japan display sufficient selenium intake, while most parts of Europe including Germany and Sweden, as well as Asia are affected by selenium deficiency (6). Due to these differences the actual daily intake can range from below 10 µg to up to 5 mg per person per day (6, 7). Recommended intake for European populations is 60 µg for females and 70 µg for males per day, as suggested by the joint German, Austrian and Swiss Nutrition Societies (8). In 2023, the Nordic Nutritional Recommendations that inform Nordic countries including Sweden have increased their recommended intake for women to 75 µg per day (9). The geographical variation is caused by differing selenium contents and availabilities in soil and crops, which in turn is driven by complex soil-plant-atmosphere interrelations. Although the mechanisms are not fully understood yet, predictive modelling of the climate-soil interactions indicates an increase in selenium deficiency in consideration of the future climate crisis (10). Severe selenium deficiency affects certain parts of China particularly

strongly, leading to two endemic diseases, namely Keshan and Kashin-Beck disease (11-13). Apart from soil availability, intraindividual dietary preferences also affect selenium intake, as the selenium content of nutrients differs according to food item and geological origin. Meat, seafood, eggs, milk, and poultry as well as Brazil nuts are among selenium rich food sources, while plant-based foods contain lesser amounts of Se and exhibit a high variability according to geographical origin (6, 14). The generally higher selenium content in animal products as compared to plant-based products is reflected in the higher selenium status of omnivores in comparison to vegetarians and vegans (15, 16).

1.1.2. Selenoprotein expression and functions

The principal mode of action of selenium in the human body is enabled via its incorporation into selenoproteins, which occurs translationally in form of the 21st proteinogenic amino acid selenocysteine (Se-Cys, Sec) (5). Dietary selenium occurs in four major forms, i.e., selenomethionine (Se-Met), Se-Cys, selenite (SeO_3^{2-}), and selenate (SeO_4^{2-}) (**Figure 1**). Following dietary intake and selenide (Se^{2-}) formation, selenophosphate is produced by selenophosphate synthetase 2 (SEPHS2). O-phosphoseryl-tRNA(Sec) selenium transferase (SEPSECS) facilitates the reaction of selenophosphate with the phosphorylated seryl-loaded Sec-tRNA (PSer-tRNA$^{[ser]sec}$), yielding the specifically designated Sec-tRNA$^{[ser]sec}$ containing the anticodon for UGA for directed insertion of Sec into the growing peptide chain (17). In the messenger ribonucleic acid (mRNA) of selenoproteins, the UGA codon, which typically serves as a signal for the termination of protein synthesis, is recognized and re-interpreted as a sense codon specifying Sec insertion (18, 19). This repurposed use of UGA, instead of a premature discontinuation of the translation is facilitated by stem-loop RNA structures called selenocysteine insertion sequence (SECIS) elements, downstream of each selenoprotein open reading frame (20, 21). As part of the mRNA transcript, the SECIS element constitutes an important and essential cis-acting component for selenoprotein biosynthesis. Besides Sec-tRNA$^{[Ser]Sec}$ and SECIS, several trans-acting elements such as SECIS binding protein 2 (SECISBP2) and the Sec-specific eukaryotic elongation factor (eEFSec) are required for translation, and constitute limiting-factors of selenoprotein expression (22, 23). Recently, the structure of this complex and unique translational process has been decoded using cryo-electron microscopy (24). Currently, 25 human selenoprotein genes have been identified, in which Sec serves as a catalytic element (**Figure 1**) (25). While functions of some selenoproteins are still unknown, glutathione peroxidases (GPx), iodothyronine deiodinases (DIO) and thioredoxin

reductases (TXNRD) as well as the selenium transporter selenoprotein P (SELENOP) are well characterized (26). The GPx family is made up by eight isoforms, three of which, namely GPx5, -7 and -8, are no selenoenzymes and do not contain a Sec residue (27). The GPx family is a key regulator of antioxidative processes in the human body, facilitated by isoforms that differ in their time- and site-specific expression patterns and the preferred substrates they act on, which ranges from hydrogen peroxide (H_2O_2) to phospholipid-, cholesterol- and other organic hydroperoxides (27). All isoforms are located and act mainly intracellularly, except for GPx3 that is most abundant in blood plasma. In a state of deficient selenium intake, expression, proper function, and activity of selenoproteins strictly rely on an adequate availability of selenium and Sec-loaded $tRNA^{[Ser]Sec}$ (28-30). This close relationship between selenium intake and selenoprotein expression can be used to monitor selenium status, by quantifying abundant selenoproteins in serum or their enzymatic activity (31, 32). Although total serum selenium is most commonly used as biomarker of selenium status, additional quantification of serum SELENOP secreted mainly by the liver and serum activity of GPx3 secreted mainly by the kidney provides a more comprehensive insight into selenium status (33-35).

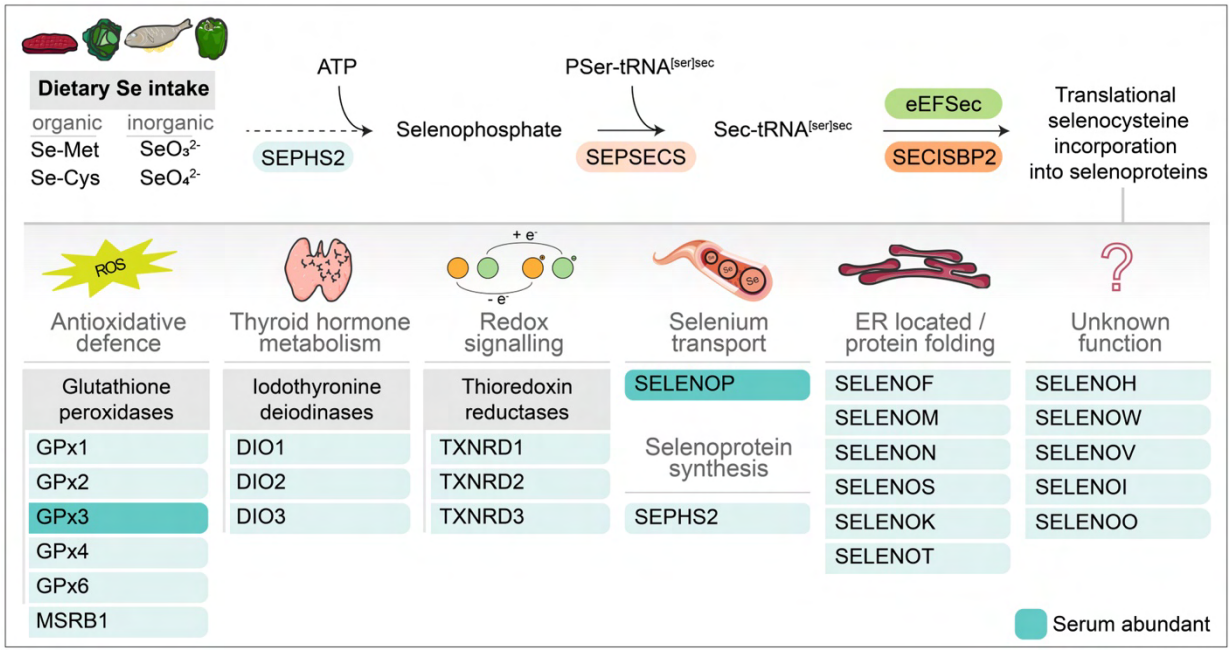

Figure 1. *Overview of selenium incorporation and selenoprotein functions. Dietary selenium occurs mainly in four major forms, i.e., selenomethionine (Se-Met), selenocysteine (Se-Cys, Sec), selenite (SeO_3^{2-}), and selenate (SeO_4^{2-}). After dietary intake and selenide (Se^{2-}) formation (not depicted), selenophosphate production is facilitated by selenophosphate synthetase 2 (SEPHS2), which is a selenoprotein itself. O-phosphoseryl-tRNA(Sec) selenium transferase (SEPSECS) facilitates the reaction of selenophosphate with PSer-$tRNA^{[ser]sec}$ to form Sec-$tRNA^{[ser]sec}$, which contains the anticodon for UGA to deliver Sec for insertion during protein biosynthesis. The translocation of Sec-$tRNA^{[ser]sec}$ to the ribosome is facilitated by SECIS binding protein 2 (SECISBP2)*

which simultaneously associates with the SECIS-element, followed by Sec incorporation by the Sec-specific eukaryotic elongation factor (eEFSec) (not depicted in detail). 25 genes encoding the selenoprotein family have been identified in humans, that can be divided into seven groups according to their functions, i.e., antioxidative defence, thyroid hormone metabolism, cellular redox regulation, selenoprotein synthesis and transport, protein folding, and those without an established function, most of which belong to the so called "alphabet" selenoproteins. GPx3 and SELENOP are abundant in blood serum and serve as accessible biomarkers. Own representation. Created on Adobe Illustrator 2021. Parts were drawn with Servier Medical Art licensed under CC BY 3.0.

1.1.3. Selenoprotein P – transporter, biomarker, and novel autoimmune target

SELENOP is unique among all the selenoproteins, as it contains more than a single Sec residue, thereby serving as the main selenium transporter from liver to peripheral tissue (36). Besides the transport role, SELENOP has enzymatic peroxidase activity (37). It serves as a meaningful biomarker of selenium status due to its role as a transporter, and as its serum concentrations are closely related to selenium intake (31). The *SEPP1* gene encoding for SELENOP includes ten UGA codons that can initiate incorporation of up to 10 Sec residues (38). However, the amount of Sec actually incorporated into SELENOP is regulated by multiple mechanisms, whereby selenium intake and availability are the strongest regulators, leading to lower than the predicted number of ten Sec incorporations in case of selenium deficiency (39, 40). In this case, other amino acids, e.g., cysteine, arginine or tryptophane are incorporated into SELENOP instead, yielding variable forms of the protein, potentially making it an autoantigen. This hypothesis of circulating autoantibodies to SELENOP was tested in a cohort of healthy individuals and patients with thyroid disease using a novel immunoprecipitation assay (41). Although without clinical implications at the time, it was found that SELENOP-aAb are present in healthy individuals, and to a higher extent in patients with thyroid disease. Patients with autoantibodies displayed a lower GPx3 activity with increasing SELENOP-aAb titers, indicating a transport disrupting role, as GPx3 expression stringently depends on selenium supply by SELENOP to the kidneys (36).

1.2. Breast cancer

1.2.1. Breast tissue and histological classification of breast cancer

Breast cancer is a heterogenous disease, particularly with respect to its pathophysiology and molecular basis (42). Hence, there are multiple ways of categorizing breast tumors. The mammary tissue is composed of multiple lobules and ducts that are embedded in the stroma, i.e., surrounding adipose and fibrous tissue. The lobules, composed of terminal duct lobular units (TDLU) are involved in milk production, and are connected to ducts which guide the milk to the mamilla and out of the breast. These structures are lined by a bilayer of epithelium, i.e. luminal cuboidal cells in the inner and myoepithelial cells in the outer section (43). Based on this histology, a simple subdivision of breast cancer can be made according to tumor origin; invasive carcinomas of no special type (NST, formerly invasive ductal carcinoma) make up around 75% of cases, followed by the invasive lobular carcinoma (ILC) (15%) and the less frequent subtypes, e.g. cribriform, tubular, mucinous, neuroendocrine, or inflammatory cancer (44).

1.2.2. Breast cancer epidemiology

Malignant disease of the mammary tissue has become the most commonly diagnosed cancer entity in both sexes according to the Global Cancer Statistics 2020 (GLOBOCAN2020), outscoring lung cancer (45). The global incidence is steadily increasing and is projected to rise from 2.3 million cases in 2020 to ~3 million in 2040 (46). In Sweden, 10222 new diagnoses of primary invasive breast cancer were made in 2020 (47). A considerable fraction of the increase arises from improved diagnosis by implementation of screening programs and higher exposure to lifestyle and hormonal risk factors (44).
Despite increasing incidence levels, breast cancer mortality has improved significantly over the last 30 years. Recent data from the National Cancer Registries in England displays a drop in five year breast cancer mortality from ~14.4% when diagnosis was made in the 1990s to less than 5% in case of diagnosis in the 2010s (48). This decrease is mostly due to the successful introduction of treatment options that are personalized and oriented towards the molecular biology of the tumor (49-51). Due to the rising incidence however, the global mortality burden still constituted ~685.000 deaths in 2020, of which 1398 were in Sweden, underlining the need for novel and improved prognostic biomarkers in order to identify those women with particularly poor prognosis risk already at time of diagnosis (45, 52).

1.2.3. Breast cancer risk factors

The heterogeneity of breast cancer is also well reflected in its diverse risk factors. Female sex (>99% of all breast cancer cases) and increasing age are the two most determinant risk factors for breast cancer. Genetic inheritability and family history of breast cancer account for ~10% of all breast cancer cases (53). Autosomal-dominant inheritable mutations in *BRCA1* and *BRCA2* are the most important drivers of family history associated cases, leading to a risk of breast cancer incidence to age 80 of 72% and 69%, respectively (54). Beside mutations in the high-penetrance DNA-repair genes *BRCA1/-2*, many other syndromes such as Li Fraumeni syndrome driven mostly by *TP53* mutations, Ataxia telangiectasia driven by *ATM* mutations as well as other germline mutations such as *PALB2*, *PTEN*, and *CHEK2* have been identified as high risk factors (55, 56). Many other low risk, low penetrance genes have been described through large scale genomic analyses and contribute to polygenic risk scores (57).

Hormonal and reproductive risk factors associated with prolonged exposure to increased circulating estrogen levels through early menarche, late menopause, higher maternal age at first pregnancy or no breast feeding have been identified as partially addressable risk factors (58-60). High density of breast tissue at mammography and previous diagnosis of benign breast disease are established risk factors (44). Beyond these, risk factors causing around 1/5 of the cases are considered to be directly modifiable and include physical inactivity, overweight and obesity, (postmenopausal) hormone therapy and poor dietary choices (61, 62). Dietary risk factors include alcohol intake as the most important risk factor, and current data consistently indicates that even moderate intake leads to increased risk of breast cancer (61). Although not consistent throughout the literature, increased intake of saturated fat, low consumption of vegetables and fibre were associated with a higher breast cancer risk, and low fibre consumption is shown to interact with alcohol intake, potentiating its disadvantageous effects (63-65).

1.2.4. Breast cancer prognostic factors

Prognosis of breast cancer is commonly evaluated based on clinical outcomes such as all-cause or breast cancer specific mortality and recurrent disease status. Age is not only an important risk factor, but also relevant for the prognosis; the association between age of onset and prognosis is rather U-shaped, as younger patients tend to have hereditary, and often more aggressive tumors (66). Currently established clinical prognostic factors

include the stage which is composed of tumor size (T), lymph node involvement (N), distant metastases (M) and lymphovascular invasion (67). However due to improved screening and, as a consequence thereof, the shift of incident breast cancer cases to earlier stages, specific biology of the tumor has gained in importance for prognostic evaluation (44). The expression of different receptors determined by immunohistochemistry (IHC) or in situ hybridization (ISH), e.g. estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are among the most important theranostic markers that characterize the tumor biology, inform about prognosis and shape further therapy (44, 68). Histological grading is made based on microscopic evaluation of tumor cells based on Nottingham Histological Grade (NHG) as the currently established grading system (69). Hereby the tumor is categorized into three categories based on mitotic count, nuclear pleomorphism and formation of tubules. Ki-67 is a marker determined by immunohistochemistry to deduce the proliferation rate of the tumor (70). Presently, a classification system based on these markers, i.e. surrogate intrinsic subtype classification, is clinically used to categorize breast cancer into five different groups considering tumor biology and providing prognostic information (**Figure 2**) (44). Mastectomy or breast conserving surgery followed by radiation are the foremost important therapies for primary invasive breast cancer without distant metastasis (71). These surgical interventions are usually augmented with systemic therapy, which are based on tumour biology as outlined by the five subtypes, e.g., HER2 positive tumors respond well to the directed anti-HER2-antibody trastuzumab, luminal tumors are efficiently treated with adjuvant endocrine therapy, while the triple negative subtype (TNBC) is associated with the worst survival chances (44).

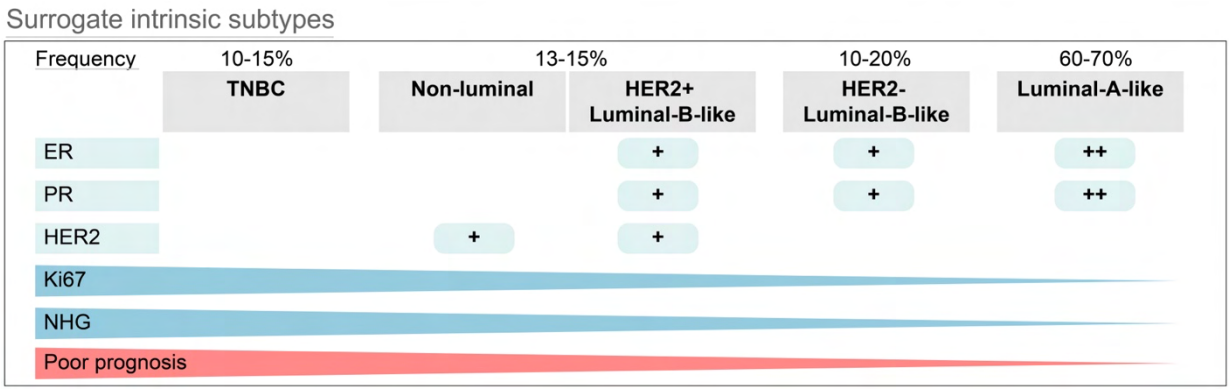

Figure 2. *Simplified surrogate intrinsic subtype classification of breast cancer. Breast cancer is currently categorized into five major groups according to tumor biology, based on expression of estrogen receptor (ER), progesterone receptor (PR) or human epidermal growth factor receptor 2 (HER2), markers of proliferation and histology, i.e., Ki67 and Nottingham Histological Grade.*

This classification provides prognostic information as well as guidance for therapy options. Own representation. Created on Adobe Illustrator 2021.

Beyond the clinically established prognostic markers, there are various ongoing efforts to identify and develop novel prognostic models based on genomic profiles of the tumors applying large-scale genomic technologies, as conducted by the Sweden Cancerome Analysis Network-Breast Initiative (SCAN-B) used in this thesis (72-74).

1.3. Selenium and breast cancer

The impact of selenium intake on tumorigenesis has been a frequent topic of interest due to the pleiotropic effects of selenoproteins on mechanisms involved in tumorigenesis and tumor progression, such as antioxidative defence, control of cellular redox signalling, modulation of the immune system and thyroid hormone metabolism (6). However, the translation of experimental insights into clinical outcomes remains complex. The potential role of selenium on preventing cancer incidence has been explored through some large-scale and costly randomized controlled trials (RCTs), mainly yielding null results (75). However, several aspects of the study designs were criticized. The two largest of these trials, i.e., the Nutritional Prevention of Cancer (NPC) and Selenium and Vitamin E Cancer Prevention Trial (SELECT) predominantly focused on male participants and centred on prostate cancer outcome (76, 77). However, it is well established, that selenium and selenoprotein expression display highly varying sex-specific tissue distribution, and sex hormones affect selenoprotein expression patterns (78). This sexual dimorphism in animal models is also reflected in clinical outcomes, as e.g., reported by our group recently (79). Hence, it is difficult to extrapolate findings from these trials to the context of the female dominant breast cancer. Another limitation in the study design concerns the study population, which is composed of participants in the US only, where the vast majority of residents have a replete selenium status (80). Various associations of selenium status with disease outcomes display a threshold effect, i.e., a lack of association beyond selenium deficiency, which may potentially explain the lack of effect in these selenium replete populations. Nevertheless, current evidence from observational data does not decisively establish a robust relationship of selenium and breast cancer risk, and a comprehensive Cochrane analysis has failed to reveal a relationship between total selenium and the incidence of various cancers (75).

Despite these inconclusive findings regarding impact of selenium on breast cancer initiation, attention has shifted towards exploring its potential as a prognostic determinant for cancer progression and survival. Two recent studies have shown associations of low serum selenium concentrations with high breast cancer mortality, although some aspects preclude establishing selenium as a prognostic factor (81, 82). One study quantified prediagnostic selenium concentrations, warranting consideration of the extent to which these concentrations mirror levels at time of diagnosis, as they have been measured many years prior (82). Meanwhile, the other study incorporated a relatively limited sample size of approximately 500 cases only (81). Notably, both studies only quantified total selenium as a biomarker. Quantification of serum SELENOP concentrations, GPx3 activity and the recently described autoantibodies to SELENOP may provide further insight into the association of selenium status with breast cancer prognosis. A simultaneous analysis of these biomarkers with selenoprotein expression in breast cancer tissues may moreover even provide some insights into potential underlying mechanisms of action.

1.4. Aims and hypotheses

Overall, this thesis aimed to characterize the potential association of selenium, selenoproteins and selenium transport for prognosis and survival of breast cancer patients. To this end, a set of established and newly developed analytical tools needed to be adapted and optimized for the analysis of a sufficiently large and well-characterized clinical cohort study, the SCAN-B (n=1996) prospective study. Three consecutive main working hypotheses have been developed, that built upon each other (**Figure 3**).

1. The first hypothesis tested whether low selenium status at time of diagnosis of breast cancer is associated with poor survival of newly diagnosed patients.
2. The second hypothesis was based on the observed strong associations of low SELENOP concentrations with a poor prognosis. It was hypothesized that autoantibodies to SELENOP may be associated with a particularly poor survival.
3. The third hypothesis aimed to gain insight into the potential mechanisms of action underlying the observed associations. It was hypothesized that the association of certain tumor selenoprotein mRNAs with prognosis depends on circulating selenium levels.

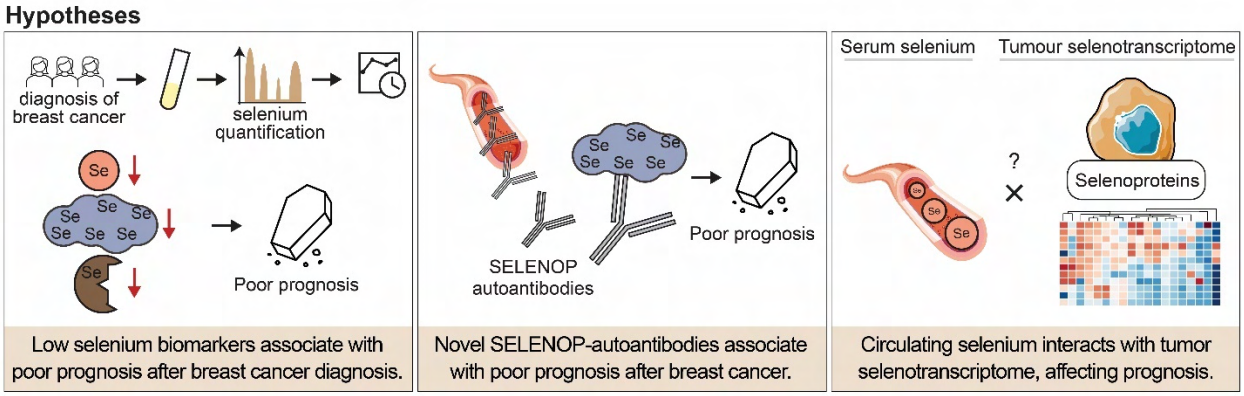

Figure 3. *Schematic overview of the three consecutive hypotheses investigated. Hypothesis 1 claims an inverse association of selenium status with an unfavourable prognosis in breast cancer patients. Hypothesis 2 tests the potential association of SELENOP-autoantibodies with a poor prognosis. Hypothesis 3 aimed to test potential interactions between circulating selenium, tumor selenoprotein expression and prognosis. Own representation. Hypotheses are from Demircan et al. 2021 (ref.35), 2022 (ref.83) and 2023 (ref.84). Created on Adobe Illustrator 2021. Parts were drawn with Servier Medical Art licensed under CC BY 3.0.*

2. Methods

Methodologies applied in this work have been published already in the publications included in the dissertation, i.e., Demircan K, et al. 2021 (35), Demircan K, et al. 2022 (83), Demircan K, et al. 2023 (84).

2.1. Study

2.1.1. Study design and study population

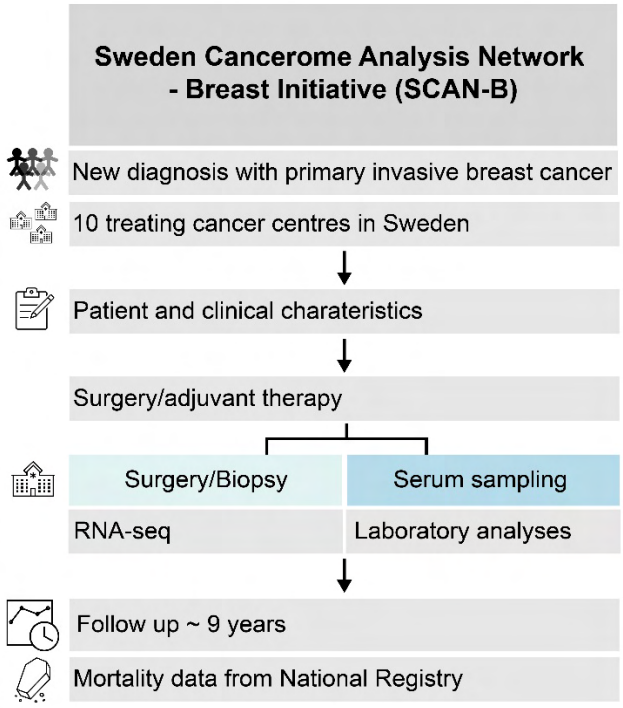

Figure 4. *Study design.*
The Sweden Cancerome Analysis Network – Breast Initiative (SCAN-B) is a prospective, multicentric, real-world study enrolling new non-metastatic primary invasive breast cancer cases. Multiple centres, mostly in South Sweden, are participating. After enrolment, patient and clinical characteristics are documented. Surgery and adjuvant therapy are conducted according to guidelines, and the study does not interfere with therapy. Serum sampling is conducted at time of enrolment and corresponding tumor tissue is collected within surgery. Patients for this study have been followed for ~ 9 years and mortality data from national registries were retrieved. (From Demircan K. et al., 2023 (ref. 84), Figure 1, with modifications.) Created on Adobe Illustrator 2021.

The study population is derived from the SCAN-B (Sweden Cancerome Analysis Network – Breast Initiative) study, a multicentric, real-world study initiated in August 30th 2010 (**Figure 4**) (73). The study has been registered at ClinicalTrials.gov under the ID NCT02306096. Following the main aim of identifying novel prognostic serum and genomic biomarkers for breast cancer survival, the study enrols patients at over ten centres

in Sweden, (e.g. Malmö, Karlskrona, Lund, Växjö, Halmstad, Ljungby, Kristianstad, Helsingborg, Varberg, and Uppsala) (74, 85). All eligible patients in the area are offered enrolment, and currently over 85% of all eligible cases have been included in the study, which corresponds to a total of 20323 participants as of August 2023. Serum sampling is conducted at time of enrolment in a fasting state and tumor tissue is sampled within the surgical procedure. Baseline characteristics and follow-up data are extracted from validated national registries, as described in detail in the next sections. Main eligibility criteria included recently diagnosed primary invasive breast cancer, and absence of distant metastases at time of enrolment, whereas patients with prior malignancy of the contralateral breast, missing information on treatment status, absence of planned treatment in a participating centre or no planned primary surgery or neoadjuvant treatment were excluded (**Figure 5**). Considering these exclusion specifications, 5417 patients were enrolled in the first ~ 5 years of the study. The earliest registered (i.e., those with the longest follow-up) 1996 patients with available serum samples were included for the purpose of this study. Corresponding RNA-sequencing (RNA-seq) data was available for 1453 patients.

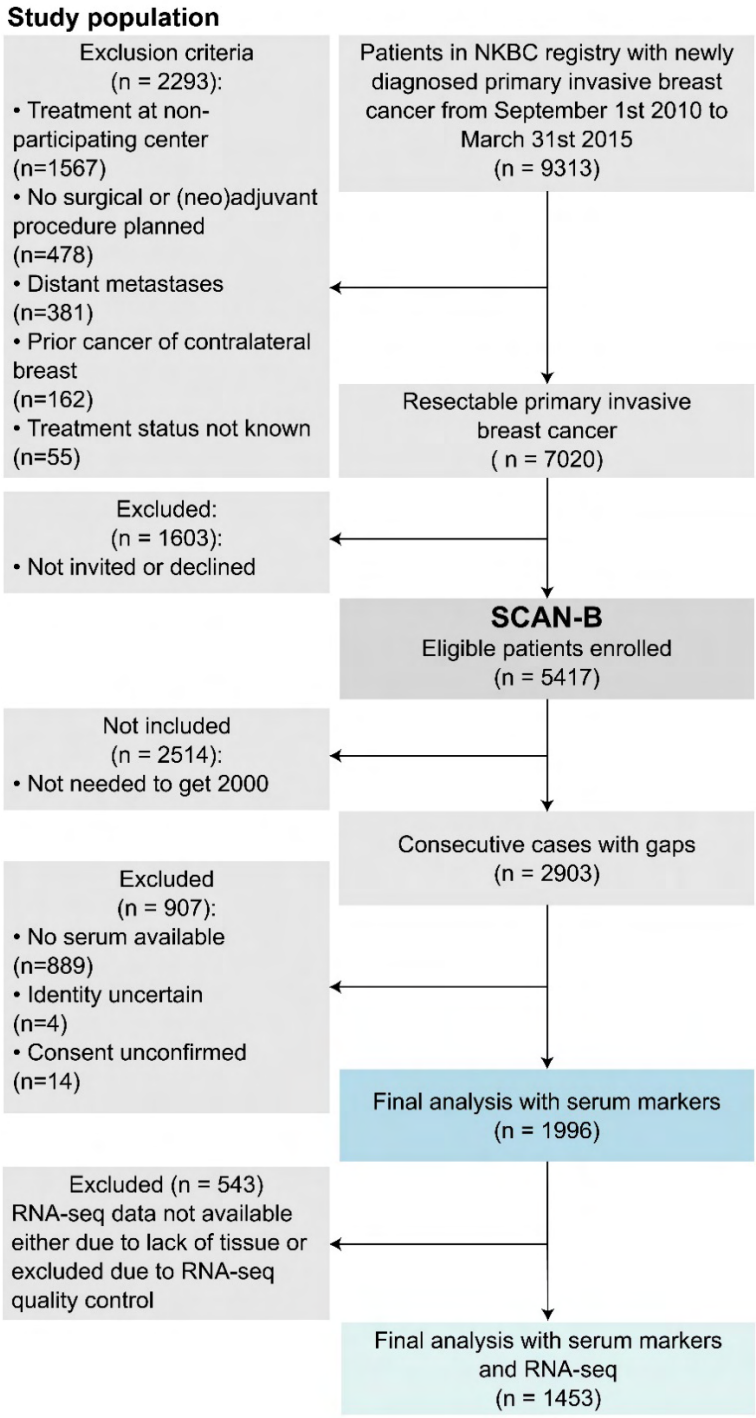

Figure 5. *Flow chart depicting composition of study population.*

From 9313 new breast cancer cases in the specified time frame, 2293 plus 1603 met one or several of the exclusion criteria as listed and were excluded. 5417 of the remaining cases were enrolled in SCAN-B. As a priori defined, ~ 2000 (exact 1996) patients in a consecutive order with available serum samples were included in the final analysis for the purpose of this study. Corresponding RNA-seq data from tumor tissue was available for 1453 of these patients. (From Demircan K. et al., 2021 (ref. 35), Figure 1, Demircan K et al., 2022 (ref. 83), Supplementary Figure 2, Demircan K, et al., 2023 (ref. 84), Supplementary Figure, Supplementary Figure 2, with modifications.) Created on Adobe Illustrator 2021.

