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Authors

Zeng, Peter YF  
Cecchini, Matthew J  
Barrett, John W  
et al.

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# Immune-based classification of HPV-associated oropharyngeal cancer with implications for biomarker-driven treatment de-intensification



Peter Y. F. Zeng,<sup>a,b</sup> Matthew J. Cecchini,<sup>b</sup> John W. Barrett,<sup>a</sup> Matthew Shamma-Toma,<sup>a</sup> Loris De Cecco,<sup>c</sup> Mara S. Serafini,<sup>c</sup> Stefano Cavalieri,<sup>d,e</sup> Lisa Licitra,<sup>d,e</sup> Frank Hoebbers,<sup>f</sup> Ruud H. Brakenhoff,<sup>g</sup> C. René Leemans,<sup>g</sup> Kathrin Scheckenbach,<sup>h</sup> Tito Poli,<sup>i</sup> Xiaowei Wang,<sup>j</sup> Xinyi Liu,<sup>j</sup> Francisco Laxague,<sup>a</sup> Eitan Prisman,<sup>k</sup> Catherine Poh,<sup>k,l,m</sup> Pinaki Bose,<sup>n</sup> Joseph C. Dort,<sup>n</sup> Mushfiq H. Shaikh,<sup>a</sup> Sarah E. B. Ryan,<sup>a</sup> Alice Dawson,<sup>a</sup> Mohammed I. Khan,<sup>a</sup> Christopher J. Howlett,<sup>b</sup> William Stecho,<sup>b</sup> Paul Plantinga,<sup>b</sup> Sabrina Daniela da Silva,<sup>o</sup> Michael Hier,<sup>o</sup> Halema Khan,<sup>a</sup> Danielle MacNeil,<sup>a,p</sup> Adrian Mendez,<sup>a,p</sup> John Yoo,<sup>a,p</sup> Kevin Fung,<sup>a,p</sup> Pencilla Lang,<sup>p</sup> Eric Winquist,<sup>a,p</sup> David A. Palma,<sup>a,p</sup> Hedyeh Ziai,<sup>q</sup> Antonio L. Amelio,<sup>r,s</sup> Shawn S-C. Li,<sup>t</sup> Paul C. Boutros,<sup>u,v,w,x,y,aa</sup> Joe S. Mymryk,<sup>a,p,z,aa</sup> and Anthony C. Nichols<sup>a,p,aa,\*</sup>



<sup>a</sup>Department of Otolaryngology - Head and Neck Surgery, University of Western Ontario, London, Ontario, Canada

<sup>b</sup>Department of Pathology and Laboratory Medicine, University of Western Ontario, London, Ontario, Canada

<sup>c</sup>Integrated Biology Platform, Department of Applied Research and Technology Development, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

<sup>d</sup>Head and Neck Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

<sup>e</sup>Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy

<sup>f</sup>Department of Radiation Oncology (MAASTRO), Research Institute GROW, Maastricht University, Maastricht, the Netherlands

<sup>g</sup>Amsterdam UMC, Vrije Universiteit Amsterdam, Otolaryngology/Head and Neck Surgery, Cancer Center Amsterdam, the Netherlands

<sup>h</sup>Department of Otolaryngology, Medical Faculty, Heinrich Heine University Düsseldorf, Düsseldorf, Germany

<sup>i</sup>Unit of Maxillofacial Surgery, Department of Medicine and Surgery, University of Parma—University Hospital of Parma, Parma, Italy

<sup>j</sup>Department of Pharmacology and Regenerative Medicine, The University of Illinois at Chicago, Chicago, IL, USA

<sup>k</sup>Division of Otolaryngology- Head and Neck Surgery, Department of Surgery, Vancouver General Hospital, Vancouver, British Columbia, Canada

<sup>l</sup>Department of Oral Biological and Medical Sciences, Faculty of Dentistry, University of British Columbia, Vancouver, British Columbia, Canada

<sup>m</sup>Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

<sup>n</sup>Division of Otolaryngology - Head and Neck Surgery, Department of Surgery, University of Calgary, Calgary, Alberta, Canada

<sup>o</sup>Department of Otolaryngology Head and Neck Surgery, McGill University, Montreal, Quebec, Canada

<sup>p</sup>Department of Oncology, University of Western Ontario, London, Ontario, Canada

<sup>q</sup>Department of Otolaryngology - Head and Neck Surgery, University of Toronto, Toronto, Ontario, Canada

<sup>r</sup>Lineberger Comprehensive Cancer Center, UNC School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

<sup>s</sup>Department of Cell Biology and Physiology, UNC School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

<sup>t</sup>Department of Biochemistry, University of Western Ontario, London, Ontario, Canada

<sup>u</sup>Department of Human Genetics, University of California, Los Angeles, CA, USA

<sup>v</sup>Department of Urology, University of California, Los Angeles, CA, USA

<sup>w</sup>Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, CA, USA

<sup>x</sup>Institute for Precision Health, University of California, Los Angeles, CA, USA

<sup>y</sup>Jonsson Comprehensive Cancer Centre, University of California, Los Angeles, CA, USA

<sup>z</sup>Department of Microbiology & Immunology, University of Western Ontario, London, Ontario, Canada

## Summary

**Background** There is significant interest in treatment de-escalation for human papillomavirus-associated (HPV<sup>+</sup>) oropharyngeal squamous cell carcinoma (OPSCC) patients given the generally favourable prognosis. However, 15–30% of patients recur after primary treatment, reflecting a need for improved risk-stratification tools. We sought to develop a molecular test to risk stratify HPV<sup>+</sup> OPSCC patients.

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\*Corresponding author. Department of Otolaryngology - Head and Neck Surgery, Victoria Hospital, London Health Science Centre, Room B3-433A, 800 Commissioners Road East, London, ON, Canada N6A 5W9.

E-mail address: [anthony.nichols@lhsc.on.ca](mailto:anthony.nichols@lhsc.on.ca) (A.C. Nichols).

<sup>aa</sup>These authors contributed equally to this paper.

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**Methods** We created an immune score (UWO3) associated with survival outcomes in six independent cohorts comprising 906 patients, including blinded retrospective and prospective external validations. Two aggressive radiation de-escalation cohorts were used to assess the ability of UWO3 to identify patients who recur. Multivariate Cox models were used to assess the associations between the UWO3 immune class and outcomes.

**Findings** A three-gene immune score classified patients into three immune classes (immune rich, mixed, or immune desert) and was strongly associated with disease-free survival in six datasets, including large retrospective and prospective datasets. Pooled analysis demonstrated that the immune rich group had superior disease-free survival compared to the immune desert (HR = 9.0, 95% CI: 3.2–25.5,  $P = 3.6 \times 10^{-5}$ ) and mixed (HR = 6.4, 95% CI: 2.2–18.7,  $P = 0.006$ ) groups after adjusting for age, sex, smoking status, and AJCC8 clinical stage. Finally, UWO3 was able to identify patients from two small treatment de-escalation cohorts who remain disease-free after aggressive de-escalation to 30 Gy radiation.

