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Serum estrone concentration, estrone sulfate/estrone ratio and BMI are associated with human epidermal growth factor receptor 2 and progesterone receptor status in postmenopausal primary breast cancer patients suffering invasive ductal carcinoma

Borbála Vincze^{1*}, Bence Kapuvári¹, Nóra Udvarhelyi², Zsolt Horváth³, Zoltán Mátrai⁴, Ferenc Czeyda-Pommersheim⁴, Krisztina Kőhalmy¹, Judit Kovács¹, Mariann Boldizsár¹, István Láng⁵ and Miklós Kásler⁶

Abstract

Background: We investigated in postmenopausal women with primary breast cancer prior to surgical intervention whether, serum levels of different steroid hormones and hormonal precursors associated with tumor tissue estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) status.

Methods: We enrolled 1,042 patients suffering invasive ductal carcinoma undergoing surgical resection in the National Institute of Oncology, Hungary between 2003 and 2011. Serum parameters were measured by RIA/IRMA assays; tumor tissue ER, PR and HER2 status was assessed histologically. Patients were classified according to tumor receptor status. Case–case analysis subjects were categorized into four subgroups based on serum hormone concentrations in ER, PR and HER2 receptor-negative cases, respectively.

Results: Serum estrone sulfate and dehydroepiandrosterone sulfate levels correlated with each other and also with serum estrone and estradiol levels. According to case–case study the odds ratios in the highest quartile were 1.517 (p = 0.0305, $P_{trend} = 0.0394$) for androstenedione, 1.495 (p = 0.0317, $P_{trend} < 0.0105$) for estrone and 0.654 (p = 0.0273, $P_{trend} < 0.0151$) for estrone sulfate/estrone ratio in PR+ vs. PR– tumors. Regarding HER2 status (HER2+ vs. HER2–), the odds ratios for estrone, estrone sulfate and estrone sulfate/estrone ratio were 0.530 (p = 0.0234, $P_{trend} = 0.0595$), 2.438 (p = 0.0042, $P_{trend} < 0.0066$) and 3.118 (p = 0.0001, $P_{trend} < 0.0001$) in the highest quartile, respectively. Of note significantly increased BMI associates with PR+ and ER +/PR+ status while significantly decreased BMI was observed in HER2+ cases.

Conclusions: Taken together, measurement of serum estrone and estrone sulfate concentrations prior to surgical intervention might support the individualization of regime in postmenopausal primary breast cancer patients.

Keywords: Postmenopausal breast cancer, Invasive ductal carcinoma, Estrone, Estrone sulfate, Progesterone receptor, HER2

*Correspondence: vincze@oncol.hu

1122 Budapest, Ráth György u. 7-9., Hungary

Full list of author information is available at the end of the article



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¹ Department of Biochemistry, National Institute of Oncology,

Background

Breast cancer is a very heterogeneous disease that can be classified to molecular, histopathologic and clinical subgroups. Anticancer therapy is determined by biological characteristics and stage of the tumor. Most important biological features determining therapy are endocrine sensitivity, human epidermal growth factor receptor 2 (HER2) expression and proliferative capability of the tumor (Láng et al. 2012).

Approximately 70% of breast cancer cases express estrogen receptor (ER) and progesterone receptor (PR) thus are referred as hormone receptor (HR)-positive. HER2 expression is presented in 20–25% of breast cancer cases that are classified as HER2-positive (HER2+) (Ross et al. 2009; Dawood et al. 2010). Approximately 50% of HER2-positive cases are also HR-positive (ER+/PR+/HER2+) (Dowsett et al. 2008; Tripathy et al. 2013; Mehta and Tripathy 2014). About 10–20% of invasive breast cancer cases do not express ER, PR or HER2 and are termed as of triple negative receptor status (ER-/PR-/HER2-) (Perou 2011; Aysola et al. 2013).

Estrogens play a crucial role in breast cancer progression through inhibition of apoptosis and stimulation of cell proliferation via ER activation (Hankinson and Eliassen 2007). Several epidemiological studies indicate that plasma estradiol (E2), adrenal androgens and testosterone (TE) levels were higher in women who developed ER-positive breast cancer later (Key et al. 2002; Zeleniuch-Jacquotte et al. 2004; Missmer et al. 2004; Kaaks et al. 2005; Cummings et al. 2005; Sieri et al. 2009; Endogenous Hormones and Breast Cancer Collaborative Group 2013). A Danish population-based prospective study revealed that the association between the risk of postmenopausal breast cancer and serum estrone (E1) or estrone sulfate (E1S) levels is stronger than that between E2 and breast cancer risk (Würtz et al. 2012). Zhang et al. (2013) demonstrated that blood sex hormone levels measured at a single time-point would predict the development of breast cancer within up 16-20 years.

Epidemiological studies focused on the association between sex hormones and breast cancers or excluded ER-negative breast cancers from the analysis (Key et al. 2002; Zeleniuch-Jacquotte et al. 2004; Kaaks et al. 2005; Cummings et al. 2005). The reason of exclusions from several cohort studies is that the number of ER-/PRcases was relatively small; the statistical power was insufficient to assess other relevant breast cancer subtypes, such as triple negative, or HER2-positive (Zhang et al. 2013).

It is well documented that ER/PR and HER2 status predict the clinical outcome and the response to adjuvant endocrine therapy or poly-chemotherapy. However, a detailed hormone profile determined before surgical intervention can also support to predict the hormone sensitivity of the tumor. Based on biological and clinical observations it was suggested that plasma levels of sulfoconjugated and unconjugated steroid hormones and tissue-specific expression of steroid sulfatase (STS) might play a significant role in breast cancer biology and might regulate the effects of endocrine therapy (Falany and Falany 2007). Kim et al. found that high preoperative serum E2 level indicate worse prognosis in postmenopausal women with breast cancer, particularly in those with ER-negative cancer (Kim et al. 2013). However, the interaction between sexual hormone levels before surgery and receptor status was not investigated widely.

Our aim was to investigate whether circulating steroid hormone levels including sexual hormones and their precursors along with sex hormone binding globulin [i.e. E1, E1S, E2, TE, dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), androstenedione (AD) and SHBG] measured prior surgical intervention show any association with tumor ER, PR and HER2 status in postmenopausal women with primary breast cancer. In our case-case study the relationship between serum sexual hormone levels and tumor ER, PR and HER2 status was retrospectively studied using data collected from postmenopausal patients treated with breast cancer between 2003 and 2011 in the National Institute of Oncology, Hungary.

Methods

Patients

Our study involved 1381 postmenopausal patients with primary breast cancer (stages ranging between 0 and III). Women were considered postmenopausal when they reported not having any menstrual cycles in the past 24 months; those with bilateral ovariectomy in medical history; and those with age above 55 years (Kaaks et al. 2005).

Distribution of the patients according to age: 485 cases (55–59 years), 528 cases (60–69 years), 271 cases (70–79 years) and 97 cases (\geq 80 years). The mean age of the population studied was 64.7 ± 9.1 years.

The diagnosis of breast cancer was confirmed by histology in all cases. Tumors had been diagnosed by experienced pathologists using standard criteria for histology and grading. All patients had resectable stage 0–III tumors according to the TNM 6.0 staging (Union International Cancer Congress, TisN0M0-T2N3M0). The histological diagnosis was mainly invasive ductal carcinoma (IDC) 1,042 (75.45%), IDC in situ (DCIS) 84 (6.08%), invasive lobular carcinoma (ILC) 140 (10.14%) and others 115 (8.33%) (including metaplastic, adenoid, papillary, apocrine, cribriform, medullary, mucinous and tubular invasive carcinomas). Patients were diagnosed mainly with IDC (1,042 cases) therefore statistical analysis was performed within this subgroup.

The study was approved by the Institutional Review Board and Ethics Committee of National Institute of Oncology, Hungary since 2003. Permission of the Hungarian Regional Committee of Science and Research Ethics was obtained for retrospective evaluation of the data (Number of permission: 322/2014).

At the time of blood sampling all patients was informed about the purpose of our study. Written informed consent was obtained from all individual participants included in the study. Patients donated 8–10 ml blood sample and completed a questionnaire about reproductive history, previous use of contraceptives, postmenopausal hormone replacement therapy (HRT). Women who used HRT at the last 6 months and/or had a diagnosis of cancer within 10 years before surgical intervention were not elected to the present study. Patients who received neoadjuvant chemotherapy were excluded, as well.

