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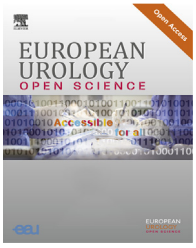

European Association of Urology

Urothelial Cancer

STAG2 Protein Expression in Non–muscle-invasive Bladder Cancer: Associations with Sex, Genomic and Transcriptomic Changes, and Clinical Outcomes

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Abstract

Background: Mutations in *STAG2* cause complete loss of STAG2 protein in approximately one-third of non–muscle-invasive bladder cancers (NMIBCs). STAG2 protein expression is easily determined via immunohistochemistry (IHC) and published data suggest that loss of STAG2 expression is a good prognostic indicator in NMIBC.
Objective: To confirm the relationship between *STAG2* protein expression and clinical outcomes and tumour characteristics in NMIBC.
Design, setting, and participants: IHC was used to determine STAG2 expression in 748 incident urothelial bladder cancers (UBCs) and recurrence-free, progression-free, and disease-specific survival were compared for patients with and without STAG2 loss. Exome and RNA sequencing were used to explore links between STAG2 loss and tumour molecular characteristics.
Results and limitations: STAG2 loss was observed in 19% of UBC patients and was 1.6-fold more common among female patients. Loss was frequent among grade 1 pTa tumours (40%), decreasing with stage and grade to only 5% among grade 3 pT2+ tumours. Loss was associated with fewer copy-number changes and less aggressive expression subtypes. In UBC, STAG2 loss was a highly significant prognostic indicator of better disease-free survival but was not independent of stage and grade. STAG2 loss was not a statistically significant predictor of NMIBC recurrence. STAG2 loss was significantly associated with better progression-free survival in NMIBC and appeared to be more prognostic for males than for females.
Conclusions: A simple IHC-based STAG2 test shows promise for identifying NMIBC patients at lower risk of progression to MIBC for whom more conservative treatments may be suitable.

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Patient summary: A protein called STAG2 is frequently lost in early bladder cancers, most often in less aggressive tumours. STAG2 loss is easily measured and could be used as a biomarker to help guide treatment decisions.

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1. Introduction

The 1231-amino-acid protein cohesin subunit A2, also known as stromal antigen 2, is encoded by the *STAG2* gene located on the X chromosome. Expression of STAG2 protein is often lost early in the development of urothelial bladder cancer (UBC) because of truncating somatic mutations and this can be easily detected via immunohistochemistry (IHC) [1–3]. It has been reported that the frequency of STAG2 loss is 24–36% in non–muscle-invasive bladder cancer (NMIBC) and 10–16% in muscle-invasive bladder cancer (MIBC) [1–6]. During the cell cycle, the cohesin complex holds sister chromatids together until anaphase, playing an important role in chromatid segregation [7]. In urothelial cell lines, STAG2 knockdown causes aneuploidy [8]; however, STAG2 mutations and loss of expression occur most frequently in low-grade NMIBC, in which aneuploidy is rare. Hence, the mechanistic role of STAG2 in bladder carcinogenesis remains unclear, although the discovery of synthetic lethality between STAG1 and STAG2 in breast cancer cell lines suggests that STAG1 may compensate for loss of STAG2 [9,10]. One explanation could be that STAG2 loss prevents senescence in normal cells, extending the window for accumulation of oncogenic mutations [11]. STAG2 also affects transcriptional programming in embryonic stem cells and bladder cancer cells [12,13], and may play a role in DNA damage repair [14].

STAG2 mutations and loss of expression in UBC were first described in 2013 in three parallel publications that reported differing associations with clinical outcomes [1–3]. Solomon et al [3] found that one out of eight NMIBCs with STAG2 loss recurred, compared to 15 out of 26 STAG2-expressing NMIBCs, but STAG2 loss was associated with recurrence, lymph node involvement, and worse disease-specific survival (DSS) in 349 MIBCs treated with cystectomy. Balbás-Martínez et al [1] also found that STAG2 loss was associated with a lower risk of recurrence and progression in 426 NMIBCs (although not an independent prognostic indicator) and reported a lower risk of progression and better DSS for 182 MIBCs. Conversely, in a study of 99 patients, Guo et al [2] reported that STAG2 loss was very strongly associated with worse overall survival in both NMIBC and MIBC. Subsequently, Qiao et al [5] studied *STAG2* expression in 91 NMIBCs and 34 MIBCS: loss of STAG2 expression was an indicator of better recurrence-free survival (RFS) in NMIBC and MIBC and of better DSS across the whole cohort [5]. In 2018, Lelo et al [4] presented perhaps the most compelling evidence regarding the potential clinical utility of *STAG2* as a prognostic biomarker: *STAG2* loss was associated with a 2.4-fold lower risk of developing recurrence and a 1.86-fold lower risk of disease progression in 335 papillary NMIBCs. Most recently, the same group reported data for 279 NMIBCs and 406 MIBCs [6]: although *STAG2* loss was not significantly associated with time to recurrence in NMIBC, it was associated with a 2.5-fold decrease in the risk of progression of low-grade NMIBC, but was not prognostic in MIBC. The results of these studies are summarised in Supplementary Table 1.

