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EARLY DIAGNOSIS OF BLADDER CANCER THROUGH THE DETECTION OF URINARY TYROSINE-PHOSPHORYLATED PROTEINS

Running title: Tyr-phospho-proteins for bladder cancer diagnosis

Amina Khadjavi[1], Franca Mannu[2], Paolo Destefanis[3], Carlotta Sacerdote[4], Antonino Battaglia[3], Marco Allasia[3], Dario Fontana[3], Bruno Frea[3], Silvia Polidoro[5], Giovanni Fiorito[5], Giuseppe Matullo[5], Antonella Pantaleo[6], Agata Notarpietro[7], Mauro Prato[1], Franco Castagno[8], Paolo Vineis[9], Paolo Gontero[3], Giuliana Giribaldi[7,#,*], Francesco Turrini[7,#]

[1]Department of Neurosciences, University of Turin, Torino, Italy, [2]Nurex, Sassari, Italy, [3]University of Turin, Urology Clinic, Torino, Italy, [4]Unit of Cancer Epidemiology, University of Turin and Centre for Cancer Epidemiology and Prevention (CPO Piemonte), Torino, Italy, [5]HuGeF Human Genetics Foundation and Department of Medical Sciences, University of Turin, Torino, Italy, [6]Department of Biomedical Sciences, University of Sassari, Sassari, Italy, [7]Department of Oncology, University of Turin, Torino, Italy, [8]Blood Center, A. O. Città della Salute e della Scienza di Torino, Hospital San Giovanni Battista, Torino, Italy, [9]MRC-PHE Center for Environment and Health, School of Public Health, Imperial College, UK

[#] equally contributed to the work

***Correspondence:** Dr. Giuliana Giribaldi. Department of Oncology, University of Turin Medical School, via Santena 5 BIS, 10126 Torino, Italy. E-mail: giuliana.giribaldi@unito.it

Abstract

BACKGROUND: A noninvasive, highly sensitive and specific urine test is needed for bladder cancer (BC) diagnosis and surveillance in addition to the invasive cystoscopy. We previously described the diagnostic effectiveness of urinary tyrosine-phosphorylated proteins (UPY) and a new assay (UPY-A) for their measurement in a pilot study. The aim of this work was to evaluate the performances of the UPY-A using an independent cohort of 262 subjects.

METHODS: UPY were measured by UPY-A test. The area under ROC curve, cut-off, sensitivity, specificity and predictive values of UPY-A were determined. The association of UPY levels with tumor staging, grading, recurrence and progression risk was analyzed by Kruskal-Wallis and Wilcoxon test. To test the probability to be a case if positive at the UPY-A, a logistic test adjusted for possible confounding factor was used.

RESULTS: Results showed a significant difference of UPY levels between patients with BC vs healthy controls. For the best cut-off value, 261.26 Standard Units (SU), the sensitivity of the assay was 80.43%, and the specificity 78.82. A statistically significant difference was found in the levels of UPY at different BC stages and grades between Ta and T1 and with different risk of recurrence and progression. A statistically significant increased risk for BC at UPY-A $\geq$ 261.26 SU was observed.

CONCLUSIONS: The present study supplies important information on the diagnostic characteristics of UPY-A revealing remarkable performances for early stages and allowing its potential use for different applications encompassing the screening of high-risk subjects, primary diagnosis and post-treatment surveillance.

Keywords: Bladder cancer; Urinary tyrosine-phosphorylated proteins; Urinary tumor markers; Diagnostic assay.

Urinary bladder cancer (BC) ranks 9th in cancer incidence worldwide (Ploeg et al, 2009; Chavan et al, 2014). The diagnosis is made after symptom observation and urethro-cystoscopy (UCS) (Boman et al, 2002; Babjuk et al, 2013). More than 50% of non-muscle-invasive (NMIBC: CIS, Ta, T1) patients will experience at least one recurrence while 10 to 15% will have a progression to an invasive form (Simon et al, 2003). Patients undergo a lifelong follow-up, and also for this reason BC is the most costly cancer from diagnosis to death (Gore and Gilbert, 2013; Hong and Loughlin, 2008).

Several markers have been proposed, but none of them was able to replace the UCS in the diagnosis and follow-up of BC (Cheung et al, 2013) as documented by the current urological guidelines (AUA, EAU, NCCN). It should be noticed that estimates of UCS false-negative range from 10% to 40% (Kriegmair et al, 1996; Zaak et al, 2001; Schneeweiss et al, 1999), and specificity can be as low as 37% (Sarosdy et al, 2002). Although available urine markers are not usually considered to possess sufficient sensitivity and specificity for the screening of BC in the general population (Cheung et al, 2013; Parker and Spiess, 2011), many markers have shown potential value in improving diagnostic accuracy when used to complement current strategies or when multiple markers are used (Miremami et al. 2014). Nevertheless, it should be noticed that some markers with comparable performances are used for the screening of other tumors (Greene et al, 2009). As a possible cause of their limited use, the urinary tests showing better diagnostic performances as uCyt+/immunoCyt, microsatellite and FISH require personnel with specific training, have low throughput and are very expensive (Cheung et al, 2013). Therefore, to be widely usable and to allow a wide clinical validation, new urinary tests for BC should be standardized, easier to interpret and cost-effective (Cheung et al, 2013).

The present study grounds on our previous proteomic analyses of BC tissue and urine revealing the presence of anomalous levels of tyrosine-phosphorylated proteins (UPY) (Khadjavi et al, 2011). UPY showed remarkable stability in urine but their low concentration initially required complex and expensive proteomic techniques for their measurement (Khadjavi et al, 2011), thus limiting

their practical utility. Substantial work was then required to miniaturize the method encompassing all purification and detection steps, to standardize the results and to limit the assay costs. Its performances were investigated in a training set of subjects (Khadjavi et al, 2013). Therefore the aim of the present study was to evaluate the UPY assay (UPY-A) in an independent set of subjects and its diagnostic performances at different tumor stages and grades, its association with tumor progression and recurrence risk and the effects of possible confounding factors such as age, smoking status and gender.