Determination of serum hormone levels

Blood samples were collected according to a standardized protocol. Briefly, the whole blood was centrifuged at 2,500 g for 15 min. The serum was removed from the blood clot and stored in aliquots at -20° C until the determination. Measurements were carried out within 2-3 months in all cases. Steroid hormone assays were performed in our Laboratory on Department of Biochemistry (NIO). Since the concentration of serum E2 is usually lower than 40 pmol l^{-1} in postmenopausal women, serum E2 level was determined by using an ultra-sensitive estradiol radioimmunoassay (RIA) kit (Immunotech, Prague, Czech Republic, detection limit: 8.14 pmol l^{-1}). E1S, DHEA and AD were measured by RIA kit (Immunotech, Prague, Czech Republic). Total TE and DHEAS were measured by Immunotech SAS RIA kit (Marseille, France); E1 concentration was measured by RIA with a Diasource Immunoassays S.A. kit (Louvainla-Neuve, Belgium). SHBG was measured by Izinta kit (Isotope Institute, Budapest, Hungary). The mean intraand inter-assay coefficient of variations were 7.5 and 9.4% for E2, 14.8 and 15.0% for TE, 6.3 and 8.6% for E1, 6.1 and 4.3% for SHBG, 9.2 and 8.8% for E1S, 5.6 and 9.8% for AD, 7.4 and 10.6% for DHEAS, 3.8 and 8.6% for DHEA. Stratec SR 300 (Birkenfeld, Germany) an automatic, open analyser system was used to detect ¹²⁵I radioactivity.

Free TE and E2 concentrations were calculated from the concentrations of E2, TE and SHBG according to previous studies (Vermeulen et al. 1999; Endogenous Hormones and Breast Cancer Collaborative Group 2003).

Assessment of tumor receptor status

The ER and PR status were evaluated histologically on immunohistochemical (IHC) slides [ER: SP1 (NeoMarkers), PR: NCL-L PGR-312 (Novocastra)]. ER and PR positivity were defined when at least 10% prevalence of malignant cells exhibiting staining characteristics.

HER2 protein overexpression was assessed by IHC method using three different antibodies: RTV-CB11 (Novocastra), C-erbB-2/Her-2/neu SP3 clone (NeoMarkers) and HercepTest (DAKO). Samples were scored using the recommended scoring system for the HercepTest.

HER2 gene amplification was tested with the Inform-HER2 test by Ventana. The updated cut-off value for positive cases is more than six copies of the gene. HER2 was scored positive if the result was 3+; 2+ was considered to be positive only if it was confirmed by fluorescence in situ hybridization (FISH).

Statistical analysis

MedCalc Software was used for statistical analysis. In the case of a normal distribution, the correlation between serum hormone parameters was calculated using the correlation coefficient (r), in the remaining cases Spearman's coefficient of rank correlation (rho) was used. 95% confidence interval (95% CI) for r or rho was computed.

During case–case study hormone receptor-positive vs. hormone receptor-negative postmenopausal breast cancer patients by quartiles of serum steroid concentration were compared. The hormone receptor negative cases were categorized into four classes according to hormone levels. Receptor-positive/receptor-negative ratio was calculated belong to the four serum hormone concentration ranges. Odds ratios (ORs) were computed taking the lowest category of hormone receptor-negative cases as reference (Begg and Zhang 1994). ORs with 95% CIs and P_{trend} are presented by quartile limits of serum parameters.

Chi-square test was used (with Yates' correction for continuity) for the investigation of independence of numerical variables and the determination of linear trends (P_{trend}) among the groups classified by tumor receptor status.

Results

Selection of case subjects

IDC patients (1,042 cases) were categorized according to histological grade (HG), stadium (St) and ER, PR, HER2 receptor status (Table 1).

We classified the cases according to their joint status of hormone and HER2 receptors. For the case–case study we defined the following tumor subtypes: ER+ (852 cases) vs. ER– (190 cases); PR+ (709 cases) vs. PR– (333 cases); ER+/PR+ (703 cases) vs. ER–/PR– (184 cases); ER+/PR– (149 cases) vs. ER–/PR– (184 cases) and

Table 1 Categorization of the subjected primary breast cancer patients suffering invasive ductal carcinoma (IDC) (total number: 1,042) according to histological grade (HG), stadium (St) and receptor status

Factor	Numbe
IDC histological grade (HG) (IDC total number: 1,042)	
HG 1	244
HG 2	425
HG 3	373
IDC stadium (St) (IDC total number: 1,042)	
1	489
2	336
2a	103
2b	43
3	47
3a	15
3b	2
3с	7
Receptor status (IDC total number: 1,042)	
ER+	852
ER—	190
PR+	709
PR-	333
ER+/PR+	703
ER—/PR—	184
ER+/PR-	149
ER—/PR+	6
HER2+	123
HER2—	949
HER2+/ER+/PR+	33
HER2-/ER+/PR+	670
HER2-/ER+/PR-	125
HER2+/ER-/PR-	65
HER2-/ER-/PR-	119

ER estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2.

HER2+ (123 cases) vs. HER2- (949 cases) (Table 1). In addition, further subgroups were also established according to the combined presence/absence of ER, PR and HER2. These subgroups were as follows: HER2+/ER+/PR+ (33 cases) vs. HER2-/ER+/PR+ (670 cases); HER2+/ER-/PR- (65 cases) vs. HER2-/ER-/PR- (119 cases) and HER2-/ER+/PR+ (670 cases) vs. HER2-/ER+/PR- (125 cases) (Table 1).

Serum hormone levels

Hormone measurements were completed for all 1,042 cases except E2. Until 2005, the IMMUNOTECH RIA kit had been used for serum E2 measurements, which had low sensitivity in the range of <40 pmol l^{-1} . The values of E2 concentration measured by the two

different kits significantly differ from each other. Therefore, the measurements done with IMMUNO-TECH RIA kit were excluded from the analysis (392 subjects).

Later on the serum E2 concentration was measured with an ultra-sensitive RIA kit in 650 (62%) patients. The average E2 value was 60.53 pmol l^{-1} (95% CI of the mean was 54.97–66.09), therefore this ultra-sensitive kit is appropriate for the detection of low serum E2 level in the majority of postmenopausal women. Only these data were used in the statistical analysis.

Serum concentrations of the steroid hormones correlated significantly with each other. DHEAS significantly correlated with DHEA r = 0.704 (p < 0.0001), TE/ SHBG ratio r = 0.441 (p < 0.0001) and AD rho = 0.391 (p < 0.0001) (data are not shown). Serum E1 and E2 levels strongly correlated with DHEAS (r = 0.543, p < 0.0001 and r = 0.463, p < 0.0001) (data are not shown) and with E1S, the major substrate for STS (r = 0.457, p < 0.0001 and r = 0.509, p < 0.0001), respectively (Fig. 1a, b). E1 and E2 correlated significantly with each other r = 0.518 (p < 0.0001) and with and rogens (AD, free TE) (data are not shown). The association between E1 and AD, as well as E2 and TE/SHBG, was r = 0.415 (p < 0.0001) and r = 0.433 (p < 0.0001), respectively (data are not shown). The serum levels of sulfoconjugated steroids (DHEAS, E1S) also correlated significantly with each other r = 0.529 (p < 0.0001) (Fig. 2).

Case-case study

To assess the impact of receptor status on serum steroid hormone levels, case–case comparisons and Chi-square test were used (OR, 95% CI, P_{trend}). IDC patients (1,042 cases) were categorized into different classes based on hormone receptor status as written earlier.

In ER+ vs. ER- tumors the alteration of ORs were significant only in case of E1 level (OR = 1.663, p = 0.0026, $P_{trend} = 0.0101$) (Table 2).

Our results showed that E1 levels were significantly elevated in the fourth quartile of PR+ vs. PR- tumors (OR = 1.495, p = 0.0317; $P_{trend} = 0.0105$). The same association was observed in case of serum AD levels (OR = 1.517, p = 0.0305; $P_{trend} = 0.0394$). Due to association between E1S and E1 levels we examined the association between E1S/E1 ratio and hormone receptor status (PR+ vs. PR-). E1S/E1 ratio significantly decreased (OR = 0.654, p = 0.0273) and a significant trend was also present ($P_{trend} = 0.0151$). There was no significant difference in serum E2, TE SHBG concentrations and E2/SHBG ratio. The TE/SHBG ratio showed only a significant trend ($P_{trend} = 0.022$). In case of BMI, we found a significant elevation in the third (OR = 1.638,



p = 0.0487) and fourth quartiles (OR = 1.658, p = 0.0409) with a significant trend ($P_{trend} = 0.0044$), as well (Fig. 3).