Taken together, the literature suggests that STAG2 loss is indicative of good prognosis in NMIBC and thus could be a useful tool for personalising NMIBC treatment and surveillance schedules. Interpretation of STAG2 immunostaining is straightforward in UBC (STAG2 is either expressed or not expressed), so reliable routine testing should be feasible.

Many IHC prognostic biomarkers have been reported for NMIBC; however, none with high performance have been sufficiently validated and adopted in clinical practice (eg, [15]). Given the existing evidence and ease of testing for *STAG2* expression, we sought to undertake independent validation of the role of *STAG2* in NMIBC prognostication. We performed STAG2 IHC on tissue microarrays (TMAs) and obtained data for duplicate cores from 748 bladder cancers and investigated associations with RFS, progression-free survival (PFS), and DSS, as well as tumour molecular characteristics determined via exome and RNA sequencing.

2. Materials and methods

2.1. Biospecimens

Formalin-fixed, paraffin embedded (FFPE) and frozen tissues were collected at initial transurethral resection of bladder tissue as part of the Bladder Cancer Prognosis Programme between 2004 and 2011 at ten hospitals across the West Midlands region of the UK (ethical approval: 06/MRE04/65) [16]. All tumours were treatment-naïve primary UBCs encompassing all disease stages and grades. Patients received contemporary European Association of Urology (EAU) guideline–directed treatment, and dates of recurrence, progression, death, and cause of death were recorded for >5 yr. We used the 1973 World Health Organization grade classification as it was in universal use in the UK at the time of patient recruitment, is the basis for the European Organization for Research and Treatment of Cancer and EAU NMIBC risk tables [17], and has comparable utility to the 2004/2016 classification [18]. For quality assurance, 10% of diagnostic FFPE tumour samples were retrieved from local histopathology departments and underwent expert pathological review. All tumours included were purely or predominantly transitional cell carcinomas. TMAs were constructed with two 2-mm cores from different regions of each FFPE tumour.

2.2. STAG2 IHC

TMAs were sectioned at 4 μm and heat-induced epitope retrieval was performed using sodium citrate. Sections were blocked with horse

serum and incubated with SA-2 (J-12) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-81852; 1:100 dilution) for 2 h at room temperature. Horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit secondary antibody (ImmPRESS HRP Universal Antibody Polymer Detection Kit Peroxidase, Vector Laboratories, Burlingame, CA, USA) was used, followed by detection using ImmPACT DAB substrate (Vector Laboratories). TMAs were scored as STAG2-positive, STAG2-negative, or mosaic by two independent observers. A positive score required immunoreactivity in >90% of tumour nuclei, a negative score required immunoreactivity in <10% of tumour nuclei, and mosaic status required distinct patches of tumour cells with and without nuclear staining. Scores were accepted for all tumours for which both observers and both cores agreed.

2.3. Exome and RNA sequencing

Nucleic acids were extracted from frozen tumours and paired blood and sequencing libraries were prepared using Nextera Rapid Capture Exome and TruSeq Stranded RNA LT kits (Illumina, San Diego, CA, USA). The RNA sequencing data are available at https://ega-archive.org (accession code EGAS00001004358).

2.4. Data analysis

Somatic mutation calling from whole-exome sequencing was performed using MuTect2 v2.2 within the GATK4 v4.1.4.0 framework. Variant effect predictor v94 and gnomAD population frequencies were used to identify mutations that were nonpolymorphic and affecting coding sequence or predicted to be splice-site altering. APOBEC mutational activity was estimated from somatic single-nucleotide variants using *maftools* v1.4.28. Tumour mutational burden (TMB) was calculated as the number of mutations per Mb of the genome. Copy number segments were identified using *cnvkit* v0.8.3, and then GISTIC v2.0.23 was used to identify statistically significant focal copy-number peaks. Copy number burden (CNB) was calculated as the fraction of autosomal genome harbouring copy-number peak regions. Gene-level counts from RNA sequencing were estimated using STAR v2.5.2b. Normalisation of the count data was performed using the *limma* v3.44.1 package. The UROMOL 2021 four-class single-sample classifier was used for subtyping [19]. The 5-yr RFS, PFS, and DSS were compared between STAG2-positive and STAG2-negative UBCs using Kaplan-Meier analysis and log-rank testing. Frequencies of events in patient groups were compared using χ^2 tests, and continuous variables were compared using Mann-Whitney tests. UBCs with mosaic STAG2 loss (n = 23) were excluded from all analyses.