**Interpretation** With additional prospective validation, the UWO3 score could enable biomarker-driven clinical decision-making for patients with HPV<sup>+</sup> OPSCC based on robust outcome prediction across six independent cohorts. Prospective de-escalation and intensification clinical trials are currently being planned.

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**Keywords:** Head and neck squamous cell carcinoma; Transcriptomics; Biomarkers; Cancer immunology; HPV; De-escalation

#### Research in context

##### Evidence before this study

HPV-associated oropharyngeal cancer (OPSCC) is one of the fastest rising cancers in North America. While cure rates can be high, up to 30% of patients recur after primary treatment. Current multimodal treatment regimens are historically derived from clinical trials for the more aggressive HPV-negative OPSCC and may be more intensive than necessary for most HPV<sup>+</sup> OPSCC patients. Thus, there is significant interest in treatment de-intensification for HPV<sup>+</sup> OPSCC.

##### Added value of this study

We defined three clinically-translatable and biologically-plausible immune classes in HPV-associated OPSCC using a

three-gene expression score. Our three gene-expression score was associated with distinct disease-free survival and overall survival in five independent cohorts which included 863 patients. More importantly, the three-gene expression score was associated with recurrence status following aggressive radiation de-escalation.

##### Implications of all the available evidence

The UWO3 immune score may identify patients with improved prognosis. Further studies in the context of a randomized trial will be needed to confirm these results.

## Introduction

The incidence of human papillomavirus-associated (HPV<sup>+</sup>) oropharyngeal squamous cell carcinoma (OPSCC) is increasing worldwide.<sup>1</sup> HPV<sup>+</sup> OPSCC is biologically and clinically distinct from non-HPV driven (HPV<sup>-</sup>) HNSCC, which is typically associated with tobacco and alcohol consumption.<sup>2–9</sup> Although HPV<sup>+</sup> OPSCC patients are usually younger and exhibit markedly improved outcomes compared to HPV<sup>-</sup> OPSCC patients,<sup>3</sup> current treatment guidelines from both the American Society of Clinical Oncology

(ASCO) and the National Comprehensive Cancer Network recommend identical treatment regimens of high-dose cisplatin and 70Gy radiation (CRT) independent of HPV status.<sup>10,11</sup> The rising incidence of HPV<sup>+</sup> OPSCC is leading to an increasing number of young survivors, making post-treatment quality of life and treatment-related morbidities a major concern. There is significant interest in treatment de-intensification for HPV<sup>+</sup> OPSCC patients to reduce morbidity rates, while maintaining excellent cure rates.<sup>12–17</sup>

Treatment de-intensification efforts have been complicated by the ~15–30% recurrence or metastasis rate of HPV<sup>+</sup> OPSCC patients treated with the current standard of care therapy.<sup>18</sup> Early efforts at de-intensification demonstrated that modification of current standard of care CRT can result in harm for patients. Both the De-ESCALaTE HPV and RTOG1016 phase III randomized trials found that substitution of cetuximab for cisplatin led to inferior survival outcomes.<sup>19,20</sup> Reflecting these unsuccessful attempts that led to poorer outcomes for patients on the experimental arm, recent treatment de-intensification guidelines from ASCO<sup>21</sup> and the Head and Neck Cancer International Group<sup>22</sup> have called for de-intensification to only be attempted in the context of a clinical trial for patients with favorable risk profiles. Thus, the ideal treatment de-intensification method and patient population remain highly controversial.<sup>10,21–26</sup>

Molecular biomarkers reflecting the biology of the tumour may better risk-identify patients who are ideal candidates for treatment de-intensification. HPV<sup>+</sup> head and neck cancer treatment failure has been linked to TP53 mutations,<sup>27</sup> tumour hypoxia,<sup>28</sup> keratinocyte differentiation,<sup>29</sup> metabolism,<sup>30</sup> chromosome 3p arm loss,<sup>31–33</sup> and HPV-related transcriptional programs.<sup>34</sup> These findings have not yet been thoroughly validated, require complex assays only available at select institutions, or generally exhibit modest effect-sizes that explain only a fraction of HPV<sup>+</sup> head and neck cancer treatment failures. There remains an urgent need for improved risk-stratification to guide therapeutic decision-making in balancing treatment toxicity and therapeutic efficacy.

We created a clinically translatable immune classification tool strongly associated with survival outcomes in HPV<sup>+</sup> OPSCC based upon the abundance of three transcripts. We validated it in five HPV<sup>+</sup> OPSCC cohorts comprising 863 patients, including two blinded cohorts and a tissue microarray (TMA) cohort using immunohistochemistry. Finally, we show this immune classification can identify patients who responded to aggressive treatment de-escalation. Taken together, our results may enable biomarker-guided personalized treatment de-intensification and intensification in HPV<sup>+</sup> OPSCC low and high-risk groups respectively.

## Methods

### Ethics

The study was approved by the Research Ethics Boards at Western University (REB 7182) and informed consent was obtained from each patient.

### Patient cohort

Primary site fresh tumour samples were prospectively collected from patients with HPV<sup>+</sup> OPSCC at Victoria Hospital, London Health Science Centre, London,

Ontario, Canada between 2010 and 2016. Patient demographics and survival outcomes were prospectively collected. Frozen section analysis was carried out to confirm tumour cellularity greater than 70%. HPV status was confirmed via p16 immunohistochemistry, as well as polymerase-chain reaction and Sanger sequencing. Detailed clinical information in the LHSC cohort is provided in [Supplementary Table S1](#). There were no other exclusion criteria. Disease free survival was defined as time from diagnosis to recurrence at any site or death. Recurrence was defined as the presence of local, regional, or distant disease after completion of treatment. The Cancer Genome Atlas (TCGA, n = 71),<sup>35</sup> Johns Hopkins University (JHU; n = 47), BD2Decide (n = 286), and Washington University (WashU) and Vanderbilt University (n = 262) cohorts are public HPV<sup>+</sup> OPSCC transcriptomic cohorts and have been described elsewhere.<sup>36–40</sup> Detailed descriptions of all other cohorts have been provided elsewhere.<sup>36–40</sup> Treatments received for each cohort are described in [Supplementary Table S2](#). All cohorts except for the TCGA cohort are HPV<sup>+</sup> tumours solely from the oropharynx region. The only cohort that contained tumours from the non-oropharynx region is that of the TCGA, which is 75% oropharynx. The reporting of the study followed guidelines from Reporting Recommendations for Tumour Marker Prognostic Studies (REMARK)<sup>41</sup> and STROBE.<sup>42</sup>

### RNA processing and sequencing

Total RNA and DNA was isolated using Qiagen AllPrep DNA/RNA kits. HPV status was confirmed by real time PCR as we have previously described.<sup>43,44</sup> One microgram of total RNA was shipped to The Center for Applied Genomics (Hospital for Sick Children, Toronto, ON) for quality control, library preparation, and sequencing. RNA quality was confirmed with a Bioanalyzer and libraries were prepared using a NEB Ultra II Directional mRNA library kit. Samples were then processed using random primers and paired end sequencing using an Illumina HiSeq 2500 yielded 50–90 million reads/sample (median: 66 million reads, [Supplementary Table S1](#)).