Materials and Methods

Patients and sample collection

Urine samples from newly diagnosed BC patients were collected at the Department of Urology of our Institution. Urine samples from healthy volunteers were obtained from the Blood Bank of the same hospital. Patients with suspected BC were enrolled in this study before undergoing transurethral resection (TUR) of the bladder. Patients with a histological diagnosis (reference standard) different from BC or with a previous BC history were subsequently excluded. The list of recruited patients included people who: 1) received a first diagnosis of BC between September 2010 to May 2012; 2) lived in the study area at the time of diagnosis; 3) were over age 18 years; 4) were able to provide interview data. Healthy controls included people that did not present symptoms or signs of BC, previous BC history and meeting the criteria 2, 3 e 4. All recruited subjects underwent the test. The study was approved by the local research Ethical Committee and was conducted according to Helsinki Declaration's prescriptions. All the subjects included in the protocol signed a declaration of informed consent and received a brief questionnaire covering detailed medical and personal information. The subjects were classified as "current smokers", "former smokers" (quit smoking for at least ten years) and "nonsmokers". A total of 260 participants provided age information, 239 smoking status and 262 gender information. BC grade and stage were determined according to WHO (1973 and 2004) criteria and TNM classification, respectively. Risk scores for recurrence and progression were calculated for each patient affected by NMIBC according to the EORTC definition. These factors comprise tumor grade, stage, size, number and concomitant CIS. Based on these scores, patients were considered to have Very low (score 0), Low (score 1-4), Moderate (score 5-9), or High (score 10-17)risk for recurrence and Very low (score 0), Low (score 2-6), Moderate (score 7-13), or High (score 14-23) risk for progression (Babjuc et al, 2014). Voided urine samples (10-50 ml) were collected from the second micturition of the morning. Samples were

stored at -20°C within 2 hours from collection. The test was performed within 6 months from collection. No significant decay of UPY levels have been noticed after two years of cold storage.

Measurement of urine tyrosine-phosphorylated proteins

Urine samples were centrifuged for 20 minutes at 700 x g at 10°C and supernatants were collected. Five hundred µl of supernatant from each patient were processed using the UPY-A (Khadjavi et al, 2013). Detection was performed by employing a standard chemiluminescence reader (Synergy HT Multi-Mode Microplate Reader, Biotek): luminescence end-point, sensitivity 100 and integration time 1.0 ss. Using an external peptide calibration curve, UPY levels were interpolated and expressed as Standard Units (SU).

Statistical analyses

Summary data are presented as means, medians and standard deviations for continuous variables and as percentages for categorical variables. Differences between BC cases and healthy controls were tested using the nonparametric Wilcoxon rank sum test or Chi-square test, for continuous variables or categorical variables, respectively. The accuracy of the UPY-A biomarker was tested computing the area under the ROC curve (AUC). Different cut-off levels were used to determine that which performs better. Positive predictive value (PPV) and negative predictive value (NPV) were also computed at each cut-off point. The Kruskal-Wallis test was used to assess if UPY levels were different between groups characterized by different tumor stage and grade as well as different recurrence or progression risks. The increase in the prediction performance in predicting recurrence and progression given by the UPY-A marker with respect to the EORTC risk class, was evaluated computing the AUC of three logistic models including age, gender, smoke: model 1: + EORTC risk class; model 2: + UPY-A marker; model 3: + UPY-A marker + EORTC risk class. The AUC of the three models were compared by means of the DeLong test (DeLong et al, 1988). To test the probability to be a case if positive at the UPY-A, we used a multivariate logistic regression adjusted for age, smoking status and gender. All tests were two-sided and we considered a 5% significance level. Analyses were performed using SAS V9.2.

Results

Evaluation and optimization of urine tyrosine-phosphorylated protein assay

To evaluate the results concerning UPY obtained in the pilot study (Khadjavi et al, 2013) in an independent cohort of subjects, 262 new urinary samples collected from 92 BC patients and 170 healthy subjects were analyzed (Table 1). UPY levels showed a significant difference ($p=1.71x10^{-23}$ Wilcoxon rank sum test) between patients with BC *vs* healthy controls (means: 434.8±258.4 SU *vs* 157.9±114.6 SU), corresponding to an approximately 4-fold increase of UPY (Figure 1A). We performed ROC curve analysis (Figure 1B) and the ROC AUC including UPY-A, age, smoking status and gender as predictors was 0.92 with a 95% confidence interval of 0.89-0.97. For the best cut-off value (261.26 SU), the performances of the UPY-A, using only the test as a predictor, were calculated, displaying a sensitivity of 80.43% and a specificity of 78.82% with a PPV of 67.3% and a NPV of 88.2%. It should be noticed that the sensitivity of the assay is still 57.61% with 95.29% of specificity. In order to evaluate possible interferences, we tested 16 urine samples from patients with cystitis and variable levels of leukocyturia and hematuria. The obtained results (mean values: 179.1 ± 117.1 SU) were not significantly different from control subjects (p=0.364 Wilcoxon rank sum test).