2.1.2. Outcomes

Primary outcomes considered in this study were endpoints of prognosis after diagnosis of primary invasive breast cancer, i.e. overall survival (all-cause mortality) and recurrent disease (local, regional or distant metastases) (86).
According to Swedish laws, every citizen is given a number in the Swedish Population Register, in which next to personal information, also information on vital status, i.e., death including date is recorded. Thus, information on mortality was reported to the Swedish Cause of Death Register (87). Recurrent disease was specified to National Quality Registry for Breast Cancer (NKBC) by the centres involved in SCAN-B (88).

2.1.3. Follow-up assessment

The main endpoints were retracted from NKBC or Swedish Cause of Death Register by authorities in SCAN-B, and anonymized data on follow-up as well as event occurrence were given to investigators of this study by the governing body of SCAN-B. Next to anonymized data, for the purpose of ensuring further patient confidentiality, SCAN-B only provided access to length of follow-up, rather than specific dates.

2.1.4. Covariates

Various covariates with regard to patient, tumor and treatment characteristics as well as treatment procedures were recorded and reported to the NKBC in analogy to primary outcomes. Age and menopausal status of the participants were recorded. Clinical stage of the tumor included tumor laterality, tumor size and lymph node involvement. Histopathological covariates included histological type, ER-, PR-, HER2 status, NHG and Ki67 expression. Mode of detection of the tumor was recorded, i.e., by clinical examination or screening. Planned treatment methods were recorded, including axillary and breast surgery procedures as well as adjuvant therapy methods (chemotherapy, immunotherapy, endocrine therapy, radiotherapy).

2.1.5. RNA-sequencing

Tumor tissue sampling for RNA-seq analyses were conducted within the surgical procedure. Remaining tissue after routine pathological assessment was stored in tubes with RNAlater (Ambion, USA) followed by further processing and RNA-seq protocols according to either Illumina NeoPrep or KingFisher system, as described in detail before (73, 89). The gene expression data yielded was transformed to fragments per kilobase per

million reads (FPKM) employing previously described pipelines. Gene annotation was conducted according to GENCODE 27 (90). Data for selenoprotein genes were extracted. After transformation of expression data to FPKM, 1 FPKM was added to every gene as an offset, followed by a logarithmic transformation for further analysis (89). Gene expression data used in this thesis is publicly available (91).

2.2. Laboratory analyses

The following laboratory analyses were conducted with the serum samples from the patients, which were collected at time of enrolment, and stored at -80°C at Skåne University Hospital until shipment to the Institute for Experimental Endocrinology at Charité University Berlin. The clinical data remained concealed from the scientists during laboratory analyses and was disclosed to them after completing all measurements.

2.2.1. Total reflection X-ray fluorescence

Total selenium concentrations in sera were determined using the total reflection X-ray fluorescence (TXRF) method with the S4 T-STAR TXRF analyzer (Bruker Nano GmbH, Berlin, Germany) (**Figure 6**) (35, 79, 92, 93). For this procedure, sera were mixed 1:2 with a double distilled water solution containing the element Gallium (Gallium-HPLC-H_2O, Alfa Aesar GmbH, Karlsruhe, Germany) at a concentration of 1000 µg/L. As Gallium is not endogenously present in human serum samples, the addition of a known concentration of Gallium served as standard when computing concentrations of the trace elements contained in the serum samples. An aliquot of 8 µL of each diluted sample was given onto polished quartz glass slides provided by the manufacturer (Bruker Nano GmbH, Berlin, Germany), and left to dry at 37°C for 24 hours. Glass slides with dried samples were then loaded into the TXRF-analyzer, which measured (750 s) characteristic emissions of light produced by electrons of each trace element that were previously excited by monochromatic, parallelized X-ray radiation. Area under the curve (AUC) for intensity of each trace element was compared to the AUC of Gallium, to determine the concentrations. Multiple runs were conducted for measurement of all samples, with each run containing 84 serum samples and two samples of a serum standard that served as control (Seronorm, Sero AS, Billingstad, Norway). Intra- and interassay coefficients of variation (CV) were less than eight percent during the measurements.

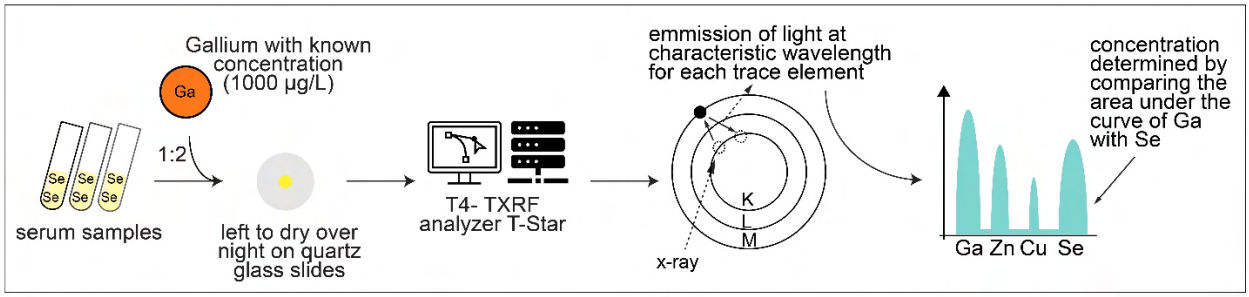

Figure 6. *Schematic overview of the total reflection X-ray fluorescence method. Sera were mixed with double distilled water containing Gallium and placed onto quartz glass slides. T-Star TXRF analyzer was used for quantification of emitted lights induced by X-ray radiation. Gallium with known concentration served as standard for calculation of concentration of other trace elements. Own representation. Created on Adobe Illustrator 2021.*

2.2.2. Selenoprotein P ELISA

Concentrations of SELENOP were quantitated with a commercial kit (selenOtest ELISA, selenOmed GmbH, Berlin, Germany) that is based on the sandwich ELISA technique (94). A total of 5 µL of serum sample was used to measure SELENOP concentration, according to instructions of the manufacturer. The supplied serum standard in three different concentrations (low, medium, high) served as quality control, covering the detection range of 0.4 – 14 mg/L. Intra- and interassay CVs were beneath 10% in case of low and medium, and beneath 20% in case of high standard concentrations.

2.2.3. NADPH-coupled enzyme assay for glutathione peroxidase 3 activity

Activity of GPx3 in serum samples was analyzed by an enzyme reaction coupled with nicotinamide adenine dinucleotide phosphate (NADPH) (**Figure 7**) (95). The enzyme GPx3 catalyzes the reduction of hydrogen peroxide (H_2O_2) to two water molecules (H_2O), enabled by oxidising reduced glutathione. Reduced glutathione is regenerated, whereby this reaction involves the oxidation of NADPH to $NADP^+$. As the consumption of NADPH takes place for regeneration of each reduced glutathione molecule by an excess of added glutathione reductase, the decrease in NADPH is proportional to the activity of GPx3. Therefore, absorbance of NADPH was measured at 340 nm. For that purpose, 5 µL serum samples were placed in 96 well-plates and each mixed with 200 µL of a buffer containing the reagents for the reaction, i.e., 0.3 U/mL glutathione reductase, 3.4 mM reduced glutathione and 0.27 mg/mL NADPH. Ten µL of ddH_2O containing 0.00375% H_2O_2 (v/v) was given to the mixture to initiate the reaction, before measurement. Measurement of NADPH absorbance was conducted over a period of 4 minutes, and per minute activity

was calculated and averaged. Serum samples were measured as triplicates. Each measurement run (31 serum samples) contained a serum standard measured in triplicates to ensure quality control. Intra- and interassay CVs were beneath 10% and 15%, respectively.

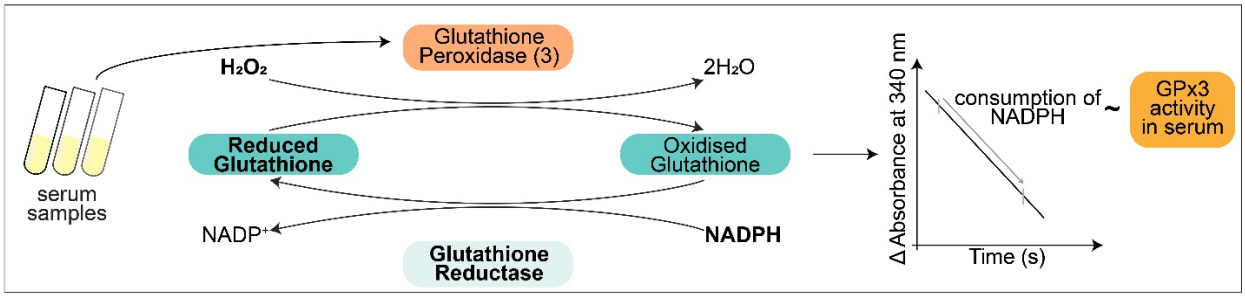

Figure 7. *Overview of the NADPH-coupled glutathione peroxidase 3 activity assay. In serum samples, consumption of NADPH was measured, which is proportional to the GPx3 activity. Own representation. Created on Adobe Illustrator 2021.*

2.2.4. Immunoprecipitation assay for SELENOP-autoantibodies

An immunoprecipitation assay was used to quantify titers of SELENOP-aAb in serum samples (35, 41). The general principle of the method is based on three steps involving (i) expression of a SELENOP protein fused with secreted embryonic alkaline phosphatase (SEAP) in HEK293 cells which was provided by selenOmed GmbH (Berlin, Germany), (ii) the process of immune complex formation between serum abundant autoantibodies and recombinant fusion proteins, and (iii) the precipitation of the immune complexes with following luminometric measurement. A modified cDNA of SELENOP was incorporated into a pIRES-neo vector containing SEAP-cDNA by linearization of the vector by restriction and following ligation of SELENOP-cDNA (**Figure 8A**). The pIRES-neo-SEAP-SELENOP vector was then transformed into competent *E. coli*. After purification of the plasmid, it was transfected into HEK293 cells using FuGENE transfection reagent (Promega, Madison USA). Selection of the clones with the transfected plasmid was conducted using 0.8 mg/mL G418 as part of the cell culture media DMEM/F12 (Biochrom, Berlin, Germany) containing 10% (v/v) fetal bovine serum (FBS). After expansion of stably transfected cells (tested by luminometric measurement of cell culture supernatants), cell culture supernatants enriched with the fusion protein were aliquoted and stored at a -80 °C freezer. The generation of the fusion protein was conducted and provided by selenOmed GmbH (Berlin, Germany). On the first day of the measurement process, 5 µL of serum samples were mixed with 40 µL of cell culture supernatants containing fusion proteins in 96 well plates and left overnight at 4°C (**Figure 8B**). Next day (**Figure 8C**), fusion

protein-autoantibody complexes formed overnight were precipitated using protein A sepharose, which can bind immunoglobulins at the fragment crystallizable (Fc) site. For that purpose, 40 µL of a phosphate buffered saline (PBS) (Thermo Fisher Scientific, Massachusetts, USA) solution containing Protein-A agarose (20% (v/v), ASKA Biotech GmbH, Berlin, Germany) were given to each well of the 96 plate, and stirred on a microplate shaker for one hour at room temperature. Non-specifically bound materials were removed by addition of 50 mM Tris/HCl, 100 mM NaCl, 10 % (v/v) Glycerin und 0,5 % (v/v) Triton X-100 (pH 7,4) wash buffer and subsequent centrifugation at 2000 rpm before removal of supernatant for a total of 5 times. After application of the chemoluminescent substrate for alkaline phosphatase (Thermo Fisher Scientific, Massachusetts, USA), luminescence was measured as relative light units (RLU) using the multimode Tecan NanoQuant infinite M200 PRO (Tecan, Männedorf, Switzerland). Based on RLUs, binding indices (BI) were calculated for each serum sample applying unbiased mathematical outlier criteria. Patients with a BI > 3.0, i.e., in good agreement to median + 1.5 interquartile ranges (IQR) of all participants, were considered positive, as described in detail before (41, 83, 96).

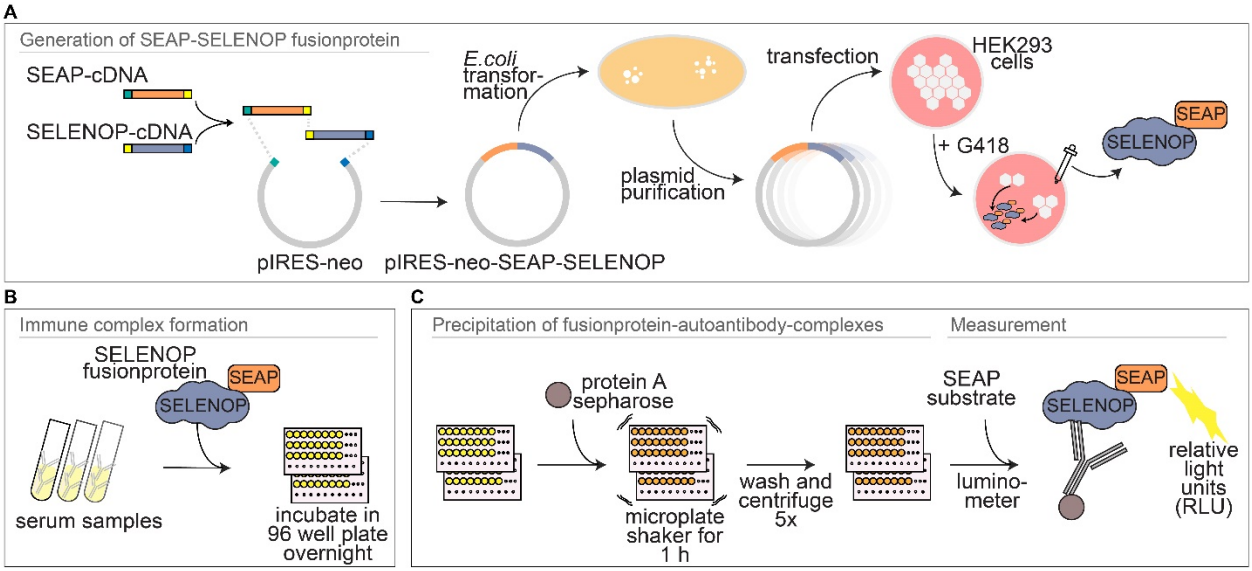

Figure 8. *Overview of the SELENOP-autoantibody immunoprecipitation assay.* ***A*** *Expression of SELENOP fused with secreted embryonic alkaline phosphatase (SEAP) in HEK293 cells (provided by selenOmed GmbH (Berlin, Germany).* ***B*** *Immune complex formation involving autoantibodies that bind the fusion protein.* ***C*** *Precipitation of immune complexes followed by luminometric measurement. (From Demircan K. et al., 2022 (ref. 83), Figure 1, with modifications.) Created on Adobe Illustrator 2021.*

2.3. Statistical analyses

Statistics were computed with the R Language (The R Foundation, Vienna, Austria, version 4.0.4.) on RStudio integrated development environment (Posit, Boston, USA, version 1.4.1106.). Adobe Illustrator (Adobe Inc., California, USA, version 25.2 2021) was used to post-process graphical outputs from R or to create illustrations. Software and packages used for the analyses are listed in **Table 1**. All statistical tests employed were two sided.

Table 1. Software and packages for statistical analyses.

Software/ Packages	Description of purpose
R	An open-source statistical computing and data analysis language with a wide range of tools for data processing and modeling.
RStudio	An integrated development environment, enhancing R programming workflows with code editing, debugging, and visualization tools.
Adobe Illustrator	A vector graphics editor used for creating precise and high-quality illustrations, diagrams, and graphics.
Dplyr	R package for data transformation; for filtering, arranging, summarizing, and mutating data frames, streamlining data preprocessing.
Tidyr	Transforming unstructured or wide datasets into a structured, analysis-friendly format, facilitating data exploration.
MICE	Imputing missing data in datasets using statistical techniques, particularly the Multiple Imputation by Chained Equations method.
Mitools	Exploring and analyzing multiply imputed datasets generated by MICE.
Ggplot2	R package based on the grammar of graphics, simplifying the creation of customizable and visually appealing data visualizations.
Ggpubr	Extension for ggplot2, simplifying the creation of publication-ready plots by adding summaries and themes.
Ggsci	Offers a variety of color palettes designed for ggplot2 visualizations, enhancing data representation.
Scales	Customization of axis scales and labels in ggplot2 plots, allowing for precise control over graphical elements.
Patchwork	Combination / arrangement of multiple plots into cohesive compositions.
Corrplot	Creating correlation plots, facilitating the visualization and analysis of variable relationships in datasets.
Survminer	Visualization of survival analysis data, including the creation of Kaplan-Meier survival curves and more.
Survival	Provides tools for survival analysis, enabling estimation of survival curves and conducting survival regression.
Gtsummary	Creation of summary tables and descriptive statistics, especially useful for summarizing regression models.
Rms	R package focusing on regression modeling, offering tools for building, evaluating, and visualizing regression models.
Hmisc	R package containing a range of functions for data analysis, including data summarization, manipulation, and reporting.
Visreg	Computing and visualizing regression model results with an interaction term, aiding in the interpretation of variable relationships.

2.3.1. Descriptive statistics

Distribution of continuous variables was tested for normality applying Shapiro-Wilk test and were confirmed by visual assessment of quantile-quantile plots. In summary tables, normally distributed numerical variables were presented as mean (standard deviation (SD)), and variables with non-normal distribution were presented as median (IQR). Frequency (percent) was used to present categorical data.

2.3.2. Missing data assessment and imputation

Missing values in the dataset were subjected to visual assessment to examine the pattern of missingness and considered to be missing at random, fulfilling the requirement for multiple imputation by chained equations (MICE) (97). The missing data accounted for a total of <1% of all variables included in the final regression models. Using the MICE algorithm, a fully conditional specification was performed, i.e., an appropriate model was defined for each variable (**Figure 9**). Predictive mean matching was specified for data of continuous nature, proportional odds model was specified for ordinal categorical variables, polytomous logistic regression model was specified for nominally ordered categorical variables, and logistic regression was adapted to impute binary categorical variables (35, 98). All variables in the regression models, outcome data including mortality and recurrence, and survival days were included in the prediction matrix for the MICE algorithm. A total of ten iterations with ten imputations were performed. Performance was evaluated by convergence, and no large deviations were observed compared with the full case analyses.

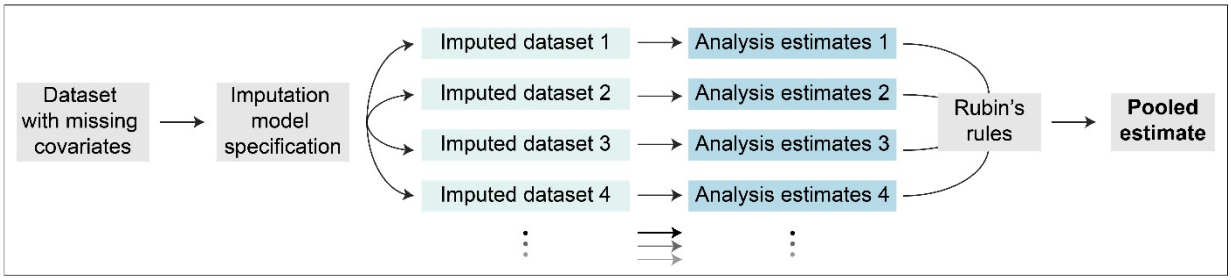

Figure 9. *Multiple imputation by chained equations. A separate model for each variable was specified. Ten imputed datasets were created with ten iterations each. Cox regression analyses were conducted with all datasets and estimates from respective analyses were pooled. Created on Adobe Illustrator 2021.*

2.3.3. Spearman's rank correlation

Correlation analyses were done by applying Spearman's Rank correlation, and p values as well as Spearman's R were reported (99). Correlation matrices were plotted based on

Spearman's R to visualize correlation between tumor selenoprotein mRNA expression and serum selenium status.

2.3.4. Kaplan-Meier-Analyses

Crude non-adjusted survival analysis was conducted and visualized using Kaplan-Meier curves. Censoring was depicted using vertical lines. Log-rank test was employed to test for survival differences between groups (100).

2.3.5. Multivariable Cox proportional hazards regression

Cox proportional hazard models were fitted to quantify survival, hazard ratios (HR) with corresponding 95% confidence intervals (CI) for each selenium biomarker, SELENOP-aAb or selenoprotein gene of interest were reported (101). Selenium biomarkers were categorized into quintiles and autoantibodies to SELENOP were categorized into negative or positive (according to the unbiased mathematical outlier criterion described before), when assessing survival with Cox models. Further, in order to deduce dose-dependent associations, continuous variables for selenium biomarkers and SELENOP-aAb were also modelled in Cox regression models and a p-value for trend (p_{trend}) over quintiles was calculated. Selenoprotein genes were entered as continuous variables to derive dose-dependent associations. For selenium biomarkers and SELENOP-aAb, a crude model containing the biomarker of interest solely, an age adjusted, and a fully adjusted model that included age (continuous), menopause (categorical), laterality of the breast tumor (dichotomous), detection of the tumor (dichotomous), tumor size (continuous), lymphonodular involvement (categorical), Nottingham Histological Grade (categorical), histological type of the tumor (categorical), tumor expression of ER (dichotomous), PR (dichotomous), and HER2 (dichotomous). In sensitivity analyses, fully adjusted models were further augmented with information on adjuvant or surgical treatment regimens applied, i.e., endocrine therapy, immune therapy, chemotherapy, radiotherapy, surgical procedures applied for the breast, and surgery for the axillary lymph nodes. The proportional hazards assumption of the Cox models was tested by examination of Kaplan Meier curves. In order to test the assumption statistically, overall Schoenfeld residuals were computed for each biomarker of interest, whereby $p < 0.05$ from Schoenfeld individual test was considered a violation of the assumption (102). No violation was observed for any of the biomarkers.

2.3.6. Non-linear multivariable Cox models with restricted cubic splines

In order to detect potential non-linear associations, the aforementioned Cox models were augmented with restricted cubic splines that are flexible at three knots, (i.e. 10^{th}, 50^{th}, and 90^{th} centiles) (103). Restricted cubic splines were related to linear Cox regressions applying the Likelihood ratio test. $P_{non\text{-}linearity} < 0.05$ was set as the cut-off for deviation from linearity.

2.3.7. Time-resolved receiver operating characteristic analyses

For determining predictive value of the composition of the three selenium biomarkers, timepoint-specific AUCs were computed using a time-dependent approach in order to account for censoring in the survival analysis (**Figure 10**) (104). Incident/dynamic model proposed by Heagerty PJ. et al. was applied, and at each time of an event in the primary outcome measure, i.e. death, a receiver operating characteristic (ROC) analysis was conducted, and the corresponding AUCt was extracted (105). AUCts for each given time of death were visualized, and a global overall integrated AUC was computed for each variable. Most clinically meaningful prognostic factors of breast cancer were compared to a composite marker of three selenium biomarkers.

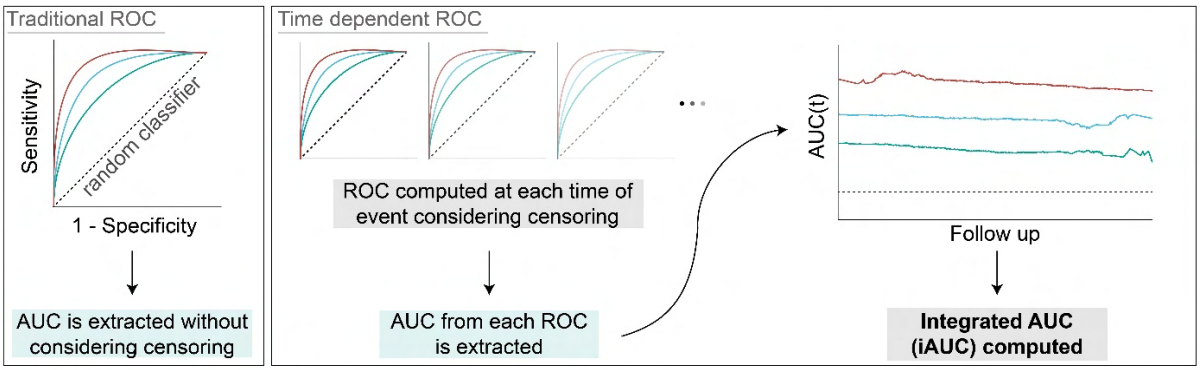

Figure 10. *Time dependent receiver operating characteristic analysis. Traditional receiver operating characteristic analysis (ROC) provides information about prognostic value of a marker, without considering censoring, which is common in survival analysis. The time dependent (ROC(t)) computes this analysis at multiple times, from which the areas under the curves (AUC(t)) are displayed over follow-up time. Based on this, an integrated AUC (iAUC) can be computed to quantify the overall prognostic value. Created on Adobe Illustrator 2021.*

3. Results

Results presented in this work have been published in the publications included in the dissertation, i.e., Demircan K, et al. 2021 (35), Demircan K, et al. 2022 (83), Demircan K, et al. 2023 (84).

3.1. Study 1: Selenium biomarker status and breast cancer prognosis (35)

3.1.1. Baseline characteristics according to mortality and recurrence

The final analyses were based on 1996 eligible patients with available measurements of total Se, SELENOP concentrations, GPx3 activity and SELENOP-aAb. Eight of the cases included were male participants and were excluded when investigating SELENOP-aAb in relation to prognosis. Follow-up of the 1996 patients consisted of a total of 13306 person years when assessing all-cause mortality as an outcome, and 13039 person years when investigating recurrent breast cancer as an outcome. Within the follow-up time window, 310 deaths and 167 recurrent breast cancer cases were documented. RNA-seq data was available in 1453 of the patients, with a total follow-up of 9701 years, and 237 deaths. In **Table 2**, baseline characteristics are presented according to stratification by all-cause mortality and recurrent breast cancer status.

Table 2. Clinical and tumor characteristics according to death and recurrence.

	Mortality		Recurrent breast cancer	
Characteristic	**Survived**	**Deceased**	**No recurrence**	**Recurrence**
	n = 1,686	N = 310	n = 1,829	n = 167
Patient age (y)	*63 (52 – 69)*	*72 (65 – 82)*	*64 (54 – 70)*	*65 (54 – 74)*
Menopause				
Pre-menopausal	342 (21)	23 (7.5)	335 (19)	30 (18)
Post-menopausal	1,246 (75)	278 (91)	1,396 (77)	128 (77)
Uncertain	79 (4.7)	4 (1.3)	75 (4.2)	8 (4.8)
Tumor side				
Left	861 (51)	177 (57)	946 (52)	92 (55)
Right	825 (49)	133 (43)	883 (48)	75 (45)
Size of tumor (mm)	*15 (11 – 21)*	*22 (14 – 30)*	*15 (11 – 22)*	*21 (14 – 30)*
Involved 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	1,066 (66)	174 (59)	1,149 (65)	91 (57)

Characteristic	Mortality		Recurrent breast cancer	
	Survived n = 1,686	**Deceased** N = 310	**No recurrence** n = 1,829	**Recurrence** n = 167
Submicrometastasis	35 (2.2)	7 (2.4)	40 (2.3)	2 (1.2)
(missing)	62	16	72	6
Histological grade				
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)	1,266	252	1,387	131
Histopathological type				
Ductal	1,356 (81)	241 (78)	1,461 (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	1,462 (88)	259 (86)	1,587 (88)	134 (83)
Positive	206 (12)	42 (14)	220 (12)	28 (17)
ER expression				
Negative	201 (12)	80 (26)	232 (13)	49 (30)
Positive	1,481 (88)	229 (74)	1,593 (87)	117 (70)
PGR expression				
Negative	423 (25)	134 (43)	488 (27)	69 (42)
Positive	1,258 (75)	176 (57)	1,337 (73)	97 (58)
Selenium (µg/l)	*72 (62 – 82)*	*63 (52 – 74)*	*71 (60 – 81)*	*69 (57 – 81)*
SELENOP (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 only shown if >2%.

y = years, HER2 = human epidermal growth factor receptor 2, ER = estrogen receptor, PGR = progesterone receptor, GPx3 = glutathione peroxidase 3

(From Demircan K. et al., 2021 (ref. 35), Table 1, with modifications.)