FASTQ files were pre-processed with trim\_galore (v0.6.4) and then quality controlled using FastQC (v0.11.9). Each sample was mapped to the human reference genome GRCh38 (v97) using STAR aligner (v2.7.2b) in two-pass mode,<sup>45</sup> and quantified using HTSeq-count (v0.12.3) intersection-strict mode.<sup>46</sup> Read normalization and differentially expressed gene testing were conducted using DESeq2 (v1.26.0).<sup>47</sup> Differentially abundant transcripts between the disease-free and recurrent patients were defined as transcripts having at least an average of normalized reads of 10, an absolute log<sub>2</sub> fold change (log<sub>2</sub>FC) greater than 2, and a Benjamini-Hochberg adjusted *P*-value of less than 0.05. Generation and processing of external cohorts have been described elsewhere.<sup>36–40</sup>

### HPV genotyping via HPV transcript quantification

HPV genotyping was performed on raw RNA-seq reads using HPVViewer (branch c62f29e, available at <https://github.com/yuhanH/HPVViewer>), on a database of 182 repeat masked HPV strains. HPV reads were then quantified using HTSeq-count intersection-strict mode to the subtype with the highest read-number.<sup>48</sup>

### Tumour microenvironment estimation

The tumour microenvironment (TME) composition of each sample was estimated using the MCP-counter score (v1.1.0).<sup>49</sup> The score was based on previously analyzed transcriptomic markers that were found to be characteristic of the specific immune population and were proportional to the abundance of each cell population within the tumour. Comparisons with other immune deconvolution methods have found the method to be highly accurate and capable of inter-sample comparisons.<sup>50</sup> The MCP-counter signatures composition are as follows: T cells: CD28, CD3D, CD3G, CD5, CD6, CHRM3-AS2, CTLA4, FLT3LG, ICOS, MAL, PBX4, SIRPG, THEMIS, TNFRSF25 and TRAT1; B lineage: BANK1, CD19, CD22, CD79A, CR2, FCRL2, IGKC, MS4A1 and PAX5; natural killer cells: CD160, KIR2DL1, KIR2DL3, KIR2DL4, KIR3DL1, KIR3DS1, NCR1, PTGDR and SH2D1B; monocytic lineage: ADAP2, CSF1R, FPR3, KYNU, PLA2G7, RASSF4 and TFEC; myeloid dendritic cells: CD1A, CD1B, CD1E, CLEC10A, CLIC2 and WFDC21P; neutrophils: CA4, CEACAM3, CXCR1, CXCR2, CYP4F3, FCGR3B, HAL, KCNJ15, MEGF9, SLC25A37, STEAP4, TECPR2, TLE3, TNFRSF10C and VNN3; endothelial cells: ACVRL1, APLN, BCL6B, BMP6, BMX, CDH5, CLEC14A, CXorf36 (also known as DIPK2B), EDN1, ELTD1, EMCN, ESAM, ESM1, FAM124B, HECW2, HHIP, KDR, MMRN1, MMRN2, MYCT1, PALMD, PEAR1, PGF, PLXNA2, PTPRB, ROBO4, SDPR, SHANK3, SHE, TEK, TIE1, VEPH1 and VWF.

### Clustering

K-means clustering was performed on Z-scores of the immune cell abundance estimation ([Supplementary Figure S1a](#)) using the kmeans wrapper of ComplexHeatmap package (v2.1.0) when generating heatmaps.<sup>51</sup> Kmeans was run 1000 times to generate consensus k-means clustering. The number of K clusters was selected using the silhouette method through the fviz\_nbclust function of the factoextra R package (v1.0.6).

### Immune gene signatures

Immune gene signatures were derived from other studies.<sup>52</sup> Briefly, each signature was computed as the geometric mean of the abundance of its included genes: immunosuppression (TGFB1, TGFB3, LGALS1 and CXCL12), regulatory T cells (FOXP3 and TNFRSF18),

T cell survival score (CD70 and CD27), T cell activation (CXCL9, CXCL10, CXCL16, IL15, and IFNG), myeloid chemotaxis (CCL2), MHC I (HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G and B2M), and tertiary lymphoid structures (CXCL13).

### UWO3 score development and prediction

Statistically significant transcripts between disease-free and recurrent tumours were filtered to only include transcripts whose abundance was independently associated with prognosis. The cohort was dichotomized into high and low abundance groups for each transcript and tested for association with prognosis in a Cox proportional hazard multivariate model including important clinical variables (age at diagnosis, sex, T stage, N stage, smoking status, alcohol abuse status). The abundance of transcripts with an FDR <0.25 were normalized using DESeq2, log-10 transformed, and Z-score standardized for score development. To facilitate the development of protein diagnostic tools for pathology, the gene lists were further filtered to contain genes for which the RNA abundance was highly correlated with its protein abundance (FDR <0.05 & rho >0.6, Spearman Correlation) using the HPV-negative HNSCC CPTAC cohort.<sup>53</sup>

For the development of the UWO3 score to differentiate between the 3 TME immune classes (immune rich, mixed, and immune desert), we used a regularized linear regression technique based on the LASSO algorithm, as implemented in the glmnet (v3.0-2) R package using 5-fold cross-validation. The score was trained with a “Gaussian” family model to minimize mean squared error (MSE). A minimal subset of 3 genes (CD3E, IRF4 and ZAP70) was outputted by LASSO, that we labeled the UWO3 score. The UWO3 score can be calculated with the following equation:  $UWO3 = 2.232 + ZAP70 \times -0.224 + IRF4 \times -0.137 + CD3E \times -0.273$ . Cut-offs were determined by maximizing the correct number of tumours assigned to the same immune subtype through clustering using the cutpointr package (v1.1.0) to find all acceptable cutpoints within a range of 0.01 by setting the tol\_metric = 0.01. Using UWO3, tumours were then assigned to an immune class as follow:  $UWO3 \leq 2$  were predicted to be “immune rich”,  $2 < UWO3 \leq 2.5$  were predicted to be “mixed”, and  $UWO3 > 2.5$  were predicted to be “immune desert”. An online UWO3 calculator is available (<https://www.nicholslab.com/uwo3calculator>).