Diagnostic performances of urine tyrosine-phosphorylated protein assay at different stages and grades of bladder cancer

The diagnostic performances of the assay have been evaluated according to tumor stages and grades. Figure 2A shows the levels of UPY-A in control, CIS, Ta, T1, T2-3 tumor stages, while panel B shows the variations observed in G1, G2 and G3. A statistically significant difference was found in UPY levels between the control group and the groups of patients at different tumor stages and grades ($p=8.10x10^{-22}$ and $p=6.99x10^{-22}$ respectively by Kruskal-Wallis test). Table 2 shows the p-values for the pairwise Wilcoxon rank sum test, evaluating different levels of UPY among different stages (A), and grades (B) and the P values (on the bottom) for the overall Kruskall-Wallis

test, taking controls samples as reference. In particular, a statistically significant difference was observed through UPY-A at early stages between Ta and T1 (p=0.008 by Wilcoxon rank sum test) (see also Figure 2A and Figure 2B). The sensitivity and specificity of the assay at various stages and grades are displayed in Table 3, with the specificity being fixed at 78.82% to facilitate the comparison of the sensitivities. Consistently with the results shown in Figure 2A, the sensitivity of the assay displayed striking increases from Ta to T1 or T2-3 (from 69.81% to 95.00% or 93.33%) and a less pronounced increase from G1 to G2 (from 68.97% to 79.31%). A remarkable increase of sensitivity was also observed from G2 to G3 (from 79.31% to 90.00%). With fixed specificity at 90% (cut-off value: 335.57 SU), the sensitivities were 50.94% for Ta, 80.00 % for T1 and T2-3. Increasing the cut-off value to 373.39 SU (thus leading specificity to 95.29%), a sensitivity decrease was observed especially for the earlier stages of BC, yet the observed values were still above 47.17% and 75.00 % in Ta and T1 respectively. We performed also the analysis of UPY levels in patients classified according to WHO 2004 classification of BC and we observed a statistically significant difference between the low grade and high grade patients as shown in Figure 3 (p=0.0005 by Wilcoxon rank sum test). Moreover, a statistically significant difference was found in the levels of UPY in groups of patients with different risk of recurrence (p=0.002 by Kruskal-Wallis test) (Figure 4A) and progression (p=0.001 by Kruskal-Wallis test) (Figure 4B). In particular, UPY levels in Very low/Low recurrence risk groups were lower than those in Moderate risk group (p=0.004 and p=0.002 respectively by Wilcoxon rank sum test), whereas levels of UPY in Very low progression risk group were lower than those in moderate risk group (p=0.001 by Wilcoxon rank sum test) strengthening the biological plausibility of the association and indicating that UPY-A could help in the prediction of progression and recurrence of BC. The increase in the prediction of recurrence and progression given by the UPY-A marker was also evaluated. The AUC of the model including EORTC risk class as predictor (model 1) was 0.61 (0.45 – 0.76); the AUC of the model including UPY-A marker as predictor (model 2) was 0.69 (0.54 – 0.83); finally the AUC of the model including both variables (model 3) was 0.70 (0.56 – 0.84). The increase in prediction

performance was evaluated by the De Long test (model 2 vs. model 1 p = 0.30; model 3 vs. model 1 p = 0.21).

Effect of possible confounding factors on the diagnostic performances of UPY-A

To assess the effect of possible confounding factors, we compared UPY levels in the healthy control group stratified for age, smoking status and gender (data are shown in Supplemental Figure S1). The differences in smoking status (p=0.51 by Kruskal-Wallis test) and gender (p=0.35 by Wilcoxon rank sum test) were not statistically significant. On the contrary, after comparing the group of healthy controls older than 55 years (mean 168.6±109.4 SU) to those younger than 55 years (mean 134.3±123.0 SU), age-related differences were statistically significant (p=0.017 by Wilcoxon rank sum test), with lower UPY levels in the younger group. Odd ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression for potential confounders. A statistically significant increased risk for BC among subjects presenting the UPY-A ≥ 261.26 SU was observed (OR= 15.30, 95% CI = 8.12–28.82). OR was still significant after the adjustment for age, smoking status and gender. Since among healthy controls the subjects older than 55 years were significantly different for UPY-A compared to younger controls, healthy controls and BC patients were stratified in four additional different age groups. Figure **5**A shows that UPY levels are significantly increased in BC patients *vs* healthy controls in all age groups. The figure also highlights that healthy controls under 55 years display lower UPY levels with respect to older controls, not showing reciprocal variations. On the other hand, BC patients showed a progressive increase of UPY with the age due to the increasing prevalence of more advanced tumor stages in older patients. In patients under 55 years we observed a large prevalence of Ta and G1 (Figure 5B and 5C). As a matter of facts the best cut-off limit in this age group was 180 SU instead of 261.26 SU. With this cut-off limit sensitivity improved from 54.5% to 81.8%.

Discussion

Changes of protein tyrosine-phosphorylation are involved in cell growth and differentiation and have been observed in many cancer types, usually as a consequence of altered tyrosine kinase activity (Lim, 2005; Blume-Jensen and Hunter, 2001; Harsha and Pandey, 2010). On the other hand, tyrosine kinases are amongst the most important oncogenes known to date, since they play a central role in cancer development and progression (Lim, 2005; Blume-Jensen and Hunter, 2001; Hunter, 1998). Robust evidence demonstrates the involvement of abnormal kinase activity in BC following mutations and/or overexpression of protein kinases (Al Hussain and Akhtar, 2013), protein hyper-phosphorylation in biopsy specimens (Khadjavi et al, 2011) and after using tyrosine kinase inhibitors for BC treatment (Wallerand et al, 2010; Mitra et al, 2006). FISH analysis of Aurora kinase A has been used as a marker for BC (Park et al, 2008).