3.1.3. Surgery and adjuvant therapy methods according to outcomes

Diagnostic and therapeutic approaches are contrasted with respect to all-cause mortality and recurrent breast cancer status in **Table 3**. Patients who succumbed to breast cancer more often had tumors that had been detected in clinical examination, more often underwent mastectomy, more often underwent axillary node dissection, and received endocrine, chemo-, or radiation therapy less frequently. Patients with recurring breast cancer had comparable diagnostic and therapeutic features, with the exception that they commonly underwent chemotherapy. A non-participation analysis revealed that the distributions of these characteristics were identical among the patients who lacked access to blood samples.

Table 3. Diagnostic/therapeutic procedures according to mortality and recurrence.

	Mortality		Recurrent breast cancer	
Characteristic	**Survived** N = 1,686	**Deceased** N = 310	**No recurrence** N = 1,829	**Recurrence** N = 167
Type of diagnosis				
Clinical	722 (43)	205 (66)	829 (46)	98 (59)
Screening	942 (57)	104 (34)	978 (54)	68 (41)
Mammary surgery				
Mastectomy	612 (36)	211 (68)	720 (39)	103 (62)
Partial Mastectomy	1,074 (64)	99 (32)	1,109 (61)	64 (38)
Axillary surgery				
Sentinel Node	1,094 (65)	176 (57)	1,181 (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)
Adjuvant therapy*				
Endocrine treatment	1,264 (75)	207 (67)	1,373 (75)	98 (59)
Chemotherapy	593 (35)	82 (27)	608 (33)	67 (40)
Immunotreatment	185 (11)	23 (7.5)	190 (10)	18 (11)
Radiotherapy	1,169 (70)	143 (46)	1,209 (66)	103 (62)

n (%), missing only shown if >2%. (From Demircan K. et al., 2021 (ref. 35), Table 2, with modifications.);

* adds up to >100% as the same patient may receive multiple treatments

3.1.4. Correlation of serum biomarkers of selenium

All three serum biomarkers of selenium status were correlated with each other, and the strongest coefficient of correlation was observed between selenium and SELENOP (R = 0.604, p < 0.001) (**Figure 11A**). Selenium and GPx3, as well as SELENOP and GPx3 displayed a moderate correlation (R = 0.295, R = 0.279, p < 0.001 for both) (**Figure 11B and C**).

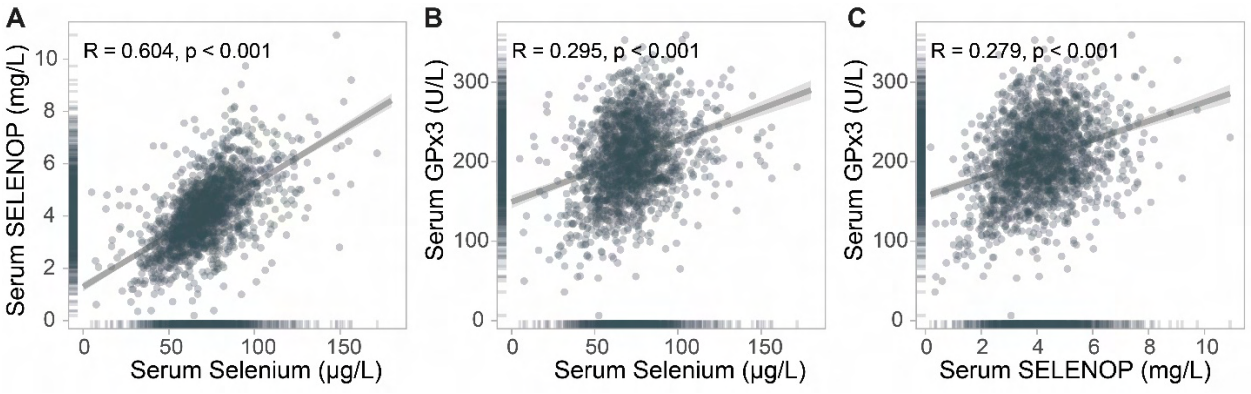

Figure 11. *Correlation of selenium biomarkers. Spearman's rank correlation test was applied. The line and transparent shadow represent the fit from linear regression model with 95% confidence intervals.* ***A*** *Correlation between total serum selenium and serum SELENOP.* ***B*** *Correlation between selenium and activity of serum GPx3.* ***C*** *Correlation of SELENOP with activity of serum GPx3. (From Demircan K. et al., 2021 (ref. 35), Figure 2, with modifications.)*

3.1.5. Survival according to baseline serum selenium biomarker status

In Kaplan-Meier analyses, survival was analyzed according to the status of each biomarker at the time of diagnosis (**Figure 12A, B and C**). For this purpose, participants were put into quintiles based on the concentration/activity of biomarkers at the time of diagnosis, and survival was compared using log-rank test. For each biomarker, the lowest concentration/activity, i.e., quintile 1, was found to have the lowest probability of survival (for all $p < 0.0001$), while survival chances increased with increasing quintile in a dose-dependent manner.

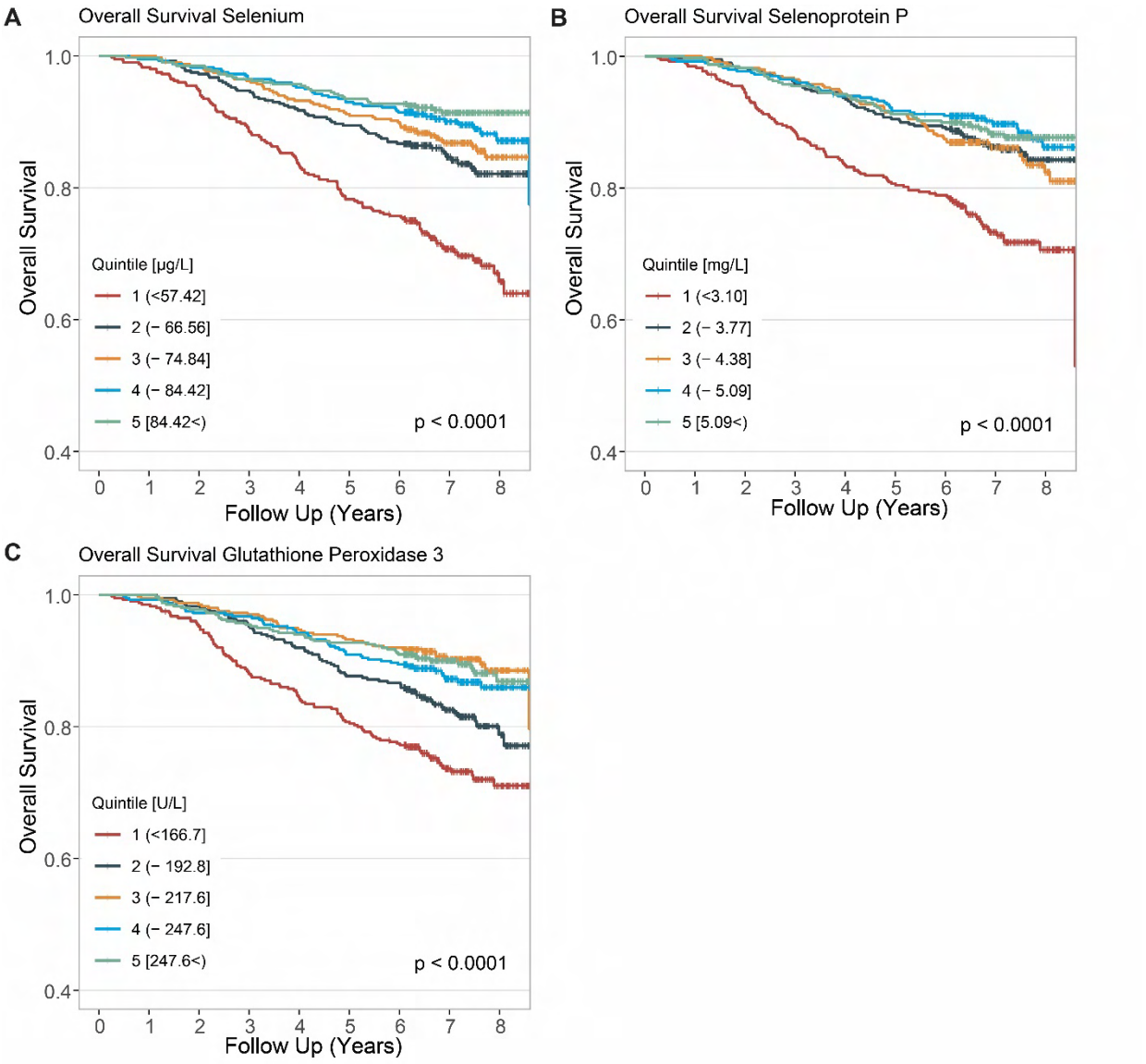

Figure 12. *Kaplan Meier analyses according to baseline selenium biomarkers. Kaplan Meier Curves visualized survival according to quintiles for concentration/activity of each biomarker at baseline, log-rank test was employed to detect differences.* ***A*** *Survival chances according to total serum Se.* ***B*** *Survival chances according to serum SELENOP.* ***C*** *Survival chances according to quintiles of GPx3 activity. (From Demircan K. et al., 2021 (ref. 35), Figure 4, with modifications.)*

The associations of biomarkers with mortality were then analyzed in Cox regression models and controlled for multiple confounders in fully adjusted models (**Table 4**). Here, the associations for mortality were robust across all biomarkers (all $p < 0.001$). In addition, dose dependency was further tested by entering the biomarkers as continuous variables in the Cox regression models and was shown to be dose-dependent (all $p < 0.001$). P for nonlinearity was < 0.05 for all three biomarkers.

Table 4. Cox proportional hazards models for mortality.

	At risk (death)	**Crude***		**+ Age†**		**Full adjustment‡**	
Characteristic	n	HR	95% CI	HR	95% CI	HR	95% CI
Selenium							
Q1 (**ref.**)	400 (119)	—	—	—	—	—	—
Q2	399 (62)	0.48	0.35 , 0.65	0.59	0.44 , 0.81	0.63	0.46 , 0.87
Q3	399 (53)	0.40	0.29 , 0.55	0.53	0.38 , 0.74	0.53	0.38 , 0.74
Q4	399 (43)	0.32	0.22 , 0.45	0.46	0.32 , 0.66	0.47	0.33 , 0.67
Q5	399 (33)	0.24	0.17 , 0.36	0.37	0.25 , 0.55	0.42	0.28 , 0.63
SD increment		0.59	0.52 , 0.66	0.71	0.63 , 0.81	0.72	0.63 , 0.82
SELENOP							
Q1 (**ref.**)	400 (106)	—	—	—	—	—	—
Q2	399 (55)	0.47	0.34 , 0.65	0.53	0.38 , 0.73	0.54	0.39 , 0.76
Q3	399 (60)	0.51	0.37 , 0.70	0.61	0.44 , 0.83	0.60	0.43 , 0.83
Q4	399 (43)	0.36	0.26 , 0.52	0.42	0.30 , 0.61	0.46	0.32 , 0.66
Q5	399 (46)	0.39	0.27 , 0.55	0.46	0.32 , 0.65	0.51	0.36 , 0.73
SD increment		0.65	0.58 , 0.73	0.71	0.63 , 0.80	0.74	0.65 , 0.83
GPx3							
Q1 (**ref.**)	400 (105)	—	—	—	—	—	—
Q2	399 (72)	0.64	0.48 , 0.87	0.80	0.59 , 1.08	0.76	0.56 , 1.03
Q3	399 (40)	0.34	0.24 , 0.49	0.45	0.31 , 0.65	0.43	0.30 , 0.63
Q4	399 (50)	0.44	0.31 , 0.61	0.60	0.43 , 0.85	0.59	0.42 , 0.84
Q5	399 (43)	0.37	0.26 , 0.53	0.53	0.37 , 0.76	0.52	0.36 , 0.75
SD increment		0.65	0.58 , 0.73	0.76	0.68 , 0.85	0.75	0.66 , 0.84

HR = Hazard Ratio, CI = Confidence Interval, ref = reference group, SD = standard deviation, Q = Quintile
p – trend computed by entering quintiles as continuous variable: all p – trend < 0.001.

* Non-adjusted.

† Adjustment for age.

‡ Adjustment for age, menopausal status, number of lymph nodes involved, diagnosis mode, size of the tumor, ER expression, PGR expression, HER2 expression, Nottingham histological grade, histopathological type. Missing values in adjustment factors were imputed via multiple imputation.

(From Demircan K. et al., 2021 (ref. 35), Table 4, with modifications.)

3.1.6. Survival in triple selenium deficiency

In further analyses, probability of survival was tested in participants who were in quintile 1 for selenium, SELENOP and GPx3 activity (**Figure 13**). This triple deficient group of patients had a ~50% chance of survival after approximately 8 years of follow-up. In comparison to triple-deficient patients, patients with a minimum of one biomarker in the fifth quintile had a HR 95%CI of 0.30 (0.21, 0.43) in fully adjusted models, and a corresponding survival chance of >85%.

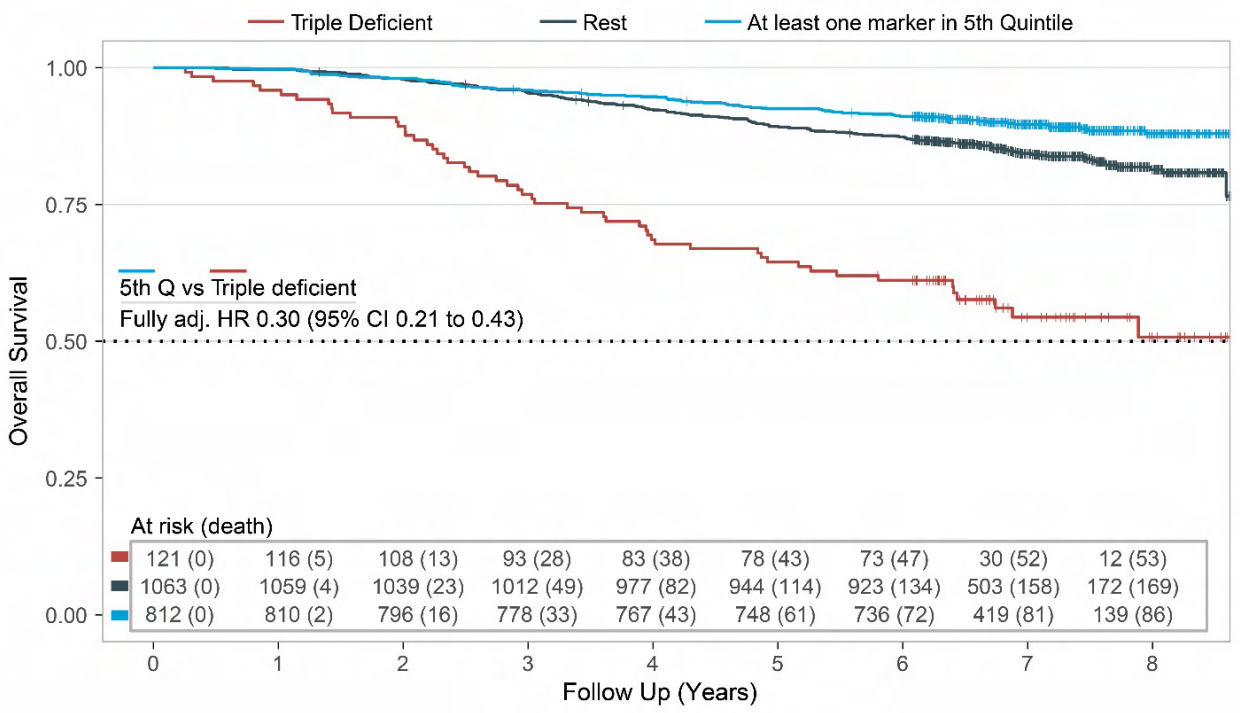

Figure 13. *Survival based on simultaneous deficiency in all three biomarkers. Survival was assessed in patients residing in the first (lowest) quintile for each biomarker, triple deficient (red line) and in those with a minimum of one biomarker in the fifth quintile (highest, dark blue) and the rest (light blue). Kaplan Meier curves visualized survival chances. Hazard ratio and 95% CI from fully adjusted Cox regression models are reported. (From Demircan K. et al., 2021 (ref. 35), Figure 5, with modifications.)*

3.1.7. Time-dependent prognostic value of baseline selenium biomarkers

In **Figure 14**, the time-resolved predictive value for overall survival of a composite marker from all three biomarkers was compared with well-established clinical predictors. In the comparison of the individual biomarkers, the selenium biomarkers performed better than stand-alone clinical predictors with an AUCt of 0.672. The addition of the composite selenium variable to the model with all established clinical biomarkers improved the predictive value from 0.754 to 0.780.

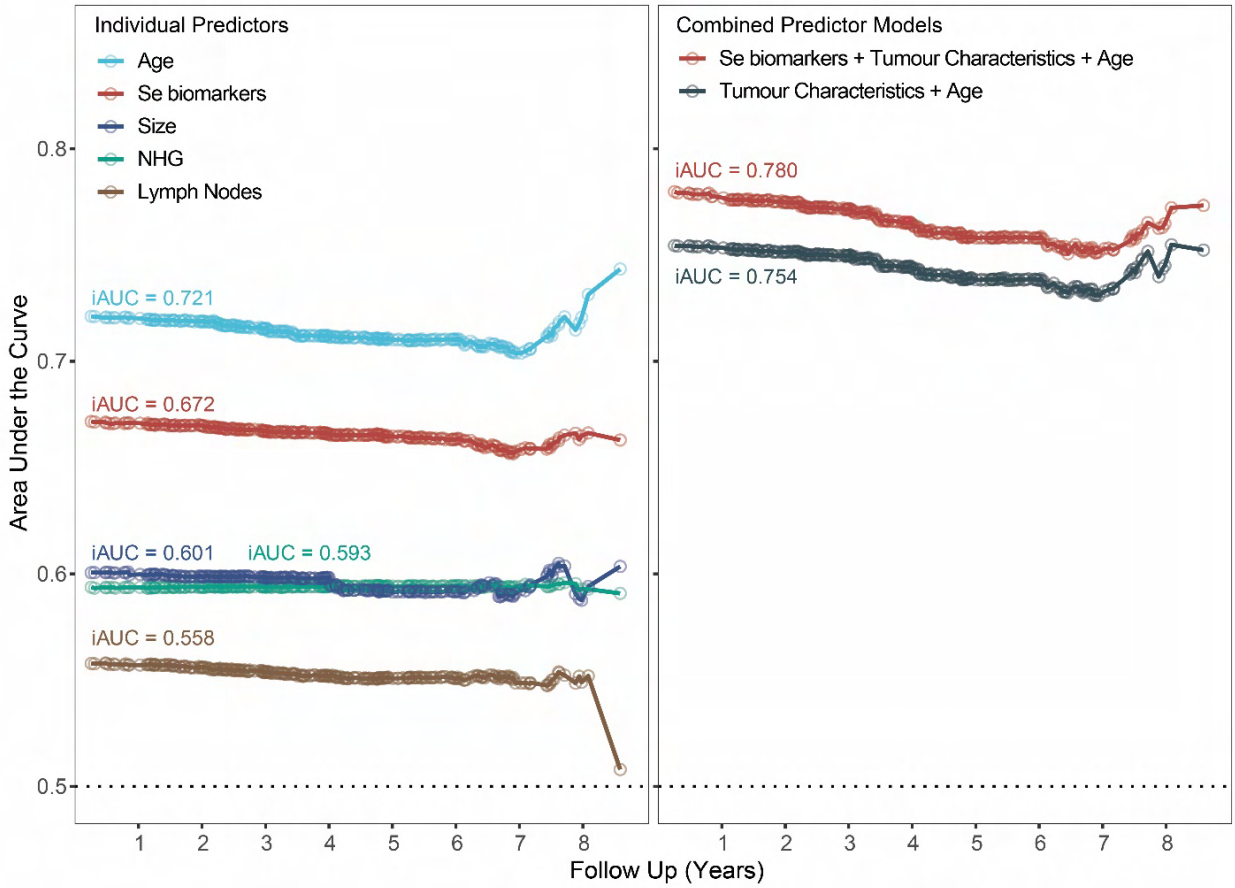

Figure 14. *Time-resolved predictive value of selenium biomarkers for survival. Time resolved receiver operating characteristic (ROCt) analyses were conducted at each time of an event(death), and areas under the curve (AUCt) for each ROC analysis were extracted, for each biomarker/model. AUCt were visualized as line plots and compared visually. An integrated AUC was computed (iAUC), in order to compare overall predictive value. The dotted line at an AUCt of 0.5 represents a predictor without value, i.e., random predictor, while an AUCt of 1.0 would represent a predictor with 100% sensitivity and specificity. (From Demircan K. et al., 2021 (ref. 35), Figure 6, with modifications.)*

3.2. Study 2: SELENOP-autoantibodies and breast cancer prognosis (83)

3.2.1. Baseline characteristics according to autoimmunity to SELENOP

Autoimmunity to SELENOP (SELENOP-aAb) was determined in 1988 patient samples. Judging by the unbiased statistical cut-off, which corresponds to the binding index of 3.0, 7.65% (152 women) exhibited SELENOP-aAb positivity. **Table 5** compared clinical and tumor characteristics according to SELENOP-aAb positivity in all patients. No clinical or tumor characteristics were different between the two groups except age, which was slightly higher in the participants positive for SELENOP-aAb

Table 5. Clinical and tumor characteristics according to SELENOP-aAb positivity.

Characteristic	**SELENOP-aAb negative** n = 1,836	**SELENOP-aAb positive** n = 152	p-value *
Age (y)	*64 (53, 70)*	*66 (56, 72)*	0.031
Menopause			0.4
Pre-menopausal	343 (19%)	22 (15%)	
Post-menopausal	1,401 (77%)	123 (81%)	
Uncertain	77 (4.2%)	6 (4.0%)	
Tumor side			0.063
Left	943 (51%)	90 (59%)	
Right	893 (49%)	62 (41%)	
Size of tumor (mm)	*16 (11, 23)*	*15 (10, 22)*	0.2
Involved lymph nodes			0.3
>=4	164 (9.3%)	10 (6.9%)	
1-3	430 (24%)	29 (20%)	
No Involvement	1,134 (64%)	101 (70%)	
Submicrometastasis	37 (2.1%)	5 (3.4%)	
(missing)	71	7	
Histological grade			0.4
I	348 (19%)	35 (24%)	
II	846 (47%)	68 (47%)	
III	591 (33%)	43 (29%)	
(missing)	51	6	
Ki67 Expression			0.2
Low	203 (46%)	22 (58%)	
High	236 (54%)	16 (42%)	
(missing)	1,397	114	
Histopathological type			0.087
Ductal	1,467 (80%)	122 (80%)	
Lobular	245 (13%)	15 (9.9%)	
Other	96 (5.2%)	9 (5.9%)	
Ductal + Lobular/Other	26 (1.4%)	6 (3.9%)	
HER2 expression			0.7
Negative	1,586 (87%)	128 (86%)	
Positive	227 (13%)	20 (14%)	
ER expression			0.2
Negative	254 (14%)	27 (18%)	
Positive	1,578 (86%)	124 (82%)	
PGR expression			>0.9
Negative	514 (28%)	43 (28%)	
Positive	1,318 (72%)	108 (72%)	

Median (IQR); n (%), number of missing values only provided if >2% of all.

HER2 = Human epidermal growth factor receptor 2, ER = Estrogen receptor, PGR = Progesterone receptor.

* Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test

(From Demircan K. et al., 2022, Table 1 (ref. 83), with modifications.)

3.2.3. Autoantibody titers in serum samples of the patients at baseline

In **Figure 15A**, serum titers of individual women at the time of diagnosis are displayed and show a right-skewed distribution, particularly visible in the non-logarithmic y-axis. In **Figure 15B**, age of positive and negative women was visualized in boxplots, and difference was tested by Wilcoxon rank-sum test. Women positive for SELENOP-aAb were slightly older (p = 0.031).

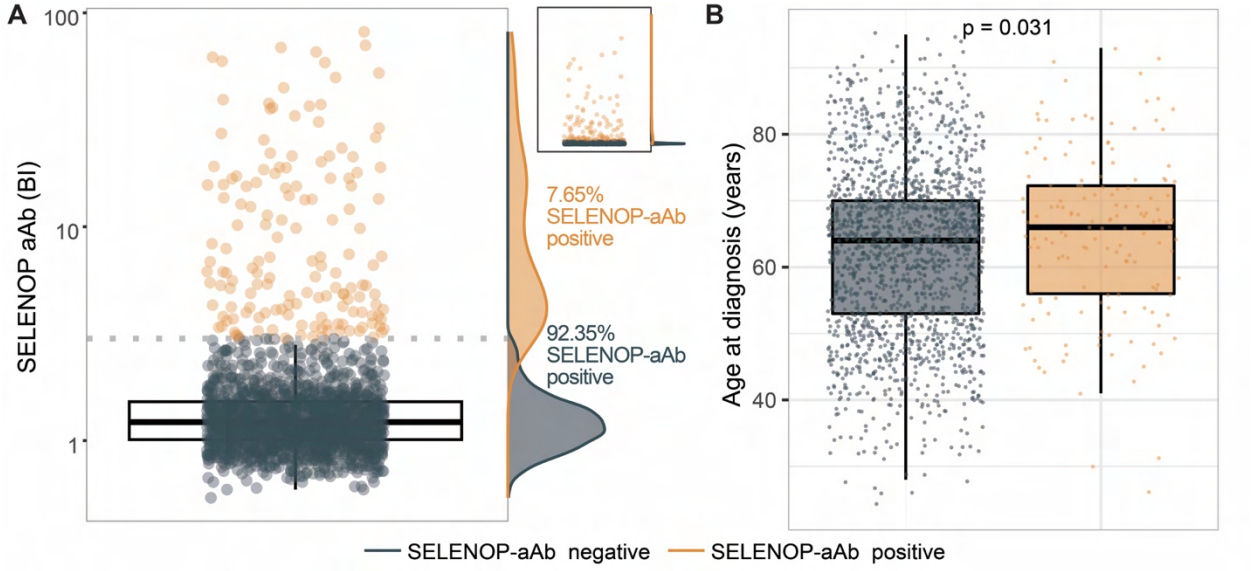

Figure 15. *SELENOP-aAb titers and age distribution according to positivity.* ***A*** *Distribution of SELENOP-aAb titers using a log-transformed y-axis. The small boxplot within displays an overview of the distribution using a non-logarithmic y-axis, in order to display the right-skewness.* ***B*** *Comparison of age according to SELENOP-aAb positivity. Wilcoxon rank sum test quantified the statistical difference. (From Demircan K. et al., 2022 (ref. 83), Figure 1, with modifications.)*

3.2.4. Correlation of the autoantibodies with other selenium biomarkers

Correlation of autoantibody titers with the three other selenium biomarkers was examined in order to detect dose-dependent effects of autoantibodies to SELENOP on Se transport potentially affecting the other Se biomarkers. In **Figure 16A** and **B** there was a positive correlation between SELENOP-aAb titers and both selenium and SELENOP above a threshold of BI=10 (R = 0.336, p = 0.009 and R = 0.273, p = 0.037, respectively). SELENOP-aAb titers were not correlated with GPx3 activity (**Figure 16C**), although in the overall population, the parameter GPx3 activity also correlates with selenium and SELENOP (**Figure 11B and C**).