3. Results

3.1. STAG2 expression across UBC stages and grades

STAG2 expression data were obtained for 748 UBCs as shown in Table 1. Total loss of expression was observed in 141 UBCs (19%) and a mosaic pattern of STAG2 loss was observed in 23 UBCs (3%). Representative IHC images are shown in Figure 1. Loss of STAG2 was fourfold more frequent for grade 1 tumours than for grade 3 tumours: complete loss of expression was observed in 40% of grade 1, 28% of grade 2, and 9% of grade 3 UBCs. Loss of expression was more frequent among tumours from female than from male patients (28% vs 17% overall; p = 0.002) and this trend was observed for all disease stages and grades (Fig. 2). Although rare, mosaic STAG2 loss was also more common among females than among males: nine of the 23 mosaic cases were female patients (the male/female patient ratio in the cohort is 3.7:1).

Table 1 – Frequency of STAG2 loss by tumour stage and grade [a]

pT stage	Patients with STAG2 loss/STAG expression (% with STAG2 loss)		
	Grade 1	Grade 2	Grade 3
pTa	41/65 (39%)	47/112 (30%)	6/45 (12%)
pT1	2/0	13/40 (25%)	20/134 (13%)
pT2+	0/0	2/7	10/176 (5%)

[a] The cohort also included four patients with carcinoma in situ, one patient with stage pT1 for which grade was not recorded, and 23 patients with mosaic expression loss (not included in the table).

3.2. Associations between STAG2 protein loss and genomic alterations

STAG2 mutation status was available from exome sequencing data for 79 tumours; high-impact *STAG2* mutations were detected in eight tumours. STAG2 expression was lost in seven of the eight mutant tumours (87.5%). Among the 71 tumours in which mutations were not detected, there were nine cases of complete STAG2 loss (14%) and two of mosaic STAG2 loss. These data confirm the link between *STAG2* mutations and protein expression as determined by IHC. TMB and APOBEC mutagenesis did not differ significantly between STAG2-negative and STAG2-positive NMIBCs (Fig. 3B,C), but STAG2-negative tumours had a significantly lower copy-number burden (p = 0.022; Fig. 3A).

3.3. Associations between STAG2 protein loss, gene expression, and NMIBC subtype

RNA sequencing data for 70 NMIBCs revealed 205 genes upregulated in NMIBCs with STAG2 protein loss (n = 13) and 77 downregulated genes (adjusted p < 0.05; Supplementary Fig. 1 and Supplementary Table 2). *STAG2* mRNA expression mirrored protein levels and was downregulated in tumours negative for STAG2 protein (Supplementary Fig. 2; p < 0.001). *STAG1* mRNA was expressed in all tumours and did not differ significantly between STAG2-positive and STAG2-negative tumours (Supplementary Fig. 2; p < 0.079). Genes downregulated in tumours with STAG2 loss included *EVX1*, *ESR2*, *CECR1*, *SYNE2*, *RFX2*, and *SETBP1*, but no pathway enrichment was observed; conversely, ribosomal protein genes were highly significantly upregulated in tumours with STAG2 loss.

The NMIBCs were classified into UROMOL subtypes 1, 2a, 2b, and 3 using an online single-sample classifier [19]. STAG2 protein expression was lost in 3/9 (33%) class 1, 4/30 (13%) class 2a, 2/11 (18%) class 2b, and 4/7 (57%) class 3 NMIBCs. The frequency of STAG2 loss was significantly higher in the lowest risk classes (classes 1 and 3) than in the higher risk classes (classes 2a and 2b; p = 0.019).

3.4. Loss of STAG2 expression and DSS

Across the entire cohort of patients, loss of STAG2 protein expression was a highly significant predictor of better DSS (p < 0.001; Fig. 4A). When NMIBCs and MIBCs were analysed separately (Fig. 4B,C), STAG2 loss appeared to remain associated with better DSS, although this association did not reach statistical significance in either case.

