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Molecular Diagnostics

A plasma derived hypoxia signature predicts benefit from hypoxia-modifying radiotherapy and reveals dynamic humoral immune modulation in bladder cancer

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INTRODUCTION: Hypoxia is a hallmark of solid tumours and drives radioresistance, yet no hypoxia biomarkers are used clinically. We explored nanoproteomics to identify a plasma protein signature for longitudinal non-invasive hypoxia monitoring.

METHODS: Twenty-three bladder cancer patients (T2–T3b) undergoing radiotherapy (55 Gy, 4 weeks) were prospectively enrolled. Plasma was collected at baseline and weekly, nanoparticle-enriched and analysed by proteomics. Hypoxia scores (HS) were derived from diagnostic biopsies using a validated 24-gene signature, and patients stratified by median HS.

RESULTS: We identified 115 differentially abundant proteins (DAPs; $|FC| > 1.5$; $p < 0.05$). Seven DAPs (IGKV3, IGLV2-18, VL_4, VL_7, FN1, IGLV2-14, CAMP) correlated with HS across multiple timepoints ($p < 0.05$; $|r| > 0.4$). A two-protein signature (FN1, CAMP) was retrospectively validated, showing prognostic value in TCGA-BLCA ($n = 404$; HR = 1.54; CI = 1.12–2.13; $p = 0.009$), BC2001 ($n = 313$; HR = 1.42; CI = 1.08–1.86; $p = 0.011$) and in meta-analysis ($n = 150$; HR = 1.48; CI = 1.23–1.78; $p < 0.001$). In BCON ($n = 150$), it predicted benefit from hypoxia-modifying therapy (HR = 0.60; CI = 0.34–1.06; $p = 0.079$). The 115 DAPs formed five temporal co-expression clusters peaking at successive treatment weeks. Clusters were enriched for humoral immune pathways, with one linked to extracellular matrix remodelling.

CONCLUSION: These findings support the use of low abundance plasma biomarkers for real-time hypoxia stratification, revealing dynamic immune responses during radiotherapy associated with hypoxia.

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INTRODUCTION

Hypoxia, a hallmark of solid tumours [1], is a key radioresistance driver and a poor prognosis marker [2]. In bladder cancer, hypoxia affects up to 70% of patients [3] and is consistently associated with poorer outcomes, irrespective of treatment [3–7]. Targeting hypoxia has been proven to improve bladder cancer outcomes [8]. The UK phase III BCON trial showed a 17% increase in 5-year overall survival (OS) rates for bladder cancer patients having concurrent hypoxia-targeting carbogen and nicotinamide (CON) when undergoing radiotherapy [8]. There is interest in developing methods for assessing tumour hypoxia, with evidence that some can identify patients who benefit from hypoxia-modifying treatment [2].

Despite considerable effort into developing hypoxia biomarkers, none are routinely used in clinical practice [9]. An ideal hypoxia biomarker would not only estimate the extent of hypoxia before treatment but also monitor changes during therapy. We

previously derived a 24-gene hypoxia-associated signature for bladder cancer, showing it predicts benefit from having CON with radiotherapy [7, 8]. While gene signatures are promising, they require invasive tumour biopsies and are not suitable for serial monitoring. A minimally invasive plasma protein biomarker panel could overcome these limitations [10, 11]. However, discovery efforts have been hampered by the dominance of high-abundance plasma proteins, which mask the identification of lower-abundance and potentially relevant protein markers [12, 13]. Consequently, previous discovery studies in radiotherapy cohorts favoured targeted antibody-based analyses of single proteins rather than unbiased proteomic discovery [14–16]. These studies showed that higher expression of hypoxia-associated blood biomarkers is linked to a poor prognostic in radiotherapy cohorts, highlighting the potential of blood circulatory biomarkers [14–16]. However, these single-protein biomarkers failed prospective validation, partially due to the variability in the protein

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abundance levels [10, 11]. As shown in RNA expression studies, signatures outperformed single-gene markers in stratifying patients, as they offer higher robustness and resolution, improving reliability [17]. Non-targeted nano-omics studies enable both signature discovery and comprehensive profiling of systemic blood protein changes linked to tumour pathways [18, 19], which can improve our understanding of hypoxia-associated tumour changes.

Here we employ for the first time a previously developed nanotechnology-enabled plasma proteomics approach [18–21], to identify novel plasma biomarkers associated with tumour hypoxia, and to characterise systemic plasma compositional changes in patients with high versus low tumour hypoxia. Our data shows that hypoxia modulates the humoral immune response, inducing a plasma immunoglobulin profile shift during radiotherapy. Related to this mechanism, we identified seven hypoxia-associated plasma biomarker candidates. Two candidates (FN1, CAMP) were evaluated at RNA level, constituting a two gene plasma-associated hypoxia signature, which was prognostic and predicted benefit from hypoxia-modifying treatment in retrospective validation across three cohorts (TCGA-BLCA, BC2001, BCON).

METHODS

Study design and patient recruitment

The study was prospective, approved by the Manchester Cancer Research Centre Biobank Ethics Committee (Reference 18/NW/0092), and informed consent was obtained from all patients. A cohort of 23 MIBC patients (stage T2 to T3b) was recruited prospectively. Two patients were female, and 21 patients were male. All patients had concurrent Gemcitabine treatment during radiotherapy. Table S1 lists the clinicopathologic and treatment details. Patients underwent radiotherapy (52.5–60 Gy) in 20 fractions over 4 weeks in combination with weekly gemcitabine at 100 mg/m² [22]. Weekly blood samples were collected, starting prior to the first radiotherapy fraction and up to the end of the treatment. Samples (10 ml) were collected into ethylenediaminetetra-acetic acid (EDTA) tubes and plasma separated through centrifugation. Paired formalin-fixed, paraffin-embedded (FFPE) pre-treatment biopsies were retrieved. Haematoxylin and eosin staining was performed on 4 µm sections, and the percentage of tumour in each FFPE section was assessed by a specialised pathologist (Table S2).

Derivation of hypoxia scores

RNA was extracted from FFPE samples, gene expression measured, and hypoxia scores (HS) calculated as previously described [7]. Briefly, two 10 µm sections were deparaffinised with xylene and RNA extracted using the High Pure FFPE RNA Isolation Kit (Roche, Basel, Switzerland). Total RNA (900 µg) was reverse transcribed using the ABI High-Capacity RNA to cDNA kit (ThermoFisher Scientific, Massachusetts, USA). Pre-amplification of the genes of interest was performed with a TaqMan Custom PreAmp Pool (Applied Biosystems). Custom TaqMan Low Density Array (TLDA) cards (Applied Biosystems) were used to measure gene expression using the QuantStudio 12 K Flex qPCR system (Applied Biosystems). Thirty-two genes were analysed: 24 hypoxia signature genes, 6 endogenous control genes, 1 internal plate control gene and 1 hypoxia marker gene (Table S3). HS were calculated as the median expression of the 24 genes.

Amplification plots were analysed using QuantStudio 12 K Flex Software (Applied Biosystems). For each sample, the geometric mean of the Ct values of the endogenous control genes (Z) was obtained, as well as the median of the Ct values of the hypoxia signature genes (Y). HS for each tumour were calculated with the following formula: $HS = (Y - Z)^2$. Finally, the median cohort HS was used as a threshold, stratifying the patients into high-HS and low-HS groups (Table S3). Patients with an HS within ±10% of the threshold value range were excluded from all analyses ($n = 2$). Additionally, one patient had no diagnostic FFPE sample available and was discarded. Of the remaining 20 patients, one had plasma samples available for only two timepoints (before radiotherapy, one week after radiotherapy).

Preparation and physicochemical characterisation of liposome NPs

HSPC: Chol: DSPE-PEG2000 (56.3:38.2:5.5) liposomes were prepared by the thin lipid film hydration method followed by extrusion using a 10 ml

LIPEX® 10 Thermobarrel Extruder (Evonik Canada Inc.), as we described previously [18]. The physicochemical properties of the liposomal nanoparticles employed (Fig. S1) were characterised using Zetasizer Pro instrument and automatically analysed by the ZS Xplorer software (Malvern Panalytical). Size and zeta potential measurements were obtained; liposomes with an average size of 105.2 nm, a polydispersity index (PDI) of 0.039, and a zeta potential of -27.4 mV were used.