Measuring the effects of abnormal protein kinase activity on protein phosphorylation takes advantage from a substantial amplification of the signal, as a consequence of increased catalytic activity of the mutated kinase. However, the instability of phospho-proteins in blood has limited their use for cancer diagnosis. On the contrary, we previously observed that protein phosphatases activity is negligible in urine, thus conferring a particular stability to urinary phospho-proteins (Khadjavi et al, 2011). Nevertheless, only a small amount of phospho-proteins is associated to BC, therefore stringent purification steps and high sensitivity detection methods are required (Khadjavi et al, 2011; Khadjavi et al, 2013).

The present report evaluates and optimizes the performances of the UPY examined in the pilot study (Khadjavi et al, 2013) by confirming the high sensitivity and specificity of the assay to detect BC in an independent and larger cohort of subjects. A statistically significant difference was found in the levels of UPY at different BC stages and grades. Sensitivity values have been measured at different fixed specificities. As shown in Table 3 UPY-A with chosen specificity comparable to cytology (> 93%) displays higher sensitivity in Ta (47% vs 26%) and T1 (74% vs 64%) respectively and more than 2-fold higher sensitivity in G1 subjects (41% vs 6%) (for the cytology data see Saad

et al. 2001). Nevertheless, the comparison with other techniques is very complex, and larger and independent studies are certainly needed. The comparison should also consider additional characteristics such as the cost, the productivity and the intra/inter-laboratory standardization of the test. At this regard, UPY-A will be in the cost range of an ELISA test such as BTA and NMP22 tests with additional advantages as UPY-A high throughput and the possibility of automated calibration in each analytical sessions, making it simpler to standardize than techniques requiring larger effort for inter-laboratory harmonization (Behrens et al, 2014).

In the present report we have also observed that UPY-A can identify patients more prone to recurrence and progression. Therefore, these patients could receive closer surveillance or more aggressive therapy. Of note, the increase in the prediction performance in predicting recurrence and progression given by the UPY-A with respect to the EORTC risk class, is not statistically significant but the association of the UPY-A and the EORTC classification improves the recurrence and progression prediction. We also have excluded that age, smoking status and gender can affect the diagnostic performances of the assay as confounding factors. Nevertheless, taking in account that the average values of the test were lower in younger healthy subjects (≤ 55 years old), we have found that lowering the cut-off limit in this group of subjects determined a significant improvement of sensitivity. Therefore, these results encourage further studies involving a wider number of patients younger than 55 year, in order to optimize the cut-off values. Interestingly, a first preliminary investigation on a group of patients with nonmalignant urological disorders did not reveal considerable interference. Large and independent studies are currently in progress to confirm the present data and to evaluate the value of UPY-A in the follow-up of BC patients.

In conclusion, the present study supplies important information on the diagnostic characteristics of UPY-A revealing remarkable performances for early stages. The efforts made to miniaturize the method markedly increased its throughput allowing its potential use for a wide range of applications encompassing the screening of high-risk subjects, primary diagnosis and post-treatment surveillance.

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Table 1. Clinical and pathologic characteristics of subjects

	Controls		**Bladder Cancer Cases**	
	N°	%	N°	%
Total (n=262)	170	65	92	35
Sex				
Women	39	15	6	2
Man	131	50	86	33
Age				
≤55	53	20	11	4
56-65	72	28	25	9
66-75	28	11	37	14
>75	15	6	19	7
Missing	2	1		
Smoking				
Current smokers	28	11	53	20
Former smokers	10	4	20	8
Non smokers	111	42	17	6
Missing	21	8	2	1
Histology at 1st diagnosis				
CIS			4	4
Ta			53	53
T1			20	22
T2-3			15	16
Grading at 1st diagnosis				
CIS			4	4
1			29	33
2			29	33
3			30	34
WHO 2004				
Low grade			43	47
High grade			43	47
Missing			6	6
Risk of recurrence (n=73)				
Very low			24	33
Low			40	55
Moderate			9	12
Risk of progression (n=73)				
Very low			40	55
Low			17	23
Moderate			16	22

Table 2. p-values for the pairwise Wilcoxon rank sum test, evaluating different levels of urinary tyrosine-phosphorylated protein (UPY) among different stages (A) and grades (B). p-values on the bottom refers to the overall Kruskall-Wallis test. Controls samples were taken as a reference

A	CTRL	CIS	Ta	T1	T2-3	**B**	CTRL	G1	G2	G3
CTRL	-	0.004	$4.26x10^{-13}$	$3.11x10^{-11}$	$1.55x10^{-7}$	CTRL	-	$3.04x10^{-7}$	$7.02x10^{-11}$	$2.83x10^{-14}$
CIS	-	-	0.532	0.439	0.230	G1	-	-	0.592	0.002
Ta	-	-	-	0.009	0.005	G2	-	-	-	0.001
T1	-	-	-	-	0.368	G3	-	-	-	-
T2-3	-	-	-	-	-	-	-	-	-	-
$p= 8.10x10^{-22}$						$p = 6.99x10^{-22}$				

Table 3. Diagnostic performances of urinary tyrosine-phosphorylated protein assay (UPY-A) at different BC stages and grades

BC stage or grade	AUC (area under ROC curve)	95% confidence interval	sensitivity (specificity 78.82 %) (cut-off value 261.26 SU)	sensitivity (specificity 90.00 %) (cut-off value 335.57 SU)	sensitivity (specificity 95.29 %) (cut-off value 373.39 SU)
CIS	0.924	0.873-0.958	100%	50.00%	50.00%
Ta	0.830	0.774-0.877	69.81%	50.94%	47.17%
T1	0.954	0.914-0.979	95.00%	80.00%	75.00%
T2-3	0.909	0.858-0.946	93.33%	80.00%	73.33%
G1	0.798	0.735-0.851	68.97%	48.28%	41.38%
G2	0.879	0.826-0.921	79.31%	51.72%	48.28%
G3	0.936	0.892-0.966	90.00%	86.67%	83.33%

Titles and legends to figures

Figure 1. Urinary tyrosine-phosphorylated protein (UPY) levels in urine samples. (A) Analysis of urinary UPY levels in samples of healthy subjects (*n*= 170) and bladder cancer (BC) patients (*n*= 92) using the UPY-A. Healthy subject (CTRL) mean levels: 157.9±114.5 SU; BC mean levels: 434.8±258.4 SU. Significance of the differences: $p=1.71x10^{-23}$ by Wilcoxon rank sum test . The solid lines indicate the mean values; the dotted line indicates the best cut-off value. **(B)** ROC curve of total UPY levels adjusted for age, smoking status and gender.