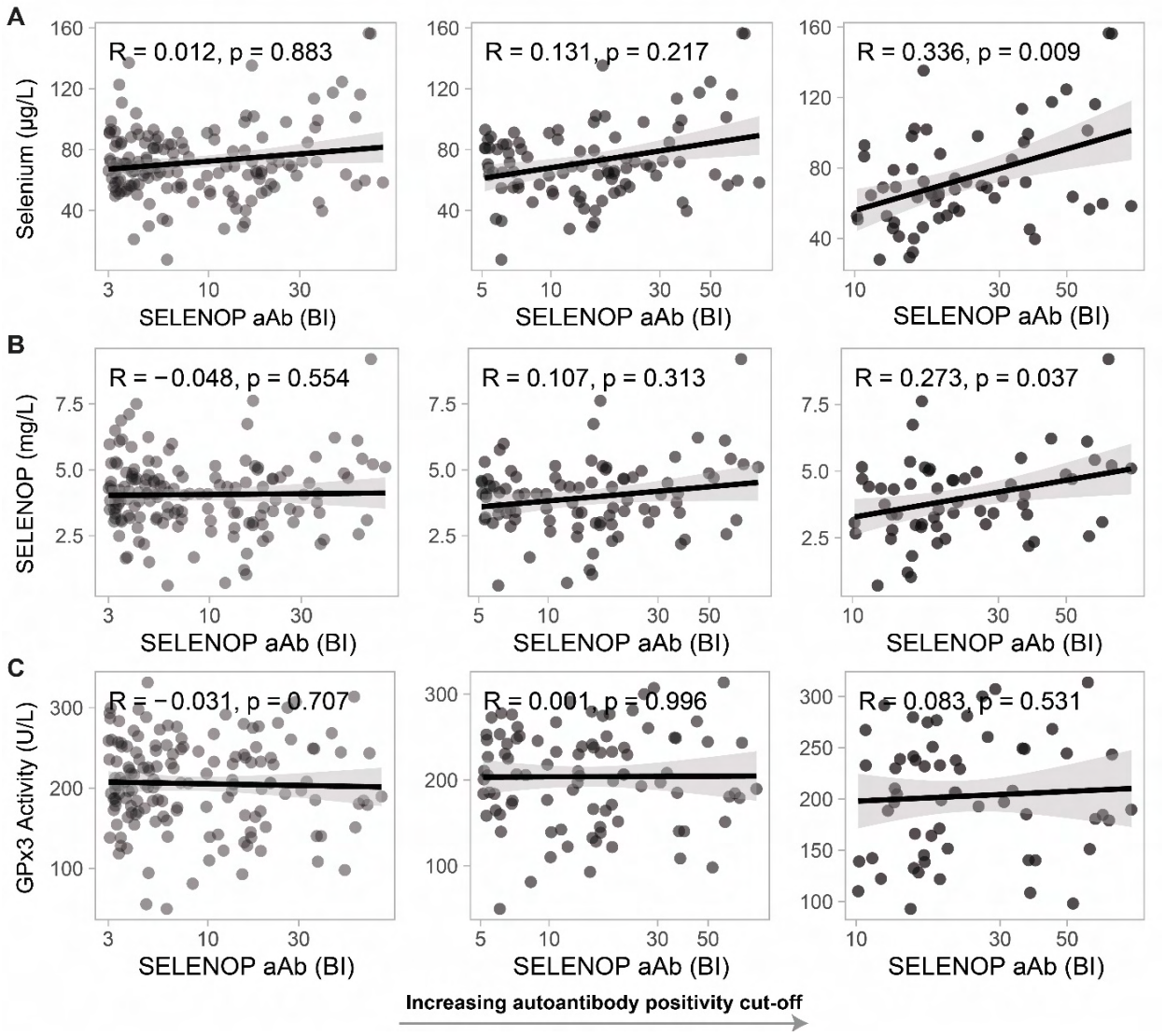

Figure 16. *SELENOP-aAb titers in relation to selenium biomarkers. Spearman's Rank correlation test was employed.* ***A*** *SELENOP-aAb titers in relation to total serum Se.* ***B*** *Correlation between SELENOP-aAb titers with serum SELENOP.* ***C*** *Correlation between SELENOP-aAb titers with GPx3 activity. (From Demircan K. et al., 2022, Figure 2 (ref. 83), with modifications.)*

3.2.5. Survival according to baseline SELENOP-aAb positivity

Next, it was investigated whether the survival of SELENOP-aAb positive patients differed from those who were SELENOP-aAb negative. In Kaplan Meier analyses (**Figure 17A**) these patients had a higher mortality (p = 0.0064) and recurrence rate (p = 0.0085) compared with SELENOP-aAb negative patients. Next, the analyses were stratified by low/high SELENOP levels, according to the median of the cohort. The poor prognosis in patients with autoantibodies was distinct in those who already had low serum SELENOP concentrations (p = 0.0014 for mortality and p = 0.00015 for recurrence) (**Figure 17B** and **C**).

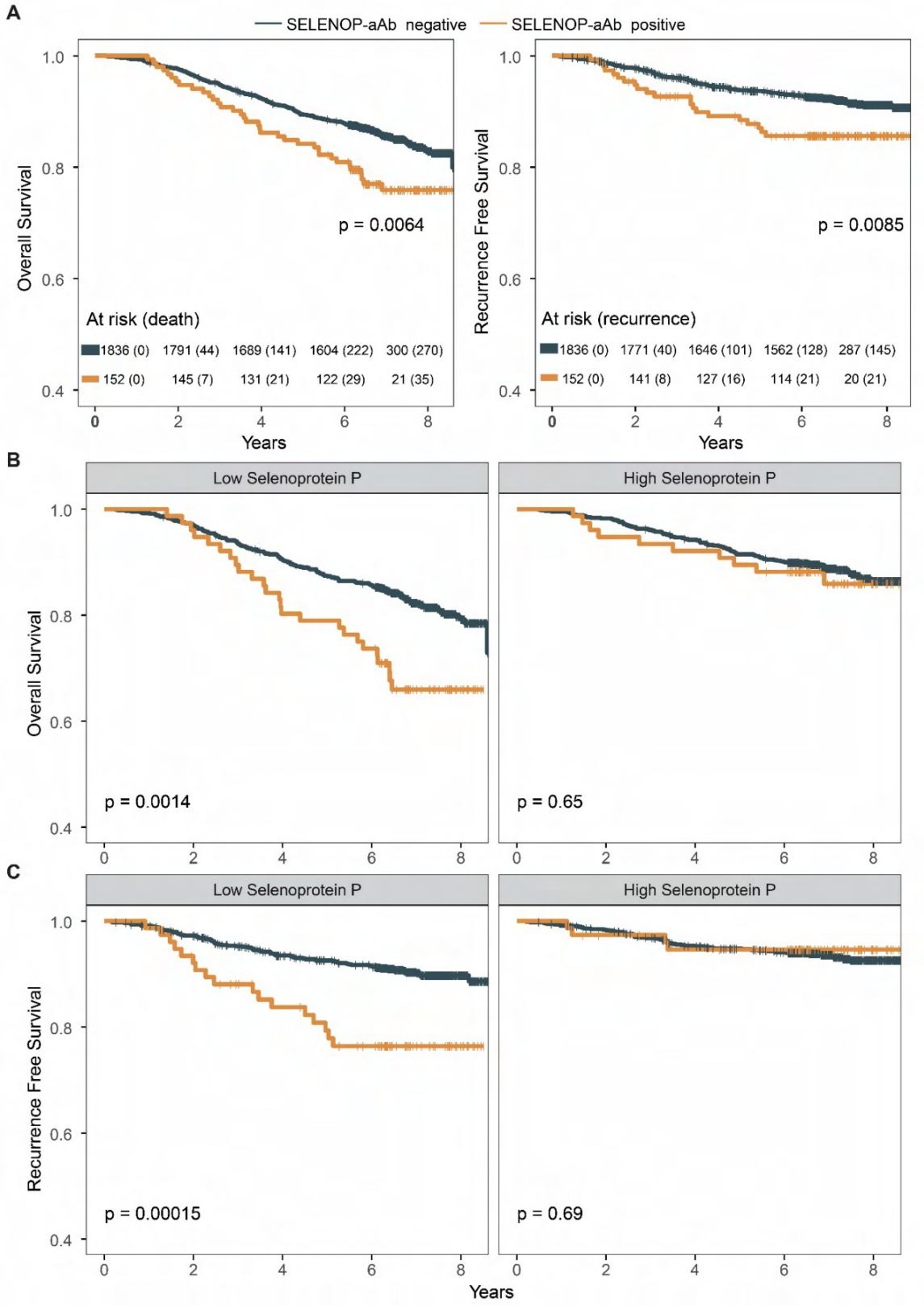

Figure 17. *Kaplan Meier analyses for survival according to SELENOP-aAb. Kaplan Meier curves served to visualize survival. Differences were detected employing the log-rank test.* ***A*** *Survival and recurrence free survival chances according to SELENOP-aAb positivity.* ***B*** *Survival chances according to SELENOP-aAb, in low and high SELENOP subgroups.* ***C*** *Recurrence free survival chances according to SELENOP-aAb, in low and high SELENOP subgroups. (From Demircan K. et al., 2022 (ref. 83), Figure 3, with modifications.)*

The analyses proved to be robust when adjusting for various confounders in fully adjusted Cox regression models, as presented in **Table 6**. Dose dependency was assessed by entering the parameter as a continuous variable in fully adjusted models. HR (95% CI) for one increment on the log-scale of SELENOP-aAb titers was 1.31 (1.13-1.51) for overall survival and 1.25 (1.01-1.55) for recurrent disease. Both associations were linear (p for nonlinearity > 0.05).

Table 6. Cox regression for SELENOP-aAb in relation to survival.

		At risk (death)	**Crude***		**+ Age†**		**Full adjustment‡**	
	SELENOP-aAb	n	HR	95% CI	HR	95% CI	HR	95% CI
Mortality								
	Neg. (**ref.**)	1,836 (272)	—	—	—	—	—	—
	Pos.	152 (35)	1.62	1.14, 2.31	1.45	1.02, 2.06	1.41	0.98, 2.02
Recurrence								
	Neg. (**ref.**)	1,836 (146)	—	—	—	—	—	—
	Pos.	152 (21)	1.83	1.16, 2.89	1.79	1.13, 2.84	1.87	1.17, 2.99
Mortality								
Low SELENOP								
	Neg. (**ref.**)	918 (166)	—	—	—	—	—	—
	Pos.	76 (25)	2.02	1.32, 3.08	1.68	1.10, 2.58	1.49	0.96, 2.33
High SELENOP								
	Neg. (**ref.**)	918 (106)	—	—	—	—	—	—
	Pos.	76 (10)	1.18	0.61, 2.27	1.16	0.60, 2.24	1.24	0.63, 2.42
Recurrence								
Low SELENOP								
	Neg. (**ref.**)	918 (86)	—	—	—	—	—	—
	Pos.	76 (17)	2.72	1.60, 4.60	2.61	1.54, 4.43	2.69	1.56, 4.64
High SELENOP								
	Neg. (**ref.**)	918 (60)	—	—	—	—	—	—
	Pos.	76 (4)	0.83	0.29, 2.32	0.83	0.29, 2.32	0.88	0.31, 2.52

HR = Hazard Ratio, CI = Confidence Interval, ref. = reference group

***** Non-adjusted.

† Adjustment for age.

‡ Adjustment for age, menopausal status, number of lymph nodes involved, diagnosis mode, size of the tumor, ER expression, PGR expression, HER2 expression, Nottingham histological grade, histopathological type. Missing values in adjustment factors were imputed via multiple imputation.

(From Demircan K. et al., 2022 (ref. 83), Table 2 and 3, with modifications.)

3.3. Study 3: Serum selenoproteins and tumor selenotranscriptome (84)

3.3.1. Cancer selenotranscriptome and correlation with serum biomarkers

In 1453 patients, RNA-seq was conducted in tumors in addition to the measurement of serum selenium biomarkers. RNA-seq included sequencing mRNA expression of the 25 human selenoprotein genes, as visualized in **Figure 18A**. *GPX6* mRNA was not expressed in breast tumor tissue, and *SELENOV* displayed a very low expression in a small number of tumors only. *GPX1 and GPX4* displayed the highest expression among all selenoprotein mRNA. The correlation matrix in **Figure 18B** displays the interrelationship between tumor selenoprotein mRNA with each other as well as their correlation with circulating selenium biomarkers. Tumor selenoprotein mRNAs displayed a heterogeneous correlation pattern, although isoenzymes within the same family, e.g., deiodinases, glutathione peroxidases or thioredoxin reductases mostly displayed positive correlations.

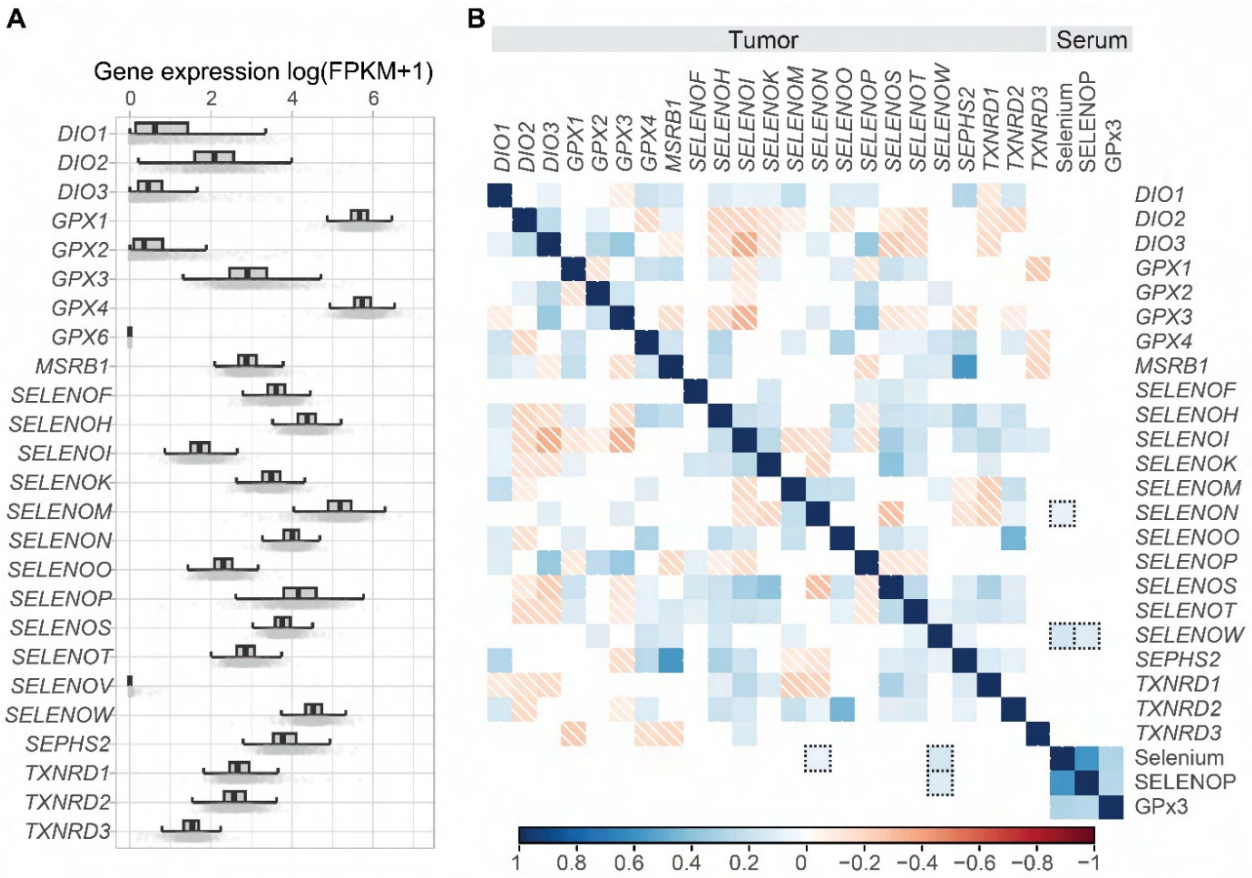

Figure 18. *Correlation of serum selenium with tumor selenoprotein mRNA.* ***A*** *mRNA expression levels of the 25 selenoprotein genes in tumor tissues of the patients.* ***B*** *Correlation of selenoprotein mRNA among each other and with serum selenium biomarkers. Correlation coefficients were detected employing Spearman's Rank correlation test. Correlations were corrected for multiple testing, considering the 23 selenoprotein genes expressed in the tumors and tested in this analysis. (From Demircan K. et al., 2023 (ref. 84), Figure 1, with modifications.)*

Among tumor selenoprotein mRNAs, highest correlation was observed between *SEPHS2* and *MSRB1* (R = 0.57, p < 0.001), highest negative correlation was observed between *SELENOI* and *DIO3* (R = -0.34, p < 0.001). Tumor selenoprotein RNA expression was mostly unrelated to circulating selenium biomarkers, except for *SELENOW*, which was positively correlated with serum selenium and SELENOP (R = 0.18 and R = 0.13, p < 0.001), and *SELENON* with serum selenium (R = 0.082, p < 0.001).

3.3.2. Interactions of serum selenium with selenotranscriptome and mortality

In an unbiased analysis, effect modification of the associations between each selenoprotein gene with mortality by circulating selenium levels was tested i.e., whether the association of certain selenoprotein mRNA with prognosis is dependent on serum selenium. Cox regression models with a multiplicative interaction term for each gene were computed (**Figure 19**), whereby all models were adjusted for various potential confounders, as described in the statistical analysis section. The unbiased analysis identified an effect modification for three genes only, namely for *DIO1*, *DIO3*, and *SELENOM*.

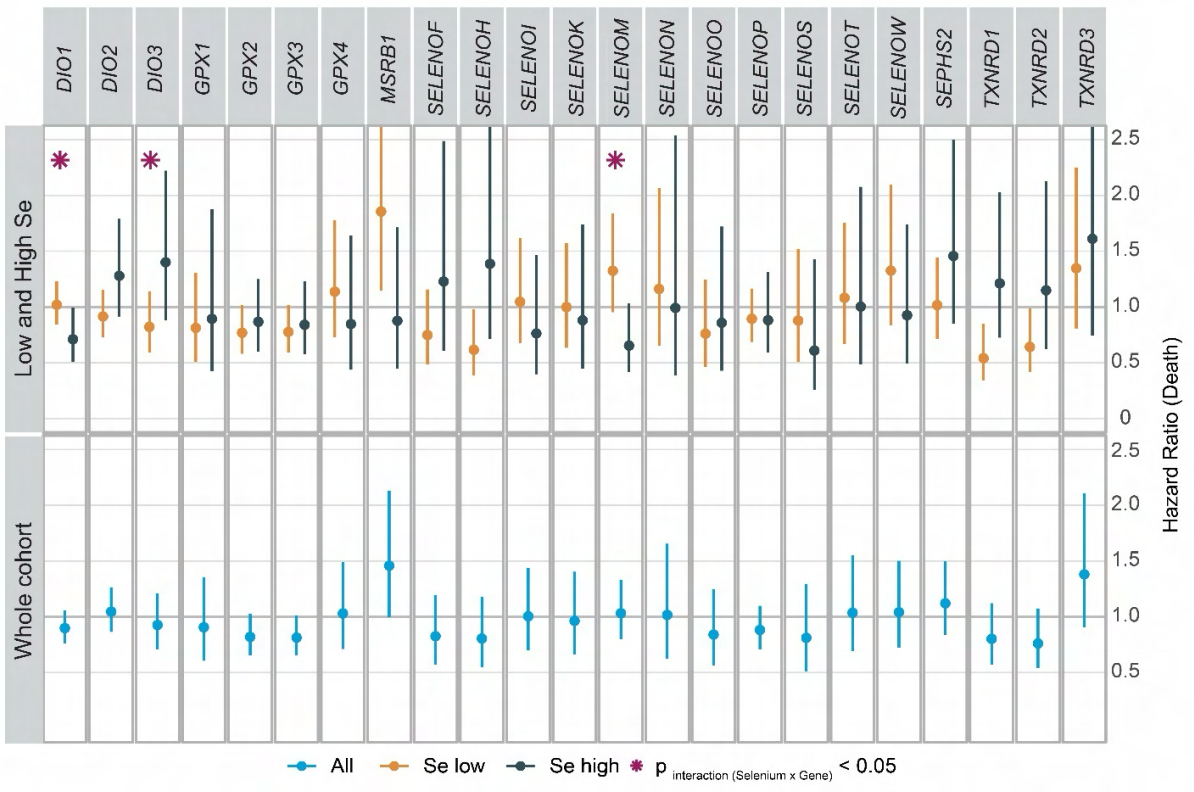

Figure 19. *Interactions of serum selenium with tumor selenoprotein mRNAs and survival. Cox proportional hazards models were employed for each selenoprotein mRNA in the whole cohort as well as in subgroups according to median of serum selenium concentrations. A multiplicative interaction term with serum selenium concentrations was included in the models to test for true dose-dependent effect modification. Purple asterisk marks models with a statistically significant interaction. (From Demircan K. et al., 2023 (ref. 84), Figure 2, with modifications.)*

3.3.3. Interactions of selenium with *DIO1*, *DIO3*, *SELENOM* and survival

The dose-dependent interactions identified were visualized as contour plots (**Figure 20**). With increasing circulating selenium, patients with higher *DIO1* mRNA expressing tumors had a favourable prognosis (**Figure 20A**, $p_{interaction} < 0.001$). In contrast, with increasing selenium, patients with tumors with increasing *DIO3* mRNA had higher mortality (**Figure 20B**, $p_{interaction} = 0.02$). The interaction between circulating selenium and *SELENOM* was similar to *DIO1* (**Figure 20C**, $p_{interaction} = 0.038$). The opposing effects of *DIO1* and *DIO3* potentiated by increasing selenium levels point to an involvement of local thyroid hormone activation, considering the opposing effects of DIO1 (positive) versus DIO3 (negative) on thyroid hormone activation (**Figure 20D**). The observed interactions were robust when including treatment methods applied as listed in Table 2 as confounders.

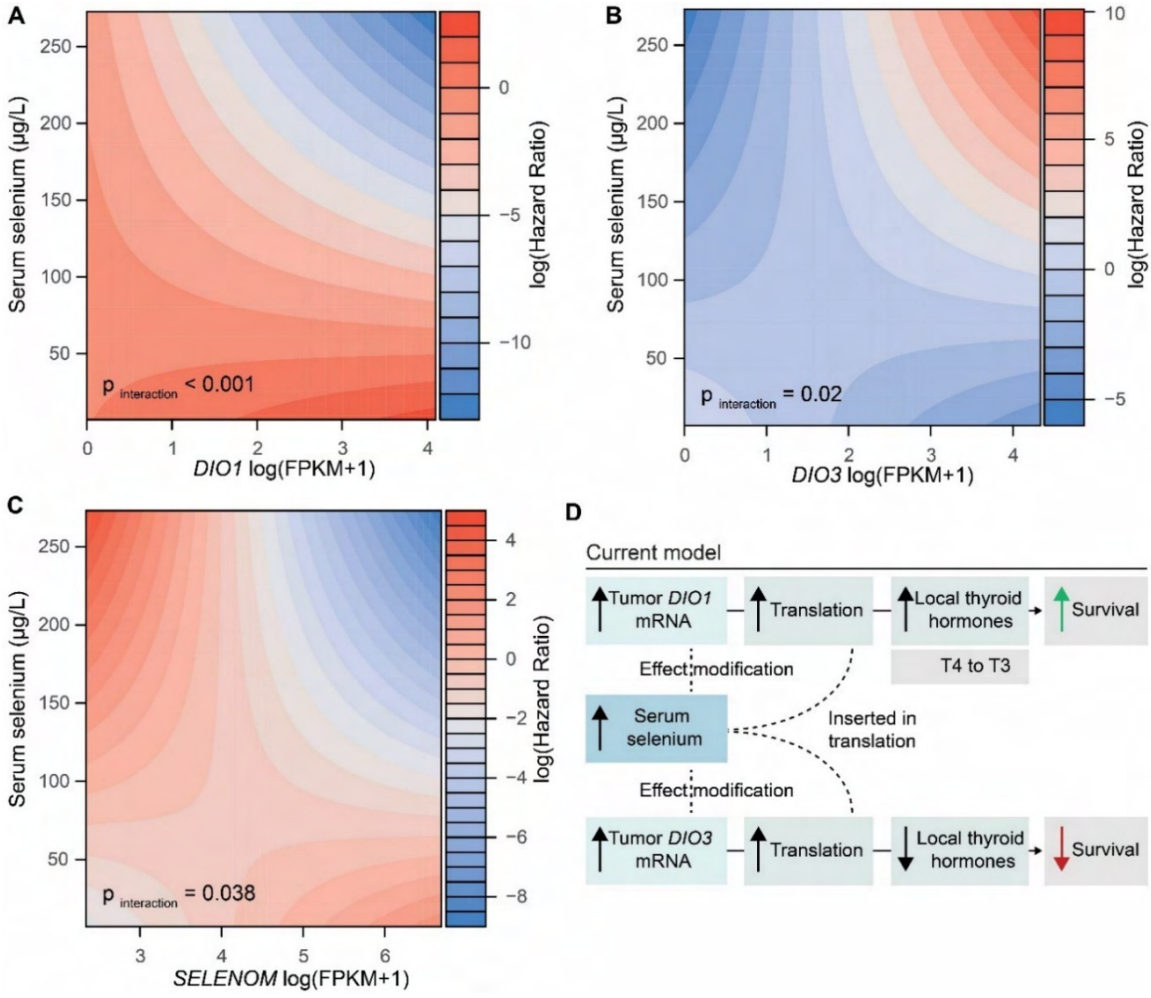

Figure 20. *Contour plots displaying the complex dose-dependent interactions of selenoprotein mRNA expression, Se status and survival.* ***A*** *Interaction of selenium and DIO1.* ***B*** *Interaction of selenium and DIO3.* ***C*** *Interaction of selenium and SELENOM.* ***D*** *Model for mechanism of action based on the opposing effects of DIO1 and DIO3 on survival, considering their inverse biological effects on thyroid hormone activation. (From Demircan K. et al., 2023 (ref. 84), Figure 3, with modifications)*

4. Discussion

4.1. Short summary of the results

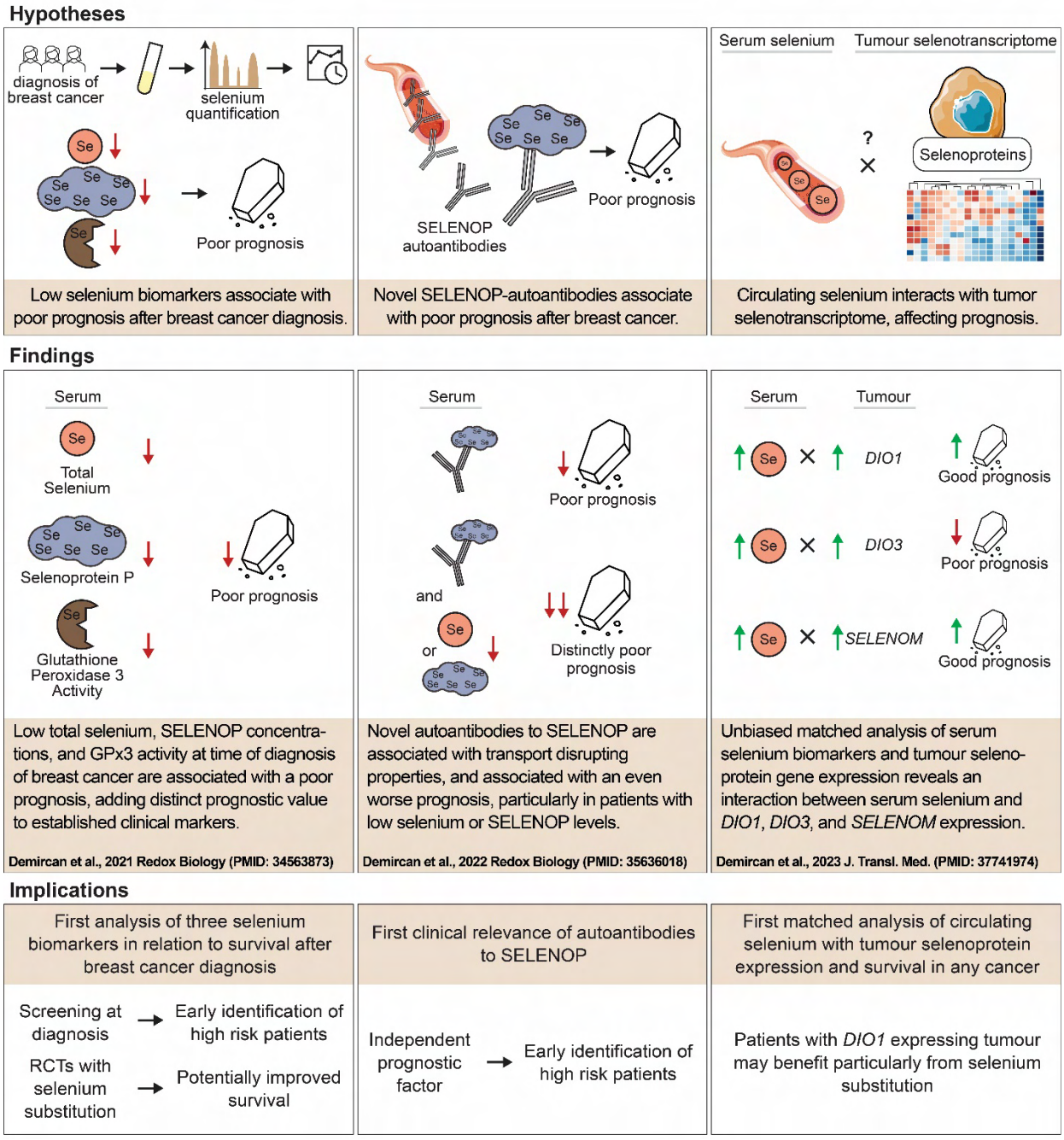

Figure 21. *Simplified summary of hypotheses, findings, and implications. Created on Adobe Illustrator 2021. Parts were drawn with Smart Servier Medical Art licensed under CC BY 3.0. Hypotheses, findings, and implications are from Demircan K, et al 2021 (ref. 35), 2022 (ref. 83), and 2023 (ref. 84).*

This thesis included three consecutive publications, together contributing to a better understanding of the association of circulating serum selenium/selenoproteins and tumor selenoprotein mRNAs with prognosis after breast cancer diagnosis (**Figure 21**).

In the first study, a comprehensive analysis of selenium status in relation to prognosis after breast cancer diagnosis was conducted. The study provides data for a negative dose-dependent association of all three selenium biomarkers with mortality after breast cancer diagnosis, which was independent of various established prognostic parameters of breast cancer, and other potential confounders. In particular, patients with low levels of all three biomarkers displayed a distinct risk of a poor prognosis, characterized by a ~50% survival chance in ~8 years, as compared to a ~85% survival probability after 8 years of follow-up when at least one selenium biomarker was in the top quintile of the cohort. A composite marker of the three biomarkers added a substantial prognostic value to the established clinical markers in determining mortality.

The second study investigated SELENOP-aAb titers as a novel biomarker of prognosis after breast cancer. The titers were associated with higher selenium and SELENOP levels, without simultaneous increase in GPx3 activity in serum, indicating transport disruption. SELENOP-aAb titers dose-dependently associated with higher mortality and recurrence, independent of various potential confounders. Particularly, these associations were most prominent selenium deficiency, additionally arguing for a potential causal relationship between poor selenium status and autoimmune selenium transport impairment with high mortality risk.

The third study conducted the first matched analysis of circulating serum selenium markers and gene expression of tumor selenoproteins. Serum selenium and SELENOP were correlated to SELENOW and SELENON, without interfering with other selenoprotein genes. Unbiased analyses revealed that serum selenium interacts with DIO1, DIO3, and SELENOM in relation to mortality. A simultaneous increase in serum selenium with increasing DIO1 or SELENOM tumor expression was associated with a favourable prognosis, while increasing selenium and DIO3 was associated with a poor prognosis.