Nanoparticle-enabled plasma protein enrichment

To perform plasma protein enrichment, 820 µl plasma was incubated with 180 µl of 12.5 mM liposomes (10 min, 37 °C, 250 rpm), inducing the formation of a protein corona. To purify unbound plasma proteins, samples were run through Sepharose CL-4B (Sigma Aldrich) separation columns (15 × 1.5 cm), eluted with HEPES buffer, and concentrated to 100 µl with VivaSpin 6 (10 kDa MWCO) centrifugal concentrator columns (9000 rpm, 60–90 min, 4 °C; Sartorius). Samples were washed three times with 100 µl HEPES buffer. Recovered protein from the protein corona samples were quantified using a BCA protein quantification kit (Thermo Fisher Scientific) [18].

Mass spectrometry sample preparation and analysis

For MS analysis, 10 µg of total protein per sample was lysed in 10 µl of buffer (50 mM TEAB, 5% SDS) at 4 °C for 1 h. Samples were reduced with 5 mM DTT at 60 °C for 10 min, alkylated with 30 mM IAA in the dark for 30 min, and quenched with DTT. After centrifugation (14,000 × g, 10 min), the supernatant was acidified to 1.2% (w/v) phosphoric acid and diluted six-fold with S-Trap binding buffer (90% methanol in 100 mM TEAB, pH 7.1). Samples were loaded onto S-Trap™ micro spin columns (ProtiFi), washed once with MTBE/methanol (10:3), and three times with binding buffer. Trypsin digestion (1 µg in 20 µl) was performed in-column at 47 °C for 1 h. Peptides were eluted, desalted using a 0.2 µm PVDF 96-well plate (Corning) with Oligo R3 resin, washed with 0.1% formic acid, eluted with 30% acetonitrile/0.1% formic acid, and lyophilized. Dried samples were reconstituted in 10 µl 5% acetonitrile 0.1% formic acid and analysed by LC-MS/MS using an UltiMate® 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to a QExactive™ Hybrid Quadrupole-Orbitrap™ (ThermoFisher Scientific, Waltham, MA) mass spectrometer.

Raw files were processed using Progenesis QI v3.0 (Nonlinear Dynamics), with alignment to a reference run, Hi-N quantification, and a ≥ 3-peptide requirement per protein. MS/MS spectra were searched against the homo species UniProt database using Mascot v2.3.0, allowing one missed cleavage, fixed carbamidomethylation (C), variable oxidation (M), and charge states +2/+3 (20mmu precursor, 5ppm fragment tolerance). Peptides were filtered at PSM > 20 and FDR < 1%. Statistical analysis (one-way ANOVA, $p < 0.05$) compared low and high HS patients.

Retrospective analysis of clinical cohorts

Three bladder cancer cohorts were used. The BCON (NCT00033436; assessing hypoxia-modifying therapy combined with radiotherapy) [23] and BC2001 (NCT00024349; assessing the addition of chemotherapy to radiotherapy) [24] cohorts were from multicentre, randomised, phase 3 trials. Trial details, including eligibility criteria, procedures, statistical analysis, histopathology, RNA extraction, transcriptomic analysis, quality control, and exon array hybridization are available for both cohorts [7, 23–26]. Similar inclusion and exclusion criteria were used in both trials as previously reported [25]. The TCGA-BLCA is a publicly available cohort, and the details, including inclusion criteria, procedures, RNA extraction, sequencing, and quality control were previously described [27]. Outcome, transcriptomics and reverse-phase protein array protein quantification data were downloaded through cBioportal [28]. The TCGA pan cancer cohort is a public cohort aggregating TCGA cohorts clinical and omics data of 11,258 cancer patients across 33 cancer types. Data curation, normalisation, and aggregation approaches have been previously described [29]. Both outcomes and transcriptomic data were downloaded using the UCSCXenaTools R package [30]. The CPTAC dataset is a public cohorts repository spanning 1,072 patients across 10 cancer types which includes both transcriptomics and proteomics data. Cohorts details, including patients' data, sample processing, and omics dataset generation, are described elsewhere [31]. Omics data for the CPTAC cohorts was downloaded using the Reticulate R package [32]. Bladder cancer is not available among the CPTAC cohorts.

Ten-years OS was used as endpoint. For the BC2001 and BCON cohort, it was defined as the time from the date of randomisation to the date of

death due to any cause up to ten years of follow-up. For the TCGA-BLCA and TCGA Pan cancer cohort, it was defined as the time from diagnosis to the date of death due to any cause up to ten years of follow-up.

Patients were dichotomised into high ($\geq 50\%$) and low ($< 50\%$) groups based on their HS for each cohort. Endpoint event estimates were calculated using the Kaplan–Meier estimator. Analyses were performed using R (v. 4.3.0) packages: *tibble*, *survival*, *survminer*.

In silico and statistical analyses

Data representation, correlation, ontology and clustering analyses were performed in RStudio (v.4.3.0) with the following packages downloaded from The Comprehensive R Archive Network (CRAN, <https://cran.r-project.org/>): *dplyr*; *purrr*; *tidyr*; *matrixStats*; *org.Hs.eg.db*; *AnnotationDbi*; *ClusterProfiler*; *DOSE*; *ggplot2*; *grid*; *gridExtra*; *dendextend*; *psych*; *heatmap*; *RColorBrewer*; *gttools*; *factoextra*; *WGCNA*. The candidate protein signature fold-change trends over time and protein overexpression odds ratio (ORs) analyses and representations were performed in GraphPad Prism (V. 9.3.1; GraphPad Software Inc, California, USA). Protein abundance values were normalised against total protein abundance. Fold changes were defined as the ratio of the normalised protein abundance of low vs high HS patients for each time point. P-values were calculated through ANOVA testing. Significant protein abundance changes were defined as $p < 0.05$ and fold change > 1.5 or < -1.5 .

Similarity scores were calculated independently for each protein by calculating cosine similarity, with protein expression levels before radiotherapy being used as a reference baseline. Resulting values were scaled from 0 to 1 using min-max normalisation. Spearman's rank correlation coefficient was used for correlation analyses, and correlation strength was classified as "high" ($r > 0.7$ or $r < -0.7$) or "low" ($-0.2 \leq r \leq 0.2$). When protein expression was correlated against HS, significant correlation was defined as $p < 0.05$ and $r > 0.4$ or < -0.4 . Canberra and Euclidean distances were used for clustering analyses. "During radiotherapy" timepoint for gene ontology enrichment analysis was defined as all significant proteins identified at one week, two weeks and three weeks after radiotherapy. Yang HS (reference hypoxia signature) were derived as the median of normalised log2-transformed expression values of genes in the Yang et al. signature [7]. Protein HS were calculated as the median of normalised protein abundance levels for IGKV3, IGLV2-18, VL_4, VL_7, FN1, IGLV2-14 and CAMP. Only two of the seven proteins were assessable at the RNA level. RNA HS for the two-gene plasma-associated candidate signature (FN1, CAMP) were calculated as the average of FN1 expression and $-1 \times$ CAMP expression.

ORs were estimated by first calculating each patient's individual fold change vs the protein ratio of the normalised protein abundance of low HS patients. The number of patients with a > 1.5 fold change increase for each protein of interest was determined within each Yang HS group (high, low). ORs and 95% confidence intervals (CIs) were then estimated using the logarithmic transformation method, with CIs calculated on the ln scale and subsequently exponentiated as previously described [33]. Protein abundance values normalised to total protein levels were quantile normalised before performing WGCNA protein clustering and timeline expression trends. Generated total protein and quantile normalised abundance values were defined as normalised protein expression values.

RESULTS

Differences in tumour hypoxia associate with different plasma protein profiles

Proteomics identified 816 plasma proteins across all time-points. Among the 816 identified plasma proteins in the 20 MIBC patients, 115 showed significant differences (fold change > 1.5 or < -1.5 ; $p < 0.05$) between high and low HS groups: 24 before radiotherapy, 46 at week one, 40 at week two, 14 at week three, and 37 at week four (Fig. S2a–e; Supplementary File 1). Twenty-seven proteins (IGKV3, IGLC6, IGLV2-18, IGHV, FN1, F13A1, VL_7, CHD8, LUM, NCCRP1, VL_2, F13B, CCDC40, IGLL1, APOC1, CAMP, KRT82, CSAD, H1-1, MTX1, IGH, VL_9, IGHV_8, C2, IGH_2, VL_4, CASP14) were highly significant (fold change > 1.5 or < -1.5 ; $p < 0.01$) in at least one time-point (Fig. S2a–e). Among all significant proteins, only three immunoglobulins (IGLV2-18, IGH, VL_7) were consistently identified as significant across time-points (Fig. S2f). Fifty-one of the 115 differentially abundant proteins (DAPs) were

immunoglobulins and complement cascade proteins (Supplementary File 2). In terms of dynamics, high HS patients ($n = 10$) had increased overall levels of the 115 DAPs, peaking one week after radiotherapy and then declining (Fig. 1a). Fifteen proteins exhibited strong linear changes ($r > 0.7$ or $r < -0.7$), with two increasing and 13 decreasing, while 37 showed stable or non-linear patterns (r between -0.2 and 0.2) (Fig. 1a).