Figure 2. UPY levels in subjects with different BC stages and grades. (A) Distribution of UPY levels in subjects with different BC stages. CTRL, *n*= 170, mean 157.9 ±114.5 SU; CIS, *n*=4, mean 400.8 ±114.8 SU; Ta, *n*= 53, mean 356.3 ±181.0 SU; T1, *n*= 20, mean 540.9 ±346.6 SU and T2-3, *n*=15, mean 579.5 ±290.6 SU. UPY levels are significantly different in groups of BC patients with different BC stages (Kruskal-Wallis test, $p=8.10x10^{-22}$). **(B)** Distribution of UPY levels in subjects with different BC grades. CTRL, *n*= 170, mean 157.9±114.5 SU; G1, *n*=29, mean 357.6±217.9 SU; G2, *n*= 29, mean 361.7±122.9 SU and G3, *n*= 30, mean 584.5±336.4 SU. UPY levels are significantly different in groups of BC patients with different BC grades (Kruskal-Wallis test, $p=6.99x10^{-22}$). The solid lines indicate the mean values; the dotted line indicates the best cut-off value.

Figure 3. UPY levels in BC subjects classified according to WHO 2004 classification of BC. Distribution of UPY levels in subjects classified according to WHO 2004 classification of bladder tumors. High grade, *n*=43, mean 528.06 ± 297.19 SU; Low grade, *n*=43, mean 354.22 ± 191.16 SU. UPY levels are significantly different in groups of BC patients with different BC grade (Kruskal-Wallis test, p=0.0005).

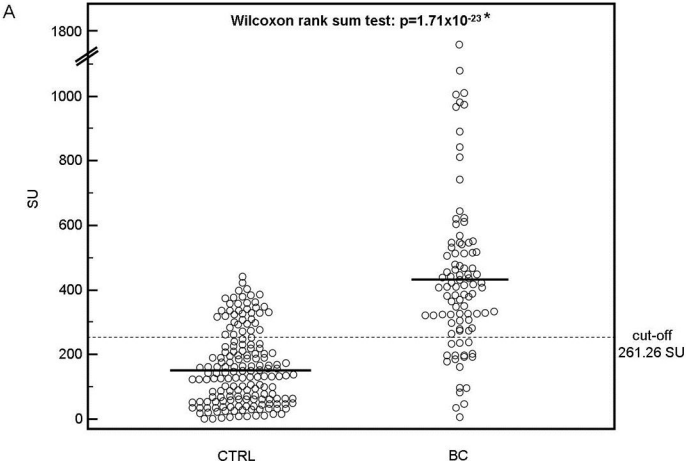
Figure 4. UPY levels in relation to classification of recurrence and progression risks. (A) Analysis of UPY levels in relation to classification of recurrence risks of BC patients (Kruskal-

Wallis test, p=0.002). **(B)** Analysis of UPY levels in relation to classification of progression risk of BC patients (Kruskal-Wallis test, **p=0.001**).

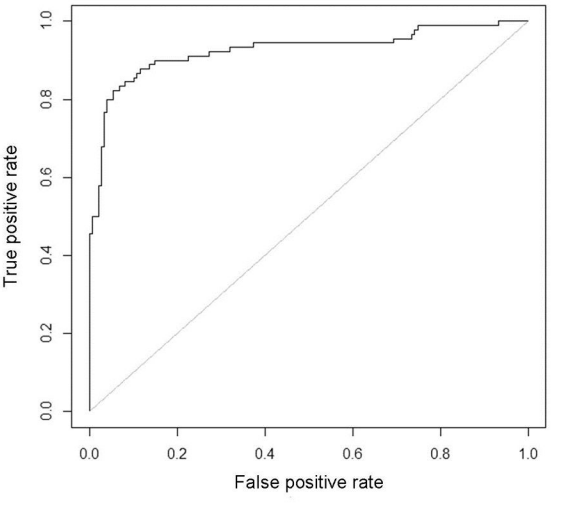
Figure 5. UPY levels in different subjects age ranges. (A) Distribution of UPY levels in CTRL and BC patients age ranges: ≤55 year-old (CTRL, *n*=53, mean 134.3±123.0 SU; BC, *n*=11, mean 326.8±164.9 SU), 56-65 year-old (CTRL, *n*=72, mean 169.2±114.0 SU; BC, *n*=25, mean 437.2±354.5 SU), 66-75 year-old (CTRL, *n*=28, mean 168.5±108.0 SU; BC, *n*=37, mean 431.2±187.1 SU), >75 year-old (CTRL, *n*=15, mean 168.2±103.4 SU; BC, *n*=19, mean 501.0±270.6 SU). UPY levels are significantly increased in BC patients in all age groups (Wilcoxon rank sum test). The solid lines indicate the mean values. **(B)** Distribution of patients with different BC stages: data are expressed as percentage in different age groups. **(C)** Distribution of patients with different BC grades: data are expressed as percentage in different age groups.