### Tissue microarray and immunohistochemistry

All samples were obtained with informed consent after approval of the Institutional Review Board at Western University, the University of Calgary, and the University of British Columbia. The TMAs from University of Calgary and University of British Columbia have been

described previously.<sup>39,54</sup> The TMA from Western University were processed as following: The formalin-fixed paraffin-embedded (FFPE) blocks for each tumour were sectioned and stained with hematoxylin & eosin (H&E) to confirm the presence of tumour tissue. A Manual Tissue Arrayer (MTA-1; Beecher Instruments Inc.) was used to punch out 3–4 cylindrical cores of 0.6 mm diameter from each tumour sample. Cores were arrayed into recipient paraffin blocks. Control tissues were also included on each block. Cores were sealed into recipient blocks by heating at 40 °C for ~40mins. Blocks were sectioned into 1.5 µm sections and affixed to glass slides. Every ninth slide was stained with H&E to provide a reference. Additional details are available in the MTA-1 Instruction Manual ([www.beecherinstruments.com](http://www.beecherinstruments.com)). IHC staining was completed at the Department of Pathology & Laboratory Medicine and the Molecular Pathology Core Facility (Western University). Tissues were examined using an Aperio ScanScope® slide scanner and staining quantification was performed using QuPath (v0.2.3).

To translate the UWO3 score for use in TMAs, we stained the TMA with anti-CD3 (IR503, Agilent Dako), anti-ZAP70 (clone 2F3.2, IR653, Agilent Dako), and anti-IRF4 (clone MUM1p, IR644, Agilent Dako) antibodies on an Omnis staining platform (Agilent Dako). The tumour was contoured by a subspecialist pathologist and the number of cells within the tumour positive for each marker was quantified using the positive cell detection function in QuPath.<sup>55</sup> The percentage of positive cells within each tumour was then used to create Z-scores and to calculate the UWO3 score using the same equation as RNA-based assays. Using UWO3, tumours were then assigned to an immune class as follow: UWO3 ≤ 2 were predicted to be “immune rich”, 2 < UWO3 ≤ 2.5 were predicted to be “mixed”, and UWO3 > 2.5 were predicted to be “immune desert”.

### Statistics

All statistical analyses were performed with R software (v4.0.5) using the following packages: survival (v3.2.10), ggpubr (v0.4.0), stats (v4.0.5), and rms (v6.2.0). Wilcoxon rank sum test (2 categories) and Kruskal-Wallis test (>2 categories) were used to examine all relationships between categorical variables and quantitative variables. *P*-values were corrected for multiple testing using the Benjamini-Hochberg method. The Fisher's Exact test was used to analyze contingency tables. Survival was analyzed with the log-rank test and Cox proportional hazards model with the survival (v3.1-12) package. The proportional hazards assumption was assessed using Schoenfeld residuals. Hazard ratio in the JHU cohort was estimated using Cox Regression with Firth's penalized likelihood,<sup>56</sup> implemented using the coxph package (v1.13.1), as monotone likelihood is

observed due to no events in the immune rich group. Meta-analysis of the association between UWO3 immune class and disease-free survival was performed using the inverse variance method through the R package meta (v5.1-1) using log<sub>10</sub>HR and SEM in a fixed-effect model. The significance of any discrepancies in the estimates of the treatment effects from the different cohorts was assessed using Cochran's test for heterogeneity and the *I*<sup>2</sup> statistic as described previously.<sup>57</sup> Heterogeneity was considered statistically significant if the *P* value was less than 0.10 for the  $\chi^2$  test. Brier's error analysis of Cox models was calculated using the package pec (v2021.10.11). The predictive accuracy of UWO3 score was evaluated by the c-index with 1000 × bootstrap resampling, as described previously.<sup>58</sup> The relative importance of each parameter to survival risk was assessed using the  $\chi^2$  from R package rms (v6.2.0). All tests were 2-sided.

### Role of funders

The funding sources had no roles in the design of the study, the collection, analysis, and interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication.

## Results

### Development and validation of the UWO3 immune classification

To reveal transcriptomic features predictive of treatment response, we performed RNA-seq on 43 HPV<sup>+</sup> OPSCC tumours, 16 of which experienced local, regional, or distant recurrence. As specific TME alterations are associated with recurrence in HPV<sup>+</sup> OPSCC,<sup>52,59–61</sup> we characterized the TME by the abundance of distinct cell populations.<sup>49</sup> Through unsupervised clustering of these estimated immune cell abundances (see [Methods](#)), we classified samples into three categories: “immune rich”, “immune desert”, and “mixed” ([Supplementary Figure S1a](#)). These three TME subtypes exhibited distinct patterns of overall (OS; *P* = 0.003, log rank test; [Supplementary Figure S1b](#)) and disease-free survival (DFS; *P* < 10<sup>−3</sup>, log rank test; [Supplementary Figure S1c](#)). To facilitate translation of these results into the clinic, we developed a minimal classifier based on using the Least Absolute Shrinkage and Selection Operator (LASSO; see [Methods](#), [Supplementary Figure S2](#)) to stratify patients into one of three immune classes. The resulting classifier, which we call University of Western Ontario 3 (UWO3), is based on the abundance of three transcripts (*CD3E*, *IRF4* and *ZAP70*) and assigns immune classes strongly associated with DFS in our discovery cohort, as expected (*P* < 10<sup>−3</sup>, log rank test, [Supplementary Figure S3a](#)).



We next tested five independent cohorts to validate the association between UWO3 immune class and survival outcomes in HPV<sup>+</sup> OPSCC. We first used The Cancer Genome Atlas (TCGA, n = 71)<sup>35</sup> and Johns Hopkins University (JHU; n = 47) HPV<sup>+</sup> OPSCC RNA-seq cohorts.<sup>40</sup> In both, the immune rich patients had improved DFS and OS (TCGA:  $P = 0.01$ , JHU:  $P = 0.02$ , log rank test, Fig. 1a, c, Supplementary Figure S3b, c).

Next, we employed clinically-validated antibodies for the proteins corresponding to the transcripts within the UWO3 score (CD3E, ZAP70 and IRF4) that are used routinely in clinical pathology labs for hematologic malignancies. As immunohistochemistry (IHC) is cost-effective and broadly available, we applied the UWO3 score to a TMA consisting of 197 independent HPV<sup>+</sup> OPSCC patients treated uniformly with radiation (70 Gy) with concurrent cisplatin (Supplementary Figure S4a). Immune desert patients experienced inferior DFS (HR = 3.1, 95% CI 1.1–9.7,  $P = 0.038$ , univariate Cox model; Fig. 1A, Supplementary Figure S4b) compared to the immune rich patients, highlighting the potential of the UWO3 immune classification to be implemented as an IHC assay.