Enrichment analysis of the 115 DAPs revealed consistent differences in humoral response pathways between HS groups across all timepoints (Fig. 1b). Although high variability in the proteins involved was found across the time points (Fig. 1c), a protein interaction network analysis of the 115 DAPs revealed strong interactions within extracellular matrix (ECM) organisation, lipoprotein assembly, coagulation, and humoral immune pathways. Two ECM-associated proteins (TF, ITIH4) were connected to the humoral cluster via C3 signalling, suggesting a role in modulating immune responses (Fig. S3). ECM related pathways were uniquely enriched before radiotherapy, ranking top three (Fig. 1b, c), whilst humoral response pathways had the highest enrichment during and at the end of radiotherapy. Humoral pathways were also dominant for proteins with both linear and non-linear dynamics, although differences in the enrichment were also found for proteins with linear (enriched for keratinocyte differentiation, epidermis development pathways) and non-linear (enriched for coagulation, fibrin clot formation pathways) dynamics (Fig. 1d, e). Collectively, these findings indicate that tumour hypoxia is associated with dynamic pathway-specific plasma protein changes before and during radiotherapy.

A candidate plasma hypoxia signature stratifies patients by tumour HS

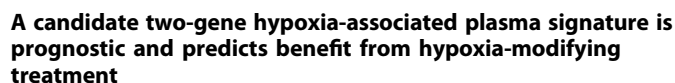
To identify a plasma-based hypoxia signature, hypoxia-associated DAPs were correlated with Yang HS across all timepoints. Sixteen DAPs were significant ($p < 0.05$; $r > 0.4$ or $r < -0.4$) before radiotherapy, 32 after one week of treatment, 15 after two weeks, 8 after three weeks, and 24 at the end of treatment (Fig. 2a; Supplementary file 1), suggesting hypoxia-associated DAPs levels fluctuate during radiotherapy.

Seven proteins (IGKV3, IGLV2-18, VL_4, VL_7, FN1, IGLV2-14, CAMP) had a significant correlation at ≥ 2 timepoints, and their correlation was maintained as positive (IGKV3, IGLV2-18, VL_4, VL_7, FN1) or negative (IGLV2-14, CAMP) across all timepoints, being selected as a candidate signature. PCA (Fig. 2b) and K-means (Fig. 2c) clustering showed the candidate signature separated HS-high and HS-low patients. The presence of patients with intermediate scores suggests a continuous relationship between circulating protein expression and tumour hypoxia severity.

A candidate plasma hypoxia signature stratifies patients by tumour HS throughout radiotherapy

We next assessed the temporal behaviour of the candidate signature during radiotherapy to study its potential for longitudinal patient monitoring. Increased ORs of upregulation were observed for IGKV3 (1.17–81), IGLV2-18 (8.17–400), VL_4 (6–31.5), VL_7 (2–21), FN1 (1–81), and of downregulation for IGLV2-14 (2.92–11.25), and CAMP (2.9–9.3) (Fig. 3a). In multivariable analyses incorporating time as a covariate, expression trends of five of the seven signature proteins (IGKV3, IGLV2-18, VL_4, VL_7, and FN1) tended to increase ($p < 0.05$), whereas IGLV2-14 and CAMP levels decreased ($p < 0.05$; Fig. 3b).

To evaluate the robustness of patient stratification over time, hierarchical clustering was performed across all timepoints. Patients consistently segregated into two major clusters corresponding to high and low tumour HS, independent of treatment timepoint (Fig. 3c). These results suggest that the candidate plasma hypoxia signature reliably differentiates patients with high intratumoral hypoxia throughout radiotherapy.



data of the immunoglobulin-related genes was not available. Ten-year OS was used as endpoint. Individually, increased CAMP expression was associated with better prognosis in the TCGA-BLCA (Fig. S4a, c) but not in the BC2001 (Fig. S4b, c) cohort. *FN1* expression was previously associated with poorer prognosis in the TCGA-BLCA and the BC2001 cohorts, but

Fig. 1 Differences in plasma protein composition in bladder cancer patients undergoing radiotherapy (RT) who have high versus low tumour hypoxia scores (HS). **a** A total of 115 differentially abundant proteins (DAPs) with a fold change ≥ 1.5 or ≤ -1.5 and $p < 0.05$ were identified across five timepoints: before RT, and at weeks 1, 2, 3, and end of RT. Each row corresponds to one protein. The top-ranking proteins have been labelled. Overall protein abundance and fold changes decreased during RT. Fifteen (red labels) of the proteins showed strong linear trends ($r > 0.7$ or $r < -0.7$), 37 (blue labels) had non-linear changes or stable expression ($r -0.2$ to 0.2). **b** Enrichment analysis based on the identified DAPs in the different time points revealed consistent involvement of humoral response pathways under hypoxia. **c** Enrichment network analysis highlights that immune-related proteins are most affected during and after radiotherapy. ECM-related pathways were most enriched before RT, while humoral response pathways dominated during and after treatment. **d** Proteins with high correlation values ($r > 0.7$ or $r < -0.7$) were enriched in cell differentiation pathways, whereas those with low correlation ($r -0.2$ to 0.2) were linked to coagulation. **e** Enrichment network shows association of low and high correlation proteins to humoral response pathways, with proteins associated with the fibrin clot pathways linking with humoral response. Correlation values are calculated using Spearman correlation.

showed no evidence of predictive capacity for CON benefit [34].

As our cohort was mainly composed of male patients, we evaluated whether sex could induce expression differences within the candidate genes (FN1, CAMP). We found no differences at RNA expression levels between male and female patients at the TCGA-BLCA cohort (Fig. S5a–c). For FN1, no sex differences at protein expression levels were found at the TCGA-BLCA cohort (Fig. S5d). Similarly, no differences in RNA expression were found across male and female at the BC2001 (Fig. S5e–g) and the BCON (Fig. S5h–j) cohorts.

To address RNA expression as surrogate for protein expression of the two candidate genes (FN1, CAMP), we correlated RNA and protein expression where available. FN1 RNA and protein expression showed high significant correlation across the TCGA-BLCA ($r = 0.7$, $p < 0.00001$; Fig. S6a), TCGA Pan cancer ($r = 0.67$, $p < 0.00001$; Fig. S6b) and CPTAC ($r = 0.683$, $p < 0.00001$; Fig. S6c) cohorts. CAMP RNA and protein expression showed a small but significant correlation in CPTAC cohorts ($r = 0.19$, $p < 0.00001$, Fig. S6d). CAMP protein expression was not available for the TCGA cohorts.

We then evaluated both genes (FN1, CAMP) as a candidate hypoxia signature. In the TCGA-BLCA cohort (cystectomy), high HS patients had poorer OS ($p = 0.0013$; Fig. 4a). Similarly, poorer OS was seen for high HS patients in the BC2001 radiotherapy cohort ($p = 0.0069$; Fig. 4b). To further validate the signature, the BCON cohort was split into radiotherapy-only and radiotherapy with hypoxia-modifying treatment (CON) arms. The candidate signature associated to poorer prognosis in the radiotherapy ($p = 0.054$; Fig. 4c) but not in the CON ($p = 0.82$; Fig. 4d) arm. In Cox multivariate analysis including age, sex and stage as clinical variables, the candidate signature retained prognostic capacity for the TGCA (HR = 1.54; CI = 1.12–2.13; $p = 0.009$) (Fig. 4e) and the BC2001 (HR = 1.42; CI = 1.08–1.86; $p = 0.011$) (Fig. 4f) cohorts.

We further evaluated the interaction between the candidate signature and CON treatment to determine its capacity to predict benefit from hypoxia-modifying therapy using Cox multivariate analysis. High HS patients had better prognosis when undergoing CON treatment (HR = 0.60; CI = 0.34–1.06; $p = 0.079$) (Fig. 4g). High HS also had poorer prognosis when compared to low HS patients undergoing (HR = 0.54; CI = 0.30–0.96; $p = 0.034$) and not undergoing (HR = 0.56; CI = 0.32–0.98; $p = 0.041$) CON treatment, suggesting the signature has prognostic capacity in the BCON cohort (Fig. 4g). Finally, we conducted a meta-analysis across all three cohorts to validate the candidate signature. The signature strongly associated with poorer prognosis (HR = 1.48; CI = 1.23–1.78; $p < 0.001$) independently, of age, sex, stage, or treatment received (Fig. 4h).