In ER+/PR+ vs. ER-/PR- cases, the same trend ($P_{trend} = 0.0078$) for E1 was observed in the fourth quartile as described in ER+ vs. ER- cases (OR = 1.766, p = 0.0158) (Table 2). Likewise PR+ vs. PR- cases, BMI presented the same tendency in the third (OR = 1.639, p = 0.0379) and in the fourth quartiles (OR = 1.697, p = 0.0261) with a positive trend ($P_{trend} = 0.0244$).

This is the first study that investigated associations between serum sexual hormones and HER2 status of the tumors in invasive ductal breast cancer. HER2 overexpression (HER2+ vs. HER2-) was assessed with significantly decreased E1 levels (OR = 0.530, p = 0.0234). Serum E1S levels showed a significant elevation and



positive trend ($P_{trend} = 0.0066$) in the fourth (OR = 2.438, p = 0.0042) quartiles in HER2-positive cases. The ratio of E1S/E1 was increased significantly (OR = 3.118, p = 0.0001) with a positive trend ($P_{trend} < 0.0001$). In addition, AD/E1 ratio also showed significant elevation (OR = 1.922, p = 0.0282) in the third quartile, but the trend was not seen. BMI significantly decreased in the fourth quartile (OR = 0.475, p = 0.0113) and the trend was also significant ($P_{trend} = 0.0027$) (Fig. 4).

confidence interval.

Contrary to ER+ vs. ER- and ER+/PR+ vs. ER-/ PR- subgroups in HER2-/ER+/PR+ vs. HER2-/ ER+/PR- cases serum E2, E2/SHBG and E1S/E1 ratios showed significant or nearly significant trend. OR of E2 (OR = 1.667) and E2/SHBG (OR = 1.992) increased in the fourth quartile; with positive trend (P_{trend} = 0.0544, P_{trend} = 0.0257). Similar to PR+ vs. PR- cases, OR of E1S/E1 decreased to 0.582 in the fourth quartile and the trend was nearly significant (P_{trend} = 0.0530). OR of BMI increased in the fourth quartile (OR = 1.887) with a nearly significant trend (P_{trend} = 0.0538) (Table 2).

HER2+/ER-/PR- vs. HER2-/ER-/PR- cases did not show any significant changes. Interestingly, E1S/E1 ratio elevated in the fourth quartile (OR = 2.083) with an almost significant positive trend ($P_{trend} = 0.0724$) (Table 2). In addition, AD/E1 (OR = 2.750, p = 0.0387) and TE/SHBG (OR = 2.667, p = 0.0363) ratios showed significant elevation in the second quartile.

Taking account of hormone receptor (HR) status next to HER2 positivity we found the greatest variances in the

Receptor status	Case–case study, odds ratio								
	E2 (pmol I ⁻¹)	E2/SHBG	E1 (pmol l ⁻¹)	E1S (pmol l ⁻¹)	E1S/E1	AD (nmol I ⁻¹)	TE (nmol l ⁻¹)	TE/SHBG	BMI (kg/m²)
(ER+)/(ER-)	0.82	1.09	1.66**	1.26	0.72	1.24	1.50	1.20	1.12
(PR+)/(PR-)	1.09	1.36	1.5*	1.08	0.65*	1.52*	1.36	1.39	1.66*
(ER+/PR+)/(ER-/PR-)	0.84	1.18	1.77*	1.23	0.66	1.31	1.56	1.29	1.70*
(HER2+)/(HER2-)	1.32	1.99	0.53*	2.44**	3.12***	0.81	0.99	1.04	0.48*
(HER2+/ER-/PR-)/(HER2-/ER-/PR-)	0.25	1.00	0.56	1.08	2.08	0.71	1.13	1.33	0.46
(HER2-/ER+/PR+)/(HER2-/ER+/PR-)	1.67	1.99	1.38	1.07	0.58	1.51	1.11	1.43	1.89
(HER2+/ER+/PR+)/(HER2-/ER-/PR-)	2.00	9.00*	1.50	17.00**	6.00*	2.14	11.60*	2.25	0.42
(HER2+/ER+/PR+)/(HER2-/ER+/PR+)	6.94	7.00	0.83	17.00*	31.19*	1.22	1.66	2.25	nd

Table 2 Case-case comparison of receptor-positive vs. receptor-negative postmenopausal IDC breast cancer patients by quartiles of serum steroid concentrations

Odds ratios in the fourth quartile are shown. Rows in italics present comparisons where number of cases is small therefore these results are only informative. Further investigations are needed.

ER estrogen receptor, PR progesterone receptor, HER2 human epidermal growth factor receptor 2, E1 estrone, E1S estrone sulfate, E2 estradiol, TE testosterone, AD androstenedione, SHBG sex hormone binding globulin, BMI body mass index, OR odds ratio, nd no data.

Statistical significance: * p < 0.05, ** p < 0.01, *** p < 0.001.

ORs of hormones and SHBG during comparison of HR– and HR+ subgroups (HER2+/ER+/PR+ vs. HER2-/ ER+/PR+ and HER2+/ER+/PR+ vs. HER2-/ER-/ PR- comparisons). It should be noted that because of the small number of triple positive cases further investigations are needed.

In the triple-positive vs. HER2-/ER+/PR+ cases the OR of E2, E2/SHBG, E1S and E1S/E1 significantly elevated in the fourth quartile with a significant trend except E2 where trend was not found. The OR of TE increased in the third quartile, while OR of TE/SHBG already elevated in the second quartile, but none of them showed a trend (Table 2).

The most interesting associations were found in the triple-positive vs. triple-negative cases. Significant elevation was found in case of E2/SHBG, E1S, E1S/E1 and TE in the fourth quartile with a significant trend. Serum AD also showed significantly elevated OR in the third quartile but the trend was not seen. The OR of BMI decreased remarkably without any trend (Table 2).

In ER+/PR- vs. ER-/PR- and HER2-/ER+/PR+ vs. HER2-/ER-/PR- cases we did not find any association between receptor status and serum steroid hormone levels.

Discussion

The strength of our study compared with previous reports is the large number of IDC cases (1,042 patients) which enables us to evaluate the association between serum parameters and receptor status not only in HR-positive, but HER2-positive and HR-negative cases.

In our study the serum level of steroid hormones, hormone-precursors and SHBG were measured prior to

surgical intervention in postmenopausal women with primary breast cancer. The associations between E1 and E1S and between E2 and E1S and between DHEAS and E1 indicate the importance of circulating E1S and DHEAS as peripheral estrogen pools in serum (Labrie 2015).

After menopause, E2 plasma level decreases by 90% (Russo and Russo 2006) and the primary estrogen is E1. Others have already reported that serum E2 level is not a reliable risk predictor for postmenopausal women. This may be due to methodological issues as E2 levels are generally around the level of detection in menopause; therefore, E1 levels are preferred (Miyoshi et al. 2003a). In patients with diagnosed breast cancer our results for E2, E1 and E1S are consistent with literary data (Würtz et al. 2012). Based on our case-case studies the serum E1 concentration and E1S/E1 ratio associated tumor HR and HER2 status. Of note, high E1 level and low E1S/E1 ratio associate with HR-positivity, particularly PR+ and ER+/ PR+ cases. Regarding HER2, decreased E1 level and elevated E1S/E1 ratio were measured in HER2-positive cases.

Our results also support the pivotal role of STS in peripheral cells (e.g. platelets and lymphocytes) (Bonser et al. 2000; Garrido et al. 2012) and tissues (i.e. normal breast and breast carcinoma cells). Presumably, serum E1S influences intratumoral estrogen biosynthesis through STS pathway. STS enzyme activity was detected in the great majority of breast carcinomas (Evans et al. 1994). In postmenopausal women the sulfatase pathway is more dominant then the aromatase route. The uptake and efflux of sulfoconjugated steroid hormones are mediated by transport proteins (Ugele et al. 2003). It was shown that organic anion transport protein 2B1