Figure 1

A



B



C

	OR	CI	p
UPY (SU)	1.109	(1.074-1.146)	3.29×10^{-10}
AGE	1.040	(0.993-1.089)	0.096
GENDER (females vs. males)	0.414	(0.108-1.591)	0.199
smoke (former vs. never)	5.577	(1.514-20.543)	0.010
smoke (current vs. never)	10.352	(4.184-25.610)	4.26×10^{-7}

Figure 2

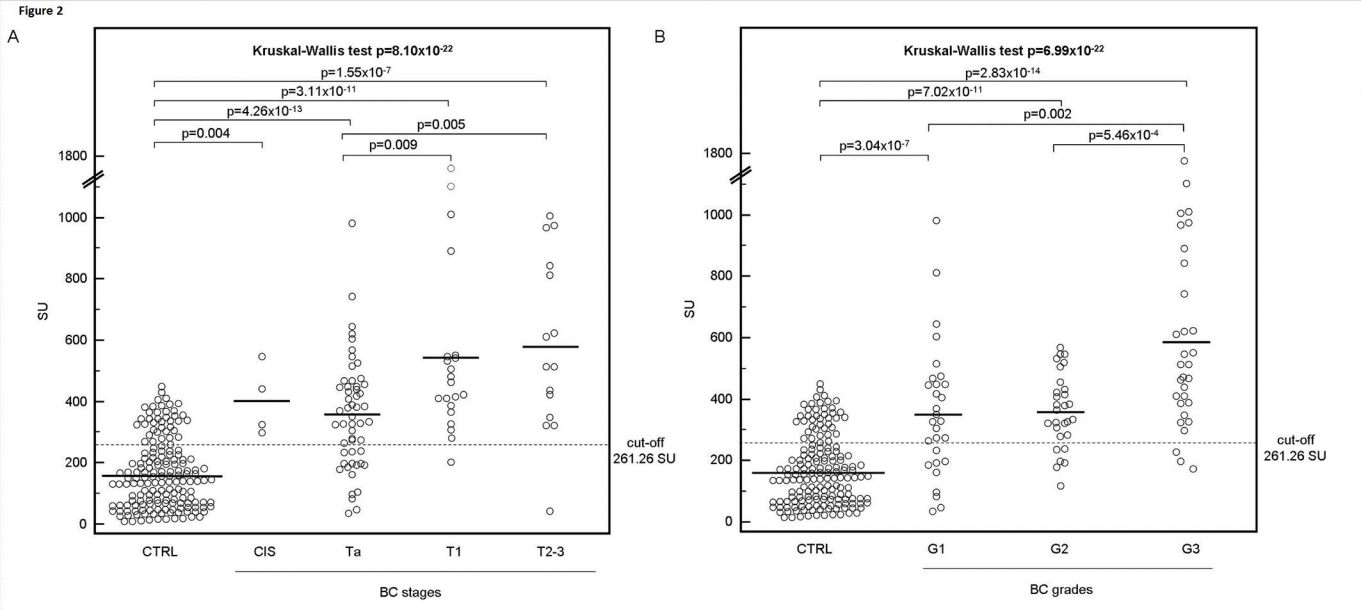

A
Kruskal-Wallis test p=8.10x10-22
p=1.55x10-7
p=3.11x10-11
p=4.26x10-13
p=0.004
p=0.005
p=0.009
SU
cut-off
261.26 SU
CTRL
CIS
Ta
T1
T2-3
BC stages
B
Kruskal-Wallis test p=6.99x10-22
p=2.83x10-14
p=7.02x10-11
p=0.002
p=5.46x10-4
p=3.04x10-7
SU
cut-off
261.26 SU
CTRL
G1
G2
G3
BC grades