As patients within the discovery cohort had radiotherapy with concurrent gemcitabine, we investigated if prognostic capacity is maintained independently of chemotherapy. In the BC2001 no chemotherapy arm, patients with high HS consistently associated with poorer prognosis ($p = 0.0057$; Fig. S7a), consistent with the result in the BCON radiotherapy arm (Fig. 4c). In multivariate

analysis, patients with high HS receiving chemotherapy (HR = 1.86, CI = 1.16–3.04, $p = 0.01$) and not receiving chemotherapy (HR = 1.50, CI = 1.08–2.06, $p = 0.013$) had poorer prognosis when compared to low HS patients not receiving chemotherapy (Fig. 7b), suggesting the signature is prognostic for patients undergoing radiotherapy with and without concurrent chemotherapy.

Hypoxia drives poor prognosis in solid malignancies [2]. Hence, we evaluated the signature's prognostic capacity at pan-cancer level. At the TCGA Pan cancer cohort, high FN1 RNA expression associated with poorer prognosis ($p < 0.0001$; Fig. S8a), whilst high CAMP RNA expression associated with better prognosis ($p < 0.0001$; Fig. S8b). When evaluated together as signature, high HS associated with poor prognosis ($p < 0.0001$; Fig. S8c), a finding validated in multivariate analysis (HR = 1.43; CI = 1.31–1.56; $p < 0.001$) independently of age, sex and stage (Fig. S8d).

Collectively, these results suggest that the candidate signature is prognostic and predicts benefit from hypoxia-modifying treatment, mimicking the Yang's signature results [7, 8, 26].

Hypoxia-associated plasma proteins are activated in sequential clusters

To explore the relationships and temporal dynamics of the 115 hypoxia-associated proteins, we performed a weighted correlation analysis, identifying five co-expression clusters (yellow, blue, brown, green, turquoise) (Fig. 5a). Each cluster significantly correlated ($p < 0.001$) with a specific timepoint: yellow (pre-RT), blue (week 1), brown (week 2), green (week 3), and turquoise (week 4), indicating stepwise activation during treatment (Fig. 5b). Cluster activation was generally accompanied by reduced co-expression in the other clusters. Specifically, broad co-expression decreases occurred before radiotherapy and during weeks 3 and 4; green and blue clusters declined in week 2, and the yellow cluster declined in week 1 (Fig. 5b).

Enrichment analysis revealed that all clusters were enriched for B cell response and immunoglobulin production, implicating humoral and complement response modulation. Additionally, the yellow cluster was enriched for ECM-related pathways, blue for fibrin coagulation, yellow and brown for metabolic processes, and turquoise for keratinocyte differentiation (Fig. 5c). These findings demonstrate that distinct hypoxia-associated protein clusters linked to humoral immunity are sequentially activated during radiotherapy, indicating that radiotherapy modulates temporal immune responses in patients with hypoxic tumours.

Radiotherapy is a main activation driver of hypoxia-associated plasma proteins

Given the sequential activation suggested by co-expression patterns, we evaluated the independent cluster dynamics for protein expression and fold-change increase in patients with intra-tumoral hypoxia. For all clusters, peak expression aligned with the timepoints showing high co-expression values (Fig. 6a,c,e,g,i). Although protein clusters showed a general fold-change increase in high HS patients, their temporal dynamics diverged from the protein cluster expression patterns, suggesting radiotherapy is the

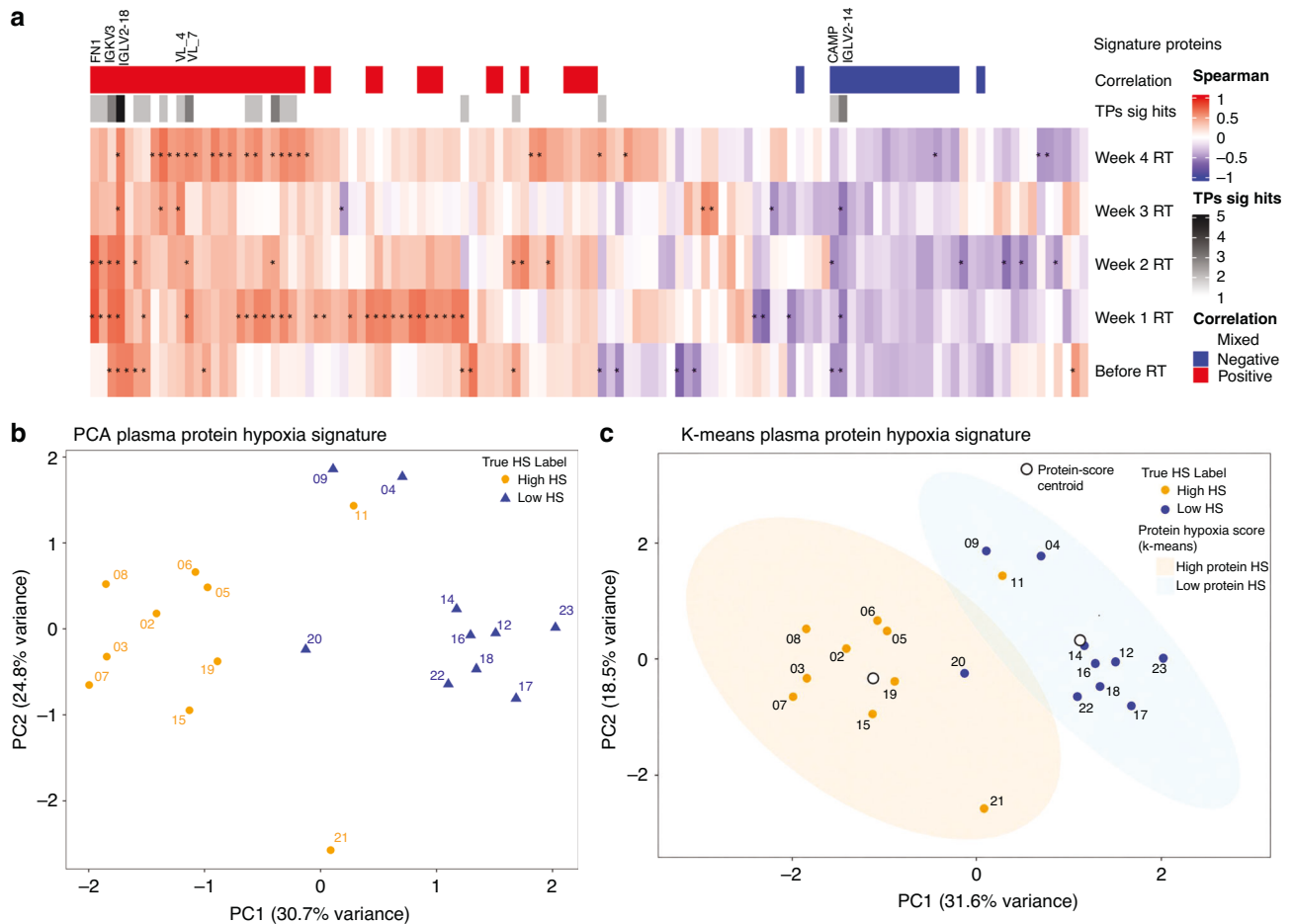


Fig. 2 Temporal correlation analysis identifies a candidate plasma protein hypoxia signature. **a** Heatmap illustrating correlation strength before and during treatment between hypoxia-associated plasma proteins and tumour hypoxia scores (HS). Seven proteins (IGLV2-18, IGLV3, IGLV2-14, VL_7, FN1, VL_4, CAMP) had significant correlations ($r > 0.4$ or $r < -0.4$; $p < 0.05$) at a minimum of two timepoints and demonstrated consistent correlation direction across all timepoints, being selected as a candidate signature. **b** Principal component analysis (PCA) and (c) K-means clustering showed that the candidate plasma protein signature effectively segregates patients with high versus low HS. Spearman correlation was used to calculate correlation coefficients. Canberra distances were applied for both PCA and K-means clustering.

main driver of the activation of identified protein clusters (Fig. 6b,d,f,h,j).

Yellow cluster expression peaked before radiotherapy and declined thereafter, while fold-change rose steadily through weeks one to three (Fig. 6a, b). Blue cluster expression surged in week one, with sustained fold-change increases to week three (Fig. 6c, d). Brown cluster expression peaked in week two, though fold-change increased at all timepoints except that week (Fig. 6e, f). Green cluster expression remained high, peaking in week three with corresponding fold-change increases (Fig. 6g, h). Turquoise cluster expression peaked in week four, with increased fold-change throughout all timepoints (Fig. 6i, j).