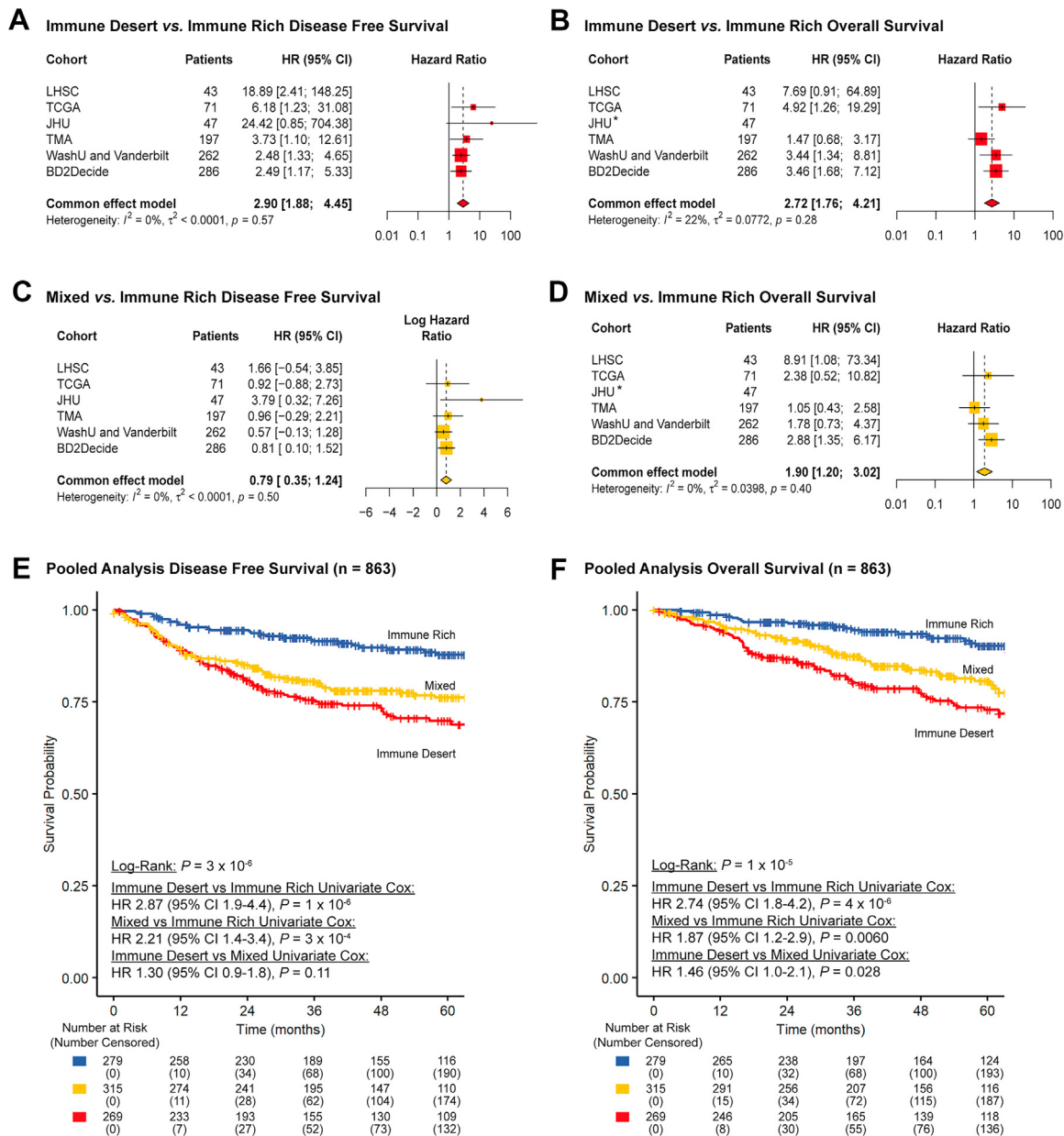
Finally, to validate the association between UWO3 score and survival outcomes, we performed blinded validations in a retrospective cohort and a prospective cohort. Patients were assigned to immune classes using UWO3 before unmasking of clinical outcomes. In a retrospective cohort of HPV<sup>+</sup> OPSCC patients (n = 262) profiled using RNA-seq of FFPE samples treated primarily with surgery at WashU at St. Louis and Vanderbilt University,<sup>37</sup> the immune classes exhibited distinct DFS ( $P = 0.01$ , log rank test; immune desert vs. immune rich: HR = 2.5, 95% CI 1.3–4.7,  $P = 0.004$ , univariate Cox model; Fig. 1a, c, Supplementary Figure S5a) and OS ( $P = 0.01$ , log rank test; immune desert vs. immune rich: HR = 3.44, 95% CI 1.59–7.44,  $P = 0.002$ , univariate Cox model; Fig. 1b, d, Supplementary Figure S5a). In the prospective Big Data and Models for Personalized Head and Neck Cancer Decision Support (BD2Decide) study (n = 286, NCT02832102) of 286 locoregionally-advanced p16-positive patients profiled using RNA-microarray and treated with radiation with or without chemotherapy at seven European institutions,<sup>38</sup> the immune classes exhibited distinct DFS ( $P = 0.03$ , log rank test; immune desert vs. immune rich: HR = 2.6, 95% CI 1.1–5.7,  $P = 0.02$ , univariate Cox model; Fig. 1a, c, Supplementary Figure S5c) and OS ( $P = 0.004$ , log rank test; immune desert vs. immune rich: HR = 5.0, 95% CI 1.6–15,  $P = 0.004$ , univariate Cox model; Fig. 1b, d, Supplementary Figure S5d). Taken together, we have shown that the UWO3 immune class is robustly associated with survival outcomes in six independent cohorts across different profiling platforms and geographic jurisdictions.

### Pooled analysis of all cohorts demonstrates that UWO3 immune class is a strong independent predictor of survival

As the association between immune groups and survival outcomes for each cohort were homogeneous between cohorts ( $I^2 < 50\%$ ,  $P > 0.10$ ; Fig. 1a–d), we performed a pooled analysis of all five validation cohorts (TCGA, JHU, TMA, WashU, and Vanderbilt, and BD2Decide; total n = 863), excluding the LHSC discovery cohort. The immune class defined by UWO3 was strongly associated with DFS ( $P = 3 \times 10^{-6}$ , log rank test, Fig. 1e) and OS ( $P = 1 \times 10^{-5}$ , log rank test, Fig. 1f) in this pooled cohort.