E1 (pmol l*) 0.000 (referent) 02 65.00 137.63 03 119.001 (66.00 128.86 04 >166.50 120.86 02 2.505 120.81 03 3.200-16.28 120.81 04 >2.505 120.81 05 3.200-17.88 120.81 04 >4.266 179.80 1.000 (referent) 1.003 0.745 to 1.574 02 1.98.90 2.11.80 1.003 0.745 to 1.574 03 3.200-12.529 120.80 0.0640 0.597 to 1.246 04 >4.286 179.80 0.0640 0.597 to 1.246 03 2.75.03-388 epi 100.00 (referent) 0.0651 0.530 to 1.384 04 >38.89 138.60 0.640 0.491 to 3.93 0.2273 10 1.000 (referent) 0.000 (referent) 0.2502 0.000 (referent) 02 0.000847 0.00046 89.49 0.346 0.530 to 1.384 04 >66.90 10.1050 (referent) 0.250 to 1.2	Et (pmol 1') 01 <86.00 154/82 1.000 (referent) 02 66.00-116.99 157/83 0.879 0.600 to 1.288 03 119.00-166.90 160/83 1.495* 1.365 0.795 to 1.677 04 >166.90 233/63 1.495* 1.000 (referent) 0.0105 E15 (pmol 1') 01 <2.505 166/60 1.000 (referent) 0.934 0.640 to 1.364 03 3.290.4.266 179/60 1.000 (referent) 0.934 0.640 to 1.364 0.8924 E15/E1 01 <19.890.7.529 162/60 0.863 0.597 to 1.246 0.934 0.640 to 1.334 02 19.890.7.529 162/60 0.863 0.597 to 1.246 0.8924 02 37.10 46.29 84/49 0.849 0.520 to 1.384 0.8273 03 46.3065.90 156/50 1.0600 (referent) 0.2502 Calculated free E2 (E2/SHBG) 1.0000 (referent) 0.2502 0.2502 04 >65.90 156/60 1.364 0.916 0.916 01 <1.180 150/62 1.0000 (referent) 0.2502 04 >0.000591
01 <06.00	01 <68.00
0.2 86.00-118.99 137/83 ••••• 0.879 0.600 to 1.288 0.3 119.00-166.90 23/83 •••••• 1.495 1.036 to 2.157 0.0105 0.1 -2.505 185/60 1.000 (referent) 1.000 (referent) 0.1 -2.505 185/61 1.088 0.745 to 1.574 0.892	122 66.00-118.99 137/83 ■ 0.879 0.6070 0.288 03 119.00-166.90 180/83 1.155 0.795 to 1.677 0.4 04 >166.90 233/83 1.495* 1.036 to 2.157 0.0105 E1S (umol I*) 01 <2.505-3.289
93 1190-165.90 130/33 1.155 0.795 to 1.677 0.0105 F15 (pmol 1*) 01 -2.505.328 12281 1.000 (referent) 0.33 2.2505.328 12281 03 3.2304.236 157/81 0.333 0.430 to 1.364 0.430 to 1.364 04 -4.286 179/80 1.000 (referent) 0.333 0.440 to 1.364 03 2.2505.328.089 120/80 0.000 1.000 (referent) 0.333 0.440 to 1.364 04 -4.286 179/80 1.000 (referent) 0.333 0.0273 101 19.890.27.529 132200 0.000 0.449 to 0.530 0.0273 11 0.000591 10.000 (referent) 0.2502 0.0273 12 10.000 (referent) 0.2501 to 1.384 0.2502 13 0.000591 0.000591 0.0273 0.2502 14 0.000591 0.000591 0.0273 0.2502 14 0.000591 0.000591 0.000591 0.000591 15 0.000591 0.000591	G3 119.00-165.90 120/83 1.155 0.795 to 2.157 0.0105 E1S (nmol I*) 01 <2.505
Q4 >165:00 233/83 1.495* 1.036 to 2.157 0.0005 C1 -2.605 165/60 1.000 (referent) 0.305 to 1.34 Q2 2.605-32.89 162/61 1.000 (referent) 0.934 0.646 to 1.364 Q4 >4.226 179/60 1.000 (referent) 0.934 0.646 to 1.364 Q2 19.890-7.523 162/60 0.853 0.597 to 1.246 0.853 Q3 27.50-3.869 10/60 0.854* 0.496 to 1.033 0.973 Q4 >38.669 10/60 0.854* 0.496 to 1.033 0.973 Q4 >38.669 10/50 0.654* 0.496 to 1.933 0.973 Q4 >58.669 165/60 1.690 0.499 0.520 to 1.334 Q4 >65.90 10/50 0.499 0.520 to 1.34 0.2502 Calculated free Z (Z/SHBG) 71/49 0.000 (referent) 0.2502 Q4 >65.90 110/50 0.000591 0.7149 0.245 to 1.477 Q2 0.000591 97/49 0.000 (referent) 0.2502 Q4 >0.000591 97/49 0.000 (referent) 0.216 Q2 0.000	Q4 >166.90 233/83 1.495* 1.036 to 2.157 0.0105 E15 (nmol I*) 01 <2.505
E15 (µmol 1 ⁴) -2505 166/60 1000 (referent) 02 2505-3289 162/61 1003 0.745 in 1574 03 3294-3286 179/60 1000 (referent) 04 >4.286 179/60 1000 (referent) 02 1980-27.529 162/60 0.683 0.597 in 1.246 03 3274.53-8689 150/60 0.654* 0.449 in 0.953 0.0273 04 >38.869 138/60 0.654* 0.449 in 0.953 0.0273 04 >38.869 1000 (referent) 0.454* 0.449 in 0.953 0.0273 04 >65.90 10050 0.654* 0.449 in 0.953 0.0273 04 >65.90 10050 1.689 0.571 in 1.763 0.2502 04 >65.90 10050 0.484 in 0.215 0.449 in 0.953 0.0264 02 0.000647 -000784 89/49 0.918 0.5821 in 1.477 0.2502 04 >65.90 10.650 1.689 0.216 in 1.337 0.2502 04 >0.000647 -000784 89/49 0.918 0.921 in 1.53 0.2502 01 -1.180 150/62 1.000	E1S (nmol I*) 01 22.505 166/80 1.000 (referent) 02 2.505 2.505 182/81 1.083 0.745 to 1.574 03 3.290.4.286 177/80 0.934 0.640 to 1.364 04 >4.286 179/80 1.078 0.741 to 1.569 0.8924 E1S/E1 01 <19.890
01 <2.505	01 <2.505
0.2 2.969-3.689 162/61 1.089 0.44 to 1.844 0.384 0.540 to 1.864 0.4 >4.286 17960 1.078 0.710 0.46 to 1.864 0.8924 E15/E1 0.10 1.9890.27.529 162/60 0.663 0.597 to 1.246 0.663 0.597 to 1.246 0.2 19.980.27.529 162/60 0.663 0.597 to 1.246 0.49 to 0.953 0.0273 0.4 >38.89 138/60 0.664* 0.49 to 0.953 0.0273 0.4 >38.89 1000 (eferent) 0.000 (eferent) 0.0273 0.4 >65.90 110/50 0.0071 to 1.49 to 0.953 0.0273 0.1 <37.10	02 2.505-3.289 182/61 1.083 0.640 to 1.574 03 3.290-4.286 179/80 1.000 (referent) 04 >4.286 179/80 1.000 (referent) 02 19.890-27.529 182/80 0.663 0.597 to 1.246 03 27.530-38.669 150/80 0.663 0.597 to 1.246 04 >38.669 138/80 0.654 0.449 to 0.953 0.0273 102 37.10-46.29 84/49 0.849 0.520 to 1.334 03 46.30-65.90 156/60 1.545 0.970 to 2.459 04 >65.90 110/50 1.000 (referent) 02 0.000591 · 97/49 0.918 0.562 to 1.497 0.250 to 1.334 03 0.000591 · 97/49 1.000 (referent) 0.252 0.250 to 1.237 04 >0.001652 132/
03 3.200-1.265 157/61 0.334 0.640 0.1364 0.680 0.8924 E15/E1 01 <19.890	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
GA 24, 200 10/90 1/900 1/900 1/900 0/11 0/12 G1 <19.890-27.529	G4 24.205 17960 10760 1076 0.741 0.3925 0.0324 E1S/E1 01 <19.890 $211/80$ 0.683 0.597 0.1246 0.0273 G2 $19.890.27.529$ $182/80$ 0.883 0.654^{+} 0.449 0.133 0.0273 G4 >38.869 $138/80$ 0.654^{+} 0.449 0.449 0.953 0.0273 E2 (pmol 1 ⁺) 0.449 0.449 0.449 0.449 0.449 0.449 0.654^{+} 0.449 0.2520 0.0273 E2 (pmol 1 ⁺) 0.344 0.520 1.000 (referent) 0.250 0.0273 G1 <37.10 $101/50$ 0.677 0.753 0.2502 Calculated free E2 (E2/SHBG) 0.000591 $97/49$ 0.0000 0.971 0.362 0.0916 G2 $0.000591 - 0.00946$ $89/49$ 0.900 0.971 0.362 0.0916 G1 <1.080 $150/62$ 0.001662 $131/60$ 0.941 0.921 0.091
F1S/E1 <19.890	E1S/E1 1 $(1 ext{ style sty$
01 <19.890	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
02 19.890-27.529 162/60 0.863 0.654 0.449 to 1.953 0.0273 04 >38.869 139/60 0.654 0.449 to 0.953 0.0273 01 <37.10	Q2 19.890-27.529 182/80 0.883 0.597 to 1.246 Q3 27.530.38.669 150/80 0.654* 0.449 to 1.033 Q4 >38.669 138/80 0.654* 0.499 to 1.033 Q2 37.10 101/50 0.654* 0.499 to 1.033 Q2 37.10-46.29 84/49 0.849 0.520 to 1.384 Q3 46.30-65.90 156/50 1.545 0.970 to 2.459 Q4 >65.90 110/50 1.089 0.677 to 1.753 0.2502 Calculated free E2 (E2/SHBG) 1.000 (referent) 0.918 0.562 to 1.497 Q3 0.000591-0.000946 89/49 0.918 0.562 to 1.497 Q3 0.000947-0.001662 131/50 1.324 0.824 to 2.125 Q4 >0.01652 132/49 1.000 (referent) Q2 0.180-1.659
03 27.530-36.869 13860 0.711 0.489 to 0.953 0.0273 E2 (pmol 1*) 01 37.10 101/50 1.000 (referent) 02 37.10-46.29 84/49 0.849 0.849 0.504 0.454 03 46.30-65.90 156/50 1.645 0.970 to 2.459 0.2502 04 >65.90 156/50 1.000 (referent) 0.989 0.507 to 1.753 0.2502 04 >0.000591-0.00346 89/49 1.000 (referent) 0.918 0.567 to 1.753 0.2502 04 >0.000591-0.00346 89/49 1.000 (referent) 0.918 0.546 to 1.497 02 0.000591-0.00346 89/49 1.000 (referent) 0.256 0.0916 TE (nmol 1*) 1.101 150/62 1.331 0.846 to 2.188 0.0916 03 0.003947-0.001662 132/49 1.000 (referent) 0.0365 0.0926 04 >2.275 207/83 1.000 (referent) 0.0365 0.0220 01 <0.0198-0.0317	$\begin{array}{c c c c c c c c c c c c c c c c c c c $
04 >388.889 13860 0.449 0.449 0.449 0.449 0.439 0.501 1384 02 37.10 101/50 0.849 0.501 1.364 0.249 0.249 0.249 0.249 0.249 0.249 0.2501 1.364 0.2499 <t< td=""><td>U4 >38.869 138/80 0.0273 U2 37.10 101/50 1.000 (referent) Q2 37.10-46.29 84/49 0.849 0.520 to 1.384 Q3 46.30-66.90 156/50 1.546 0.970 to 2.459 Q4 >65.90 110/50 1.649 0.970 to 2.459 Q4 >65.90 110/50 1.686 0.970 to 2.459 Q1 <0.000591</td> 97/49 1.000 (referent) Q2 0.000591-0.000946 89/49 0.918 0.562 to 1.497 Q3 0.0000947-0.001662 131/50 1.361 0.846 to 2.168 0.0916 TE (nmol I¹) 1.180 150/62 1.000 (referent) 0.221 to 2.128 0.0916 TE (nmol I¹) 1.180 150/62 1.000 (referent) 0.364 to 2.168 0.0916 Calculated free TE (TE/SHBG) 1.017 1.027 to 1.753 0.0850 Q1 <0.198</t<>	U4 >38.869 138/80 0.0273 U2 37.10 101/50 1.000 (referent) Q2 37.10-46.29 84/49 0.849 0.520 to 1.384 Q3 46.30-66.90 156/50 1.546 0.970 to 2.459 Q4 >65.90 110/50 1.649 0.970 to 2.459 Q4 >65.90 110/50 1.686 0.970 to 2.459 Q1 <0.000591
$ \begin{array}{c c c c c c c c c c c c c c c c c c c $	E2 (pmol 1 ¹) 01 37.10 101/50 Q2 $37.10.46.29$ $84/49$ 0.849 0.520 to 1.384 Q3 $46.30.65.90$ $156/50$ $1.56/50$ 1.645 0.970 to 2.459 Q4 > 65.90 $110/50$ 1.089 0.677 to 1.753 0.2502 Calculated free E2 (E2/SHBG) 1.000 (referent) 0.918 0.562 to 1.497 Q2 $0.000591 \cdot 0.000946$ $89/49$ 0.918 0.562 to 1.497 Q3 $0.000947 \cdot 0.001662$ $131/50$ 1.324 0.824 to 2.125 Q4 > 0.001662 $132/49$ 0.846 to 2.188 0.0916 TE (nmol 1 ¹) 1.801 $150/62$ 1.000 (referent) 0.220 Q2 1.180 $150/62$ 1.000 (referent) 0.9316 0.854 0.583 Q3 $1.660 \cdot 2.275$ $183/84$ 1.91 0.820 to 1.729 0.0850 Calculated free TE (TE/SHBG) 0.0198 0.6873 0.584 to 1.251 0.0854 0.592 to 1.237 Q3 0.0198 $158/83$ 0.927 0.6
u1 -37.10 101/50 1.000 (referent) u2 37.10-46.23 84/49 0.849 0.520 to 1.334 u3 46.30-65.90 110/50 1.646 0.970 to 2.459 u4 >65.90 110/50 1.089 0.677 to 1.753 0.2502 Calculated free E2 (E2/SHBG) 97/49 0.918 0.552 to 1.497 0.918 0.552 to 1.497 u2 0.000591-0.003946 89/49 0.918 0.552 to 1.497 0.250 u4 >0.001662 131/50 1.344 0.824 to 2.125 0.0916 TE (nmol I*) 1.190 150/62 1.000 (referent) 0.324 to 2.125 u4 >2.275 207/63 1.361 0.846 to 2.188 0.0916 Calculated free TE (FE/SHBG) 1.000 (referent) 0.0360 0.0314 to 1.975 0.0850 Q2 0.198 156/83 1.000 (referent) 0.0220 0.0314 to 1.975 0.0850 Q2 0.0198 156/83 0.854 to 1.251 0.854 to 1.251 0.974 0.0220 Q3 4.00-71.89 156/83	Q1 < 37.10 101/50 1.000 (referent) Q2 $37.10.46.29$ $84/49$ 0.849 0.520 to 1.384 Q3 $46.30.65.90$ $156/50$ 1.545 0.70 to 2.459 Q4 >65.90 $110/50$ 1.645 0.77 to 1.753 0.2502 Calculated free E2 (E2/SHBG) (referent) 1.000 (referent) Q2 0.000591 $97/49$ 0.918 0.652 to 1.497 Q3 $0.000947.0.001662$ $131/60$ 0.918 0.624 to 2.125 Q4 >0.001662 $132/49$ 0.918 0.684 to 2.188 0.0916 TE (nmol l ¹) (referent) 0.252 0.001662 $132/49$ 0.824 to 2.125 Q4 >0.001662 $132/49$ 0.810 0.846 to 2.188 0.0916 TE (nmol l ¹) (1.180 $150/62$ 1.000 (referent) 0.0250 Q2 0.1986 $158/83$ 1.934 0.846 0.594 to 1.583 Q3 $1.660.2.275$ $183/83$ 0.854 0.584 to 1.251 0.0850