These data suggest that radiotherapy primarily drove the timing of cluster activation, whereas tumour hypoxia primarily influenced the protein clusters' baseline abundance and the magnitude of changes, suggesting synergetic temporal modulation.

DISCUSSION

In this study, we identified 115 hypoxia-associated plasma proteins predominantly linked to humoral immune response pathways, with immunoglobulins and complement cascade proteins (e.g. C3) comprising ~45% of the proteins identified. From these, we identified seven plasma hypoxia-associated biomarker candidates, which stratified bladder cancer patients

according to tumour hypoxia both before and during radiotherapy. A two-gene signature version (CAMP, FN1) was retrospectively validated, being associated with poor prognosis at the RNA level in three independent bladder cancer cohorts (TCGA-BLCA, BC2001, BCON radiotherapy arm), and in meta-analysis. The two-gene signature also predicted benefit from hypoxia-modifying therapy, mimicking the performance of the validated Yang et al. signature [7, 8]. In addition, we identified five hypoxia-associated protein clusters that were sequentially activated during radiotherapy, providing insight into the temporal regulation of systemic hypoxia-associated humoral pathways alterations. To the best of our knowledge, this is the first study exploring the use of nanoparticles enrichment in the identification of plasma-associated hypoxia biomarkers in cancer, acting a proof-of-concept of the utility of the technique.

The identified plasma hypoxia-associated biomarker candidates comprised six immune-related proteins (IGKV3, IGLV2-18, VL_4, VL_7, IGLV2-14, CAMP) and one extracellular matrix (ECM) protein (FN1). This signature closely recapitulated patient stratification achieved by the validated Yang hypoxia signature [7, 8], despite being derived from plasma proteins rather than tumour transcripts. Importantly, the plasma signature stratified patients independently of treatment stage, with stable expression throughout radiotherapy, supporting its potential utility for longitudinal patient monitoring and real-time treatment

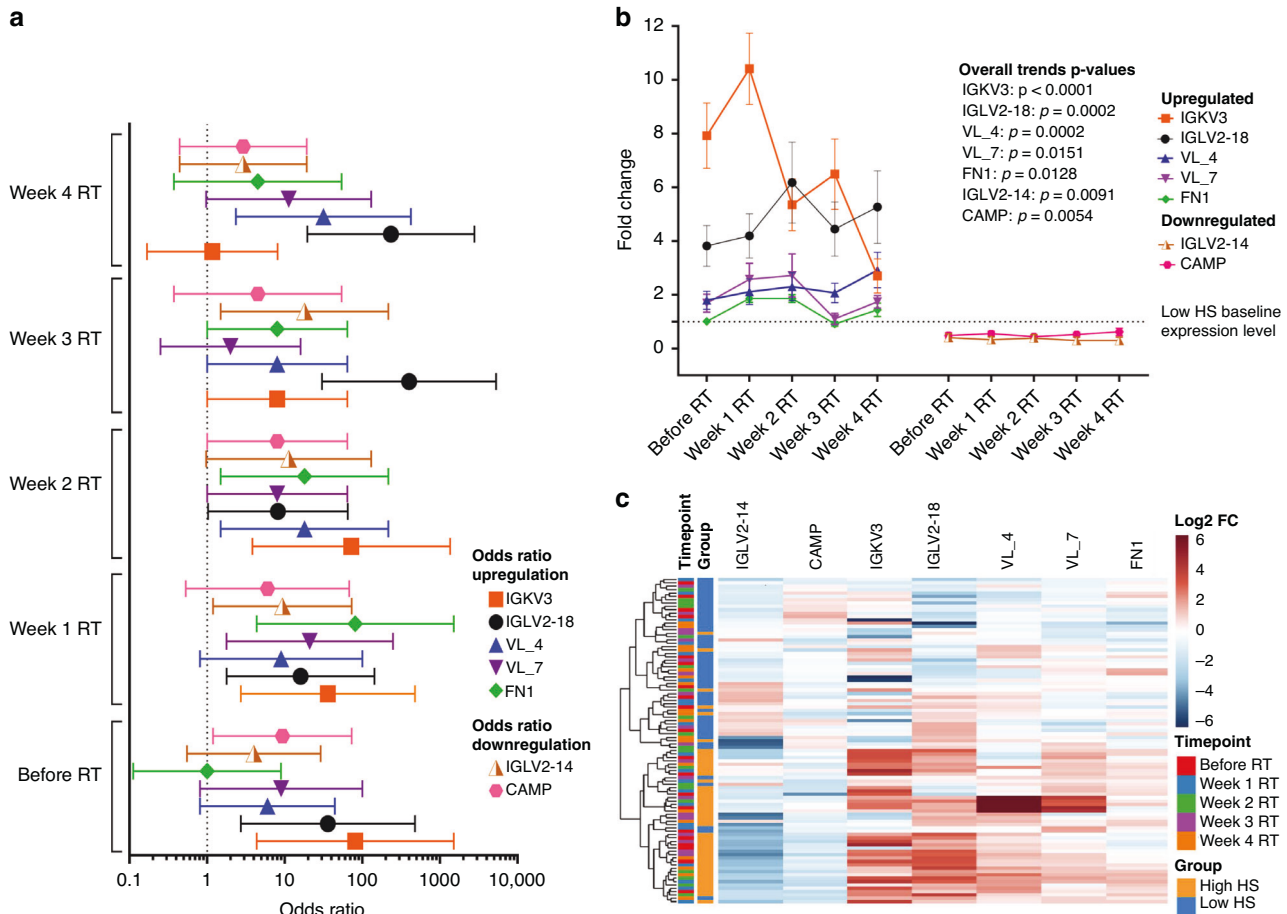


Fig. 3 Plasma levels of a candidate 7-protein hypoxia signature are consistently upregulated during radiotherapy and stratify patients with high tumour hypoxia scores (HS). **a** Forest plot highlighting increased odds ratio (OR) of IGLV2-18, IGKV3, IGLV2-14, VL_7, FN1, VL_4 and CAMP overexpression (fold change >1.5) for MIBC patients with high HS before and during treatment. **b** Trend plots showing significantly increased plasma protein signature levels for high HS vs low HS patients during radiotherapy. **c** Heatmap of log2 fold change (FC) expression for the candidate plasma signature for high vs low HS patients. The signature consistently stratified high HS patients before and during treatment. Fold changes were calculated using normalised total protein abundance values. Statistical significance was assessed using ANOVA with Sidak correction for multiple testing.

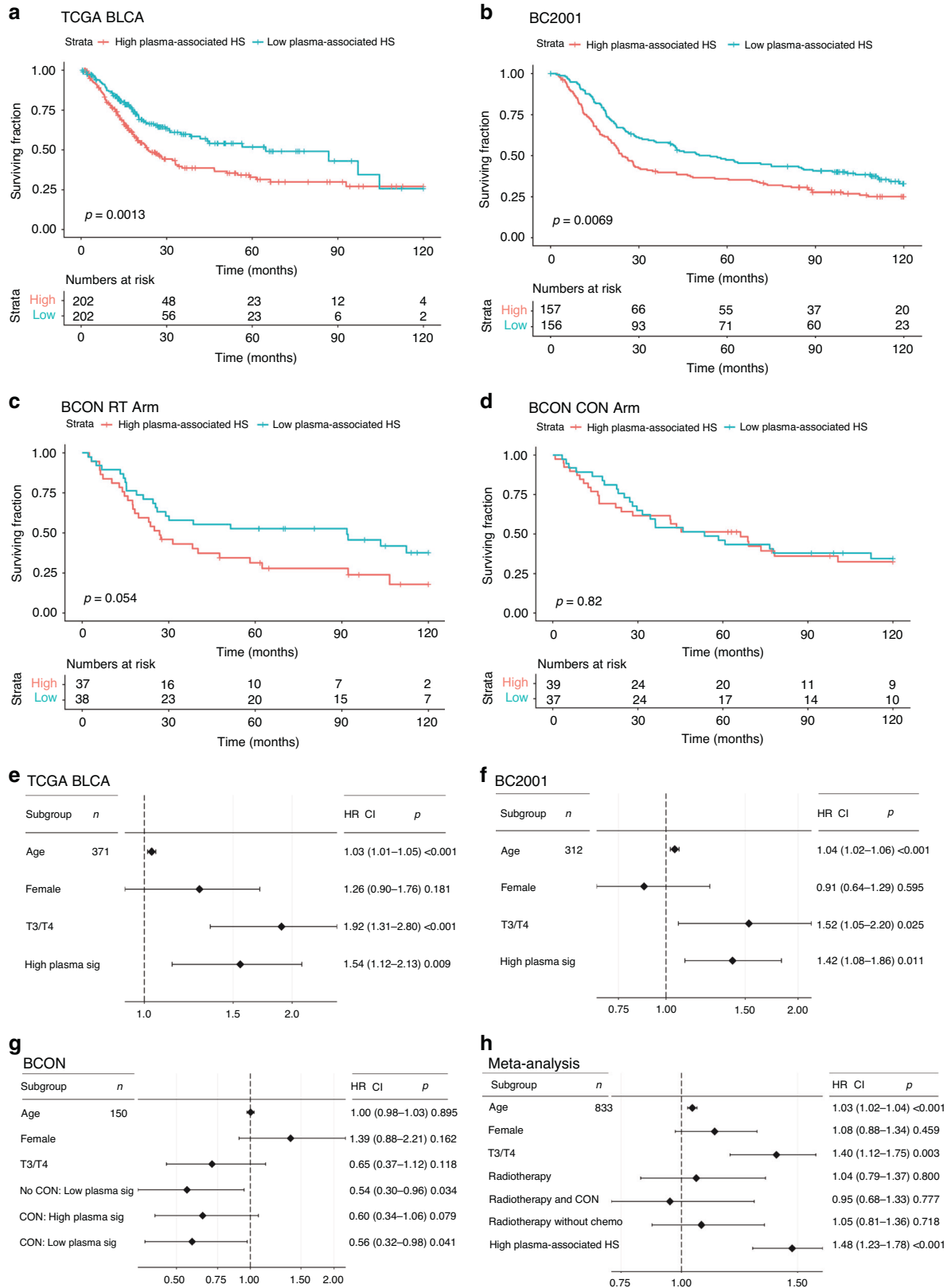
adaptation. While Rühle et al. employed imaging-based hypoxia stratification to identify circulating hypoxia biomarkers such as osteopontin and galectin-3 in head and neck cancer [15], osteopontin subsequently failed validation in a phase III trial [11] despite earlier promising results [10]. To date, no plasma hypoxia-associated signature has been proposed for bladder cancer.