The 5-year DFS probabilities for the immune rich, mixed, and immune deserts group were 87.8% (95% CI 83.5%–92.3%), 76.1% (95% CI 71.2%–81.4%), and 69.9% (95% CI 64.2%–76.1%), respectively. Immune desert patients exhibited inferior DFS over the immune rich (HR = 2.87, 95% CI 1.9–4.4,  $P = 1 \times 10^{-6}$ , univariate Cox model; Fig. 1e). The mixed patients also exhibited worse DFS over the immune rich patients (HR = 2.21, 95% CI 1.4–3.4,  $P = 3 \times 10^{-4}$ , univariate Cox model; Fig. 1e). The 5-year OS probabilities for the immune rich, mixed, and immune deserts group were 90.2% (95% CI 86.2%–94.4%), 80.7% (95% CI 75.8%–85.9%), and 72.8% (95% CI 67.1%–79.0%), respectively. Immune desert patients exhibited inferior OS over the immune rich (HR = 2.74, 95% CI 1.8–4.2,  $P = 4 \times 10^{-6}$ , univariate Cox model; Fig. 1f) and mixed (HR = 1.46, 95% CI 1.0–2.1,  $P = 0.028$ , Fig. 1f) patients. The patients with mixed tumours also exhibited worse OS than immune rich patients (HR = 1.87, 95% CI 1.2–2.9,  $P = 0.0060$ , univariate Cox model; Fig. 1f). The survival differences by UWO3 immune group persisted for patients undergoing both primary surgery (n = 324; Immune rich vs. immune desert DFS: HR = 3.12, 95% CI 1.7–5.9,  $P = 0.0004$ , univariate Cox model; Supplementary Figure S6a, b) or primary radiation (n = 293; immune rich vs. immune desert DFS: HR = 4.81, 95% CI 2.0–11.4,  $P = 0.0003$ , univariate Cox model; Supplementary Figure S6c, d).

In a multivariate Cox proportional hazards model stratified for cohort, the association between the UWO3 immune class and DFS was independent of other clinical factors (immune desert vs. immune rich: HR = 9.0, 95% CI 3.17–25.5,  $P = 3.6 \times 10^{-5}$ ; mixed vs. immune rich: HR = 6.4 (95% CI 2.2–18.7,  $P = 0.0006$ ; Table 1). Brier score analysis demonstrates that the UWO3 immune group alone had a lower prediction error for disease-free survival than a Cox model of clinical factors (AJCC8 stage, sex, smoking status, and age) ( $P = 0.049$ ,  $\chi^2$  test; Fig. 2a). Furthermore, integration of the UWO3 immune group with other clinical factors (UWO3 + Full Clinical) further decreased prediction error ( $P = 5 \times 10^{-7}$ ,  $\chi^2$  test; Fig. 2a). We analyzed the relative contribution of each parameter to predict DFS and identified the UWO3 immune group classification



**Fig. 1: UWO3 immune group is a strong predictor of survival outcomes across six independent cohorts.** Patients from the immune desert and mixed group show inferior disease free survival and overall survival compared to the immune rich patients (A–D). Hazard ratio (HR) are based on the univariate Cox model and were combined using a Mantel-Haenszel fixed-effect model. Heterogeneity between studies was analyzed with  $\chi^2$  and  $I^2$  statistics. \*Hazard ratio for overall survival in the JHU cohort excluded from the analysis due to only one event in the cohort. Pooled Kaplan-Meier analyses of disease-free survival (E) and overall survival (F) of HPV+ HNSCC patients show that UWO3 immune groups are associated with distinct survival outcomes. LHSC samples are not included in the meta-analysis or pooled analysis. P values from two-sided log rank tests and Cox proportional regression model.

(50.9%, Fig. 2b) as the strongest parameter, compared to other clinical factors. Finally, the UWO3 score was also superior at predicting DFS compared to levels of tumour infiltrating lymphocytes (as determined by

CD3E abundance) (Supplementary Figure S7). Thus, UWO3 immune class is a strong independent prognostic factor that can improve the risk-stratification of HPV+ OPSCC.



	Disease free survival	
	HR (95% CI)	P value
<b>UWO3 Immune Class</b>		
Mixed vs. Immune Rich	6.4 (2.21-18.7)	0.0006
Immune Desert vs. Immune Rich	9.0 (3.17-25.5)	$3.6 \times 10^{-5}$
<b>Sex</b>		
Female vs. Male	1.29 (0.63-2.63)	0.47
<b>Age</b>		
Age	1.04 (1.01-1.07)	0.0035
<b>AJCC 8th edition stage</b>		
Stage II vs. Stage I	2.04 (1.11-3.75)	0.022
Stage III vs. Stage I	2.28 (1.26-4.09)	0.006
Stage IV vs. Stage I	$5.41 \times 10^{-7}$ (0-Inf)	1.00
<b>Smoking</b>		
Yes vs. No	1.43 (0.79-2.57)	0.24

AJCC: American joint committee on cancer. HR: hazard ratio. CI: confidence interval.

**Table 1: Multivariate analysis of the UWO3 immune class.**

**UWO3 immune classification has implications for treatment de-intensification**

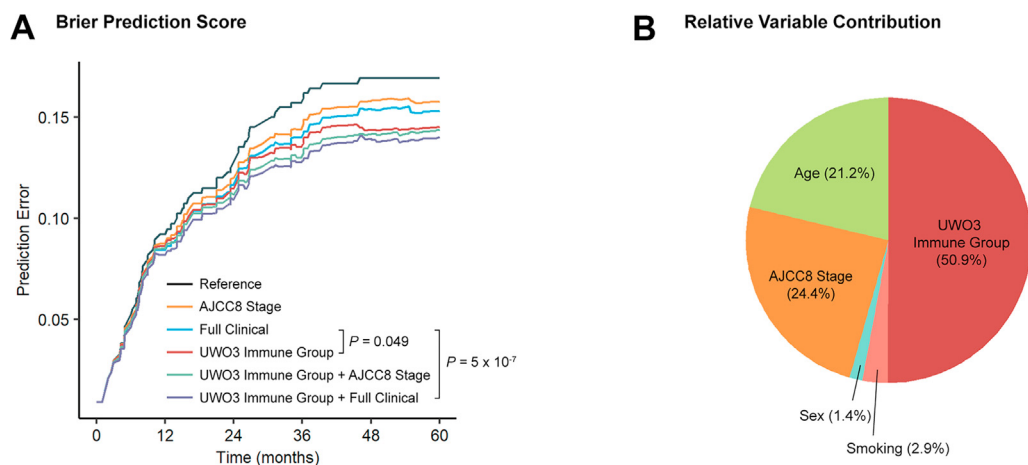
As the immune rich patients exhibited excellent survival outcomes, we hypothesized that the UWO3 score could identify patients who respond favourably to de-intensified treatment. We used RNA-seq data from the phase II MC1273 (NCT01932697) trial,<sup>25</sup> which tested an aggressive de-escalation regimen of 30 Gy radiation with concurrent docetaxel post-surgery and the 30ROC trial (NCT00606294),<sup>62</sup> in which patients received 30 Gy radiation and cisplatin. The UWO3 score was associated with higher odds of recurrence following aggressive treatment de-escalation (Odds ratio: 24.9,  $P = 0.0147$ , logistic regression with UWO3 as continuous variable stratified for cohort; Fig. 3). Strikingly, 7 out of 9

patients (77.8%, Fig. 3) in the immune desert group developed recurrence, while only 4 out of the 24 patients (16.6%) in the immune rich and mixed groups recurred. While limited by the sample size, these results support the potential ability of the UWO3 score to identify patients who will maximally benefit from aggressive treatment de-escalation.