Figure 3

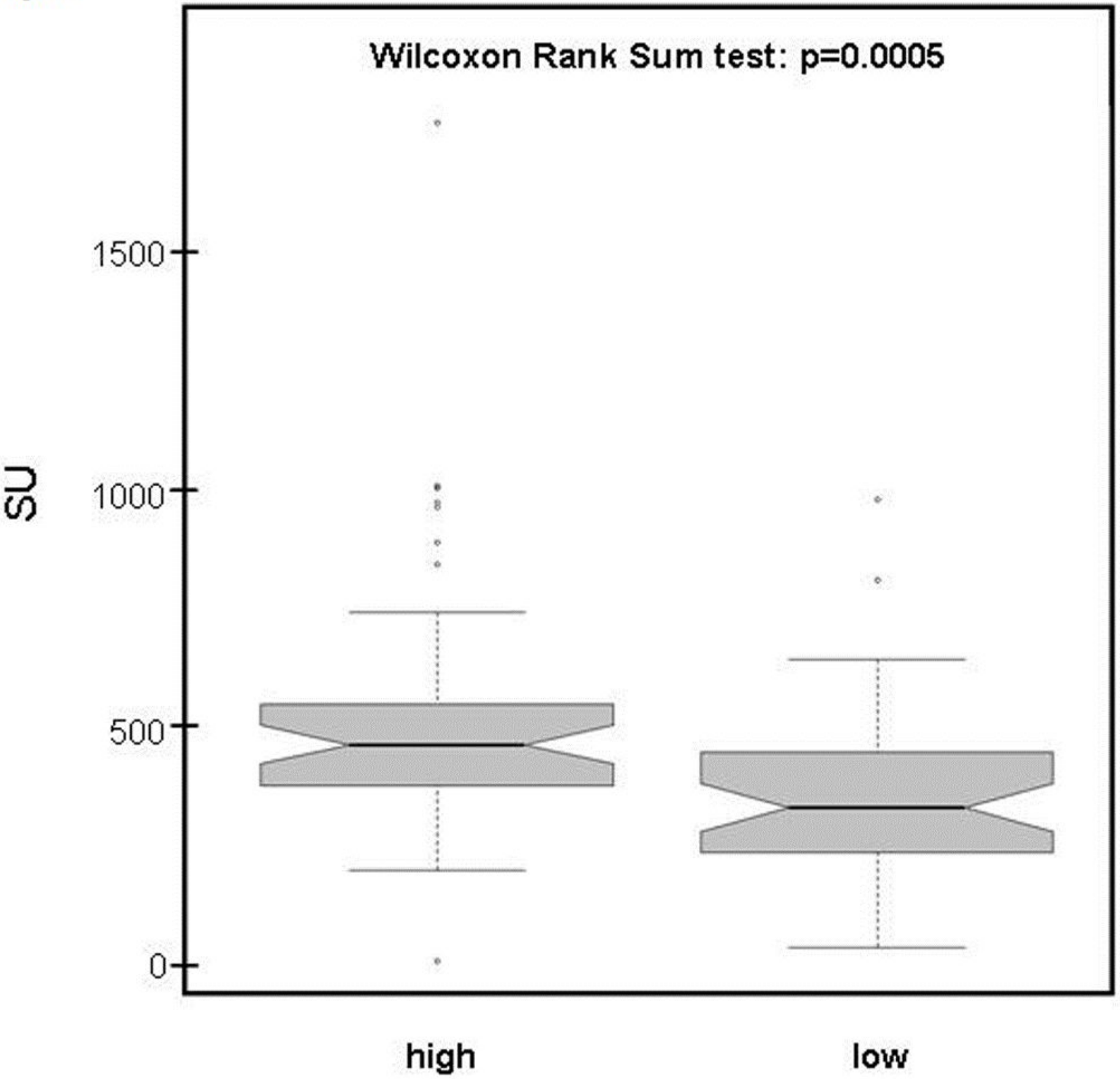
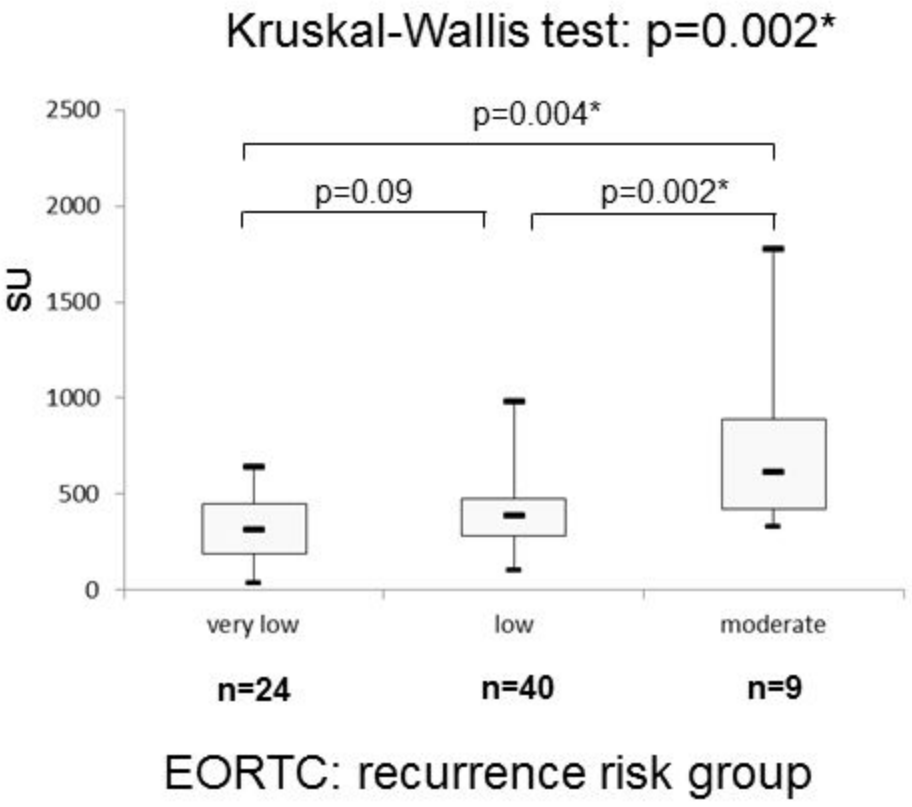


Figure 4


Kruskal-Wallis test: p=0.002*
p=0.004*
p=0.09
p=0.002*
SU
2500
2000
1500
1000
500
0
very low
low
moderate
n=24
n=40
n=9
EORTC: recurrence risk group