Here, we demonstrate that a two-genes signature (CAMP, FN1), measurable in plasma at protein level, retains prognostic capacity at RNA level. The candidate signature also predicted benefit from CON treatment, being prognostic within the patients of the radiotherapy-only arm, mimicking Yang's signature stratification capacity [7, 8, 26]. The candidate signature was also prognostic at the TCGA Pan cancer cohort, supporting the well-established fact of hypoxia being a poor prognostic factor in cancer [2]. We showed strong correlation between FN1 expression at RNA and protein level in bladder cancer patients, suggesting RNA is a good surrogate for FN1 protein expression. Although limited, and without available data in bladder cancer, CAMP also showed significant correlation between RNA and protein expression. These results demonstrate that circulating hypoxia biomarkers are both detectable and measurable in plasma, representing a promising area for further investigation.

Five of the seven signature genes (IGKV3, IGLV2-18, VL_4, VL_7, IGLV2-14), all immunoglobulins, have no prior evidence of direct hypoxia regulation or hypoxia-response elements. CAMP has been

described as a hypoxia-responsive gene containing a functional HIF-1 binding site [35]. However, its regulation under hypoxia appears highly context- and time-dependent. Short-term hypoxia models support transient activation of CAMP-associated PKA/CREB signalling [36]. Other studies demonstrated hypoxia can activate CREB independently of CAMP through alternative mechanisms, including repression of PRKAR2B [37] and reduced PP1 expression, leading to CREB hyper-phosphorylation and subsequent proteasomal degradation [38]. There are also studies reporting CAMP expression is not increased under hypoxia [37, 39]. In a panel of six bladder cancer cell lines (J82, T24, UMUC3, HT1376, RT4, RT112) and across three different hypoxia conditions (1%, 0.2%, 0.1% O_2 , 24 h exposure) we found no evidence of CAMP RNA expression being increased under hypoxia [40]. To our knowledge, no previous reports associated hypoxia with decreased CAMP expression at protein or RNA level.

Most mechanistic studies assessing CAMP regulation have been limited to acute hypoxia (≤ 24 h), which may capture early short-time adaptive rather than sustained responses. Prolonged hypoxia induces dynamic fluctuations in CREB activity [41]. These fluctuations promotes a shift from HIF-1- to HIF-2 signalling [42], potentially disengaging HIF-1-dependent CAMP transcription. In tumours, where hypoxia is chronic and spatially heterogeneous, these long-term adaptations may favour CREB activation through CAMP-independent pathways while



simultaneously suppressing CAMP expression. These mechanisms provide a biologically plausible explanation for the consistently reduced CAMP levels observed in hypoxic tumours, although definitive characterisation of these links will require

mechanistic validation in long-term hypoxia studies using in vivo models.

FN1 has been reported to be upregulated under hypoxia through modulation of ECM remodelling, promoting fibrosis,

Fig. 4 A candidate plasma-associated hypoxia signature is prognostic and predicts benefit from hypoxia-modifying treatment. Patients with high hypoxia scores (HS) exhibit poorer prognosis when stratified into “high” and “low” at the TCGA (a) and BC2001 (b) cohorts. High HS patients had poorer prognosis in the BCON radiotherapy arm (c) but no in the carbogen and nicotinamide (CON) arm (d). Cox multivariate analysis associates high HS with poorer prognosis in the TCGA (e) and the BC2001 (f) cohorts, independently of age, sex, and stage. Cox multivariate interaction analysis shows patients with high HS without CON treatment have poorer prognosis, with high HS patients having better prognosis when undergoing CON treatment (g). Meta-analysis (TCGA-BLCA, BC2001, BCON) confirms high HS patients have worse prognosis, independently of age, sex, stage and treatment received (h). Patients were dichotomised into “high” and “low” groups based on the median signature RNA expression. Univariate survival differences were evaluated using Kaplan–Meier analysis. Multivariate survival differences were estimated using Cox hazard-risk multivariate analysis.

tumour progression, and metastasis [9, 43, 44]. At the RNA level, it has recently been associated with a poor prognosis in bladder [34], breast [45], head and neck [46], gastric [47], thyroid [48], and colon [49] cancers. Two previous studies proposed FN1 as circulatory diagnosis biomarker for kidney [50] and thyroid [51] malignancies, highlighting its potential as a plasma-based marker. However, FN1 has not previously been described as a circulating hypoxia-associated biomarker, nor have its temporal fluctuations during radiotherapy been investigated, both of which represent novel contributions of this study.

From a biological perspective, hypoxia has been extensively linked to tumour immune suppression [52, 53] and can directly regulate humoral immune responses. Burrows et al. showed *in vivo* that HIF-1 directly modulates the immunoglobulin heavy chain editing mechanism, affecting B cell maturation, reducing B cell levels, and inducing different phenotypes depending on the duration of HIF-1 activity [54]. *In vitro*, Abbott et al. demonstrated that hypoxia promotes GL-7⁺ activated B cells, inducing an immunoglobulin production switch (e.g. IgM to IgG1) [55]. Firmino et al. also showed that hypoxia induces Immunoglobulin class-switching, leading to impaired humoral immune response in breast cancer models [56]. Our findings extend these observations by providing the first clinical plasma-level evidence of hypoxia-associated immunoglobulin modulation. The increased activation of specific B-cell subtypes described by Abbott et al. provides mechanistic context for the elevated immunoglobulin levels observed in patients with hypoxic tumours [55]. In addition, hypoxia alters the phenotype of other immune populations, indirectly shaping B-cell behaviour [55]. Cao et al. provide supporting evidence, demonstrating across five bladder cancer cohorts ($n = 1343$) that hypoxia affects myeloid infiltration and antigen presentation, influencing humoral immune responses [57]. In bladder cancer, Smith et al. showed that hypoxia promotes immunosuppression and increased myeloid cell infiltration, which is associated with poor prognosis [58]. Collectively, these findings suggest that hypoxia modulates innate and humoral immunity synergistically, contributing to tumour immune evasion.

We further observed that ECM-related pathways were uniquely enriched in patients with high HS before radiotherapy. Hypoxia-induced ECM remodelling promotes the release of immune-regulatory proteins and enhances immune cell recruitment and polarisation [9, 43, 44]. We have previously shown at the pan-cancer level that hypoxia-driven ECM remodelling is linked to immune pathway activation [44]. Lipoprotein and coagulation pathways were also affected and activated up to the second radiotherapy week. APO activity is also associated with hypoxia signalling, promoting immune suppression and inflammation [59]. More recently, the fibrin coagulation cascade was linked to increased expression of immune checkpoint inhibitors (e.g. PD-1/PD-L1) in circulating tumour cells, promoting immune evasion and metastasis [60, 61]. Our data shows strong interactions among ECM, lipidic and fibrin-associated proteins, with TF and ITIH4 identified as regulators of the complement cascade through the C3 protein. ITIH4 has been reported to regulate complement activation through protease inhibition [61], while TF has long been known to induce C3 secretion and inflammatory responses [62].