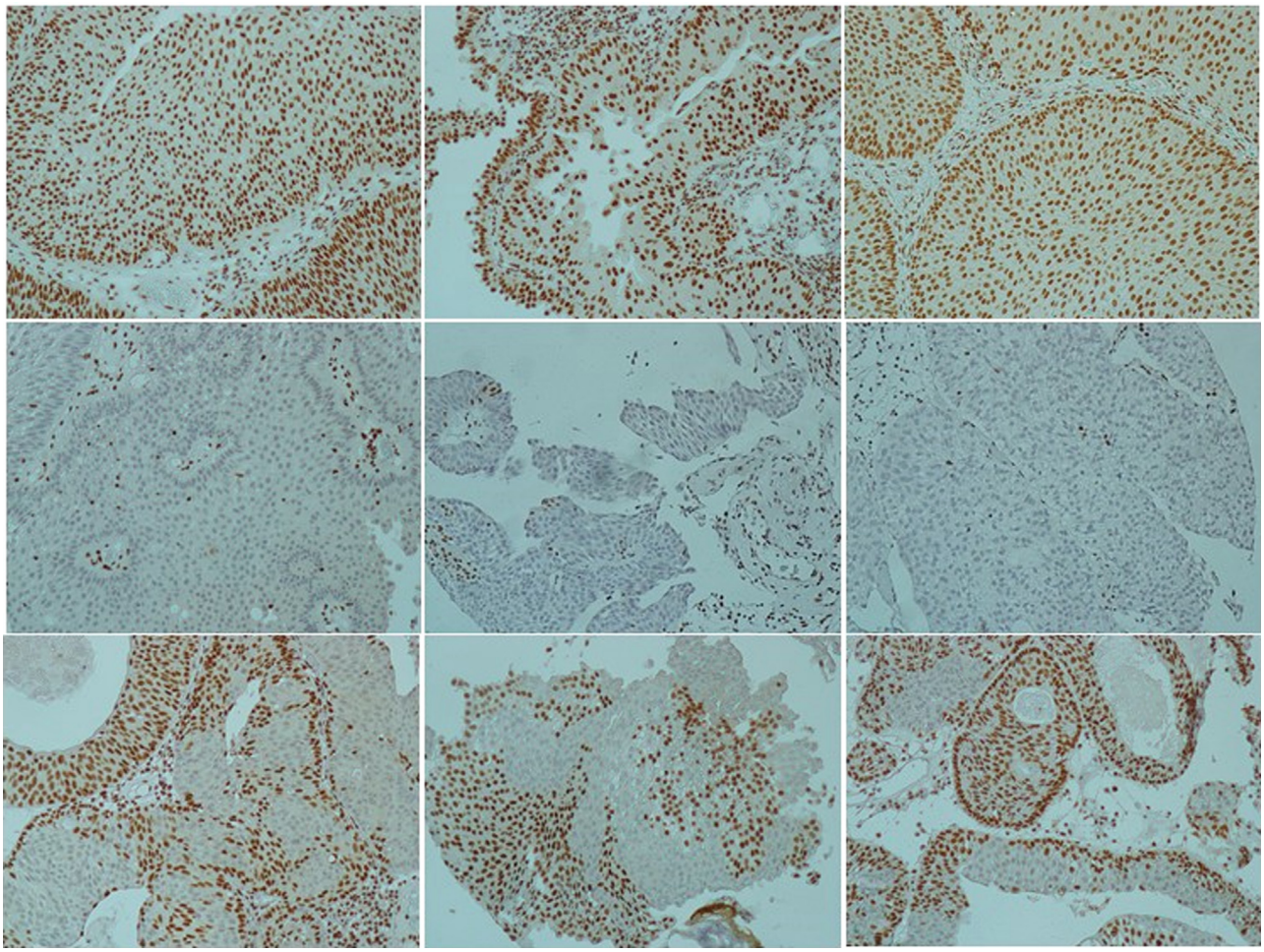

Fig 1 – Representative images of STAG2 immunohistochemistry. Top row: tissue microarray cores from three urothelial bladder cancers with intact STAG2 expression. Middle row: urothelial bladder cancers with complete STAG2 loss. Bottom row: mosaic STAG2 loss.

3.5. Loss of STAG2 expression and RFS in NMIBC

Analysis of 530 NMIBCs (129 STAG2-negative) did not reveal a significant association between RFS and STAG2 protein expression (Fig. 4D). Additional analyses stratified by stage, grade, or EAU risk group also failed to identify associations between RFS and STAG2 expression (data not shown).

3.6. Loss of STAG2 expression and PFS in NMIBC

STAG2 protein expression was significantly associated with progression (p = 0.031; Fig. 5A). With no evidence of an association with progression in low- and intermediate-risk NMIBCs (Fig. 5B), the data suggest that this is because STAG2 loss is a good prognostic indicator in high-risk NMIBC and grade 3 pT1 disease (Fig. 5C,D).