02 3/10-46.29 84/49 0.849	Q_2 $37, 10.46.29$ $84/49$ 0.849 0.520 to 1.334 Q_3 $46.30.65.90$ $156/50$ 1.645 0.970 to 2.459 Q_4 >65.90 $110/50$ 1.089 0.677 to 1.753 0.2502 Calculated free E2 (E2/SHBG) 97/49 0.918 0.562 to 1.497 0.918 0.562 to 1.497 Q_3 $0.000591 \cdot 0.000946$ $89/49$ 0.918 0.562 to 1.497 0.324 0.2125 Q_4 >0.001662 $131/50$ 1.324 0.824 to 2.125 0.9716 Q_4 >0.001662 $132/49$ 1.361 0.846 to 2.188 0.0916 TE (nmol I^1) 0.001662 $132/49$ 1.361 0.846 to 2.188 0.0916 Q_2 1.180 $150/82$ 1.000 (referent) 0.916 0.846 to 1.563 Q_3 $1.660 \cdot 2.275$ $183/84$ 1.097 0.746 to 1.583 0.916 Q_4 >2.275 $207/83$ 1.000 (referent) 0.0854 0.584 to 1.251 Q_3 0.0198 $158/83$ 1.234 0.854 to 1.251 0.37
US 46.30405.30 10950 1.345 0.970 to 2.459 04 >65.90 10059 0.677 to 1.753 0.2502 Calculated free E2 (E2/SH8G) 97/49 0.000 (referent) 0.918 0.957 to 1.753 0.2502 02 0.000591-0.000946 89/49 0.918 0.956 to 1.497 0.056 to 1.497 03 0.000947-0.001662 131/50 0.256 to 1.497 0.364 to 2.189 0.0916 TE (nmol 1 ⁺) 01 <1.180 1.509	G3 46.50-55.90 150/50 1.545 0.970 to 2.459 Q4 >65.90 110/50 1.089 0.677 to 1.753 0.2502 Calculated free E2 (E2/SHBG) 97/49 1.000 (referent) 0.918 0.562 to 1.497 Q3 0.000591 97/49 1.324 0.824 to 2.125 0.0916 Q4 >0.001662 131/50 1.324 0.824 to 2.125 0.0916 Q4 >0.001662 132/49 1.361 0.846 to 2.188 0.0916 TE (nmol 1 ⁻¹) 1.324 0.824 to 1.497 0.331 0.667 to 1.583 0.916 Q2 1.180-1.659 167/84 1.087 0.746 to 1.583 0.916 Q3 1.660-2.275 183/64 1.191 0.820 to 1.729 0.44 2.275 0.0650 Calculated free TE (TE/SHBG) 1.000 (referent) 0.854 to 1.251 0.363 to 1.786 Q4 >0.0536 195/83 1.234 0.854 to 1.251 0.372 Q3 0.0198-0.0317 135/83 1.234 0.854 to 1.251 0.364 to 1.286 Q4 >0.0536 217/82 </td
C4 205.30 11050 11050 11050 0.2502 Calculated free E2 (E2/SHBG) 01 01000591 97/49 0.918 0.552 to 1.457 C3 0.000591-0.000946 89/49 1.324 0.824 to 2.125 0.0916 C4 >0.001652 132/49 1.331 0.846 to 2.185 0.0916 C4 >0.001652 132/49 1.361 0.846 to 2.185 0.0916 C4 >0.001662 132/49 1.361 0.846 to 2.185 0.0916 C4 >0.001662 132/49 1.361 0.846 to 2.185 0.0916 C4 >0.00198 158/63 1.000 (referent) 0.200 0.946 to 1.251 C4 >0.0536 195/63 1.330 0.941 to 1.975 0.0860 C4 >0.0536 217/62 1.300 0.963 to 2.008 0.0220 SHBG (moil 1*) 1 1.000 (referent) 0.011 0.564 to 1.251 0.305 C4 >0.0536 17/82 1.000 (referent) 0.0220 0.011 0.565 to 1.175 0.3549 C4	G4 265.50 110/50 1.085 0.67/16.1.753 0.2502 Calculated free E2 (E2/SHBG) 97/49 1.000 (referent) 0.918 0.562 to 1.497 G3 0.000591-0.000946 89/49 9 0.918 0.562 to 1.497 G4 >0.001662 131/50 1.324 0.824 to 2.125 0.0916 G4 >0.001662 132/49 1.361 0.846 to 2.188 0.0916 TE (nmol 1°) 01 <1.180
Calculated free E2 (E2/SHBG) C1 <0000591 0.000246 88/49 C2 0.000591 0.000246 88/49 C3 0.000591 0.000246 83/49 C3 0.000591 0.0001662 131/50 C4 > 0.001662 131/50 C4 > 0.001662 132/49 C1 <1.180 150/62 C2 1.180-1569 167/84 C3 1.680-2275 183/84 C3 1.680-2275 183/84 C4 > 2.275 207/83 Calculated free TE (TE/SHBG) C1 <0.0198 158/83 C2 0.0198-0.0377 155/83 C3 0.0318-0.0536 195/83 C4 > 0.0536 217/82 C4 > 0.0536 12.008 C4 > 0.0536 217/82 C4 > 0.0536 217/82 C4 > 0.0536 210.000 (referent) C1 <2.7830 138/9 C4 > 0.1000 (referent) C2 2.2660 37.039 173/82 C4 > 0.632 to 1.769 C3 3.3.570-4.289 113/80 C4 > 0.632 to 1.769 C3 3.3.510-47.899 140/60 C4 > 47.889 188/83 C5 0.618 to 1.305 C4 > 0.618	Calculated free E2 (E2/SHBG) Q1 <0.000591
01 ≤0.000591 -0.00364 89/49 1.000 0.918 0.552 to 1.497 03 0.000947-0.001662 131/50 1.324 0.824 to 2.125 04 >0.001662 132/49 1.361 0.846 to 2.188 0.0916 TE (mol 1*) 01 <1.160	G1 <0.000591
02 0.0009847-0.001662 131/50 1.324 0.524 10.437 03 0.000947-0.001662 132/49 1.361 0.846 to 2.125 0.0916 04 >0.001662 132/49 1.361 0.846 to 2.126 0.0916 02 1.180-1.659 167/84 1.000 (referent) 0.0916 02 1.180-1.659 167/84 1.087 0.746 to 1.583 0.832 to 1.729 04 >2.275 207/83 1.363 0.941 to 1.975 0.0850 Calculated free TE (TE/SHBG) 01 <0.0198	G2 0.000947-0.001662 131/50 - 0.916 0.52 to 1.497 G3 0.000947-0.001662 131/50 - 1.324 0.824 to 2.125 G4 >0.001662 131/50 - 1.324 0.824 to 2.125 G1 <1.180
G3 0.000947-0.001662 13/50 1.361 0.624 10.2125 0.0246 0.02168 0.0916 TE (nmol 1*) 1 <1.180	G3 $0.000947-0.001662$ $137/90$ 1.324 0.324 0.244 0.2125 Q4 >0.001662 $137/90$ 1.361 0.846 to 2.125 0.0916 TE (nmol I ¹) 1 1.361 0.846 to 2.188 0.0916 Q2 1.180 $150/82$ 1.000 (referent) Q3 1.660-2.275 $183/84$ 1.191 0.820 to 1.729 Q4 >2.275 207/83 1.363 0.941 to 1.975 0.0850 Calculated free TE (TE/SHBG) 1.000 (referent) 0.0198 $156/83$ 0.054 to 1.251 Q3 0.0198-0.0317 $135/83$ 0.054 to 1.251 0.0850 0.0220 Calculated free TE (TE/SHBG) 1.234 0.854 to 1.261 0.0220 Q4 >0.0536 217/82 1.390 0.963 to 2.008 0.0220 SHBG (nmol I ¹) 1 1.000 (referent) 0.0220 0.043 to 1.337 0.3549 Q2 34.80-49.39 169/83 0.856 0.592 to 1.237 0.3549 Q3 49.40-71.69 183/83 0.811
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Cal 20.001002 $152/43$ $102/43$ 1000 0.040 to 2.100 0.0310 TE (nmol 1 ⁻¹) 01 <1.180 $150/82$ 1.000 $(referent)$ 0.0310 Q2 $1.180 \cdot 1.659$ $167/84$ 1.087 0.746 to 1.583 0.729 Q3 $1.660 \cdot 2.275$ $183/84$ 1.191 0.820 to 1.729 0.0850 Calculated free TE (TE/SHBG) 0.0198 $158/83$ 0.941 to 1.975 0.0850 Q1 <0.0198 $158/83$ 0.854 0.694 to 1.251 Q3 $0.0316 \cdot 0.0536$ $195/83$ 0.854 0.694 to 1.251 Q3 $0.0318 \cdot 0.0536$ $195/83$ 0.854 0.694 to 1.251 Q4 >0.0536 $217/82$ 1.390 0.963 to 2.008 0.0220 SHBG (nmol I ⁻¹) $Q2$ $34.80 \cdot 195/82$ 0.0856 0.592 to 1.237 Q3 $49.40.71.69$ $183/83$ 0.927 0.643 to 1.377 0.3549 Q4 >71.69 $160/83$ 0.811 0.560 to 1.173 0.3549
TE (nmol 1 ⁻¹) Q1 <1.180 150/82 1.000 (referent) Q2 1.180-1.659 167/84 1.087 0.746 to 1.583 Q3 1.660-2.275 183/84 1.191 0.820 to 1.729 Q4 >2.275 207/83 1.363 0.941 to 1.975 0.0850 Calculated free TE (TE/SHBG) Q1 <0.0198	TE (nmol $1^{-1})$ 150/82 1.000 (referent) Q2 1.180 150/82 1.000 (referent) Q3 1.660-2.275 183/84 1.191 0.820 to 1.729 Q4 >2.275 207/83 1.363 0.941 to 1.975 0.0850 Calculated free TE (TE/SHBG) Q1 <0.0198
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Q1 <1.180
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	$\begin{array}{c ccccccccccccccccccccccccccccccccccc$
G3 1.03024 1.191 0.220 0.17.29 G4 >2.275 207/83 1.363 0.941 to 1.925 0.0950 Calculated free TE (TE/SHBG) 1 1.363 0.941 to 1.975 0.0950 G1 <0.0198	G3 1.860-2.275 163/64 1.191 0.820 t0 1.729 Q4 > 2.275 207/83 1.363 0.941 to 1.975 0.0850 Calculated free TE (TE/SHBG) 1.000 (referent) 0.0980 0.0317 135/83 0.854 0.564 to 1.251 Q3 0.0318-0.0536 195/83 0.854 0.564 to 1.251 0.0920 0.0963 to 2.008 0.0220 SHBG (nmol I ¹) 1 1.390 0.963 to 2.008 0.0220 0.856 0.592 to 1.237 0.3549 Q2 34.80-49.39 169/83 0.856 0.592 to 1.237 0.3549 Q4 >71.69 160/83 0.811 0.560 to 1.173 0.3549
C4 72.275 20//83 1.365 0.941 10.1375 0.0080 Calculated free TE (TE/SHBG) 1 1.000 (referent) 0.854 0.594 10.251 02 0.0198-0.0317 135/83 0.854 0.594 1.231 0.854 0.594 1.251 03 0.0318-0.0536 195/82 1.390 0.963 1.200 (referent) 04 >0.0536 217/82 1.390 0.963 1.237 0.356 0.0220 SHBG (nmol I*) 1 1.000 (referent) 0.927 0.643 1.337 0.3549 04 >1.69 160/83 0.825 0.592 to 1.237 0.3549 04 >71.69 160/83 0.825 to 1.769 0.3549 01 <2.7830	Calculated free TE (TE/SHBG) 1.000 (referent) Q1 <0.0198
Calculated free TE (TE/SHBG) Q1 <0.0198	Calculated free TE (TE/SHBG) Q1 <0.0198
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Q1 <0.0198
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Q_2 0.0198-0.0517 1.35/83 - 0.854 0.684 to 1.251 Q_3 0.0318-0.0536 195/83 - 1.234 0.853 to 1.786 Q_4 >0.0536 217/82 - 1.390 0.963 to 2.008 0.0220 SHBG (nmol I ⁴) Q_1 <34.80
$\begin{array}{c ccccccccccccccccccccccccccccccccccc$	Q3 0.0316-0.0336 139/03 1.234 0.033 to 1.766 Q4 >0.0536 217/82 1.390 0.963 to 2.008 0.0220 SHBG (nmol 1 ⁴) 1 1.000 (referent) 0.856 0.592 to 1.237 Q2 34.80-49.39 169/83 0.856 0.592 to 1.237 Q3 49.40-71.69 183/83 0.927 0.643 to 1.337 Q4 >71.69 160/83 0.811 0.560 to 1.173 0.3549
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	SHB6 (nmol I ⁻¹) 1.500 0.505 0.2005 0.0220 Q1 <34.80
SHBG (nmol l ¹) Q1 <34.80 195/82 1.000 (referent) Q2 $34.80.49.39$ 169/83 0.856 0.592 to 1.237 Q3 $49.40.71.69$ 183/83 0.811 0.560 to 1.1337 Q4 >71.69 160/83 0.811 0.560 to 1.173 0.3549 AD (nmol l ⁻¹) Q2 $2.7830.3.8789$ 173/82 1.208 0.825 to 1.769 Q3 $3.8790.5.4280$ 172/82 1.201 0.820 to 1.759 Q4 >5.4280 212/80 1.517* 1.040 to 2.213 0.0305 AD/E1 1.040 0.720 to 1.503 0.749 0.512 to 1.094 Q4 >47.889 168/80 0.498 0.618 to 1.305 0.2871 BMI (kg/m ²) Q1 <23.46 127/83 1.000 (referent) Q2 23.46-26.66 164/83 1.291 0.881 to 1.893 0.2871 BMI (kg/m ²) Q3 26.67-30.29 208/83 1.638* 1.125 to 2.385 0.0044	SHBG (nmol I ⁻¹) Q1 <34.80
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Q2 34.80-49.39 169/83 0.856 0.592 to 1.237 Q3 49.40-71.69 183/83 0.927 0.643 to 1.337 Q4 >71.69 160/83 0.811 0.560 to 1.173 0.3549
$\begin{array}{cccccccccccccccccccccccccccccccccccc$	Q3 49.40-71.69 183/83
$\begin{array}{c c c c c c c c c c c c c c c c c c c $	Q4 >71.69 160/83 - 0.811 0.660 to 1.173 0.3549
AD (nmol 1 ⁻¹) Q1 <2.7830 138/79 Q2 2.7830-3.8789 173/82 Q3 3.8790-5.4280 172/82 Q4 >5.4280 212/80 AD/E1 Q1 <22.660 187/80 Q2 2.2660-33.509 197/81 Q2 2.2660-33.509 197/81 Q2 2.2660-33.509 197/81 Q4 >47.889 168/80 Q4 >47.889 208/83 Q3 26.67-30.29 208/83 Q4 >30.29 208/83	
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Q3 33.510-47.899 140/80 0.749 0.512 to 1.094 Q4 >47.889 168/80 0.898 0.618 to 1.305 0.2871 BMI (kg/m²) 01 <23.46	Q2 22.660-33.509 197/81 1.040 0.720 to 1.503
Q4 >47.889 168/80 0.898 0.618 to 1.305 0.2871 BMI (kg/m²) 01 <23.46	Q3 33.510-47.899 140/80 — 🔳 🕂 0.749 0.512 to 1.094
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Q1 <23.46	BMI (kg/m²)
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Fig. 3 Case–case comparison of progesterone receptor-positive (PR+) vs. progesterone receptor-negative (PR–) postmenopausal IDC breast cancer patients by quartiles of serum steroid concentration. Odds ratios (ORs) were computed taking the lowest category of hormone receptor-negative cases as reference. ORs with 95% CIs and P_{trend} are presented by quartile limits of serum parameters. *Black squares* show ORs in quartiles (Q1–Q4), and the *horizontal lines* show 95% CIs. ORs are shown on a log scale. Chi-square test was used for determination of linear trends (P_{trend}) among the groups classified by tumor receptor status. *ER* estrogen receptor, *PR* progesterone receptor, *E1* estrone, *E1S* estrone sulfate, *E2* estradiol, *TE* testosterone, *AD* androstenedione, *SHBG* sex hormone binding globulin, *BMI* body mass index, *OR* odds ratio, *CI* confidence interval. Statistics: *p < 0.05.