The immunoglobulin switching observed may arise from synergetic alterations in ECM, lipoprotein, and coagulation pathways during early treatment, which are then maintained during later radiotherapy stages. This suggests a potential therapeutic intervention window in hypoxic tumours, with TF and ITIH4 representing candidate targets, although mechanistic validation using knock-out and *in vivo* approaches is required. Hypoxia-induced ECM alterations and irradiation-induced ECM changes have both been linked to immune modulation through cytokine and matrix remodelling [34, 44], supporting this model. However, mechanistic validation using knock-out approaches and *in vivo* models is required to confirm the findings.

Finally, the sequential activation of five distinct hypoxia-associated protein clusters indicates that radiotherapy exerts strong temporal control over systemic hypoxia-associated signalling. While radiotherapy appeared to drive the timing of cluster activation, hypoxia elevated the overall baseline expression of these clusters up to the third week of treatment, after which overall expression declined. Radiotherapy is known to modulate humoral immune responses and can activate both anti-tumour and immunosuppressive pathways [63]. Specifically, radiotherapy promotes the activation of hypoxia-associated factors such as TGF- β , CXCL12, and VEGF through tumour re-oxygenation [9]. Re-oxygenation generates reactive oxygen species that promote HIF-1 recruitment and activation, a protective mechanism supporting vascular integrity and cell survival [64]. As tumour re-oxygenation occurs rapidly during radiotherapy [65], hypoxia signalling may diminish during later treatment stages, consistent with the reduced protein levels observed. Our findings expand on this knowledge, providing a proteomic map of systemic changes modulated by the interplay of hypoxia and radiotherapy. However, these complex dynamics and synergies require further investigation. Future circulatory biomarker studies should also consider these synergies, as they can heavily affect biomarker discovery and validation.

A limitation of the study the lack of direct validation of the identified biomarkers at plasma level. The small discovery cohort ($n = 23$ patients) does not provide enough statistical power to establish direct associations of the signature with outcomes. Retrospective cohorts (e.g. TCGA) have limited protein expression data. The complexity of linking immunoglobulin protein expression to RNA changes in corresponding genomic regions further impairs retrospective validation. Consequently, a signature of only two candidates (FN1, CAMP) could be retrospectively validated at RNA level. Addressing this limitation, we showed significant correlation for FN1 and CAMP between protein and RNA expression levels. However, CAMP correlation was limited, with no protein expression data available for bladder. Prospective validation at protein level is therefore required. Future clinical studies should aim to generate protein plasma expression datasets, allowing for retrospective validation of protein signatures.

The discovery cohort included two patients with small cell bladder carcinoma, a rare histological subtype accounting for <1% of bladder cancer patients [66]. These patients were retained to maximise statistical power in the proteomics exploratory analysis.

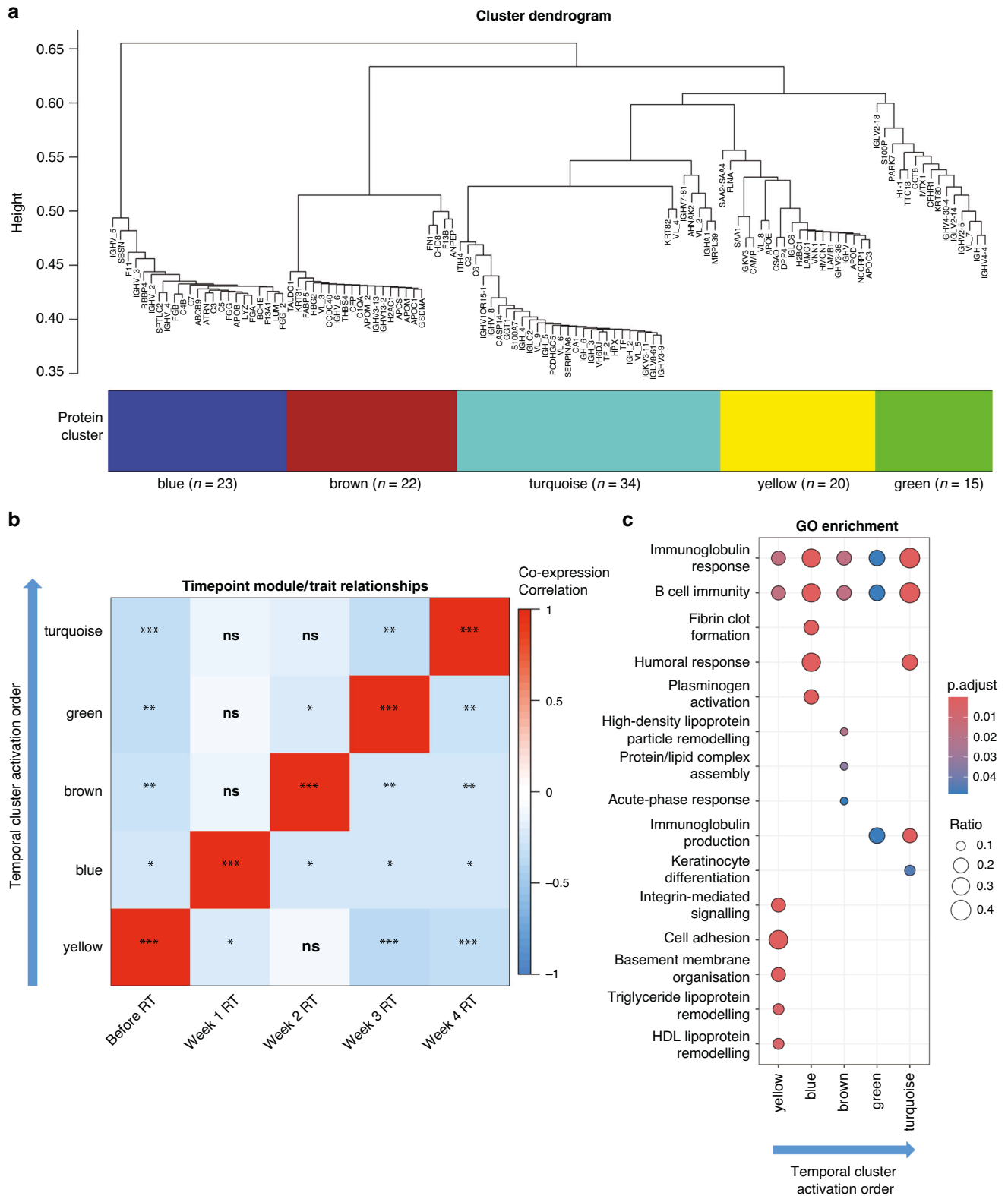
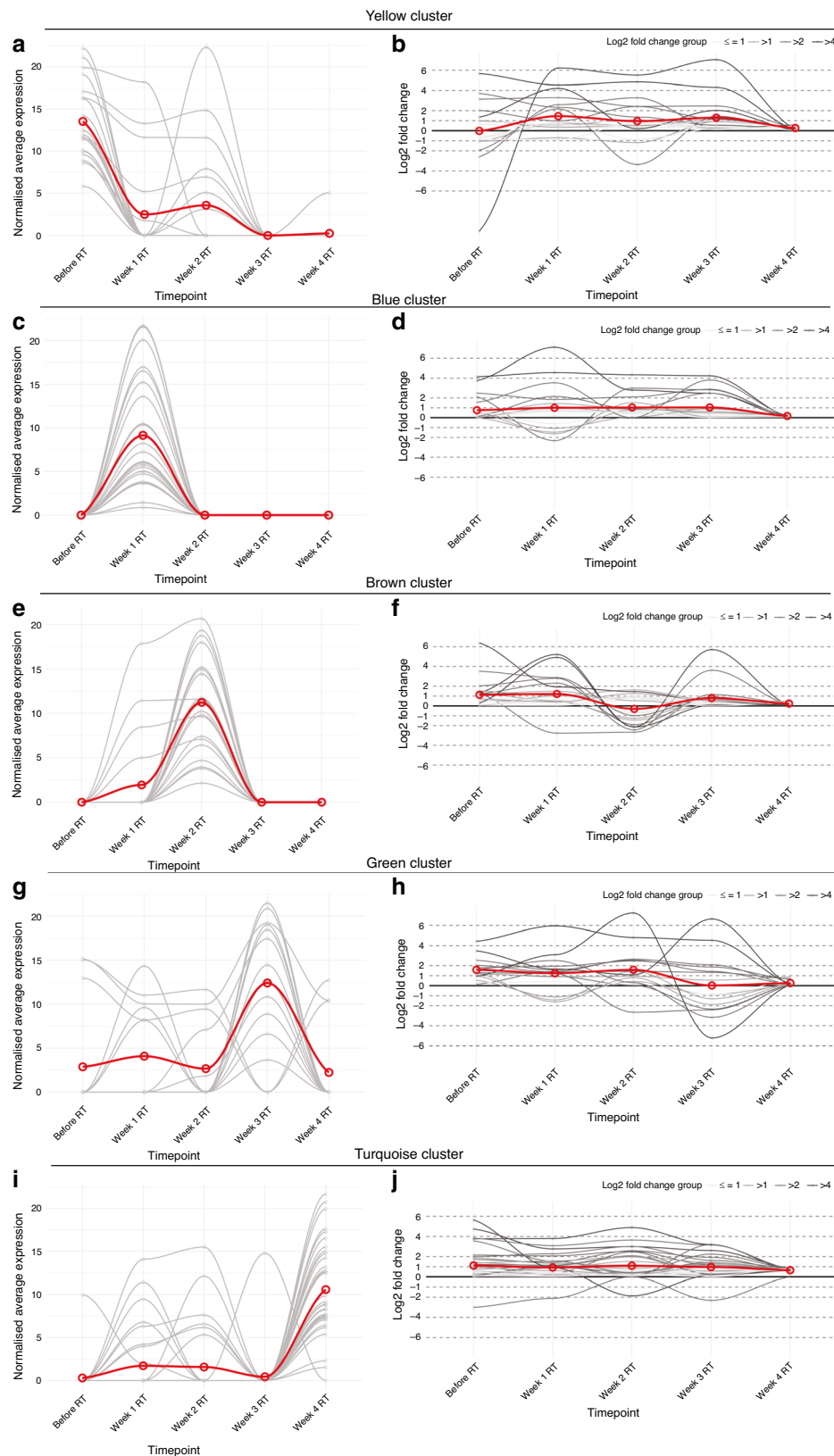