3.7. Loss of STAG2 expression and DSS, RFS, and PFS in male and female patients

As the *STAG2* gene is located on a sex chromosome and loss of expression occurs at significantly different frequencies among male and female patients, we investigated whether STAG2 loss might have different associations with clinical outcomes in males and females. All NMIBC survival analyses were repeated separately for male and female patients (n = 422 and 108, respectively). While there were no associations between STAG2 loss and outcomes for females (Supplementary Fig. 3; $p > 0.6$ in all analyses), the p values for the associations between STAG2 loss and DFS, RFS, and PFS were all smaller for male patients when compared to the whole cohort (Supplementary Fig. 4). The associations of STAG2 loss with better PFS in male high-risk NMIBC (p = 0.028) and grade 3 pT1 disease (p = 0.044) were both statistically significant.

4. Discussion

We used IHC on TMAs to detect expression/loss of expression in 748 UBCs, the largest single *STAG2* study to date. Our data show loss of expression in 19% of UBCs, with significantly higher loss for low-grade compared to high-grade tumours, consistent with previous studies. We also confirmed that loss of *STAG2* expression results from stop gain mutations and frameshift indels and showed that tumours with loss of STAG2 protein on IHC also have much lower levels of *STAG2* mRNA. STAG2 loss is more common in

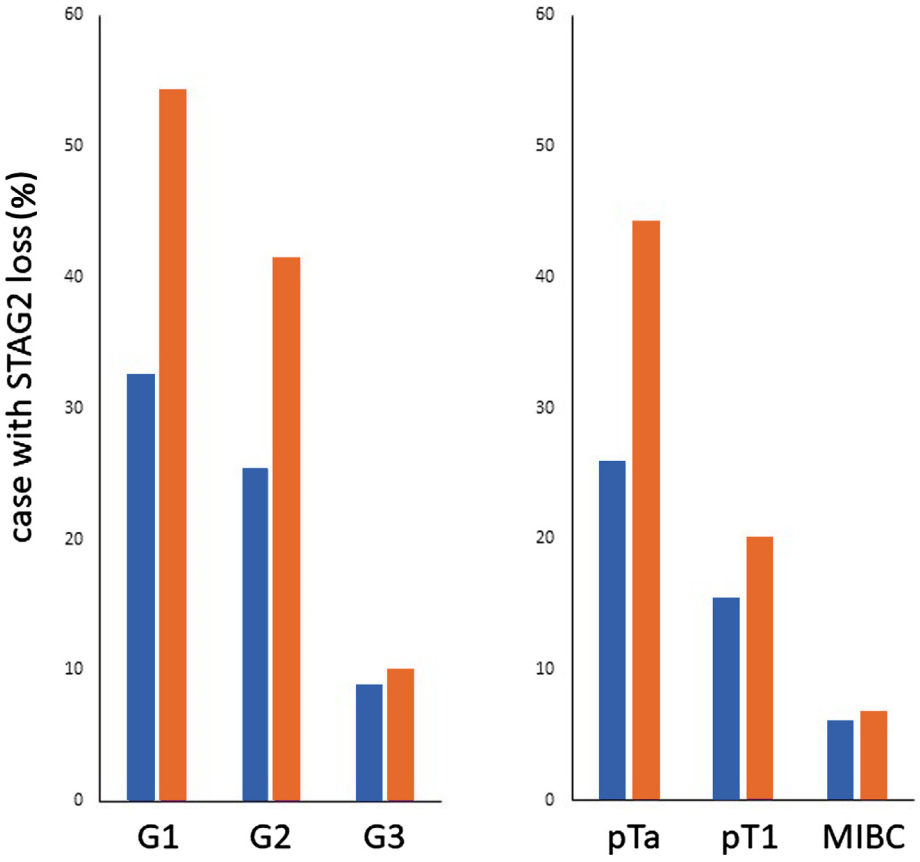

Fig. 2 – Frequency of STAG2 loss among male (blue bars) and female (orange bars) patients with urothelial bladder cancer across disease stages and grades. MIBC = muscle-invasive bladder cancer.