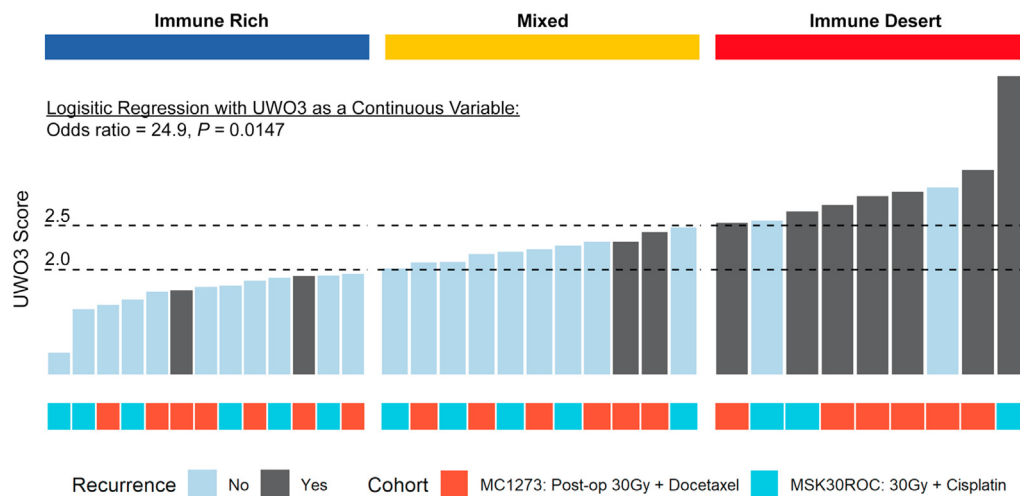
**Discussion**

Although HPV<sup>+</sup> OPSCC patients have improved prognosis over their HPV-negative counterparts, a significant portion of patients still recur after initial treatment and are at risk of death. Molecular biomarkers that reflect the unique biology of HPV<sup>+</sup> OPSCC such as tumour hypoxia, mutation status, or immune activation may refine patient prognosis and selection for de-escalation clinical trials.<sup>63</sup> In the current study, we demonstrate that the pre-treatment TME has dramatic effects in determining the prognosis of HPV<sup>+</sup> OPSCC patients. Further, we describe a clinically translatable, extensively validated UWO3 immune classification tool that may allow biomarker-driven individualized treatment in HPV<sup>+</sup> OPSCC (Fig. 4).

The proposed immune classification reflects HPV<sup>+</sup> OPSCC biology. CD3E is part of the T-cell receptor complex and its down-regulation on T-cells has been linked to worse prognosis in OPSCC.<sup>64</sup> ZAP70 plays an important role in T-cell receptor signaling but is also highly expressed on NK-cells.<sup>65</sup> IRF4 directs the development, affinity maturation, and terminal differentiation of B cells, but also plays important roles in monocyte differentiation.<sup>66-70</sup> Accumulating evidence suggests that rich immune infiltration distinguishes



**Fig. 2: UWO3 immune class outperforms clinical factors in predicting disease-free survival.** (A) Brier prediction score analysis shows lower error rate, thus higher prediction accuracy of disease-free survival for the UWO3 immune class than major clinical factors combined (AJCC8 stage, age, sex, smoking status). Integration of UWO3 immune group with other clinical factors further decreased prediction error rate. (B) Relative importance of each risk parameter to survival risk using the Pearson  $\chi^2$  test for clinical parameters plus UWO3 immune group shows that immune group is the most important factor. AJCC: American Joint Committee on Cancer.



**Fig. 3: UWO3 immune classification has implications with respect to aggressive radiation de-intensification.** The UWO3 score preferentially identifies patients who recur following aggressive radiation de-escalation from 70 Gy to 30 Gy in the Mayo Clinic MC1273 trial (NCT01932697) and the Memorial Sloan Kettering (MSK, NCT00606294) 30ROC trial. Recurrence is defined as patients who have developed locoregional or metastatic disease as of last follow-up. Odds ratio and  $P$ -value are from logistic regression with UWO3 as a continuous variable and stratified for cohort.

HPV<sup>+</sup> OPSCC from both HPV-negative OPSCC and cervical cancer, a cancer type with similar HPV-viral etiology.<sup>71–73</sup> There could be several reasons underlying the difference in the TME specifically in HPV<sup>+</sup> OPSCC. The oropharynx region includes the Waldeyer's tonsillar ring of lymphoid organs that are densely infiltrated with immune cells. Furthermore, HPV<sup>+</sup> OPSCC is virally-driven and thus expresses unique viral antigens. The presence of viral-host fusion proteins following HPV integration in some tumours may be strongly immunogenic and stimulate anti-tumour immunity.<sup>74</sup> Recent work using single-cell RNA-sequencing has identified high proportions of HPV antigen-specific tumour infiltrating lymphocytes in HPV<sup>+</sup> OPSCC,<sup>75,76</sup> including a subset of PD1<sup>+</sup> TIM3<sup>+</sup> (the protein encoded by the gene *HAVCR2*) terminally differentiated CD8 T-cells within HPV<sup>+</sup> OPSCC tumours that fail to proliferate following antigen stimulation. Multiple studies are ongoing to assess the relevance of TIM3 inhibition in a variety of tumour types.<sup>77,78</sup> Further work will be needed to investigate whether the addition of immunotherapy (such as anti-PD1 and/or anti-TIM3) to CRT can stimulate anti-tumour immunity and improve survival outcomes for HPV<sup>+</sup> OPSCC patients with an immune desert TME.

Patients with immune rich pre-treatment tumours have improved prognosis compared to the mixed and immune desert groups consistently across six different cohorts regardless of treatment. Thus, patients who are immune rich and have favorable clinical factors may be ideal candidates for aggressive treatment de-intensification. The inflammatory TME of the immune rich group and dense infiltration of PD1<sup>+</sup> CD8 T cells

pre-treatment supports further exploration of substitution of chemotherapy with immunotherapy in de-escalation settings. The three-arm phase III randomized controlled trial NRG-HN005 (NCT03952585) is currently evaluating such a regimen (60 Gy plus nivolumab) against the standard treatment of 70 Gy with cisplatin and a de-intensified regimen of 60 Gy plus cisplatin. Furthermore, the association between patients who have immune desert pre-treatment tumours and poor survival outcomes supports the investigation of neoadjuvant immunotherapy approaches, which have been found to increase T-cell density within OPSCC tumours.<sup>79</sup>

The key limitation of our study is that the treatment protocols delivered in each cohort were not uniform. However, the strong association of UWO3 with DFS in six cohorts, which span different geographic jurisdictions, treatment methods, and patient populations suggests that the immune classes can treatment-agnostically predict survival outcomes. Another limitation of the current study is that the TCGA cohort contains 25% of tumours from outside of the oropharynx. Given that over 98% of the tumours used in this study (888/906) are from the oropharynx, and the HPV<sup>+</sup> oropharyngeal patients have similar survival patterns as the TCGA cohort including both HPV<sup>+</sup> non-oropharynx and oropharynx tumours, we believe the inclusion of data from the very small number of tumours from other subsites present within the TCGA cohort will be very unlikely to influence the interpretation of this classification system for HPV-associated OPSCC. Finally, another limitation of the current study is that cohorts were profiled using different