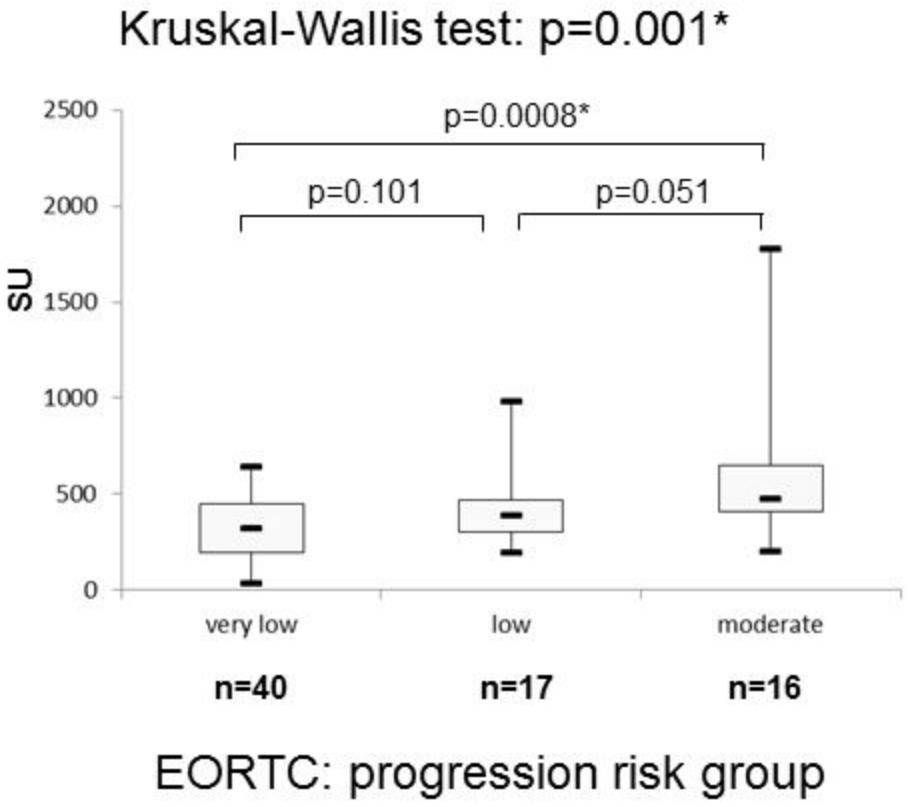
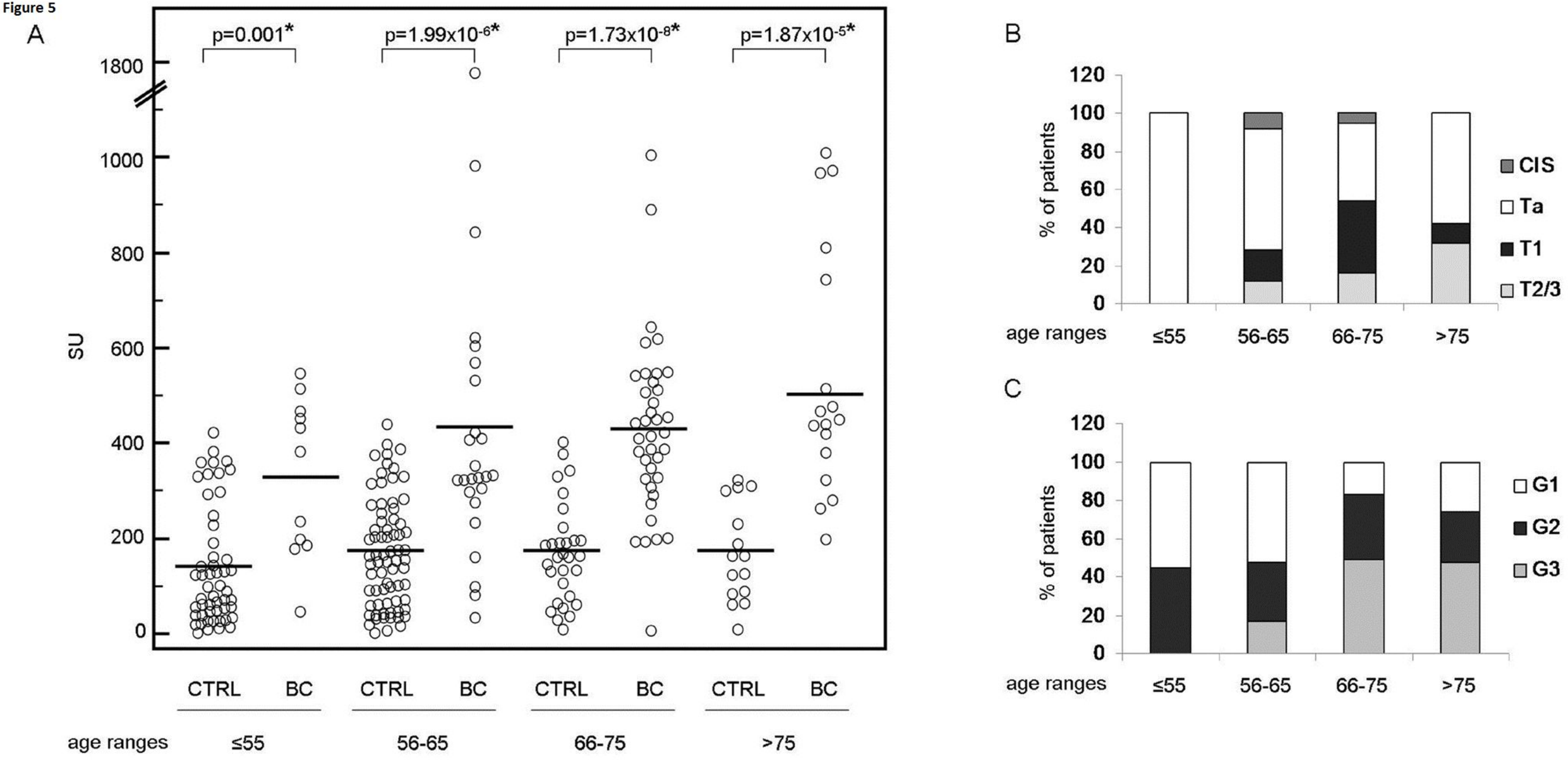

Kruskal-Wallis test: p=0.001*
p=0.0008*
p=0.101
p=0.051
SU
2500
2000
1500
1000
500
0
very low
low
moderate
n=40
n=17
n=16
EORTC: progression risk group

Figure 5


A
p=0.001*
p=1.99x10-6*
p=1.73x10-8*
p=1.87x10-5*
SU
1800
1000
800
600
400
200
0
CTRL
BC
CTRL
BC
CTRL
BC
CTRL
BC
age ranges
≤55
56-65
66-75
>75
B
% of patients
120
100
80
60
40
20
0
CIS
Ta
T1
T2/3
age ranges
≤55
56-65
66-75
>75
C
% of patients
120
100
80
60
40
20
0
G1
G2
G3
age ranges
≤55
56-65
66-75
>75