4.2. Interpretation and potential mechanisms

This thesis is the first to investigate different biomarkers of selenium in association with prognosis after breast cancer diagnosis, and the first to match blood selenium biomarkers with data of tumor selenotranscriptome. Nevertheless, it is in line with the current literature assessing total selenium or selenium intake as biomarkers in relation to breast cancer prognosis in smaller studies (81, 82, 106). The association of low selenium with a poor prognosis was also reported for other cancer entities such as melanoma, lung cancer,

larynx cancer and colorectal cancer (107-111). Hence, the biomarkers and potential mechanisms identified in this thesis may not be specific to breast cancer, but also provide prognostic value for other malignancies. Beside prognosis assessed in this thesis, more data are needed to establish whether the biomarkers of selenium status quantified in this thesis are linked with developing malignancies, although the current literature including a recent Cochrane Review argues against a general relationship between selenium and cancer incidence (75). In our recent analysis, however, there was an association of low GPx3 activity with an increased risk of breast cancer incidence in premenopausal women, which was not observed in case of total selenium or SELENOP levels (112).

Physiologically, selenium has been proposed to act in a chemopreventive way through selenoproteins via multiple mechanisms including antioxidative defence, regulation of cellular redox status, the immune system or cellular thyroid hormone status (80). Cellular oxidative stress acts as a key contributor to DNA mutations and instability of the genome, leading to tumor initiation (113). Hence, the pivotal mechanism of action by which selenium may act on tumorigenesis is through increasing expression and function of glutathione peroxidases that are involved in antioxidative defence (114). When investigating associations between selenium biomarkers and prognosis in this thesis, the most prominent associations particularly for recurrent disease as an outcome were observed for GPx3 activity, supporting this notion (35). Indeed, *Gpx2* and *Gpx3* knockout mouse models have displayed increased tumor numbers and higher degree of dysplasia in various independent experimental studies (115-118). Although selenium mediates expression of these enzymes, selenium partly rescued the phenotype in *Gpx2* knockout models assessing intestinal cancer, indicating that selenium acts through other pathways additionally (116). GPx4 is the key regulator of a newly identified form of cell death called ferroptosis (119). It has been shown that selenium in form of Sec in the catalytic centre is needed for the proper hydroperoxidase activity of GPx4, and that enzyme variants containing cysteine (Cys) instead of Sec are conferring high sensitivity to the cells to ferroptosis initiated by peroxides (120). Selenium supplementation was shown to be an essential regulator of GPx4 expression and protective function in cells sensitive to ferroptosis, e.g., follicular helper T-cells, and potentially related immune cells within the tumor microenvironment (TME) (121, 122). Ferroptosis associates with poor prognosis in cancer by acting on antitumorigenic cells of the TME, proposing a potential mechanism through which beneficial effects of selenium may act on cancer initiation and progression, although there is no experimental clinical data directly supporting this mechanism (121).

Selenium regulates expression and activity of DIOs that are involved in systemic thyroid hormone metabolism and control of local thyroid hormone action (123). DIOs serve as pivotal regulators of thyroid hormone metabolism, which is a key regulator of differentiation and proliferation of malignant and normal cells (124). Hence, thyroid hormone status was investigated thoroughly as a potential prognostic factor in cancer, yet hitherto conducted studies have yielded conflicting results. Both hyper- and hypothyroidism have been linked to cancer progression in epidemiological studies (125-128). This lack of clear evidence may be a reflection of the manifold mechanisms involved in thyroid hormone metabolism, as circulating thyroid hormone status does not necessarily mirror local thyroid hormone action, and hence effects on cancer cells or cells within the TME. DIO expression is altered across many cancer types, and DIO regulated increase in local thyroid hormone action has been shown to promote enhanced differentiation, transitioning cancer cells into a less aggressive phenotype in various cancer entities (129). Nevertheless, the effects of DIO and thyroid hormones on tumorigenesis and progression appear to be pleiotropic and to change across different stages of dysplasia and carcinogenesis (129). In the third study included in this dissertation, of all selenoproteins, interactions of circulating selenium were observed with *DIO1* and *DIO3*. This finding is in line with the fact that within selenoproteins, DIOs are among the most sensitive targets to regulation by selenium intake (30). Elements involved in local thyroid hormone regulation, such as DIO3 or thyroid hormone receptor 2 alpha expression have already been implicated as prognostic factors in breast cancer (125). *Dio3* knockdown in a murine basal cell carcinoma model displayed reduced tumor growth (130). DIO1 and DIO3 are involved in mostly opposing actions with regard to thyroid hormone regulation. DIO3 is the most important regulator of thyroid hormone inactivation, catalyzing deiodination of T4 to rT3 or of T3 to 3,3'-diiodothyronine (T2) (124). DIO1 catalyzes outer ring deiodination of thyroxine (T4) to triiodothyronine (T3), and in the liver, it can also catalyze deiodination of T4 to reverse triiodothyronine (rT3), i.e. contributing to both thyroid hormone activation and inactivation (124). In this thesis, favourable associations for *DIO1* and detrimental effects for *DIO3* were observed, dependent on circulating selenium levels. In contemplation of the physiological, opposing effects of these two deiodinases, the results point to a favourable association of increased local thyroid hormone activation with improved survival. This potential mode of action needs to be further investigated in mechanistic studies. In summary, the potential mechanisms of the observed associations are not fully established yet, however the findings from this thesis in the context of existing literature argue

for a role of the GPx family as well as the selenium-mediated modulation of the DIO expression in tumor tissue as an important mode of action involved in the positive association of selenium status and serum selenoproteins in favourable prognosis of patients with breast cancer.

4.3. Implications

The findings from this thesis offer promising opportunities for clinical practice in breast cancer care. Specifically, the first study established a strong association between serum selenium deficiency at diagnosis of breast cancer and patient outcomes including mortality and recurrence rates. This suggests that assessing selenium deficiency could become an important aspect of breast cancer management, adding prognostic value and identifying patients with specific nutrition-related risks. Notably, the blood-based biomarkers identified a subset of patients characterized by selenium deficiency in all biomarkers (triple selenium deficient), facing a remarkably low relative survival probability of approximately 50% only, which is exceptionally low for non-metastatic breast cancer (48). This identification can be made already at the time of diagnosis, i.e., very early in the disease course, offering clinicians an additional readily available tool to identify high-risk patients in need of particular attention and care, and to be considered for intensified (neo)-adjuvant therapeutic approaches for improving their survival.

The independent, coherent, and dose-responsive associations with prognosis across total Se, SELENOP, GPx3 activity and the novel SELENOP-aAb argue for a potential clinical efficacy of selenium substitution in improving survival outcomes of patients with proven selenium deficiency. While using selenium status as a surrogate prognostic tool can readily be implemented into clinical routine, however, sufficiently powered RCTs are needed to establish a potential causal link and demonstrate clinical benefit from correcting the deficit.

The second study of this thesis did not only establish a new biomarker for selenium status (SELENOP-aAb) and for predicting breast cancer prognosis, but also provides a tool to identify patients with a “functional” selenium deficiency that is not directly accessible using the other three biomarkers. Accordingly, when conducting RCTs, it should be considered that baseline stratification of patients according to selenium status as assessed by one of the three established biomarkers and according to SELENOP-aAb could be crucial, as

particularly those with a deficient selenium status or autoimmunity to SELENOP may benefit most. This notion is supported by the NPC trial, which observed a benefit of selenium on chemoprevention only in participants displaying low baseline selenium (77). Albeit this finding referred to prostate cancer, as most of the subjects were men. The observed threshold-effects in the first study of this thesis also supports the need for baseline stratification. Finally, the third study of this thesis identified tumor selenoprotein mRNA expression patterns that can guide personalized therapy with selenium supplementation based on tumor biology. While there was a favourable association of higher selenium with improved prognosis in the overall population, patients with *DIO1* and *SELENOM* expressing tumors may be even more likely to benefit from selenium supplementation, which should be considered in future RCTs.

4.4. Strengths and limitations

4.4.1. Sample size, type I and II errors

Firstly, all studies were conducted as part of the SCAN-B trial, which is currently among the largest prospective studies investigating breast cancer survival in a consecutive, population-based manner. The sample size of close to two thousand patients for serum analyses and 1.5 thousand patients for tumor RNA-seq analyses provided a sample size large enough to study the most relevant endpoint of mortality, despite improving breast cancer survival rates. Nevertheless, the statistical power was lower for the endpoint of recurrent disease. Therefore, the risk for type two error, i.e., a lack of null hypothesis rejection despite a potential relationship was increased. The comprehensive analyses in the three studies with many comparisons increase the risk of a type one error. However, the consistent concurrence of the results across all selenium biomarkers, their dose dependent associations and the high biological plausibility largely minimize the potential possibility of chance discoveries.

4.4.2. Outcomes/covariates and missing data

Correct classification of the primary outcome measure could be ensured due to extraction of data via Swedish National Registries, where mortality data is documented for each Swedish citizen, also in case of change of residence within the study period. However, for recurrence as an outcome, hospital records were used and may be subject to underreporting. Nevertheless, an association between the under-registration of recurrences with

selenium status and hence the risk for a bias is unlikely. Beside outcome measures, clinical and histopathological covariates used for adjusting the statistical analyses also derived from National Registries. Information bias is low, as the covariates reported to National Registries were assessed by the physicians, rather than relying on self-report by the patients. An independent study on data validity from the used registries was conducted previously, confirming outcome data completeness of > 99.9%, and an exact agreement of reported covariate data of > 90% (87, 88). Missing covariate data in the study consisted of <1% of all variables. In the main analyses, the state-of-the-art multiple imputation method was used in order to not exclude those patients.

4.4.3. Adjustment for confounding variables

Overall, the valid and large database of covariates facilitated an extensive adjustment for confounding, and hence the possibility to study true independent effects. Most important clinical prognostic determinants of breast cancer survival were adjusted for in all analyses presented. Despite the efforts, a risk for residual confounders remains a general issue of concern in observational prospective studies (131). Body mass index, alcohol intake, tobacco use and socioeconomic status, have been linked to both selenium and mortality, but were not accessible for this analysis (132, 133). However, it is important to note that these potential confounders are unlikely to largely affect the results, based on the complex interplay observed. For instance, while higher BMI has been shown to associate with elevated selenium levels, overweight and obesity are linked to increased mortality, which would even enlarge the observed associations (134). Furthermore, moderate to high alcohol intake, which was shown to increase mortality, was linked to increased serum selenium and SELENOP levels, i.e., would also rather potentiate the associations observed (135). However, smoking is associated with a lower selenium status and increased overall mortality, and hence remains an important residual confounder (133).

4.4.4. Validity of laboratory measurements

Three established complementary biomarkers and SELENOP-aAb were measured in order to assess selenium status (33). Importantly, laboratory analyses were performed without access to any clinical information, and the unblinding process occurred only after the data from the laboratory analyses were transferred to the collaborating team in Sweden. The agreement between the biomarkers as assessed by coherent positive correlations (**Figure 11**) highlights the internal validity of the measurements, sufficient quality of the

serum samples and minimizes the chance for incorrect classification of the main exposure variable. Internal validity of the measurements was further ensured by including control sera in every measurement run of each assay, enabling assessment of intra- and inter-assay variation, which were in a low range across all assays. The correlation across all three biomarkers allowed classification of the study population as selenium deficient, as no correlation is observed in populations with ample selenium status (6). Results observed in this cohort were in a similar range compared with other large European studies, as measured in our laboratory or by others (34, 79, 93, 136-138).

4.4.5. External validity: generalizability

Multiple aspects of SCAN-B ensure a high generalizability of the observed results. (i) The study is population-based and covered the vast majority of eligible cases (85%) within the study time frame (73). This ensures a high representativity of the study population, minimizes selection bias. (ii) Multiple centres (ten) located in different cities in Sweden were involved in the study, minimizing the risk of sampling bias, and ensuring coverage of participants from rural/urban areas or differing socioeconomic backgrounds (73). (iii) SCAN-B underlies a real-world design and is fully embedded into clinical routine, therefore does not interfere with clinical decision making for the study participants.

Nevertheless, several aspects should be considered when extrapolating the results of this study to other populations. The majority of the participants are European, which limits extension of the results to diverse ethnic backgrounds, considering the breast cancer survival differences observed across different ethnic groups (139, 140). Most importantly, the results are only generalizable to populations with a similar, deficient selenium status. While this includes most of Europe, parts of Asia and Africa, most of the USA display a replete selenium status (6, 14). The potential benefit of selenium supplementation on survival may be subject to a threshold effect, therefore only providing a beneficial effect in populations with low selenium status. Patients with lower selenium status may have a lower socioeconomic status and participate less often in screening after treatment of the breast cancer, leading to diagnosis of recurrence at a more advanced stage. This detection bias may act as an intermediate step contributing to the association of low selenium and high mortality. On the other hand, non-participation in screening in patients with low socioeconomic status followed by concurrent oversight of the recurrent disease would attenuate the association of low selenium with higher recurrence. The prevalence of

7.65% of SELENOP-aAb should not be extrapolated to male subjects, as it was investigated in female participants only. In consideration that most autoimmune diseases affect predominantly women, the prevalence can be expected to be lower in males (141).

5. Conclusions

In conclusion, the three studies conducted as part of this thesis establish selenium deficiency as an independent determinant for a poor prognosis with breast cancer. Offering assessment of selenium status by Se, SELENOP, GPx3 or SELENOP-aAb in serum at time of diagnosis could be used in clinical routine to identify patients with a poor prognosis, at the earliest time of disease course, i.e., at time of diagnosis. These patients may benefit from an intensified therapy regimen. The observed associations were consistent throughout the different biomarkers, after adjustment for various confounders, in sensitivity analyses and displayed dose-dependency, suggesting a potential survival benefit of selenium substitution in deficient patients, which however needs evaluation in well-designed RCTs. The analysis of circulating selenium with selenoprotein expression in breast tumors identified an association of *DIO1* with improved prognosis and an association of *DIO3* with a poor survival, suggesting local thyroid hormone action controlled by DIOs as a possible mechanism for the observed associations. Clinically, selenium substitution may have a particular benefit for patients with *DIO1* expressing tumors.

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Statutory Declaration

“I, Kamil Demircan, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [Selenoproteins as predictors of prognosis after breast cancer diagnosis. Selenoproteine als Prädiktoren der Prognose nach Brustkrebsdiagnose.], independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of your own contribution to the publications

Kamil Demircan contributed the following to the below listed publications:

Publication 1:
Demircan K, Bengtsson Y, Sun Q, Brange A, Vallon-Christersson J, Rijntjes E, Malmberg M, Saal LH, Rydén L, Borg Å, Manjer J, Schomburg L*. Serum selenium, selenoprotein P and glutathione peroxidase 3 as predictors of mortality and recurrence following breast cancer diagnosis: A multicentre cohort study. **Redox Biology** (Impact Factor 2020: **11.799**), 2021 Nov
Contribution (please set out in detail): Study design. Pre-processing of serum samples. Laboratory analyses including TXRF measurement for total selenium concentrations, SELENOP ELISA for SELENOP concentrations and glutathione peroxidase 3 assay for glutathione peroxidase 3 activity in all 1996 serum samples. Data extraction, cleaning and manipulation. All statistical analyses including descriptive statistics, Spearman's correlation analyses, Kaplan-Meier analyses, multivariable Cox proportional hazards models, time dependent receiver operating characteristic analysis. Data visualization. Writing of the first draft of the manuscript, review and editing. All figures and tables were created according to my statistical analyses, under supervision of Prof. Lutz Schomburg and Prof. Jonas Manjer.

Publication 2:
Demircan K, Sun Q, Bengtsson Y, Seemann P, Vallon-Christersson J, Malmberg M, Saal LH, Rydén L, Minich WB, Borg Å, Manjer J, Schomburg L*. Autoimmunity to selenoprotein P predicts breast cancer recurrence. **Redox Biology** (Impact Factor 2020: **11.799**), 2022 Jul
Contribution (please set out in detail): Study design. Pre-processing of serum samples. Laboratory analyses including the selenium biomarker analyses from the first paper and in addition quantification of autoantibodies using the SELENOP-aAb immunoprecipitation assay developed in our lab. Data extraction, cleaning and manipulation. All statistical analyses including descriptive statistics, Spearman's correlation analyses, Kaplan-Meier analyses, multivariable Cox proportional hazards models, restricted cubic spline regression analyses, likelihood ratio test for assessing non-linearity. Data visualization. Writing of the first draft of the manuscript, review and editing. All figures and tables were created according to my statistical analyses, under supervision of Prof. Lutz Schomburg and Prof. Jonas Manjer.

Publication 3:
Demircan K, Bengtsson Y, Chillon TS, Vallon-Christersson J, Sun Q, Larsson C, Malmberg M, Saal LH, Rydén L, Minich WB, Borg Å, Manjer J*, Schomburg L*. Matched analysis of circulating selenium with the breast cancer selenotranscriptome: a multicentre prospective study. **Journal of Translational Medicine** (Impact Factor 2021: **8.440**), 2023 Sept
Contribution (please set out in detail): Study design. Pre-processing of serum samples and measurement of laboratory markers as mentioned in publication 1 and 2. Extraction of RNA-seq and single cell RNA-seq data, preprocessing of data, data cleaning, manipulation. All analyses including descriptive statistics, correlation matrices, RNA-seq and scRNA-seq analyses, unbiased interaction analyses in multivariable Cox proportional hazards models, interaction analyses and their visualization as contour plots. Data visualization. Writing of the first draft of the manuscript, review and editing. All figures and tables were created according to my statistical analyses, under supervision of Prof. Lutz Schomburg and Prof. Jonas Manjer.

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

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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 antioxidative 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

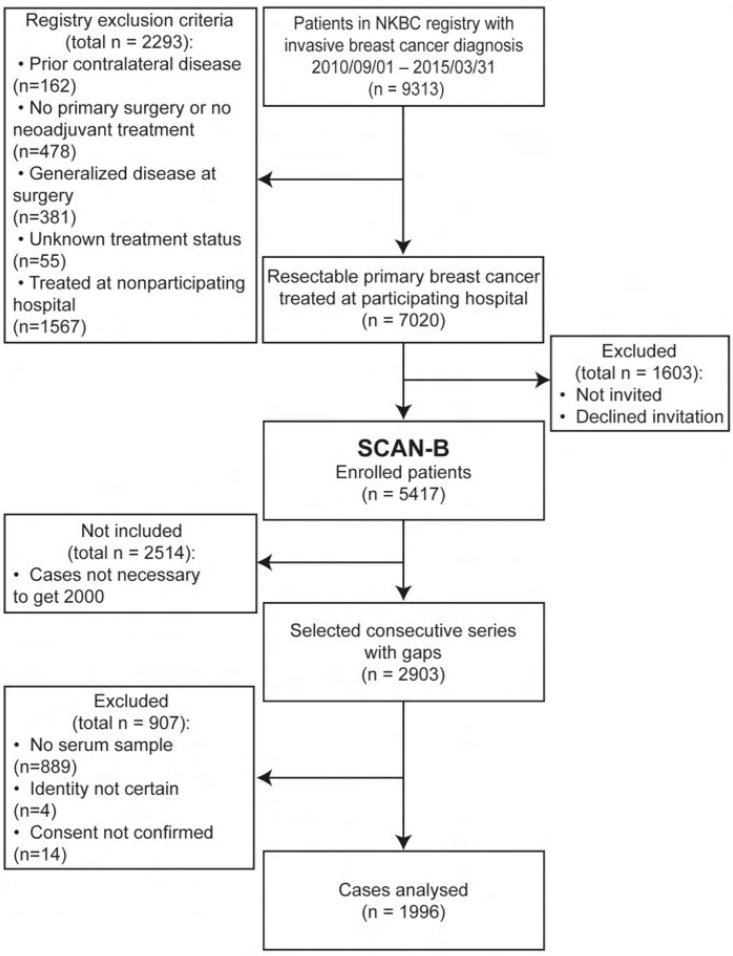

Fig. 1. Flow chart explaining inclusion and exclusion criteria.

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

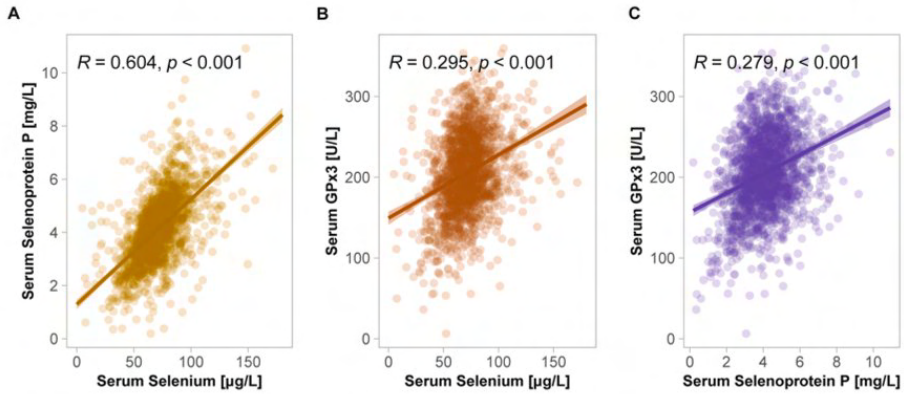

Fig. 2. Spearman's correlation analysis in patients with primary invasive breast cancer. In the total cohort, all three biomarkers of Se show significant correlations, p<0.001. (A) Total serum Se and SELENOP display a tight correlation, R = 0.604. (B) Total serum Se correlates with the GPx3 activity 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.

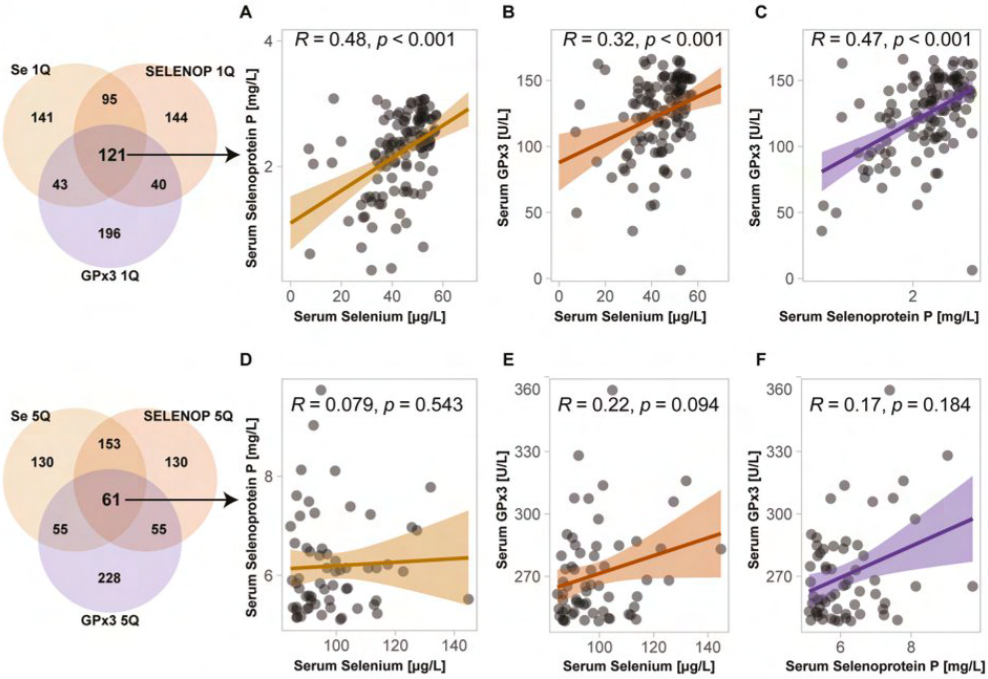

Fig. 3. Spearman's correlation of biomarkers in patients who display all biomarkers in the first or last quintile. The Venn diagram and the plots in the top section of figure (A,B,C) highlight the significant correlations between the biomarkers in samples of the 121 patients who are assigned to the first 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.

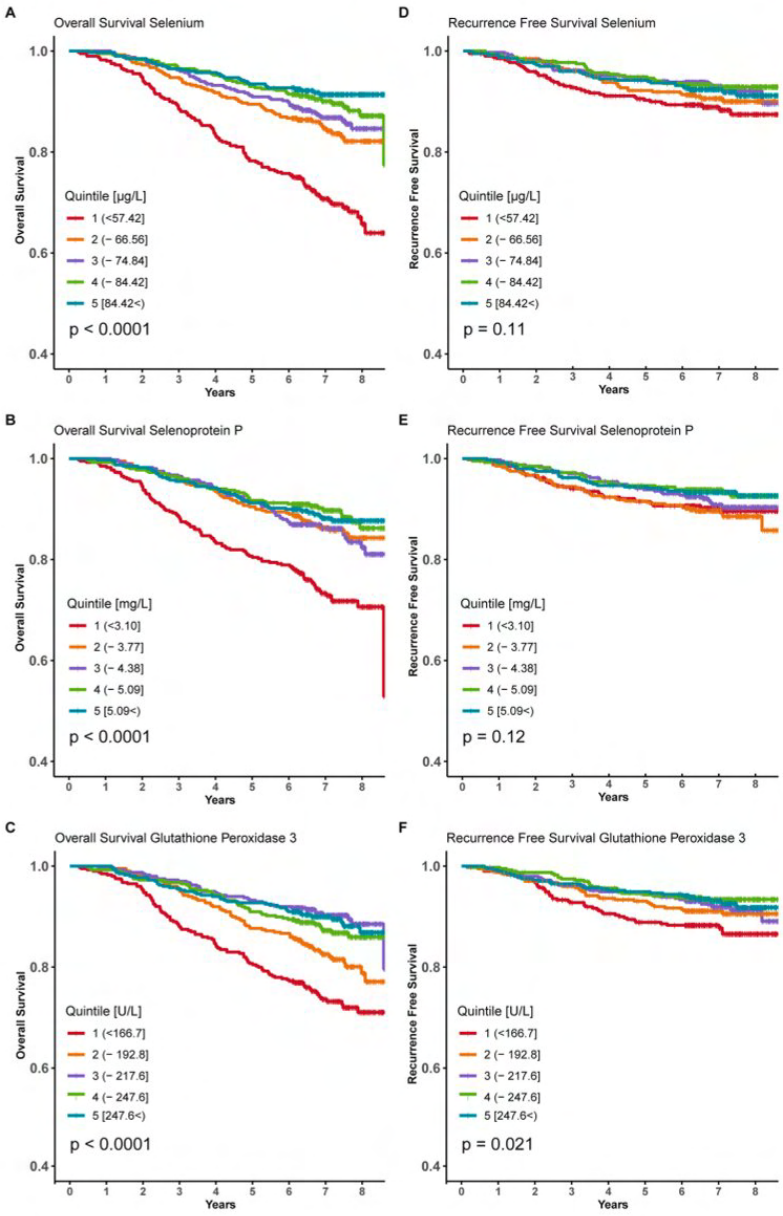

Fig. 4. Kaplan Meier curves for overall survival (A,B,C), and for recurrence free survival (D,E,F), by quintiles of the three biomarkers. Log-Rank-Test was used to evaluate differences. Y-axis-limits were set between 0.4 and 1.0 for better visualization, no points are missing. P-value was calculated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

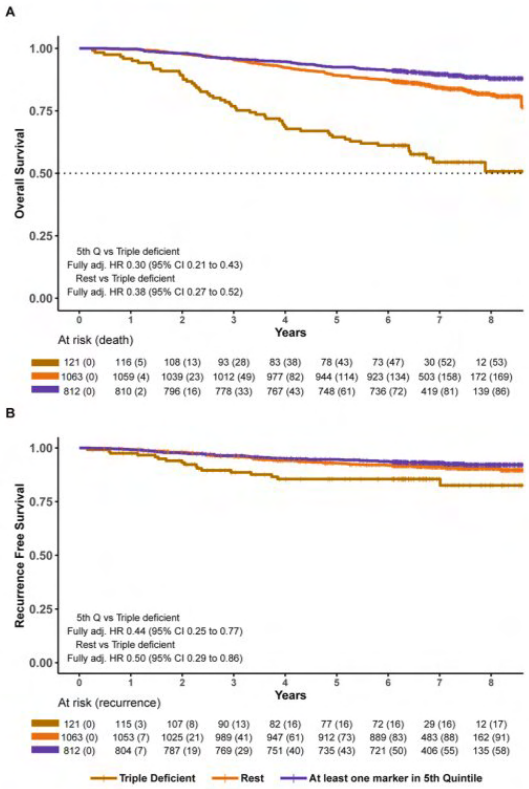

Fig. 5. Overall survival (A) and recurrence free survival (B) assessed for the triple deficient group. Triple deficient stands for patients who are in the first quintile regarding all three biomarkers; ~ (Se < 57 μg/l and SELENOP <3 mg/l and GPx3 <167 U/l). Purple curve stands for patients who are in 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

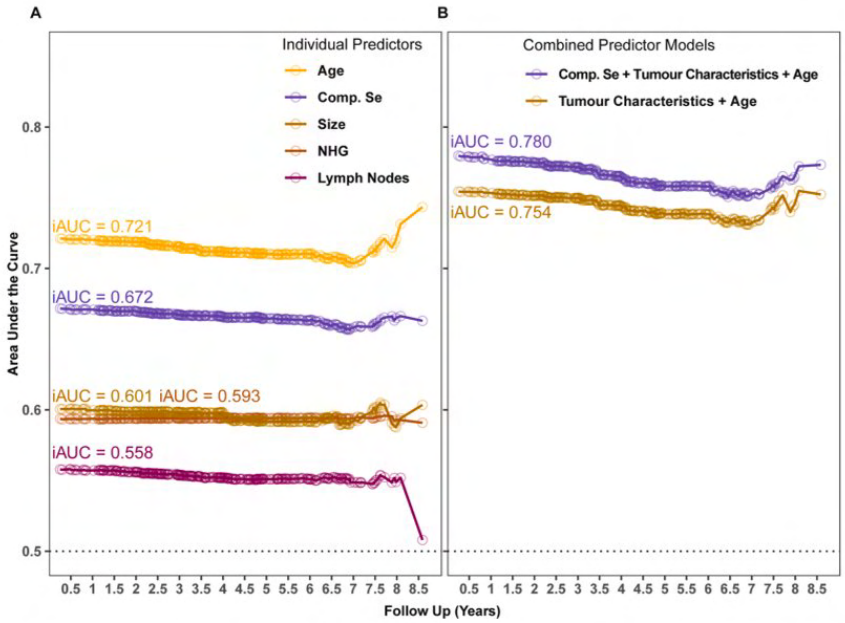

Fig. 6. Predictive value of Se status for mortality. The first panel (A) compares the predictors individually, (B) compares the predictors in combined models. AUCt (y-axis) was computed at each time of death, marked with the symbol ○. An AUC of 0.5, depicted by the dotted line, represents a random predictor 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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Autoimmunity to selenoprotein P predicts breast cancer recurrence

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ABSTRACT

Background: Low concentrations of serum selenium (Se) and its main transporter selenoprotein P (SELENOP) are associated with a poor prognosis following breast cancer diagnosis. Recently, natural autoantibodies (aAb) with antagonistic properties to SELENOP uptake have been identified in healthy subjects, and in patients with thyroid disease. Given the potential transport disrupting properties, we hypothesized that breast cancer patients with SELENOP-aAb may have a poor prognosis.