Hormone or protein	Quartile limits	No. Case HER2+/HER2-			OR	95% Cl	P _{trend}	
E1 (pmol l⁻¹) Q1 Q2 Q3 Q4	<91.00 91.0-127.00 127.1-182.09 >182.09	41/225 25/234 33/227 22/228			1.000 0.586 0.798).530*	(referent) 0.345 to 0.996 0.487 to 1.308 0.306 to 0.918	0.0595	
E1S (nmol l' Q1 Q2 Q3 Q4) <2.485 2.485-3.222 3.223-4.2779 >4.2779	16/223 30/223 30/222 39/223		■ 2	1.000 1.875 1.883 2 .438 ***	(referent) 0.994 to 3.536 0.999 to 3.553 1.323 to 4.490	0.0042	
E1S/E1 Q1 Q2 Q3 Q4	<18.620 18.620-25.319 25.320-34.919 >34.919	17/222 20/222 23/222 53/222		 	1.000 1.176 1.353 .118****	(referent) 0.600 to 2.306 0.704 to 2.602 1.751 to 5.552	0.0001	
E2 (pmol l⁻¹) Q1 Q2 Q3 Q4	<38.00 38.00-49.99 50.00-65.09 >65.09	15/144 21/146 12/147 20/145			1.000 1.381 0.784 1.324	(referent) 0.685 to 2.785 0.355 to 1.732 0.652 to 2.688	0.7762	
Calculated f Q1 Q2 Q3 Q4	ree E2 (E2/SHBG) <0.000620 0.000620-0.001040 0.001041-0.001727 >0.001727	12/144 18/146 13/144 24/145	-		1.000 1.479 1.083 1.986	(referent) 0.688 to 3.182 0.478 to 2.455 0.957 to 4.123	0.1110	
TE (nmol l⁻¹) Q1 Q2 Q3 Q4	<1.220 1.220-1.709 1.710-2.399 >2.399	25/227 35/236 38/224 25/230			1.000 1.347 1.540 0.987	(referent) 0.781 to 2.322 0.900 to 2.636 0.550 to 1.770	0.8965	
Calculated f Q1 Q2 Q3 Q4	ree TE (TE/SHBG) <1.2200 1.2200-0.0358 0.0359-0.0593 >0.0593	26/228 43/229 26/229 27/228			1.000 1.647 0.996 1.038	(referent) 0.979 to 2.771 0.561 to 1.767 0.588 to 1.834	0.5918	
SHBG (nmol Q1 Q2 Q3 Q4	l ¹) <33.50 33.50-48.99 49.00-68.99 >68.99	25/228 37/228 24/230 36/230			1.000 1.480 0.952 1.427	(referent) 0.863 to 2.539 0.528 to 1.716 0.830 to 2.455	0.4714	
AD (nmol l^{:1}) Q1 Q2 Q3 Q4	<2.9580 2.9580-4.0709 4.0710-5.7759 >5.7759	31/226 23/228 41/221 25/226			1.000 0.735 1.353 0.806	(referent) 0.416 to 1.300 0.819 to 2.234 0.461 to 1.409	0.9714	
AD/E1 Q1 Q2 Q3 Q4	<21.890 21.890-31.629 31.630-47.129 >47.129	19/223 31/222 37/226 31/224		1	1.000 1.639 I.922* 1.624	(referent) 0.899 to 2.988 1.072 to 3.443 0.891 to 2.961	0.1092	
BMI (kg/m²) Q1 Q2 Q3 Q4	<24.21 24.21-27.51 27.52-30.79 >30.79	40/229 40/229 24/229 19/229 —	_ _		1.000 1.000 0.600).475 *	(referent) 0.622 to 1.608 0.350 to 1.028 0.267 to 0.845	0.0027	
		.	0.5 1	5	ךי 10			