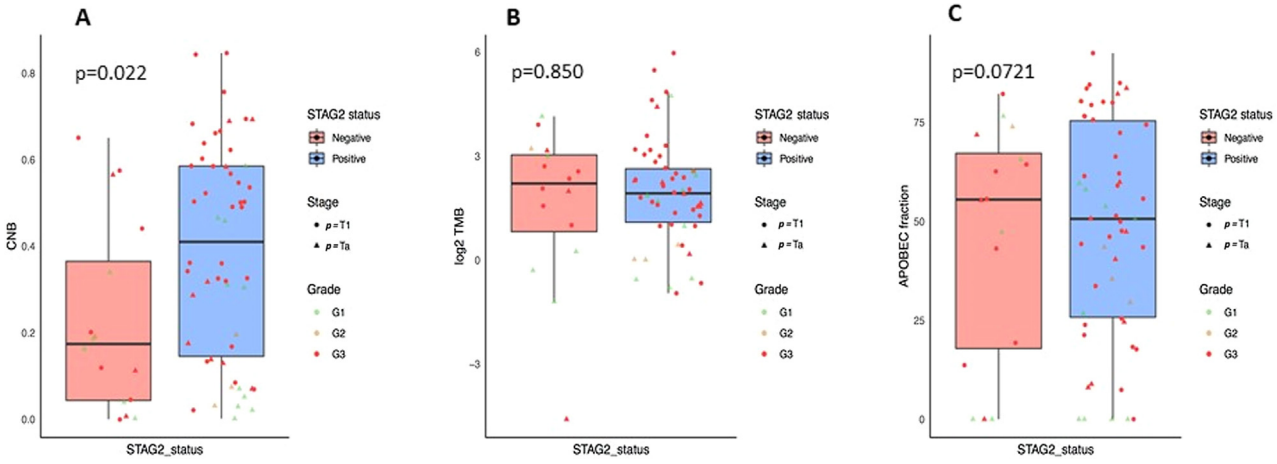

Fig. 3 – Genomic changes in non–muscle-invasive bladder cancers with or without STAG2 loss (n = 70). (A) Copy number burden (CNB), (B) tumour mutation burden (TMB), and (C) APOBEC single-base substitution signatures.

less aggressive NMIBC subtypes and is associated with fewer copy-number changes, suggesting an association with more favourable disease. There is a female bias, with loss of expression 1.6 times more frequent among female versus male patients. This echoes the higher frequency of *KDM6A* mutations (also on the X chromosome) among female UBCs [20,21].

Our objective was to confirm that STAG2 protein could be a useful prognostic biomarker in UBC. With the exception of Guo et al [2], previous studies have reported that STAG2 loss is a good prognostic indicator in NMIBC, although there is disagreement as to whether the effect is observed for RFS, PFS, and/or DSS. In our data overall, STAG2 loss was a strong predictor of DSS in UBC; however, the