Methods: SELENOP-aAb along with serum Se, SELENOP and GPX3 activity were determined in serum samples of 1988 patients with a new diagnosis of breast cancer enrolled in the multicentre SCAN-B study. Patients were followed for ~9 years and multivariate Cox regression models were applied to assess hazard ratios.

Results: Applying a cut-off based on outlier detection, we identified 7.65% of patients with SELENOP-aAb. Autoantibody titres correlated positively to total Se and SELENOP concentrations, but not to GPX3 activity, supporting a negative role of SELENOP-aAb on Se transport. SELENOP-aAb were associated with age, but independent of tumor characteristics. After fully adjusting for potential confounders, SELENOP-aAb were associated with higher recurrence, HR(95%CI) = 1.87(1.17–2.99), particularly in patients with low Se concentrations, HR(95%CI) = 2.16(1.20–3.88). Associations of SELENOP-aAb with recurrence and mortality were linear and dose-dependent, with fully adjusted HR(95%CI) per log increase of 1.25(1.01–1.55) and 1.31(1.13–1.51), respectively.

Conclusion: Our results indicate a prognostic and pathophysiological relevance of SELENOP-aAb in breast cancer, with potential relevance for other malignancies. Assessment of SELENOP-aAb at time of diagnosis identifies patients with a distinctly elevated risk for a poor prognosis, independent of established prognostic factors, who may respond favourably to Se supplementation.

1. Introduction

Breast cancer accounts for one quarter of all cancers, and one sixth of all cancer deaths in women [1]. Given the high incidence, most effort for reducing mortality over recent years has been put on early detection with screening programs [2,3]. However, the established prognostic factors including mainly tumor characteristics (histological grade, receptor expression) and tumor stage remained widely unchanged. Discovery of additional factors for the early identification of patients at high risk for breast cancer recurrence and subsequent intensified adjuvant therapy may improve prognosis.

The trace element selenium (Se) is essential for life, owing to its effects executed as active constituent of selenoproteins [4,5]. Mainly due to the function of several of the selenoproteins controlling redox status, antioxidative reactions and protective pathways, a beneficial role of Se for maintaining health and avoiding disease has been discussed since more than 40 years [6,7]. While no consistent results were obtained for

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Abbreviations

BI	binding index
CI	confidence interval
CV	coefficient of variation
ER	oestrogen receptor
GPX3	glutathione peroxidase
HER2	human epidermal growth factor receptor
HR	hazard ratio
IQR	interquartile range
Ki67	Kiel-antigen nr. 67
NHG	Nottingham Histological Grade
NKBC	Swedish National Quality Registry for Breast Cancer
OS	overall survival
PGR	progesterone receptor
PMM	predictive mean matching
RCS	restricted cubic spline
RFI	recurrence free interval
RLU	relative light units
SCAN-B	Swedish Cancerome Analysis Network – Breast
SEAP	secreted alkaline phosphatase
SELENOP	selenoprotein P
SELENOP-aAb	selenoprotein P autoantibodies
Se	Selenium
TXRF	total x-ray fluorescence

cancer incidence [8–10], several independent studies reported dose-dependent associations of low Se status with poor prognosis. An inverse association of Se status with cancer-prognosis is described for multiple cancer sites, including laryngeal [11], colorectal [12,13], lung [13,14], prostate [13], skin [15], and breast [16–20], and it was also observed in large-scale studies assessing all-cancer mortality including NHNAES III [13].

Most of the studies that analysed prognosis of patients with breast cancer by Se used blood sampling to determine Se concentrations in serum or plasma. In our recent study, the association with prognosis was assessed using three different serum biomarkers, namely total Se, the Se transport protein selenoprotein P (SELENOP), and the enzymatic activity of extracellular Se-dependent glutathione peroxidase (GPX3). All three biomarkers were inversely associated with prognosis. Besides these interrelated biomarkers of Se status, natural autoantibodies to SELENOP (SELENOP-aAb) have recently been reported in healthy subjects and thyroid patients, obviously capable of interfering with regular Se transport by SELENOP [21].

The aim of the study was to assess the prognostic value of SELENOP-aAb in a large multicentre population-based cohort of newly diagnosed breast cancer patients.

2. Methods

2.1. Study population

Since August 30th 2010, the multicentric prospective Sweden Cancerome Analysis Network – Breast (SCAN-B) study (ClinicalTrials.gov ID NCT02306096) enrols patients with a new diagnosis of primary invasive breast cancer systematically, with the aim of identifying novel genomic and serum prognostic factors [16,22,23]. With multiple participating hospitals in Sweden in Malmö, Lund, Helsingborg, Kristianstad, Växjö, Halmstad, Uppsala, Karlskrona, Varberg, and Ljungby, SCAN-B included almost 85% of all cases in the catchment region since its initiation [22]. Patients with a pre-surgical diagnosis or suspicion of primary invasive breast cancer were eligible. Among this group, patients with a previous history of contralateral breast cancer, without planned treatment, without planned treatment in any of the participating hospitals, with an unclear treatment status or with a generalized disease state at time of diagnosis, i.e. with distant metastases, were excluded. A total of 5417 patients meeting the eligibility and exclusion criteria were registered between September 1st 2010 and March 31st 2015. For the purpose of our study, we aimed to include 2000 patients. Hence, the first 2903 consecutive cases were selected. After excluding 915 cases, mainly due to missing serum, samples of 1988 female patients were finally included in the current analyses (Supplementary Fig. 1).

2.2. Follow up and endpoint retrieval

For all patients, follow-up started at time of diagnosis and serum sampling, before initiation of surgical treatment. Patients were followed until death, recurrent event (local, regional, distant), or end of follow up. In order to maintain and protect patient confidentiality, the SCAN-B steering committee provided only the number of follow-up days to the authors, instead of exact date of follow-up start and date of event of interest. Thus, end of follow-up time is a date between April 1st 2019 and June 30th 2019. Retrieval of endpoint data in the case of recurrence and all-cause mortality was conducted by linkage with the Swedish National Quality Registry for Breast Cancer (NKBC). NKBC retrieves mortality data from the Swedish Population Registry, and recurrence data from reports of treating centres.

2.3. Clinical data and tumor characteristics

Clinical data and tumor characteristics collected by the surgical and pathological department of each participating centre were obtained from the NKBC. Patient-related data comprised age, sex, and menopausal state if applicable. Tumor characteristics as assessed for the purpose of this study were size, histopathological type, Nottingham Histological Grade, Ki67 expression, oestrogen receptor overexpression, progesterone receptor overexpression, HER2 receptor overexpression, and lymph node involvement.

2.4. Modality of diagnosis and treatment

Data on diagnosis modality, surgical procedure with regard to the breast and with respect to the axilla, adjuvant endocrine therapy, chemotherapy, immunotherapy, and/or radiotherapy were reported to and retrieved from NKBC.

2.5. Quantification of selenium status biomarkers

Serum sampling was conducted within the SCAN-B infrastructure. In brief, blood was drawn at time point of breast cancer diagnosis, before initiation of treatment, and 200 μL aliquots of serum were prepared and kept at −80 °C at the Department of Clinical Chemistry, Skåne University Hospital. The laboratory analyses took place in an off-site laboratory in Berlin, Charité University, Germany, while clinical data was entirely blinded to the receiver of the samples as well as to scientists and technicians conducting laboratory analyses. Linkage of the results to clinical phenotype, i.e. unblinding took place after all measurements were completed, and no additional quantification was conducted after unblinding.

Three complementary Se status biomarkers in the serum samples, i.e., total serum Se and SELENOP concentrations along with GPX3 enzyme activity, have been assessed and were described earlier [16]. Total

reflection X-ray fluorescence (TXRF) was used for total serum Se [24], a validated sandwich ELISA (selenOtest™-ELISA,selenOmed GmbH, Berlin, Germany) for serum SELENOP concentrations [25,26], and an NADPH-coupled enzymatic test for serum GPX3 activity [27,28]. Inter- and intraassay coefficients of variation were below 15% at all times, as reported earlier [16].

2.6. Assessment of SELENOP autoantibodies

Natural SELENOP-aAb in the serum samples were detected and assessed as described recently [21]. Briefly, serum samples were incubated with a fusion protein consisting of a secreted alkaline phosphatase (SEAP) fused in frame to recombinant SELENOP variant in which selenocysteine has been replaced by cysteine residues as reporter (SEAP--SELENOP, selenOmed GmbH). Samples were incubated overnight at 4 °C, and the immune complexes formed (SELENOP-aAb) bound to SEAP-SELENOP fusion protein) were precipitated with protein A-sepharose, washed and analysed for SEAP activity in a luminometer. Luminescence corresponding to SELENOP-aAb concentration in the original sample is recorded as relative light units (RLU), and analysed in relation to background signals. Inter- and intra-assay CV using a positive sample as standard were below 15% and 11%, respectively, during the analyses.

2.7. Statistical analysis

2.7.1. Classification of autoimmunity to SELENOP

Patients were assigned as SELENOP-aAb positive or negative based on the signals obtained from serum by assessing the binding of immunoglobulins to recombinant SELENOP as described above. Final classification as positive or negative was carried out applying a mathematical outlier criterion. Based on the assumption of SELENOP-aAb being prevalent in less than 50% of samples, the arithmetic mean of the low 50% of signals per measurement plate was calculated, defined as background and assigned as a binding index (BI) of $BI = 1$. All values equal or above 3-fold of this signal, i.e. $BI \geq 3$ were considered positive. Distribution of the resulting BI of single 96-well plates and the full set of results was assessed by dot-plots and density plots.

2.7.2. Autoimmunity to SELENOP in relation to baseline patient and tumor characteristics

Baseline patient and tumor characteristics were described as mean (standard deviations) in case of normal, or as median (interquartile range) in case of non-normal distribution. Distribution was evaluated based on the Shapiro-Wilk test and visual inspection of histogram plots. Patient characteristics were compared in relation to SELENOP-aAb. Wilcoxon rank sum test was used to test differences in continuous variables, Fisher's exact test was used to test differences between categorical variables in a 2×2 contingency format, and Pearson's Chi-squared test was used to test differences in categorical variables with more categories.

2.7.3. Correlation of SELENOP-aAb to Se status biomarkers

Correlation between SELENOP-aAb and total Se or selenoproteins was tested with Spearman's rank correlation, and a visual trend was investigated by linear regression plots with 95% confidence intervals (CI). A potential dose-dependent relationship was assessed by applying different cut-offs for the signal strength, i.e., $BI \geq 3$, $BI \geq 5$ and $BI \geq 10$, respectively.

2.7.4. SELENOP-aAb in relation to mortality and recurrence

Prognosis was assessed based on overall survival (OS) and recurrence free interval (RFI). Starting time-point of the follow-up for both endpoints was the time at diagnosis, before surgery. Mortality of any cause was the event for OS. Breast cancer recurrence (local, regional or distant) was the event for RFI, while death was censored. Survival probability was visualized with Kaplan-Meier plots, and the log-rank test was used to detect differences between SELENOP-aAb positive and negative patients. Cox regression models were conducted to calculate HR and 95% confidence interval (CI). Proportional hazards assumption was checked by visual inspection of Kaplan-Meier plots and by computing Schoenfeld residuals (Supplementary Fig. 2), without observing any violations. Three models were applied. First model was univariate, the second model was adjusted for age at diagnosis, and the third model was additionally adjusted for various potential confounders of mortality and recurrence, including Nottingham Histologic Grade (NHG), histological type of the tumor, expression of HER2 receptor, progesterone receptor, or oestrogen receptor, tumor size, modality of diagnosis of breast cancer, and menopausal state of the patient. As Ki67 evaluation was not part of clinical routine, Ki67 variable has high number of missing values and was not included in the model. In all analyses, the negative patient group was set as reference. Dichotomizing a continuous variable makes it easy to interpret and apply the parameter in clinical decision making. However, statistical power is sacrificed, and a dose-dependent relationship – which is a factor suggesting a causal relationship – cannot be investigated [29,30]. Therefore, SELENOP-aAb concentrations were also modelled as a continuous variable in relation to OS and RFI using linear Cox regression. As the values were right skewed, the variable was log-transformed applying natural logarithm to approximate a normal distribution. Shape of association was assessed with restricted cubic spline regression (RCS) modelling. Three knots at the 10^{th}, 50^{th} and 90^{th} percentile were fitted to the RCS models. RCS models were compared to linear models by applying likelihood-ratio test and p-value for non-linearity was evaluated.

2.7.5. Evaluation and handling of missing data

As described previously, the fraction of the missing data included in the models constituted less than 1% of all data [16]. When applying fully adjusted models, those were imputed by multiple chained imputation, applying ten imputations iterated 10 times each. All variables included in the fully adjusted model as well as total Se, SELENOP, GPX3, both outcome measures and time from diagnosis to endpoint were entered into the prediction matrix. Fully conditional specification was applied with proportional odds model for ordinal variables, predictive mean matching (PMM) for continuous variables, logistic regression for binary categorical covariates, and polytomous logistic regression for nominal data. Robustness of the imputation model was solid, as assessed by checking convergence, as well as comparing regression results to complete case analysis, as shown before [16].

2.7.6. Subgroup and sensitivity analyses

The association of positivity and SELENOP-aAb titres with mortality and recurrence was tested in patients with low and high SELENOP concentrations separately. For that purpose, the cohort was divided into two groups based on the median value of SELENOP, which equals 4.08 mg/L. All analyses were also conducted in low and high total serum Se concentration groups, where the median Se corresponds to 70.4 μg/L. Association of SELENOP-aAb with mortality and recurrence was repeated in the fully adjusted models, by adding serum Se status biomarkers one by one. In order to rule out potential reverse causality, the main analyses were repeated excluding patients with an event or censoring within the first 12 months of follow up. In a further sensitivity analysis, the fully adjusted main analyses for all surgical and adjuvant treatment options was adjusted one-by-one to detect potential adjustment effects by treatment modality.

All statistical analyses were two-sided and were conducted with the R language (version 4.1.2.) on the RStudio environment. Packages used for main analyses are provided in the supplement section.

3. Results

3.1. Study design and prevalence of autoantibodies to SELENOP

Final analysis comprised 1988 patients with an incident diagnosis of primary invasive breast cancer. Serum sampling was conducted for each patient at time of breast cancer diagnosis, before surgical intervention (Fig. 1a). The follow-up time corresponded to a median (IQR) of 6.94 (6.28–7.63) years for OS comprising 13,290 person years, and 6.87 (6.25–7.61) years for RFI comprising 13,023 person years. In total, 307 deaths and 167 recurrent events occurred during the follow-up.

The quantification of SELENOP-aAb in serum was conducted via

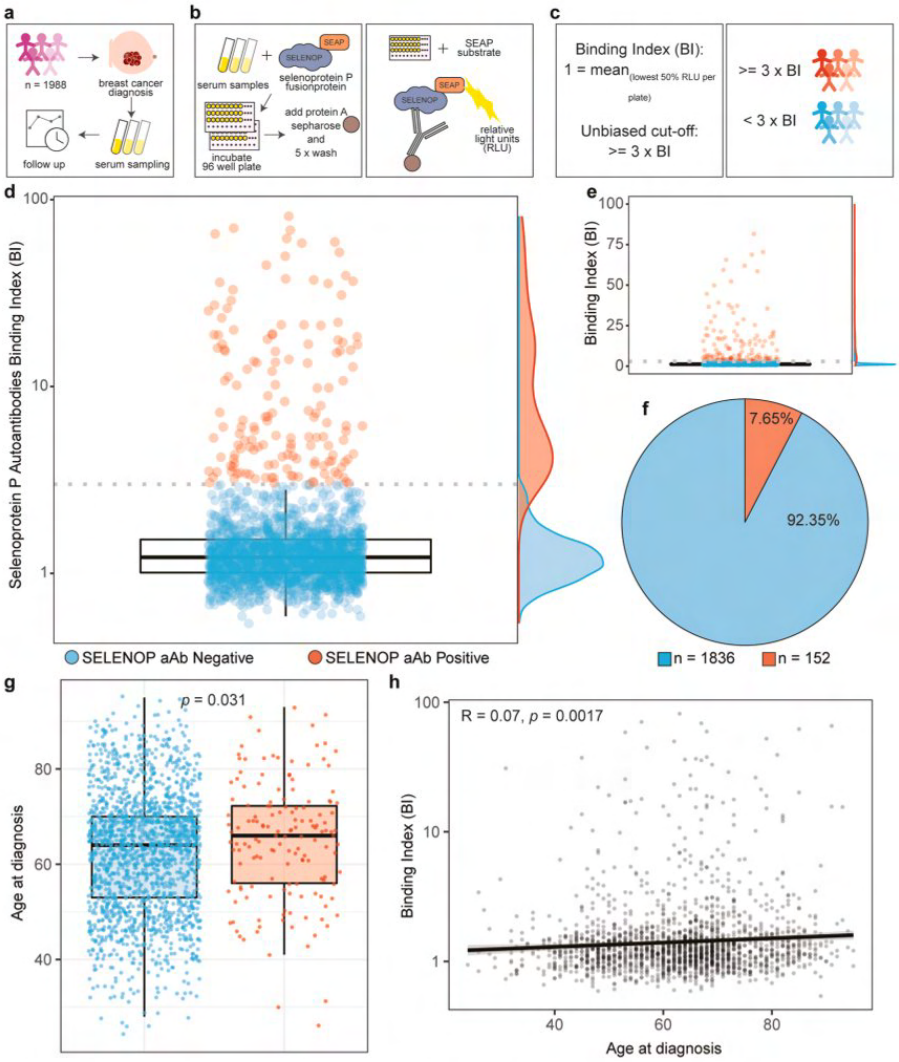

Fig. 1. Study design and prevalence of autoimmunity to SELENOP. a 1988 patients with an incident diagnosis of primary invasive breast cancer were included in this study. Serum sampling was conducted at time of diagnosis, and follow up encompassed approximately 9 years **b** Samples were analysed for SELENOP-aAb in 96 well plates by immunoprecipitation of complexes formed in serum with protein A-sepharose, and detection of luminescence as light units (RLU) from precipitated SELENOP-SEAP-aAb complexes. **c** An outlier criterion for cut-off definition of autoimmunity was applied, and values exceeding 3-fold of binding index (BI $\geq$ 3, dotted line) were considered positive. **d** Binding indices of SELENOP-aAb are displayed on a logarithmized y-axis, and plotted as density on the right y-axis. Patients above the cut-off are marked red **e** SELENOP-aAb displayed a right skew, as emphasized by the marginal density plot. BI was displayed on non-logarithmized y-axis. **f** Applying the unbiased cut-off (BI $\geq$ 3), a total of 7.65% of patients were identified as SELENOP-aAb positive. **g** Age at diagnosis was compared to aAb-positivity, applying the Wilcoxon-Rank-sum test. **h** Correlation of the continuous SELENOP-aAb titre and age at diagnosis was assessed, using Spearman's rank correlation test. Blue points indicate SELENOP-aAb negative patients, and red points indicate SELENOP-aAb positive patients. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein A-mediated precipitation of a recombinant SEAP- SELENOP fusion protein (Fig. 1b). Serum samples were assessed for their signal in relation to background, with signals exceeding three times background signal (BI ≥ 3) classified as outliers and SELENOP-aAb positive samples (Fig. 1c). The signals obtained showed a skewed distribution (Fig. 1d). This result is highlighted by the dot-plot and marginal density plot analysis presented (Fig. 1e). The unbiased cut-off (depicted by the dotted grey line in Fig. 1d and **e**) was in agreement with an alternative outlier criterion (3rd quartile + 1.5 * interquartile range), which is depicted by the upper whisker of the black boxplot (Fig. 1d). According to this analysis, the prevalence of SELENOP-aAb in the full set of samples was 7.65% (152/1988), including a fraction of 3.05% (61/1996) with particularly high titres of BI ≥ 10 (Fig. 1f). Age of patients was higher in the SELENOP-aAb positive group (Fig. 1g), with a weak correlation of the BI to age (Fig. 1h).

3.2. *SELENOP autoantibodies are associated with higher serum SELENOP but not higher GPX3 expression*

A potential dose-dependent association of SELENOP-aAb with total Se, SELENOP and GPX3 was tested next (Fig. 2). SELENOP-aAb were dose-dependently correlated to serum SELENOP and total serum Se

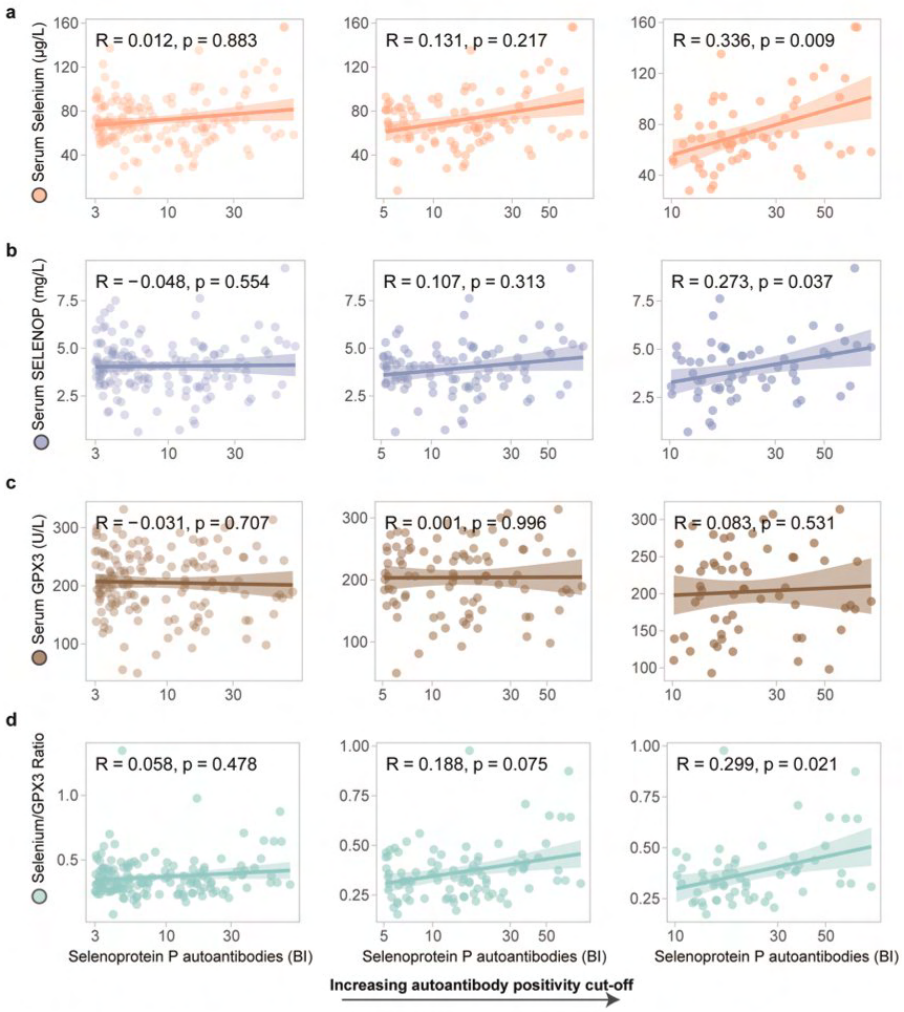

Fig. 2. Correlation of SELENOP-aAb with total serum selenium and selenoproteins. Linear regression (line) with 95% confidence intervals (shadow) was used to visualize the relationship. **a** Correlation of autoantibody titres to total serum selenium was assessed, with increasing cut-offs for autoantibody titres from left to right. Slope of the linear regression line has shown an increasing trend with increasing antibody titres. Above BI = 10, SELENOP-aAb were significantly correlated with total serum selenium, R = 0.336, p = 0.009. **b** A similar trend was seen with regard to serum SELENOP levels, which also was statistically significant above BI = 10, R = 0.273, p = 0.037. **c** No association was seen for serum GPX3, although it is tightly correlated to serum selenium and serum SELENOP concentrations in this study cohort. **d** SELENOP-aAb were significantly associated with selenium/GPX3 ratio above BI = 10, R = 0.299, p = 0.021. Spearman's rank correlation was used to assess correlation.

concentrations, with an increasing gradient over increasing autoantibody titres. Above a cut-off of BI = 10, this association was significant, R = 0.336, p = 0.007 (total Se) (Fig. 2a), and R = 0.273, p = 0.037 (SELENOP) (Fig. 2b). No association was observed for GPX3 activity in relation to SELENOP-aAb (Fig. 2c), supporting a role of SELENOP-aAb in disruption of Se transport. This notion was supported by a stringent association of SELENOP-aAb with the Se/GPX3 ratio (Fig. 2d).

3.3. Tumor characteristics do not differ according to SELENOP autoimmunity

Patient and tumor characteristics were analysed with respect to SELENOP-aAb (Table 1). On average (median(IQR)), SELENOP-aAb positive patients were older at time of diagnosis than negative patients, 66 (56–72) vs. 64 (53–70) years. Classical tumor characteristics including Nottingham Histologic Grade, expression status of common receptors (ER, PGR, HER2), tumor size, or lymph node involvement did not differ between SELENOP-aAb positive and negative patients. Similarly, the mode of diagnosis, the surgical method conducted with regard to the breast or lymph nodes as well as the applied adjuvant therapy (chemo-, radio-, immune-, or endocrine therapy) did not differ according to SELENOP-aAb status (Supplementary Table 1).

Table 1
Patient and tumor characteristics in relation to SELENOP-aAb positivity.

Characteristic	SELENOP-aAb negative N = 1836	SELENOP-aAb positive N = 152	p-value[a]
Age (years)	*64 (53, 70)*	*66 (56, 72)*	0.031
Menopausal Status			0.4
Pre-menopausal	343 (19%)	22 (15%)	
Post-menopausal	1401 (77%)	123 (81%)	
Uncertain	77 (4.2%)	6 (4.0%)	
Laterality			0.063
Left	943 (51%)	90 (59%)	
Right	893 (49%)	62 (41%)	
Size (mm)	16 (11, 23)	15 (10, 22)	0.2
Lymph Nodes			0.3
≥4	164 (9.3%)	10 (6.9%)	
1-3	430 (24%)	29 (20%)	
No Involvement	1134 (64%)	101 (70%)	
Submicrometastasis	37 (2.1%)	5 (3.4%)	
(Missing)	71	7	
NHG			0.4
I	348 (19%)	35 (24%)	
II	846 (47%)	68 (47%)	
III	591 (33%)	43 (29%)	
(Missing)	51	6	
Ki67 Expression			0.2
Low	203 (46%)	22 (58%)	
High	236 (54%)	16 (42%)	
(Missing)	1397	114	
Histological Type			0.087
Ductal	1467 (80%)	122 (80%)	
Lobular	245 (13%)	15 (9.9%)	
Other	96 (5.2%)	9 (5.9%)	
Ductal + Lobular/ Other	26 (1.4%)	6 (3.9%)	
HER2 Expression			0.7
Negative	1586 (87%)	128 (86%)	
Positive	227 (13%)	20 (14%)	
ER Expression			0.2
Negative	254 (14%)	27 (18%)	
Positive	1578 (86%)	124 (82%)	
PGR Expression			>0.9
Negative	514 (28%)	43 (28%)	
Positive	1318 (72%)	108 (72%)	

Median (IQR); n (%).
Missing not shown if <2%.
NHG = Nottingham Histological Grade, Lymph Nodes = Number of lymph nodes involved, HER2 = Human epidermal growth factor receptor 2, ER = Oestrogen receptor, PGR = Progesterone receptor.
[a] Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test.

3.4. SELENOP autoantibodies are associated with poor breast cancer prognosis

Survival probability was compared between patients positive and negative for SELENOP-aAb using Kaplan-Meier plots (Fig. 3). OS probability was significantly lower in SELENOP-aAb positive as compared to SELENOP-aAb negative patients, log-rank p = 0.0064 (Fig. 3a). RFI was also lower in patients positive for SELENOP-aAb, log-rank p = 0.0085 (Fig. 3b). Cox regression analyses were carried out for OS and RFI in relation to SELENOP-aAb (Table 2). Three models were fit to assess the hazard ratio, namely univariate, age adjusted and fully adjusted. Patients negative for SELENOP-aAb were set as reference. In univariate models, HR for mortality (OS) and recurrence (RFI) was significantly higher in SELENOP-aAb positive patients, HR = 1.62 (95% CI = 1.14 to 2.31) and HR = 1.83 (95%CI = 1.16 to 2.89), respectively. HR for RFI remained significantly elevated in the age adjusted and fully adjusted models, and HR for OS was borderline significant after full adjustment (Table 2).