Fig. 4 Case–case comparison of HER2-positive vs. HER2-negative postmenopausal IDC breast cancer patients by quartiles of serum steroid concentrations. Odds ratios (ORs) were computed taking the lowest category of hormone receptor-negative cases as reference. ORs with 95% Cls and P_{trend} are presented by quartile limits of serum parameters. *Black squares* show ORs in quartiles (Q1–Q4), and the *horizontal lines* show 95% Cls. ORs are presented on a log scale. Chi-square test was used for determination of linear trends (P_{trend}) among the groups classified by tumor receptor status. *HER2* human epidermal growth factor receptor 2, *E1* estrone, *E1S* estrone sulfate, *E2* estradiol, *TE* testosterone, *AD* androstenedione, *SHBG* sex hormone binding globulin, *BMI* body mass index, *OR* odds ratio, *CI* confidence interval. Statistics: *p < 0.05, **p < 0.01, ***p < 0.001.

(OATP2B1) is responsible for the uptake of E1S in the cell (Ugele et al. 2003). OATP2B1 protein is strongly expressed in the epithelial cells in invasive ductal carcinomas of the breast. Its expression level correlated with the grade and stage of the disease (Al Sarakbi et al. 2006). Several studies reported that the expression of STS in tumor cells might imply the progression of the tumor and indicate a poor clinical outcome (Evans et al. 1994; Utsumi et al. 1999; Miyoshi et al. 2003b; Suzuki et al. 2009). In addition, increased STS expression has also been associated with clinical resistance to endocrine therapies (Chanplakorn et al. 2010) and higher histological grades (Al Sarakbi et al. 2006; Geisler et al. 2011).