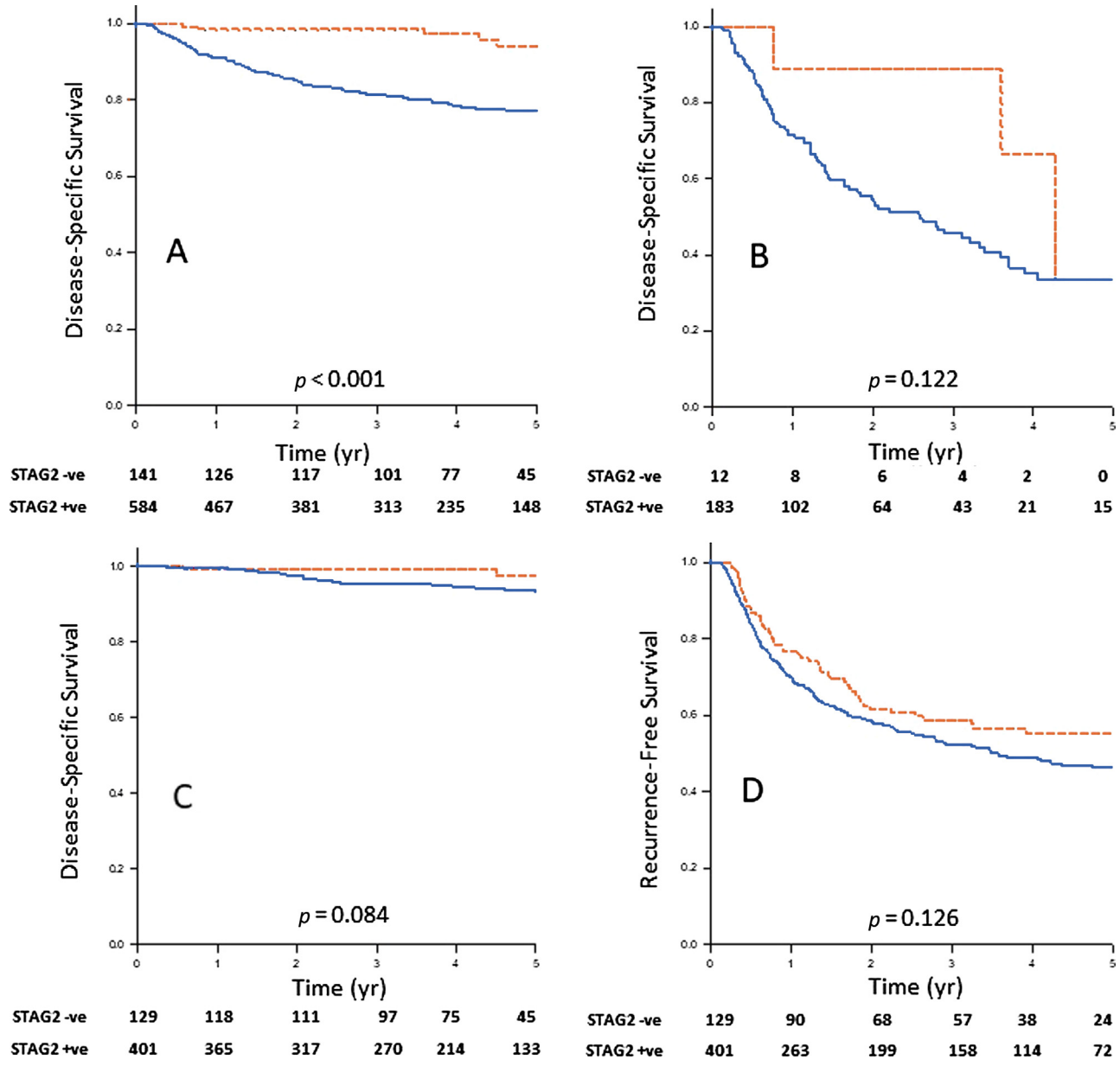

Fig. 4 – Disease-specific survival in (A) urothelial bladder cancer, (B) non–muscle-invasive bladder cancer (NMIBC), and (C) muscle-invasive bladder cancer. (D) Recurrence-free survival in NMIBC. Solid blue line = cases with STAG2 expression; dashed red line = cases with STAG2 loss. The numbers of patients at risk are shown beneath each plot.

association of STAG2 loss with low-grade NMIBC (rather than high-grade NMIBC and MIBC) meant that significant relationships with DSS were not as evident when NMIBCs and MIBCs were analysed separately. RFS curves for NMIBC patients also suggested that STAG2 loss plays at best a very minimal role in recurrence. Our most clinically relevant finding is that STAG2 loss in NMIBC indicates a lower risk of progression to MIBC. Thus, our study confirms that STAG2 loss is a good prognostic factor in the context of PFS. To a greater or lesser extent, all three previous reports that investigated PFS also found that STAG2 loss was a good prognostic indicator, although this did not reach conventional statistical significance (p = 0.163) in the study by Balbás-Martínez et al [1], and the more recent studies by Lelo et al [4] and Taber et al [6] share patients and so are not independent.

The identification of prognostic biomarkers in UBC, especially high-risk NMIBC, has long been identified as a clinical research priority [22,23]. At present, NMIBCs are currently risk-stratified according to algorithms that are based on clinical and pathological data rather than molecular characteristics. Multiple individual protein biomarkers [15], DNA mutations [24], methylation markers [25], and, more recently, TMB and expression subtypes [19] have all been proposed as prognostic biomarkers, but none have been rigorously validated and incorporated into clinical risk calculators. It is likely that many reports on prognostic biomarkers have not properly accounted for associations between stage,

