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Humoral complementomics – exploration of noninvasive complement biomarkers as predictors of renal cancer progression

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ABSTRACT

Despite the progress of anti-cancer treatment, the prognosis of many patients with solid tumors is still dismal. Reliable noninvasive biomarkers are needed to predict patient survival and therapy response. Here, we propose a *Humoral Complementomics* approach: a work-up of assays to comprehensively evaluate complement proteins, activation fragments, and autoantibodies targeting complement proteins in plasma, which we correlated with the intratumoral complement activation, and/or local production, focusing on localized and metastatic clear cell renal cell carcinoma (ccRCC). In two prospective ccRCC cohorts, plasma C2, C5, Factor D and properdin were elevated compared to healthy controls, reflecting an inflammatory phenotype that correlated with plasma calprotectin levels but did not associate with CRP or with patient prognosis. Conversely, autoantibodies against the complement C3 and the reduced form of FH (a tumor neo-epitope reported in lung cancer) correlated with a favorable outcome. Our findings pointed to a specific group of patients with elevated plasma C4d and C1s-C1INH complexes, indicating the initiation of the classical pathway, along with elevated Ba and Bb, indicating alternative pathway activation. Boostrapped Lasso regularized Cox regression revealed that the most predictive complement biomarkers were elevated plasma C4d and Bb levels at the time of surgery, which correlated with poor prognosis. In conclusion, we propose *Humoral Complementomics* as an unbiased approach to study the global state of the complement system in any pathological plasma sample and disease context. Its implementation for ccRCC revealed that elevated C4d and Bb in plasma are promising prognostic biomarkers, correlating with shorter progression-free survival.

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Activation fragments; cancer; clear cell renal cell carcinoma; complement proteins; complement system; humoral complementomics; plasma

Introduction

Despite the tremendous progress in understanding molecular and cellular mechanisms driving cancer progression and the development of novel cancer therapies, there is still an unmet need to establish noninvasive biomarkers to predict patients' prognosis. Moreover, the intricate relationships between the immune response in the solid tumor and the circulating immune effectors are not fully understood. The complement system is an innate immune defense mechanism against pathogens and altered host cells.[1] Mirroring complement evasion strategies by some pathogens and "self" cells, "altered self" cancer cells can employ complement regulators to evade complement-mediated damage while allowing the pro-tumoral chronic inflammation.[2,3]

We previously identified a fraction of patients suffering from clear cell renal cell carcinoma (ccRCC) with poor prognosis, presenting elevated plasma levels of the complement activation fragment C4d and intense intratumoral C4d deposits on tumor cell surfaces,[4–6] but what is the overall complement profile of the patients and whether C4d is an optimal biomarker remains to be defined. These results echo previous findings in lung cancer and malignant pleural mesothelioma, where plasma C4d also correlated with poor prognosis.[7,8] Therefore, quantifying complement activation fragments in the plasma of cancer patients could reveal novel, noninvasive biomarkers to predict prognosis and potentially adapt the therapeutic strategy.

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Tumor cells from many types of cancer, including ccRCC, express complement proteins that can lead to the production of autoantibodies against modified forms of these proteins. Autoantibodies against neo-epitopes of the complement regulator Factor H (FH) secreted in a reduced form by lung tumors[9–11] were described in early-stage cancer patients who control the disease.[9] One such anti-FH antibody (GT103) was cloned from a patient, found to confer protection against tumor growth in a mouse model of lung cancer[10] and is now undergoing testing in a clinical trial for advanced lung cancer in combination with anti-PD1 (NCT05617313). Apart from FH, C3,[12] and FB[13,14] are also potential candidates as they are also secreted by the tumor cells. Interestingly, autoantibodies against both C3 and FB have been described in kidney diseases with complement deposits such as Lupus Nephritis[15,16] or C3 Glomerulopathy,[17,18] but they have not been investigated in kidney cancer.

Despite these findings, no data on an unbiased systematic exploration of the complement components, activation fragments, and anti-complement autoantibodies in the plasma of cancer patients and their correlation with the status of complement activation within the tumor is available. Such data are necessary to understand the mechanism(s) of complement activation within the tumors and to identify novel biomarkers for prognosis and treatment response.