Fig. 5 Hypoxia-associated proteins form five clusters sequentially activated before and during radiotherapy (RT). **a** Identified 115 hypoxia-associated proteins from five different clusters (yellow, blue, brown, green, turquoise) based on their normalised weekly expression level before and during radiotherapy. **b** Each cluster has very high positive co-expression levels ($r > 0.9$, $p < 0.001$) at different timepoints, indicating overall increases of the expression of the proteins within each cluster at a specific time-point, suggesting clusters sequentially activate weekly before and during treatment. **c** Humoral response pathways are consistently enriched in each temporal cluster, with ECM-related pathways being enriched only in the yellow cluster. Clusters were established using quantile-normalised log2 protein expression levels. Correlation values are calculated using Pearson correlation. All patients received radiotherapy with concurrent gemcitabine treatment.



Their inclusion may contribute to additional biological heterogeneity, which should be evaluated in future studies. Regardless, the candidates identified within our study showed prognostic capacity in meta-analysis across three distinct bladder cancer

cohorts and at the TCGA Pan cancer cohort, suggesting our findings are yet translatable in a broader context.

All patients within the discovery cohort received Gemcitabine, a standard chemotherapeutic agent in bladder-preservation

Fig. 6 Radiotherapy (RT) and hypoxia drive distinct temporal protein expression patterns. **a** Yellow cluster proteins are expressed before and during the first two radiotherapy (RT) weeks and have increased baseline expression in high hypoxia-score (HS) patients during RT weeks 1–3 (**b**). **c** Blue cluster proteins activate in RT week 1, **d** with baseline expression levels being increased in high HS patients across all time-points, except for RT week 4. **e** Brown cluster proteins expression peaks in RT week 2, (**f**) with high HS patients having increased expression before RT, and in RT weeks 1, and 3. **g** Green cluster proteins have the highest baseline expression, which peaks in RT week 3. **h** Green cluster proteins expression is increased in high HS patients up to the RT week 2. **i** Turquoise protein expression levels peak during RT week 4, **j** with their baseline expression consistently increased across all timepoints in patients with high HS. Temporal protein expression graphs (**a**, **c**, **e**, **g**, **i**) represent the protein expression levels for all patients after quantile/total protein log2 normalisation. Temporal fold change graphs represent the log2-scaled protein ratio of the normalised protein abundance of low vs high HS patients for each time point. All patients received radiotherapy with concurrent gemcitabine treatment.

radiotherapy with comparable outcomes to platinum-based approaches [67, 68]. Whilst there is evidence of gemcitabine as immunomodulatory agent, it is unclear whether it can enhance of impair antitumour immunity, with contradictory evidence within the literature [69]. There is general evidence of chemotherapeutic agents modulating immunity. For example, BC2001 patients were treated with 5-fluorouracil [24], which targets myeloid immunosuppressive cells and enhances immunity [70]. Of the patients receiving chemotherapy within the retrospective validation cohorts, no patient underwent gemcitabine treatment. Furthermore, our proposed signature was prognostic in retrospective cohorts with and without chemotherapy treatment, showing no significant interaction with chemotherapy in the BC2001, suggesting that the proposed signature is independent of chemotherapy. Future studies should still confirm whether distinct chemotherapeutic agents may alter the hypoxia-associated alterations reported in this study concurrently with radiotherapy.

An additional limitation is the low proportion of female patients in the discovery cohort. In bladder cancer, females have four times lower incidence than men and poorer outcomes [71]. Molecularly, females have higher expression of the X chromosome linked demethylation enzyme *KDM6A*, protecting against tumour initiation [72], with recent studies showing >400 genes being sex-biased in males vs female bladder cancer patients [73]. Within our study, we found no significant differences in gene expression between male and female for the retrospectively validated signature, which retained significance in multivariate analyses including sex as variable in the bladder cancer cohorts and in the TCGA Pan cancer cohort.

The small discovery cohort also limits the robustness of the co-expression clustering analysis. Although distinct hypoxia-associated protein clusters were identified, their biological interpretation should be considered exploratory and requires validation further validation through in vivo studies and/or at larger prospective cohorts.

CONCLUSION

Here we identified seven hypoxia associated biomarker candidates at plasma levels, of which we generated a two gene plasma-associated hypoxia signature. The candidate two gene signature is prognostic in meta-analysis and predicts benefit from hypoxia-modifying treatment. Our findings also demonstrate that hypoxia influences dynamic humoral immune response pathways during radiotherapy. Humoral response alterations in patients with hypoxic tumours was associated with extracellular matrix remodelling, lipoprotein metabolism, and coagulation pathways. These effects evolved throughout treatment, with a sequential activation of different protein clusters associated with those pathways during radiotherapy. Our study highlights the potential of nanoproteomics to identify hypoxia circulatory biomarkers and provides a foundation for non-invasive monitoring strategies to personalise therapies in bladder cancer, whilst highlighting dynamic immune alterations during radiotherapy associated to hypoxia.

DATA AVAILABILITY

The mass spectrometry proteomics data analysed in this study were previously deposited with the ProteomeXchange Consortium via the PRIDE partner repository under dataset identifier PXD071492 (<https://doi.org/10.6019/PXD071492>) [74]. The present study reports a distinct analysis of these existing data focusing on hypoxia-associated circulatory biomarkers for the bladder cancer patients of this previous study. No new mass spectrometry data were generated in this study.

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AUTHOR CONTRIBUTIONS

Conceptualisation: CGQ, HAM, MH, VB, CMW, AC. Methodology: CGQ, HAM, VB, MH, CMW, AC; Software: CGQ, HAM, VB; Validation: CGQ, HAM; Formal analysis: CGQ, HAM, VB, TL; Investigation: CGQ, HAM, JGA, VB, TL; Resources: TL, MH, NDJ, EH, RAH, NP, PH, CMW, AC; Data curation: CGQ, TL, KR; Visualisation: CGQ, HAM; Supervision: VB, AC, PH, MH, CMW; Project administration: MH, AC, CMW; Funding acquisition: MH, KR, CMW, AC; Writing (original draft): CGQ; Writing (review & editing): All authors.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVALS

The Manchester Cancer Research Centre (MCRC) Biobank Sample Access Committee reviewed and approved the ethics for this study. All samples were collected under the MCRC Biobank Research Tissue Bank ethical approval (study number 18CAWE03; Reference-18/NW/0092), with patient consent obtained in accordance with the Declaration of Helsinki. All described methods within the study were performed in accordance with relevant guidelines and regulations.

ADDITIONAL INFORMATION

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