Interestingly in triple receptor-positive cases (HER2+/ ER+/PR+) the elevated steroid hormone concentrations and the significantly increased E1S/E1 ratio compared with HER2-/ER+/PR+ or triple-negative cases were associated with HR-positivity and HER2-positivity, respectively. This phenomenon might be due to molecular cross-talk between ER and the HER2 pathways (Dowsett et al. 2008; Tripathy et al. 2013; Mehta and Tripathy 2014). Several studies indicated that the estrogenmediated activation of G-protein coupled ER1 (GPER1) was associated with several rapid cellular signaling events including activation of epidermal growth factor receptor (EGFR) (Filardo et al. 2006; Ignatov et al. 2010; Ignatov et al. 2011; Jiang et al. 2013). According to De Francesco et al. (2014) estrogenic GPER signaling is able to trigger hypoxia-inducible factor 1A (HIF1A)-dependent vascular endothelial growth factor (VEGF) expression that supports angiogenesis and progression in breast cancer (De Francesco et al. 2014).

Another possible explanation is that estrogens promote the growth, stromalization and angiogenesis of an ER-negative breast cancer cell line (Gupta and Kuperwasser 2006; Gupta et al. 2007). These studies suggest that E2 can act as a potent metastasis-promoter in ERnegative tumors by a novel mechanism involving the host microenvironment.

Best to our knowledge we are the first who demonstrated that elevated E1S concentration and E1S/E1 ratio may be linked with HER2-positive tumors and possibly may indicate an impact of STS pathway. Because of the small number of HER2+/ER+/PR+ cases further investigations are needed to verify this hypothesis.

Our findings that serum E1 level and BMI are significantly elevated in PR+ and ER+/PR+ cases is in line with literary data and supports the role of aromatase route (Cauley et al. 1989). In HER2+ cases a decreased E1 level is associated with a significantly decreased BMI. In postmenopausal breast cancer patients obesityassociated higher estrogen levels might be explain with that aromatization is the major source of estrogens in contrast to an ovarian source in premenopausal women. An increasing volume of adipose tissue in obesity is associated with an increase in total body aromatase activity (Goodwin 2013). The surrounding adipose tissue has an influence to the steroid biosynthesis in the tumor itself. Our hypothesis is that instead of aromatase, STS pathway will be preferred in HER2+ cases.

These results raise the notion that macro-environmental concentrations and conversion of conjugated-unconjugated E1 in peripheral tissue and aromatization of AD and TE to E1 and E2 in adipose tissue might influence the micro-environmental (normal breast) and intrinsic (breast tumor) biosynthesis of estrogens. Our result draws attention to the importance of STS pathways. Measurement of serum E1 and E1S concentrations, and their ratio, and the assessment of tumor receptor status might support the selection of the appropriate therapy, and the individualization of regime.

Therefore, the measurement of serum estrone and estrone sulfate concentrations prior to surgical intervention in postmenopausal primary breast cancer patients might be of benefit.

Abbreviations

AD: androstenedione; BMI: body mass index; CI: confidence interval; DHEA: dehydroepiandrosterone; DHEAS: dehydroepiandrosterone sulfate; EGFR: epidermal growth factor receptor; E2: estradiol; ER: estrogen receptor; E1: estrone; E1S: estrone sulfate; FISH: fluorescence in situ hybridization; GPER1: G-protein coupled ER1; HRT: hormone replacement therapy; HR: hormone receptor; HER2: human epidermal growth factor receptor 2; HIF1A: hypoxia-inducible factor 1A; IHC: immunohistochemical; IDC: invasive ductal carcinoma; OATP2B1: organic anion transport protein 2B1; OR: odds ratio; PR: progesterone receptor; SHBG: sex hormone binding globulin; STS: steroid sulfatase; TE: testosterone; VEGF: vascular endothelial growth factor.

Authors' contributions

BV conception and design, project development, analysis and interpretation of data, manuscript writing: BK analysis and interpretation of data, performed the statistical analysis, figure editing; NU histochemical, genetical determination of steroid receptors and HER2. Consultations concerning to molecular markers of breast carcinomas (phenotype, genotype); ZsH participant as a physician, consultations concerning to treatment modalities and clinical impacts; ZM participant as a physician, project development, responsible for selection of patients (blood taken) and clinical impacts; FCP participant as a physician, responsible for selection of patients (blood taken); KK acquisition of data, project development, drafting and editing manuscript; JK design of experiments, evaluation of the results, acquisition of data; MB evaluation of the results, editing manuscript; IL participant as a physician, consultations concerning to treatment modalities and clinical impacts, revising manuscript critically for important intellectual content, has given final approval of the version to be published; MK project development. All authors read and approved the final manuscript.

Author details

¹ Department of Biochemistry, National Institute of Oncology, 1122 Budapest, Ráth György u. 7-9., Hungary. ² Surgical and Molecular Tumor Pathology Centre, National Institute of Oncology, Budapest, Hungary. ³ Clinic of Oncology, Centre of Clinics, University of Debrecen, 4032 Debrecen, Nagyerdei krt. 98., Hungary. ⁴ Department of General and Thoracic Surgery, National Institute of Oncology, Budapest, Hungary. ⁵ Medical Oncology and Clinical Pharmacology "B", National Institute of Oncology, Budapest, Hungary. ⁶ National Institute of Oncology, Budapest, Hungary.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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