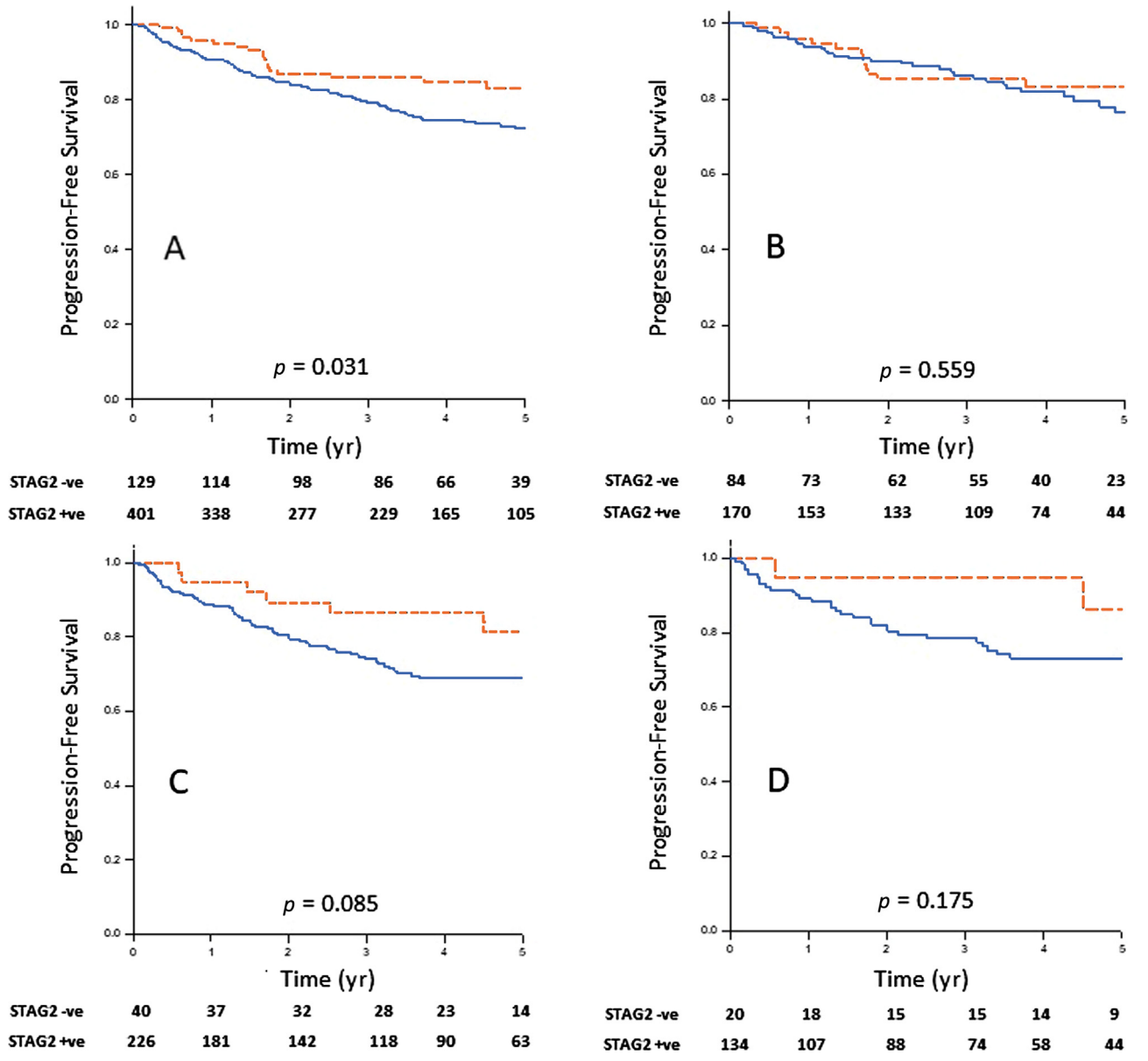

Fig. 5 – Progression-free survival in STAG2-positive and STAG2-negative non–muscle-invasive bladder cancer (NMIBC): (A) all NMIBC; (B) low- and intermediate-risk NMIBC; (C) high-risk NMIBC; and (D) grade 3 T1 disease. Solid blue line = cases with STAG2 expression; dashed red line = cases with STAG2 loss. The numbers of patients at risk are shown beneath each plot.

grade, and biomarker expression, preventing biomarker candidates from providing additional prognostic value. Furthermore, as these risk calculators work reasonably well, very large studies will be required to demonstrate incremental gains in performance on addition of biomarkers [26,27].

5. Conclusions

We propose that STAG2, which is easily measured owing to its two-state nature (present vs absent) and was found to be indicative of NMIBC progression to MIBC by three independent research groups in three independent patient cohorts, could contribute to biomarker panels for NMIBC prognostication. Nonetheless, uncertainties remain. Why are results variable across studies? Why do we find the prognostic effect confined to high-risk NMIBC? Why is STAG2 loss more frequent among females but apparently more prognostic for males and why is STAG2 expression most frequently lost in low-grade tumours in which aneuploidy is rare?

Author contributions: Douglas G. Ward had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Ward, Bryan.
Acquisition of data: Ward, Gordon.
Analysis and interpretation of data: Ward, Gordon, Goel, Humayun-Zakaria, Arnold.

Drafting of the manuscript: Ward, Bryan, Goel, Humayun-Zakaria.
Critical revision of the manuscript for important intellectual content: Arnold, Bryan.
Statistical analysis: Ward, Goel, Humayun-Zakaria.
Obtaining funding: Bryan, James, Zeegers, Cheng.
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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.euros.2022.02.004.

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