Here, we introduce the concept of Humoral Complementomics–the simultaneous exploration of a large number of complement proteins, activation fragments, and autoantibodies against complement proteins in plasma samples of patients, using two well-characterized prospective cohorts of ccRCC. Autoantibodies against C3 and reduced FH correlated with a favorable outcome, while patients with elevated C4d and Bb had shorter progression-free survival, supporting the relevance of these biomarkers for predicting renal cancer prognosis.

Materials and methods

Patient cohort

Plasma-EDTA samples collected at the time of surgery from two prospective ccRCC cohorts of adult patients were included in this study: a discovery cohort "ExhauCRF" ($n = 25$, patients with clinical data available for 22) and a validation cohort "ImmPro" ($n = 98$ patients, with tumor specimens, progression and other clinical data for 49 of them). Histopathologic and clinical features such as Fuhrman grade, TNM stages, sex, age and cohort size are summarized in Supplementary table S1. Both cohorts have been described previously, and we correlated the new measurements here with the available results from tissue staining for C1q, C4, and FH published before.[4–6] As controls, 28 and 59 plasma-EDTA samples from healthy donors from the French Blood Bank (EFS) were used for ExhauCRF and Immpro, respectively. For the C4d measurements, we extended our previous ImmPro cohort[5] by adding 17 more patients (for a total of 75). Of note, from the 58 previously published patients with C4d measurements, for 8, no more plasma samples were available, and they were excluded from the analyses, as the remaining complement components could not be tested in these cases. The patients included in this study signed an informed consent form before inclusion. The research was approved (N° CEPAR 2014–001, CPP Ile-de-France II n°2016-07-08) by the medical ethics board and conducted according to the recommendations in the Helsinki Declaration.

Complement multiplex assays

We have selected the MicroVue Complement multiplex (QuidelOrtho) assay based on a good correlation of the measured values with the gold standard monoplex methods (nephelometry for C3 and C4; ELISA for FH, FI, sC5b-9), the capacity to distinguish patients with genetic deficiency of complement components and their accordance with the expected normal range in the literature.[19] Two panels were used: Panel 1: Ba, Bb, C3a, C5a, sC5b-9, C4a, Factor H, Factor I (8 plex kit HQ1M210421 16,400 01) and Panel 2: C1q, C2 Intact, C3 Intact, C4 Intact, C5 Intact, FD, FP (7 plex kit HQ2M210316 16,400 03). Samples were diluted 1/100th for Panel 1 and 1/1000th for panel 2 and processed as recommended by the manufacturer. Specific chemiluminescence signal for each protein was detected by Q-View Imager LS, and results were analyzed using Q-View™ Software. C4d was detected by ELISA (Complement C4d – RUO – Svar Life Science) as well as MASP2 (HK326–01, Hycult Biotech). C1s/C1INH and MASP1/C1INH complexes were measured using ELISA kits (HK399–01 Hycult Biotech and HK3001–01 Hycult Biotech, respectively).[20] C4a measurements were excluded from this study due to the significant variability in the dynamic range observed across healthy control measurements which has been previously reported.[19] Calprotectin levels were measured by ELISA (CalproLab ELISA (ALP) – RUO – Svar Life Science). CRP was measured by immunoturbidimetry (Beckman-Coulter).

Detection of anti-complement proteins autoantibodies

Preparation of reduced FH (RedFH)

Purified FH (1 mg/mL, Quidel, #A410) has been incubated with 10 mM TCEP (Tris(2-CarboxyEthyl) Phosphine, Hydrochloride [SigmaAldrich]) in Tris 0.4 M pH 7.4 in a ratio 1 :1 during 30 min at 20°C as previously described.[21] The reduction was verified by western blotting before use.

Multiplex particle-based assay

Five separate batches of magnetic microbeads (Bio-Plex Pro Magnetic COOH Beads and Related Reagents™, Bio-Rad) were respectively covalently coupled to 12 µg of five separate human complement proteins: C3 (ComplementTech, #A113C), FB (Quidel, #A408), C4bp (ComplementTech, #A109), FH (Quidel, #A410) and RedFH, by using the Bio-Plex™ amine coupling kit (Biorad, #171406001) according to the manufacturer's protocol. Plasma samples of each patient were screened for IgG, IgM and IgA isotypes of the five autoantibodies in a multiplex particle-based assay according to the protocol described below. Around 3000 of each four coated microbeads were deposited in wells of a 96 wells LUMINEX plate. After two washes with 200 µL with a buffer composed of

phosphate-buffered saline (PBS) Tween 0.02%, bovine serum albumin (BSA) 0.1% (TB/PBS), microbeads were saturated with 100 µL of PBS BSA 1% (B/PBS) for 30 minutes, under agitation, and at room temperature (RT). After two washes with TB/PBS, 50 µL of patients' samples pre-diluted to 1/50th in B/PBS, were deposited, and then were incubated for 30 minutes, under agitation at RT.