3.5. Association of SELENOP autoantibodies and prognosis in relation to selenium deficiency

In the low Se group, the OS was significantly lower for SELENOP-aAb positive patients as compared to SELENOP-aAb negative patients (log-rank p = 0.0021), while OS did not statistically differ in the high Se group (Fig. 3c). Similarly, RFI probability of positive patients was significantly lower than of negative patients only in the low Se group (log-rank p = 0.0051) (Fig. 3d). Next, Cox regression was implemented to adjust for confounders. The observed differences were retained in fully adjusted models, and HR for RFI in positive patients was 2.16 (95% CI = 1.20 to 3.88) and for OS 1.58 (95% CI = 1.04 to 2.40) in the low Se group (Table 3).

In a sensitivity analysis, we evaluated OS and RFI stratified by total SELENOP concentrations, which yielded very similar results (Supplementary Fig. 3). After full adjustment, HR for RFI (Supplementary Table 2) and OS (Supplementary Table 3) was strongly elevated in SELENOP-aAb positive as compared to SELENOP-aAb negative patients in the low SELENOP group.

3.6. Association of autoantibody titres with poor prognosis is dose-dependent

SELENOP-aAb concentrations were modelled as a continuous variable in relation to OS and RFI using linear Cox regression to evaluate a potential dose-dependent relationship. After full adjustment for confounders, one natural logarithmic increase of SELENOP-aAb titres was associated with an HR of 1.31 (1.13–1.51) for OS and 1.25 (1.01–1.55) for RFI, when including the whole cohort (Supplementary Table 4). All associations assessed were of linear shape, i.e. $p_{non\text{-}linearity} > 0.05$ (Supplementary Fig.4). The association of SELENOP-aAb and mortality or recurrence was very similar after adjusting for any of the other Se biomarkers one-by-one or all together (Supplementary Table 5).

When stratified for SELENOP concentrations, the continuous variable SELENOP-aAb was associated with OS and RFI in the low SELENOP group. HR per log increase was 1.32 (95% CI = 1.10 to 1.58) for mortality, and 1.37 (95%CI = 1.06 to 1.77) for breast cancer recurrence in the fully adjusted model (Supplementary Table 6). Conducting the linear Cox regressions analyses stratified by total Se concentrations yielded very similar results (Supplementary Table 7).

In order to investigate a potential reverse causality of the effects, fully adjusted models for SELENOP-aAb and the continuous autoantibody variable in relation to OS and RFI were repeated, excluding patients with an event/censoring within the first 12 months. Autoimmunity or log increase in continuous variable remained significantly associated with both mortality and recurrence (Supplementary Table 8).

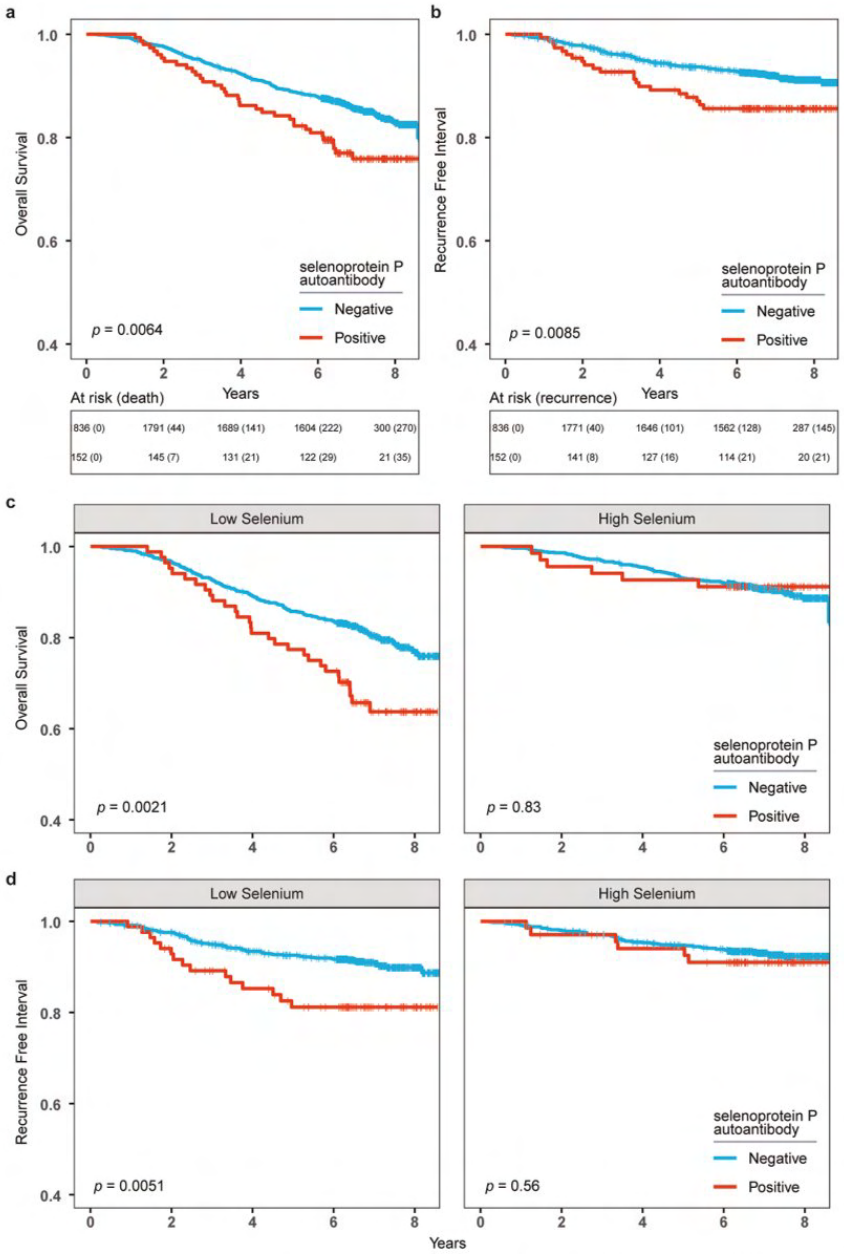

Fig. 3. Kaplan Meier plots for overall survival and recurrence free interval. **a** Overall survival according to autoantibody positivity was assessed with Kaplan Meier plots and log-rank test. Overall survival differed significantly between the two groups. **b** Recurrence free interval was also lower in SELENOP-aAb positive patients. **c** Overall survival probability stratified by Se status, cut-off was set at median of the cohort, corresponding to 70.4 μg/L Se. **d** Recurrence free interval stratified by Se status.

Table 2
Cox regression according to positivity of autoantibodies in the whole cohort.

		At Risk	Event	Univariate[a]		Age Adjusted[b]		Fully Adjusted[c]	
Endpoint	SELENOP-aAb	N	N	HR	95% CI	HR	95% CI	HR	95% CI
Mortality									
	Negative	1836	272	—	—	—	—	—	—
	Positive	152	35	1.62	1.14, 2.31	1.45	1.02, 2.06	1.41	0.98, 2.02
Recurrence									
	Negative	1836	146	—	—	—	—	—	—
	Positive	152	21	1.83	1.16, 2.89	1.79	1.13, 2.84	1.87	1.17, 2.99

HR = Hazard Ratio, CI = Confidence Interval.
[a] Crude model. Complete case.
[b] Adjusted for age at diagnosis. Complete Case.
[c] Fully Adjusted Model. Missing covariates were imputed using multiple imputation by chained equations. Adjusted for age at diagnosis, menopausal Status, ER expression, PGR expression, HER2 expression, Nottingham Histologic Grade, histological type, number of lymph nodes involved, modality of diagnosis, and size of tumor [mm].

Table 3
Cox regression according to positivity of autoantibodies stratified by selenium status.

		At Risk	Event	Univariate[a]		Age Adjusted[b]		Fully Adjusted[c]	
Group (Endpoint)	SELENOP aAb	N	N	HR	95% CI	HR	95% CI	HR	95% CI
Low Selenium **Mortality**									
	Negative	908	181	—	—	—	—	—	—
	Positive	84	29	1.83	1.23, 2.72	1.65	1.11, 2.45	1.58	1.04, 2.40
High Selenium **Mortality**									
	Negative	928	91	—	—	—	—	—	—
	Positive	68	6	1.18	0.61, 2.27	0.87	0.38, 2.00	0.88	0.38, 2.06
Low Selenium **Recurrence**									
	Negative	908	81	—	—	—	—	—	—
	Positive	84	15	2.16	1.23, 3.77	2.11	1.21, 3.69	2.16	1.20, 3.88
High Selenium **Recurrence**									
	Negative	928	65	—	—	—	—	—	—
	Positive	68	6	0.83	0.29, 2.32	1.27	0.54, 2.98	1.25	0.53, 2.97

HR = Hazard Ratio, CI = Confidence Interval.
[a] Crude model. Complete case.
[b] Adjusted for age at diagnosis. Complete Case.
[c] Fully Adjusted Model. Missing covariates were imputed using multiple imputation by chained equations. Adjusted for age at diagnosis, menopausal Status, ER expression, PGR expression, HER2 expression, Nottingham Histologic Grade, histological type, number of lymph nodes involved, modality of diagnosis, and size of tumor [mm].

For the purpose of assessing potential adjustment effects of surgical and adjuvant therapy to the association, fully adjusted models were augmented with each treatment method one at a time, without observing considerable changes in the HR (Supplementary Table 9).

4. Discussion

In this manuscript, we describe the prognostic relevance of autoimmunity to the Se transporter SELENOP in patients with a new diagnosis of primary invasive breast cancer. The association of SELENOP-aAb with poor prognosis was most distinct in Se deficient patients. Patients positive for SELENOP-aAb displayed elevated total Se and SELENOP concentrations in serum, but no elevated GPX3, indicating a disrupting effect of the autoantibodies on regular Se transport and homeostasis. The potential causality is supported by the dose-dependent relationship between SELENOP-aAb concentration and mortality or recurrence, which maintained after adjusting for potential confounders of breast cancer prognosis, and the other three biomarkers of Se status. We conclude that an assessment of SELENOP-aAb identifies patients at high risk for breast cancer recurrence, independent of the commonly assessed prognostic factors.

Beside the need for further studies with regard to risk of developing breast cancer, our results are highly congruent for survival, the main objective of our study. In line with prior observations of the inverse association of Se status biomarkers and mortality/recurrence following breast cancer, and in line with potential antagonistic properties of the SELENOP-aAb, we have observed a poor prognosis in SELENOP-aAb positive patients. Even though our study is investigating this matter for the first time, our results are backed up by several supportive backbones. Firstly, our study describes the postulated occurrence of autoimmunity to SELENOP in female patients, with an expected association to higher patient age [31]. Secondly, the autoantibodies were dose-dependently associated to higher SELENOP and Se concentrations, without a rise in GPX3 activity, which is mainly controlled by SELENOP-dependent Se supply to the kidney [32]. This is in line with our previous study in an independent cohort, and supports the hypothesis of potential antagonistic properties of the autoantibodies to Se uptake [21]. Thirdly, equal to the three Se status biomarkers, SELENOP-aAb were not related to any tumor characteristics, only to age of patients at diagnosis. These three points are coherent in themselves, and support the quality of the quantitative analysis of the main exposure, SELENOP-aAb. Further, the association with prognosis was particularly severe in patients with low serum concentrations of SELENOP, which accords with the hypothesis that SELENOP-aAb bind and inhibit uptake of SELENOP in a dose-dependent matter. Lastly, modelling the autoantibody titres as a continuous variable revealed a dose-dependent relationship of SELENOP-aAb with prognosis, similar to the observations with the other Se status biomarkers. The observed dose-dependency argues against a chance finding, and supports a potential causal relationship.

Current prognostic factors with an established clinical role mostly require invasive methods and sampling of tumor tissue, e.g.,

immunohistochemical, gene expression profile or epigenetic pattern analyses [33–35]. The assessment of SELENOP-aAb at the time of cancer diagnosis offers some promising perspectives, as the biomarker would be accessible directly from a serum sample, requiring little volume only, and would not depend on very elaborate, cost- or labour-intensive instrumentation. Still, the robustness and reproducibility of the results presented needs some independent replication in additional sufficiently-large cohort studies.

The SCAN-B study is fully integrated into the clinical routine with a high rate of coverage of all breast cancer cases in Southern Sweden, with all procedures regarding diagnosis and treatment proceeding regularly, without alterations in clinical decision making [22]. Thus, a high generalisability of the results is ensured with regard to study characteristics, coverage and design. However, although a considerable part has non-European origin, majority of patients is genetically similar and of European origin, environmental factors and nutritional patterns are similar, and the Se status of the population is accordingly marginal, similar to other European countries [36–38]. Considering this aspect and in view that our results were most distinct in patients with low Se/SELENOP concentrations, the findings may be of specific relevance to populations with insufficient Se intake. Further studies are needed to assess the results in such Se-deplete areas in comparison to Se rich countries, such as the USA, where the contribution from SELENOP-aAb to disease course may be rather marginal.

The observed prevalence of 7.65% autoimmunity to SELENOP in the patients with primary invasive breast cancer is slightly higher than reported before from patients with autoimmune thyroid disease (6.6%), and healthy subjects (0.3%), respectively. Part of this difference may be explained by the more than two-fold higher median age in this study, and the exclusive enrolment of women [39]. In how far a predisposition to breast cancer is exerted by SELENOP-aAb, or whether modified SELENOP is secreted from malignant breast tissue is unknown at present. Biosynthesis of potentially modified SELENOP by malignant cells may cause the development of SELENOP-aAb, and patients with breast cancer may consequently tend to develop autoimmunity to Se transport. Notably, the mammary gland has been described as actively secreting SELENOP, hereby enabling targeted Se supply to the offspring via mother's milk [40]. The higher prevalence observed in the patients may also be due to a higher risk of breast cancer development in the presence of SELENOP-aAb. Whether and in how far SELENOP acts as a tumour associated antigen promoting autoimmunity or whether the SELENOP-aAb rather constitute a risk factor contributing to the higher prevalence in this cohort must be investigated in further studies.

Our study has several strengths. Particularly, while most studies exploring potential clinical relevance of novel biomarkers include a rather small sample size, this investigation was conducted with one of the largest current prospective breast cancer studies in the world. As fortunately, the recurrence rate of breast cancer is relatively low, and in view that the prevalence of SELENOP-aAb is relatively moderate, the large study size was a crucial prerequisite for providing sufficient statistical power for the analyses conducted. Even though we characterize the potential prognostic relevance of very novel autoantibodies, exploration was not conducted arbitrarily, but based on prior findings that originated from the same patient group in the SCAN-B study, and without arbitrary cut-off determination. Hereby, both the congruency to our prior results on the relationship between Se and surviving breast cancer within the SCAN-B study cohort and the complete and precisely constructed database with a very low number of missing values in confounders argue for a high solidity of the findings reported. From a methodological standpoint, the large set of available covariates that were corrected for granted a focused investigation of an independent effect of serum SELENOP-aAb on mortality and recurrence. The availability of corresponding serum Se and SELENOP concentrations along with GPX3 activity levels did provide important contextual value, and was a relevant control for correct measurements of the main exposure, i.e., the SELENOP-aAb concentrations and their prognostic relevance.

Our study also has several limitations. Due to the observational study type, residual confounding cannot be fully ruled out. Even though we have controlled for the most important potential confounders of breast cancer recurrence, information on other autoimmune diseases, prior inflammatory events or other potential triggers for autoimmunity against SELENOP are missing. While in particular prevalent systemic autoimmune disease has been shown to associate with higher overall mortality, the association of autoimmunity and cancer risk and survival is not conclusive at present [41]. Thus, while it may modify the results for mortality, we do not think that it affects the main endpoint, i.e., recurrence of breast cancer. Another limitation concerns the notion that the data are retrieved from a single blood sample per patient. As the data indicate that SELENOP-aAb are associated with age, some patients who developed SELENOP-aAb within the follow-up time might have been missed. However, this limitation would rather lead to a higher association than reported, and not challenge the main results. From our experience, naturally occurring autoantibodies are relatively stable over time, once developed, supporting the notion that the initial blood sample provides relevant information for SELENOP-aAb during the time after diagnosis [42].

Beside the novel findings in relation to breast cancer, our results also outline promising paths of future research. An important aspect would be to replicate our findings in a Se rich population, such as e.g. in the USA, where the contribution from SELENOP-aAb to a poor prognosis might be minimal. The higher prevalence of SELENOP-aAb in breast cancer patients as compared to healthy subjects or Hashimoto patients implies a potential role of SELENOP-aAb in risk for developing breast cancer. This hypothesis needs to be tested in an adequate longitudinal case-control study. The dose-dependency of the results, as well as the distinctness of the findings in Se-deficient subjects indicate a causal relationship. Thus, an interventional study to test the potential benefit of Se-supplementation for correcting the deficit and poor prognosis should be considered, with applying baseline stratification for general Se deficiency and SELENOP-aAb deficiency.

5. Conclusion

We conclude that SELENOP-aAb are of pathophysiological relevance and provide an independent predictive value for prognosis in patients with breast cancer diagnosis. The assessment of an additional biomarker of Se status in combination with SELENOP-aAb analysis will stratify a given patient, and inform about a particularly elevated recurrence risk. The relevance of SELENOP-aAb for cancer prognosis may also apply to other malignancies, which should be tested in future analyses.

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Conflict of interest disclosure statement

LS and PS hold shares, and PS serves as CEO of selenOmed GmbH, a company involved in Se status assessment, LS is listed as inventor on a related patent application; no other relationships or activities that could appear to have influenced the submitted work.

Data availability statement

The data generated in this study are available upon reasonable request from the corresponding author.

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).

Author contributions

K. Demircan: Conceptualization, formal analysis, data curation, software, formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **Q. Sun**: Methodology, data curation, investigation, Writing-Review & Editing. **Y. Bengtsson**: Methodology, formal analysis, data curation, investigation, writing-review & editing. **P. Seemann**: Methodology, writing-review & editing. **J. Vallon-Christersson**: Data curation, resources, investigation, writing-review & editing. **M. Malmberg**: Resources, investigation, writing-review & editing. **L.H. Saal**: Data curation, resources, investigation, writing-review & editing. **L. Rydén**: Data curation, resources, investigation, writing-review & editing. **Å. Borg**: Supervision, project administration, resources, investigation, writing-review & editing. **W.B. Minich**: Methodology, writing-review & editing. **J. Manjer**: Conceptualization, supervision, project administration, resources, formal analysis, investigation, writing–original draft. **L. Schomburg**: Conceptualization, formal analysis, supervision, funding acquisition, project administration, resources, investigation, writing–original draft.

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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.2022.102346.

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Matched analysis of circulating selenium with the breast cancer selenotranscriptome: a multicentre prospective study

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Abstract

Introduction Low serum selenium and altered tumour RNA expression of certain selenoproteins are associated with a poor breast cancer prognosis. Selenoprotein expression stringently depends on selenium availability, hence circulating selenium may interact with tumour selenoprotein expression. However, there is no matched analysis to date.

Methods This study included 1453 patients with newly diagnosed breast cancer from the multicentric prospective Sweden Cancerome Analysis Network – Breast study. Total serum selenium, selenoprotein P and glutathione peroxidase 3 were analysed at time of diagnosis. Bulk RNA-sequencing was conducted in matched tumour tissues. Fully adjusted Cox regression models with an interaction term were employed to detect dose-dependent interactions of circulating selenium with the associations of tumour selenoprotein mRNA expression and mortality.

Results 237 deaths were recorded within ~ 9 years follow-up. All three serum selenium biomarkers correlated positively ($p < 0.001$). All selenoproteins except for GPX6 were expressed in tumour tissues. Single cell RNA-sequencing revealed a heterogeneous expression pattern in the tumour microenvironment. Circulating selenium correlated positively with tumour *SELENOW* and *SELENON* expression ($p < 0.001$). In fully adjusted models, the associations of *DIO1*, *DIO3* and *SELENOM* with mortality were dose-dependently modified by serum selenium ($p < 0.001$, $p = 0.020$, $p = 0.038$, respectively). With increasing selenium, *DIO1* and *SELENOM* associated with lower, whereas *DIO3* expression associated with higher mortality. Association of *DIO1* with lower mortality was only apparent in patients with high selenium [above median (70.36 µg/L)], and the HR (95%CI) for one-unit increase in log(FPKM + 1) was 0.70 (0.50–0.98).

Conclusions This first unbiased analysis of serum selenium with the breast cancer selenotranscriptome identified an effect-modification of selenium on the associations of *DIO1*, *SELENOM*, and *DIO3* with prognosis. Selenium substitution in patients with *DIO1*-expressing tumours merits consideration to improve survival.

Keywords Selenoproteins, SELENOP, Glutathione peroxidase, Thyroid hormones, Prognosis

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Introduction

Breast cancer remains a significant global health challenge, with an estimated 2.3 million new cases and 685,000 deaths annually [1]. Discovery of novel prognostic factors may improve prognosis by identifying high risk women early and by personalizing or intensifying therapy regimens [2].

Recently, there has been growing scientific interest in the role of the essential trace element selenium (Se) in risk, progression and prognosis of breast cancer [3]. Several large epidemiological studies have reported an independent association of low dietary intake or marginal serum levels of Se with a distinctly poor prognosis [4–8]. Se affects various physiological pathways by being translationally incorporated into the products of 25 human selenoprotein genes, that mainly act in antioxidative defense systems, quality and structure control, and by activating or inactivating thyroid hormones [9–11]. The biosynthesis of selenoproteins depends on their RNA expression level and on Se availability, and hereby correlates with Se intake and serum Se concentrations [12]. Accordingly, marginal Se intake correlates to low serum concentrations of the Se transporter selenoprotein P (SELENOP), resulting in suboptimal systemic Se supply [13]. This deficit is e.g. translated in kidney into relatively low biosynthesis and secretion, and hence lower activity of the plasma glutathione peroxidase 3 (GPx3). Accordingly, low serum Se and SELENOP concentrations as well as low serum GPx3 activity have been associated with higher risk of mortality and recurrence after breast cancer diagnosis [4]. At the same time, tumour tissue gene expression levels of several selenoproteins, such as iodothyronine deiodinases (DIO), glutathione peroxidases (GPx) or thioredoxin-reductases (TXNRD) have also been reported as prognostic factors for breast cancer in large-scale genomic profiling studies [14–16].

Despite the evidence that both circulating Se and tumour gene expression of selenoproteins are associated with breast cancer prognosis, there is a lack of data from a matched analysis of serum Se biomarkers and tumour selenoprotein expression. Se availability constitutes a key factor for RNA stability of certain selenoprotein transcripts, and for translation, i.e., Se controls the rate of biosynthesis of the different selenoproteins from a given RNA expression level [17, 18]. Therefore, the association between selenoprotein transcript expression and breast cancer prognosis may be modified by Se availability, and a sufficiently high Se status may be required for translating differences in RNA expression levels into the corresponding gene products and disease-modifying selenoprotein activities.

To test this hypothesis, we simultaneously analysed three complementary biomarkers of Se status (total serum Se, SELENOP, GPx3) and conducted bulk RNA-sequencing of tumours of 1453 patients with a new diagnosis of primary invasive breast cancer. The patients were followed for ~9 years. The main aim was to assess whether the association between tumour RNA expression of selenoprotein genes with breast cancer prognosis is modified by circulating Se and selenoprotein levels.

Methods

SCAN-B study design

The Sweden Cancerome Analysis Network Breast Study (SCAN-B) is a multicentre population based prospective study that intends to identify novel biomarkers for early identification of patients with a poor prognosis based on serum and tumour tissue (genomics) as a matrix. In brief, the study, which is still ongoing since 2010 enrols patients with a newly diagnosed or suspected primary breast cancer in the catchment area of Southern Sweden at multiple cancer centres. The study was approved by the Regional Ethical Review Board in Lund, Sweden (Registration numbers 2009/658, 2010/383, 2012/58, 2013/459, and 2015/277) and registered under the ClinicalTrials.gov ID NCT02306096. All enrolled participants have given written informed consent for inclusion in the study and analyses/procedures, which were conducted as an integrative part of clinical routine, as described before [19].

Follow up and covariate assessment

All clinical data was collected by standardized procedures by clinicians and referred to the Swedish National Quality Registry for Breast Cancer (NKBC). Vital status was obtained from the Swedish National Population Registry, which maintains records for all Swedish citizens. Covariates included patients' characteristics, tumour characteristics, and information on treatment procedures, as described before [5, 19]. These variables comprised age, sex, and information on menopausal status of the patients was reported. Histopathological information comprised tumour size, the side of involved breast, histopathological subtype, histological grade (Nottingham Histological Grade, NHG), Ki67 expression, HER2 expression, ER expression, PGR expression, and number of involved lymph nodes. Information on therapy comprised the surgical procedure regarding the breast, regarding the axillary lymph nodes, application of endocrine or immune therapy, radiation or chemotherapy. Mode of diagnosis, i.e. either clinical or by screening was also reported. The almost full completeness (99.9%) and over 90% validity of NKBC with regard to information on vital status, and other have been externally validated before [20].

Serum selenium biomarkers

Laboratory analyses and results for quantification of Se biomarkers in this study have been described before [4, 5]. In brief, total Se was measured using total reflection X-ray fluorescence (TXRF) spectroscopy, SELENOP was measured using a validated commercial ELISA (selenOtest, selenOmed GmbH, Berlin, Germany), and activity of glutathione peroxidase 3 was measured using an established coupled enzyme reaction. All analyses were conducted by scientists and technicians blinded to any clinical information, with samples arranged in a randomized order with regard to order of enrolment in the study, as reported before [4, 5].

Selenoprotein gene expression in patient's tumours

The detailed protocols for RNA-sequencing have been described before, i.e. either the protocol as shown before [19] or using the Illumina stranded TruSeq mRNA procedure. Protocols were established using the Illumina NeoPrep system or the KingFisher system. For the purpose of this study, gene expression values in Fragments Per Kilobase per Million reads (FPKM) was generated. An established analysis pipeline was used to extract FPKM values by alignment and estimation of gene expression data. The pipeline has been described in detail before [21], and involves the tools picard tools, trimmomatic, bowtie, hisat2, stringtie, dbSNP56 and GENCODE. Genes were annotated based on gene and transcript definitions contained in GENCODE Release 27. After adding an offset of 1, FPKM data were log-transformed for the analyses in this study. Single cell RNA sequencing data was accessed from the Single Cell Portal of the Broad Institute (https://singlecell.broadinstitute.org/single_cell), and included 100,064 single cells from 26 primary breast cancer tumours [22]. Access and visualization was conducted on March 21st 2023.

Statistical analyses

Descriptive statistics were presented as median along with interquartile range (IQR) for continuous variables, and as frequencies along with percent for categorical data. Correlation matrices were generated to depict the relationship between RNA expression of selenoproteins and serum Se biomarkers. Non-parametric Spearman's rank correlation test was applied to compute Spearman's R and p values for the correlations, and cut-off for p-values were adjusted in correlation matrices taking into account the number of genes tested (0.05:23).

Linear multivariable Cox proportional hazards models were employed to calculate hazard ratios along with 95% confidence intervals (CI) for one increment in log(FPKM + 1) in gene expression for each gene. Models were adjusted for established clinical predictors of breast cancer prognosis, i.e. age at diagnosis (years), menopausal status (pre-menopausal, post-menopausal, uncertain), tumour size (mm), NHG (I, II, III), lymph node involvement (at least 4, 1 to 3, submicrometastasis (< 0.2 mm) or no involvement), HER2 expression (positive, negative), ER expression (positive, negative), PGR expression (positive, negative), histological type (ductal, lobular, ductal + lobular/other, other), laterality (right or left breast). Regression analyses were conducted in the entire cohort, and separately in the low and high Se group, based on each different biomarker, while median level of the cohort served as unbiased cut-off. In order to detect potential effect modification by Se biomarkers, an interaction term with the continuous Se biomarker variable was added. An interaction was considered statistically significant in case of $p_{\text{interaction}} < 0.05$. Significant interactions between continuous variables were visualized using contour plots, and by visualizing the association based on tertiles of Se biomarker. Missing variables made up only a small portion (0.4%) of all values contained in variables in the adjusted models (Additional file 1: Fig. S1), therefore Cox regression models were computed using complete cases.

All analyses were conducted using the R language (The R Foundation for Statistical Computing, version 4.3.0) on the RStudio environment (RStudio, PBC, version 2022.2.3.492).

Results

Based on availability of tissue and RNA-sequencing data, as well as serum sampling and Se status assessment, a total of 1453 patients with complete RNA-sequencing and data on serum Se biomarkers were included in the final analyses. A detailed description of the study flow chart is included in Additional file 1: Fig. S2. Follow-up time comprised 9,701 years in total, corresponding to a mean follow-up time of 6.68 years, and 237 deaths were recorded in this time frame.