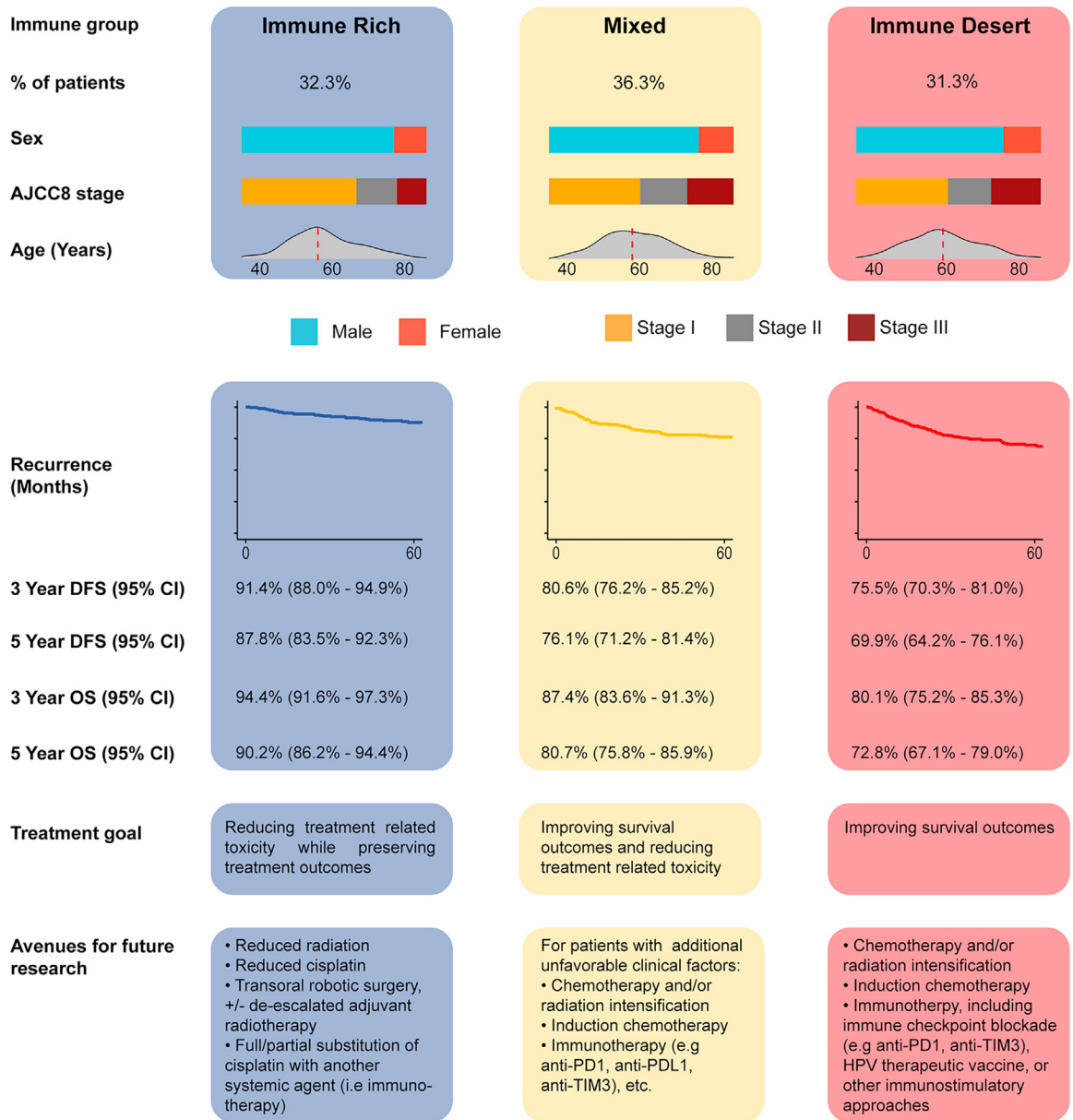


Fig. 4: UWO3 immune classification of HPV+ HNSCC has implications for treatment de-intensification and immunotherapy. CI: confidence interval; DFS: disease-free survival; OS: overall survival.

technologies such as RNA-seq, RNA microarray, and immunohistochemistry. However, the strong association between the UWO3 and survival outcomes despite these differences highlights the independence of the UWO3 score with respect to profiling technology and may facilitate clinical implementation. Development of a College of American Pathologists (CAP) and Clinical Laboratory Improvement Amendments (CLIA) approved assay is underway to further assess its analytical and clinical validity. Ultimately, validation of the UWO3 score using a CLIA-CAP approved assay

within the context of a randomized controlled trial population will be required before clinical adoption.

In summary, the ideal patient population for treatment de-intensification and intensification in HPV+ OPSCC remains controversial.<sup>21</sup> Upon further analytical validation, our retrospectively and prospectively-validated immune classification may provide a readily implemented means to identify patients with improved prognosis. Further prospective studies in the context of a randomized controlled trial will be needed.

**Contributors**

P.Y.F.Z, M.J.C, M.S, M.I.K, J.W.B, A.C.N designed the study and experiments. P.Y.F.Z, M.J.C, M.S-T, L.D.C, X.L performed the bioinformatic analysis. P.Y.F.Z, M.J.C, M.S-T, L.D.C, X.L, A.C.N verified the underlying data. J.W.B performed RNA extraction. M.S.S, S.C, L.L, F.H, R.H.B, C.R.L, K.S, T.P, X.W, F.L, E.P, C.P, S.D.DS, M.H, J.C.D, P.B., D.M, C.J.H, W.S, J.Y., K.F., A.D, S.E.B.R, P.P, H.K, D.A.P., P.L, S.D.S, A.M, P.L, E.W, H.Z, A.A, A.C.N provided patients materials and clinical data. P.Y.F.Z, M.S, M.I.K, J.W.B, S.S-C.L.A.A, P.C.B, J.S.M, and A.C.N discussed data and wrote the text. P.C.B, J.S.M, and A.C.N supervised the study. All authors read and approved the manuscript before submission.

**Data sharing statement**

Data from the TCGA HNSC cohort included in the current study is available from the Genomic Data Commons Data Portal (<https://portal.gdc.cancer.gov/projects/TCGA-HNSC>). Data from the JHU, Washington University at St. Louis, and BD2Decide cohort could be accessed from GEO with accession numbers GSE112026, GSE171898, and GSE163173. Data from the MC1273 and 30ROC trials could be accessed at GSE157517. All other data available via request to corresponding author.

**Declaration of interests**

PYFZ, JWB, PCB, JSM and ACN have a US patent pending for the UW03 score. All other authors declare no conflict of interest.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2022.104373>.

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