Baseline patient and tumour characteristics

Baseline patient characteristics and tumour characteristics as well as applied therapy regimens according to vital status during the study are presented in Table 1. Patients were divided based on whether they died during the follow-up period or survived. Patients that died over the course of the follow-up were older at time of diagnosis, more frequently post-menopausal, had larger tumours, more lymph nodes involved, lower serum Se and SELENOP concentrations and a lower serum GPx3 activity. The association of serum Se biomarkers with prognosis have been assessed in this cohort previously, displaying dose-dependent associations of low serum Se with a

Table 1 Baseline patients characteristics according to vital status

Characteristic	Alive, n = 1216	Dead, n = 237
Age	63 (52, 69)	73 (65, 82)
Menopausal status		
Post-menopausal	896 (74%)	215 (91%)
Pre-menopausal	258 (21%)	18 (7.6%)
Uncertain	56 (4.6%)	3 (1.3%)
(Missing)	6	1
Laterality		
Left	618 (51%)	135 (57%)
Right	598 (49%)	102 (43%)
Size (mm)	17 (12, 22)	22 (15, 31)
(Missing)	7	2
Number of lymph nodes involved		
≥ 4	102 (8.7%)	38 (17%)
1–3	336 (29%)	46 (20%)
No involvement	708 (60%)	136 (60%)
Submicrometastasis	25 (2.1%)	6 (2.7%)
(Missing)	45	11
Nottingham histological grade		
I	212 (18%)	24 (10%)
II	569 (48%)	95 (41%)
III	414 (35%)	114 (49%)
(Missing)	21	4
Ki67 expression		
High	163 (56%)	27 (71%)
Low	128 (44%)	11 (29%)
(Missing)	925	199
Histological type		
Ductal	999 (82%)	187 (79%)
Lobular	146 (12%)	30 (13%)
Other	49 (4.0%)	18 (7.6%)
Ductal + lobular/other	20 (1.6%)	2 (0.8%)
(Missing)	2	0
HER2 expression		
Negative	1,041 (86%)	202 (86%)
Positive	164 (14%)	33 (14%)
(Missing)	11	2
ER expression		
Negative	158 (13%)	61 (26%)
Positive	1,056 (87%)	176 (74%)
(Missing)	2	0
PGR expression		
Negative	321 (26%)	99 (42%)
Positive	893 (74%)	138 (58%)
(Missing)	2	0
Selenium (µg/L)	72 (62, 82)	63 (52, 74)
SELENOP (mg/L)	4.10 (3.34, 4.90)	3.71 (2.75, 4.49)
GPx3 (U/L)	208 (177, 240)	189 (152, 229)

Median (IQR); n (%)

Table 2 Therapy regimens according to vital status

Characteristic	Alive, n = 1216	Dead, n = 237
Diagnosis		
Mammography	629 (52%)	74 (31%)
Other	570 (48%)	162 (69%)
(Missing)	17	1
Surgical procedure breast		
Mastectomy	476 (39%)	164 (69%)
Partial mastectomy	740 (61%)	73 (31%)
Surgical procedure axilla		
Clearence only	137 (11%)	49 (21%)
No axillary surgery	3 (0.2%)	4 (1.7%)
Sampling	14 (1.2%)	5 (2.1%)
Sentinel node + clearence	328 (27%)	41 (17%)
Sentinel node surgery	733 (60%)	137 (58%)
(Missing)	1	1
Endocrine therapy	976 (80%)	162 (69%)
(Missing)	1	2
Chemotherapy	486 (40%)	64 (27%)
(Missing)	1	2
Immunotherapy	148 (12%)	17 (7.2%)
(Missing)	1	2
Radiotherapy	826 (68%)	107 (46%)
(Missing)	1	2

n (%)

poor prognosis, independent of various confounders [5]. Table 2 depicts the mode of clinical diagnosis, and the treatment regimens applied in the study cohort, comprising both adjuvant and surgical methods.

Serum selenium and tumour selenoprotein expression

Figure 1 A depicts the study design. Selenoprotein mRNA expression levels within the tumours are displayed in Fig. 1B. As *GPX6* was not expressed in the tumour and *SELENOV* displayed only a low expression, both genes were excluded from further analyses. Analyses included the deiodinase family involved in thyroid hormone regulation (*DIO1-3)*, the glutathione peroxidases involved in antioxidative defence (*GPX1-4*), thioredoxin reductases involved in cellular redox regulation (*TXNRD1-3)*, selenoproteins located within the endoplasmic reticulum (e.g. *SELENOM*, *SELENOF* etc.), as well as the other selenoproteins with specific functions [9]. Figure 1 C displays the correlation between tumour selenoprotein expression and serum Se and selenoprotein levels. Within the group of selenoprotein genes, the highest correlation was between *MSRB1* and *SEPHS2* (R = 0.57), while *SELENOI* and *DIO3* displayed the most prominent negative correlation (R = -0.34). All three serum biomarkers correlated positively, with serum Se and SELENOP displaying the

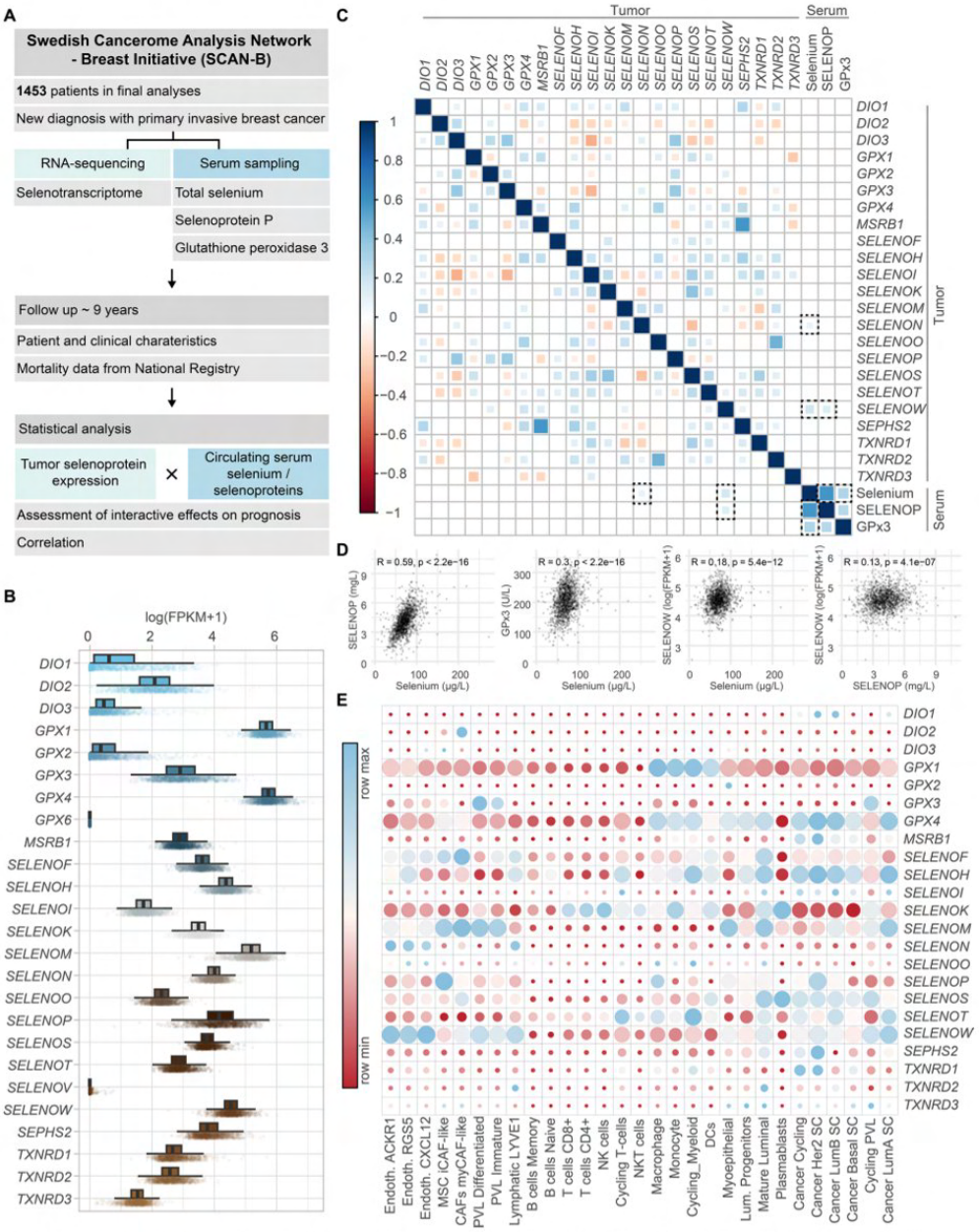

Fig. 1 **A** Study scheme. **B** Gene expression of selenoproteins in tumour samples of 1453 patients. **C** Spearman's correlation matrix of tumour gene expression of selenoprotein genes and circulating selenium biomarker concentrations. **D** Spearman's correlation of serum biomarkers with each other and serum biomarkers with SELENOW expression in the tumour. **E** Single cell RNA-expression of selenoprotein genes in different cells in breast cancer

highest correlation (R = 0.59). Serum Se biomarkers were mostly not correlated with selenoprotein gene expression levels, except for a weak correlation between serum Se and SELENOP with *SELENOW* (R = 0.18 and R = 0.13, respectively) (Fig. 1D), and between serum Se and *SELENON* (R = 0.082). Figure 1E displays the selenoprotein gene expression in different cells in breast cancer.

Selenoprotein gene expression and survival based on selenium biomarkers

Figure 2 displays the association of selenoprotein mRNA expression of each gene with survival, in the whole cohort and in patients with low or high serum Se levels. There were significant interactions between serum Se with *DIO1*, *DIO3*, and *SELENOM*, $p < 0.001$, $p = 0.020$, and $p = 0.038$, respectively. Association of *DIO1* with lower mortality was only apparent in patients with high Se [above median (70.36 µg/L)], HR (95%CI) for one-unit increase in log(FPKM + 1) was 0.70 (0.50–0.98).

The complex interaction between serum Se and *DIO1*, *DIO3 and SELENOM* is depicted in Fig. 3. Figure 3 A displays lower hazard ratios for a simultaneous increase in serum Se and *DIO1* expression. This relationship is emphasized in Fig. 3B, which shows a decreased hazard ratio with increasing *DIO1* levels, however only in patients with a relatively high Se level, i.e. residing in the 2nd or 3rd tertile. On the other hand, Fig. 3C displays an inverse interaction of Se with *DIO3* levels, where increasing serum Se and simultaneous increase in *DIO3* are associated with an elevated hazard ratio. Accordingly, *DIO3* is associated with higher mortality in patients with high Se only, i.e., solely in the 3rd tertile (Fig. 3D). The interaction of serum Se with *SELENOM* was similar to *DIO1*, where *SELENOM* associated with a lower mortality rate with increasing Se levels (Fig. 3E and F).

Interaction analyses were conducted for serum SELENOP concentrations (Additional file 1: Fig. S3) and GPx3 activity levels (Additional file 1: Fig. S4). The interaction with *DIO1* remained prominent with the Se transporter SELENOP ($p_{interaction} = 0.001$). In patients with relatively high serum SELENOP, i.e. above the cohort median of 4.05 mg/L, HR for one-unit increase in log(FPKM + 1) for *DIO1* was 0.64 (0.48–0.86). There was no interaction between serum GPx3 activity and *DIO1*, *DIO3*, or *SELENOM* expression, but for *GPX1* RNA.

In further sensitivity analyses, interactions of serum Se with DIO1, DIO3 and SELENOM were tested after further adjusting for treatment methods used. Endocrine therapy, immune-, chemo- and radiotherapy as well as surgical procedures regarding the breast and axilla were added to the fully adjusted models one by one, and all combined, and nearly no changes were observed in p values for interaction (Additional file 1: Table S1).

An analysis of The Cancer Genome Atlas Program (TCGA) data displayed no overall associations of *DIO1*, *DIO3*, *SELENOM*, *SELENOW*, and *SELENON* with survival, when not incorporating serum Se (Additional file 1: Fig. S5), highlighting the need for consideration of both serum Se and tumour selenoprotein expression in order to ascertain effects of selenoprotein mRNA expression on prognosis.

Discussion

In this large multicentric prospective study, the first matched analysis of circulating serum Se levels with the breast cancer selenotranscriptome was performed. As expected, serum Se levels were mostly unrelated to selenoprotein mRNA expression levels. Serum Se dose-dependently interacted with the association of *DIO1*, *DIO3*, and *SELENOM* and survival. With increasing serum Se, *DIO1* and *SELENOM* associated with lower, and *DIO3* expression associated with higher mortality. These opposing Se-dependent effects of *DIO1* and *DIO3* imply a mechanism of action involving alteration of local thyroid hormone status, in agreement with prior data and experiments with model systems [23, 24]. Selenium substitution particularly in patients with *DIO1* expressing tumours may improve survival, which should be considered for testing as an adjuvant therapy in randomized controlled trials.

Aberrations in the genome of breast cancer lead to alterations in expression of various genes, and hence to alterations in protein levels. Some of these proteins, such as HER2, ER, PGR are involved in key-regulatory mechanisms of breast cancer progression [25, 26]. Therefore, assessment of RNA-expression as indirect measure of actual protein levels constitutes an increasingly popular tool to predict prognosis [27–29]. For most proteins, RNA-expression has been shown to be a reliable proxy for actual protein levels [27]. Expression of selenoproteins however, is subject to more complex regulation involving a key limiting Se-dependent step in the translational process [30]. The incorporation of the characteristic Sec residue(s) via recoding of the UGA stop codon to a sense codon is an important regulator of translation during the biosynthesis of functional selenoproteins, which is mainly regulated by both transcript abundance and dietary intake of Se [31, 32]. Thus, RNA-sequencing of selenoproteins may reliably reflect true protein expression only in patients where Se intake is sufficiently high, ensuring saturated levels of circulating Se needed for optimal supply of tissues and high intracellular Se concentrations. In statistical terms, this hypothesis is described as a testable interaction between serum Se and gene expression levels in relation to survival.

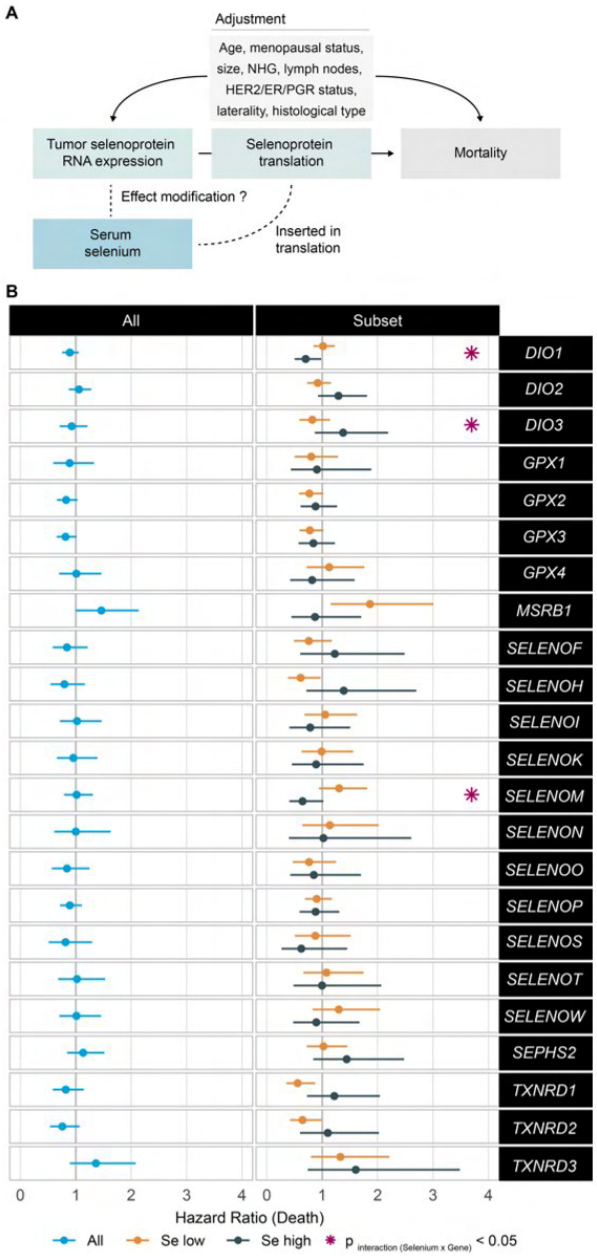

Fig. 2 **A** Analysis scheme. **B** Cox regression models in the whole cohort and in low and high selenium subsets, divided according to median selenium concentration of the cohort, i.e. 70.36 µg/L. All models were adjusted for age, tumour size, histological grade, lymph node involvement, expression of HER2/ER/PGR-Receptor, laterality of the tumour, and histological type. P for interaction was tested by adding an interaction term between serum selenium and the gene of interest, marked by purple asterisk

In line with this hypothesis, we observed strong interaction effects, particularly for *DIO1* and *DIO3*. Deiodinases are the most important regulators of thyroid hormone activity, which is essential for cellular proliferation and differentiation, and hence implicated in cancer progression and cancer mortality [14, 33, 34]. Conflictingly, both increased and decreased circulating thyroid hormone levels have been linked to breast cancer survival, which may be attributed to the complex nature of thyroid hormone transport, metabolism and action [35–38]. Local regulators, such as thyroid hormone transporters, receptors and deiodinases play a critical role in thyroid hormone metabolism and action. Recent studies have demonstrated that DIO3 is a prognostic factor in breast cancer [14], and that e.g. low thyroid hormone receptor alpha 2 expression is associated with higher breast cancer mortality [35]. In addition to promoting proliferation, thyroid hormone action also mediates cellular differentiation in physiological processes [39]. Active thyroid hormones have been found to induce differentiation into a more benign phenotype in hepatic cancer cells [40]. In murine models of basal cell carcinoma, *Dio3* knockdown with concomitant increase in local T3 led to a five-fold decrease in tumour growth [41]. Our findings are in agreement with these studies, and suggest a Se-mediated potentiation of the favourable effects of DIO1 and the unfavourable effects of DIO3 on breast cancer survival. These findings indicate potential involvement of local thyroid hormone action in breast cancer progression, as DIO3 is the primary thyroid hormone inactivating enzyme, and DIO1 plays a crucial role in the deiodination of T4 to T3 [33]. Further epidemiological and mechanistic studies are necessary to investigate this hypothesis in more detail.

The association of SELENOM with mortality was also modified by circulating Se levels, potentiating the favourable association with survival. *SELENOM* encodes selenoprotein M, which is located in the endoplasmic reticulum and involved in protein folding [42]. Although little is known about the specific roles of selenoprotein M, its functional homolog selenoprotein F (SELENOF) has been shown to be involved in cancer progression [43, 44]. In line with our findings of favourable effects of *SELENOM* on survival, SELENOF was recently described as a tumour suppressor in breast cancer, and enhancing its expression reduced tumour growth both in vivo and in murine breast cancer models [45]. Similar to SELENOF, and in line with our findings higher expression of SELENOM has been shown to be a protective prognostic factor in other cancer types, such as gastric cancer and cholangiocarcinoma [46, 47].

Interactions with circulating Se were observed for three of the 23 tested selenoprotein genes only. One likely explanation for this finding is based on the organ- and selenoprotein-specific hierarchical regulation of selenoprotein expression, which serves to provide regular functioning of essential tissues in a Se deficient state. Hence, some of the selenoproteins are preferentially sustained in case of Se deficiency, while others display a more responsive decrease in activity and reduced expression when the supply is low [48]. Although this hierarchy is further regulated specific to different tissues, DIO1 has been shown to be one of the most responsive selenoproteins in the liver, displaying 95% decrease in activity in rats with severe Se deficiency [49]. This is in line with our findings, as the interaction between *DIO1* and circulating Se levels was the most prominent. Another explanation is the heterogenous distribution of selenoprotein gene expression within cells residing in the tumour and its microenvironment, as outlined in Fig. 1E. While *DIO1* as an instance is mostly abundant in cancer cells, *DIO2* shows nearly no expression in malignant cells, but rather in cancer associated fibroblasts, which may explain the lack of interaction for this member of the deiodinase family in our study.

Previously in the cohort used in this study, serum Se, serum SELENOP concentrations, activity of the serum GPx3 as well as novel autoantibodies targeting SELENOP were shown to be independent predictors of survival after breast cancer diagnosis, outperforming established clinical prognostic markers [4, 5]. Other large breast cancer studies are in line with these findings [6–8], and the effects seem to be also consistent for some other cancer entities [50–54]. In this study, we aimed to further exploring the potential mechanisms of action underlining the consistent associations, and to examine whether patients with tumours displaying certain selenoprotein

(See figure on next page.)

Fig. 3 **A** Contour plot of the interaction between *DIO1* expression and serum selenium concentrations on mortality. **B** Cox regression models depicting the association of DIO1 expression with mortality according to 10th, 50th and 90th quantiles of circulating selenium. **C** Contour plot of the interaction between *DIO3* expression and serum selenium concentrations on mortality. **D** Cox regression models depicting the association of DIO3 expression with mortality according to 10th, 50th and 90th quantiles of circulating selenium. **E** Contour plot of the interaction between *SELENOM* expression and serum selenium concentrations on mortality. **F** Cox regression models depicting the association of *SELENOM* expression with mortality according to 10th, 50th and 90th quantiles of circulating selenium. All models were adjusted for age, tumour size, histological grade, lymph node involvement, expression of HER2/ER/PGR-Receptor, laterality of the tumour, and histological type

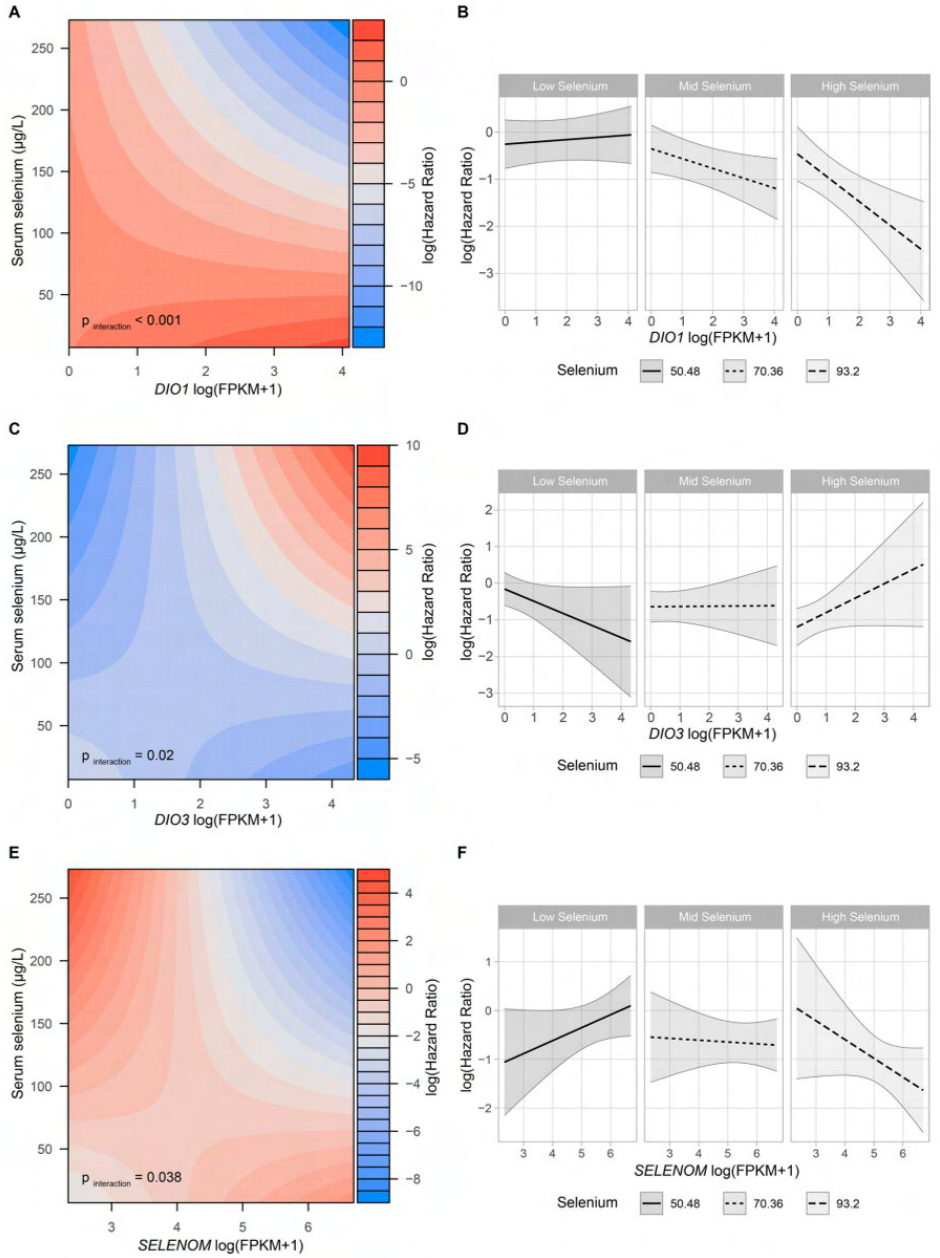

Fig. 3 (See legend on previous page.)

gene expression profiles are particularly likely to benefit from a higher Se status. Our results indicate a potential mechanism of action in local thyroid hormone action due to significant and inverse interactions with *DIO1* and the thyroid hormone inactivating *DIO3*. Clinically, our results indicate that Se-deficient patients with *DIO1* expressing tumours may distinctly benefit from Se substitution, whereas patients with high DIO3 may not.

To the best of our knowledge, this study represents the first attempt to investigate the relationship between serum Se levels and RNA levels of selenoproteins in tumour tissues simultaneously in relation to survival, providing a more comprehensive understanding of the complex relationship between Se, selenoproteins, and cancer progression. Another noteworthy strength includes the large sample size as well as the large number of covariates assessed by physicians and pathologists. This allowed for conducting complex interaction analyses that require large sample sizes. The nearly complete database enabled studying effects independent of established clinical prognostic factors. The primary outcome, all-cause mortality derives from the Swedish National Registry, and the covariates were extracted from NKBC, which exhibited over 99.9% completeness and over 90% validity in an independent validation study conducted at time of participant recruitment of this study [20]. SCAN-B has been fully integrated into clinical routine, without interfering with clinical decision making, which increases generalizability of the results. Importantly, Se status was measured by multiple biomarkers, all linearly correlating, which ensures a correct quantification of the main exposure. All Se measurements were made in a double-blinded fashion, reducing risk of bias and increasing internal validity of the results.

Despite the strengths, a significant limitation is the observational nature, which precludes the ability to infer causality. Although adjustment was done for most important potential confounders, there may be residual confounding. A set of patients were excluded due to missing variables, although missingness was very low (Additional file 1: Fig. S1). The study's population sample from Sweden may limit its generalizability to other populations and ethnicities other than European. Further studies in other populations are necessary to confirm the findings.

Conclusion

Our unbiased analysis of circulating Se levels and the breast cancer selenotranscriptome revealed that Se modifies (potentiates) the associations of type 1 deiodinase expression with a favourable prognosis. On a mechanistical aspect, the study contributes to a growing evidence of an importance of thyroid hormones on cancer progression. Specifically, the favourable effects of *DIO1* and opposing effects of *DIO3* together argue for a beneficial effect of in-tissue local thyroid hormone action.

Clinically, the results provide a translational value, as serum Se measurement and (histo)pathological assessment of *DIO1* expression in tumours of breast cancer patients undergoing surgery can be integrated into clinical routine to stratify patients according to potential benefit from Se substitution. As our data provides observational evidence, however, this potential improved survival remains to be tested in well-designed RCTs. Nevertheless, this approach can readily be used in clinical routine in order to provide (surrogate) prognostic value.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-023-04502-y.

Additional file 1: Figure S1. Missing values in variables used in Cox regression models. **Figure S2.** Study flow chart. Adapted from Demircan K, Bengtsson Y, Sun Q, Brange A, Vallon-Christersson J, Rijntjes E, Malmberg M, Saal LH, Rydén L, Borg Å, Manjer J, Schomburg L. Serum selenium, selenoprotein P and glutathione peroxidase 3 as predictors of mortality and recurrence following breast cancer diagnosis: A multicentre cohort study. Redox Biol. 2021 Nov;47:102145. **Figure S3.** Cox regression models in the whole cohort and in low and high SELENOP subgroups. Subgroups were divided according to median SELENOP concentration of the cohort, i.e. 4.05 mg/L. All models were adjusted for age, tumour size, histological grade, lymph node involvement, expression of HER2/ER/PGR-Receptor, laterality of the tumour, and histological type. P for interaction was tested by adding an interaction term between serum SELENOP and the gene of interest, marked by purple asterisk. **Figure S4.** Cox regression models in the whole cohort and in low and high GPx3 subgroups. Subgroups were divided according to median GPx3 concentration of the cohort, i.e. 205 U/L. All models were adjusted for age, tumour size, histological grade, lymph node involvement, expression of HER2/ER/PGR-Receptor, laterality of the tumour, and histological type. P for interaction was tested by adding an interaction term between serum GPx3 and the gene of interest, marked by purple asterisk. **Figure S5.** Kaplan Meier analyses of genes interacting with serum selenium in TCGA-BRCA data. Patients were compared according to mRNA expression for each candidate gene, based on being in the highest quartile (Q4) vs lowest (Q1). Log-rank test was applied to detect differences. GEPIA2 was used to plot survival, accessed on 25th August 2023, on http://gepia2.cancer-pku.cn/). Tang, Z. et al. (2019) GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. Nucleic Acids Res, 10.1093/nar/gkz430. **Table S1**. P values for interaction between DIO1, DIO3, SELENOM and serum selenium, further adjusted for therapy regimens.

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Authors' contributions

KD: conceptualization, formal analysis, data curation, software, formal analysis, investigation, visualization, methodology, writing–original draft, writing—review and editing. YB: methodology, formal analysis, data curation, investigation, writing—review and editing. TSC: methodology, data curation, investigation, writing—review and editing. JV-C: data curation, resources, investigation, writing—review and editing. QS: methodology, data curation, investigation, writing—review and editing. CL: data curation, resources, investigation, writing—review and editing. MM: resources, investigation, writing—review

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Availability of data and materials
RNA sequencing data are fully openly accessible at Mandalay Data [55]. Clinical and pathological tumour data are fully publicly available [21]. Data on trace elements can be applied for at the SCAN-B steering committee. R code for statistical analyses can be applied for from the corresponding authors.

Declarations

Ethics approval and consent to participate
The study was approved by the Regional Ethics Review Board of Lund at Lund University (diary numbers 2007/155, 2009/658, 2009/659, 2010/383, 2012/58, 2013/459) and registered under the ClinicalTrials.gov ID NCT02306096. All participants gave written consent to participate in the study.

Consent for publication
All authors have approved the manuscript for submission.

Competing interests
LS holds shares of selenOmed GmbH, a company involved in selenium status measurement. The remaining authors have no competing interests to declare.

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Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

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Publication list

Peer reviewed original research articles

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