After three washes with TB/PBS, 100 µl of PE-coupled anti-human IgG, IgM, IgA antibodies (SouthernBiotech, 2020–09) pre-diluted at 1/100th in B/PBS, were deposited in the wells for 30 minutes, under agitation at RT. Finally, after three washes, microbeads in each well were resuspended with 100 µL of TB/PBS for 10 minutes, under agitation at RT.

The reading was done on the Bio-Plex 200 system^{TM} (Bio-Rad) and analyzed using the software Bio-Plex Manager 6.1.1. Results were expressed as mean fluorescence intensity (MFI).

Immunostaining

Formalin-fixed paraffin-embedded human ccRCC primary tumors were cut into 3 µm-tick sections and were stained for C1q, C4d, C5b9 and FH. The primary and secondary antibodies, as well as the antigen retrieval and staining conditions, are detailed in Supplementary Table S2. Antigen retrievals were carried out on PT-link (Dako) using EnVision Target Retrieval Solutions (Dako pH Low catalog no. K8005, pH High catalog no. K8004) with high or low pH depending on the antibody. Endogenous peroxidases and nonspecific staining were blocked with 3% H_2O_2 (Gifrer) and protein block (Dako, X0909). The staining was revealed by the 3-amino-9-ethylcarbazole substrate (Vector Laboratories, SK4200). The slides were then counterstained using hematoxylin (Dako, CS700), mounted with Glycergel (Dako, C0563), and scanned with NanoZoomer (Hamamatsu).

For the C1s/C4d/C1q/IgG-4plex, the FB/FH/C3d-3plex and the FB/C4d/C3d-3plex, staining was performed manually, using the tyramide system amplification from Akoya (NEL82001KT and FP1501001KT). The signal is revealed by the addition of OPAL molecule according to the manufacturer's protocol. Antibodies and their staining conditions (pH retrieval and dilution) are detailed in Supplementary Table S2. Nuclei are stained with 2 µg/mL of DAPI (ThermoFisher Technologies 62,248). The slides were then mounted with Prolong Glass antifade reagent (Invitrogen, P36980) and scanned with AxioScan (Zeiss).

Statistical analyses

Software R studio and Prism were used to generate statistical analyses.

Association between one categorical variable and one continuous variable was evaluated by the Kruskal–Wallis test, the correlation between two continuous variables by the Spearman test and the association between two categorical variables by a two-sided Fisher's exact test. The R software version 4.2.1 was used to generate heatmaps, correlation heatmaps, Kaplan–Meier curves and Cox regression analyses with the "pheatmap", "corrplot", and "survival" packages, respectively. A log-rank test was applied to examine the survival difference depending on the optimal cutoff calculated with the "survminer" package, p-values were adjusted using the Benjamini–Hochberg (BH) method for controlling False Discovery Rate (FDR) with a defined statistical significance at q-value <0.05. For ImmPro, auto-antibodies were measured in two separated batches and optimal cutoffs were calculated within its corresponding batch. Unsupervised hierarchical clustering was performed with a similarity between patients calculated based on the Manhattan distance and complete linkage. The difference between healthy donors and ccRCC patients with regard to complement plasma concentrations was assessed by the Mann–Whitney test. * p-value <.05, ** p-value <.01, *** p-value <.001, **** p-value <.0001.

Least Absolute Shrinkage and Selection Operator (LASSO) regularized multivariate Cox Regression was used to select key complement variables for patient progression-free survival. For this analysis, we removed features without events in at least one of the groups and set a VIF cutoff <10 to remove colinear features. LASSO is a strategy that employs a fitting procedure which sets the coefficients of irrelevant or redundant variables to zero by applying a penalty parameter that must be chosen.[22] The optimal penalty parameter was selected by 3-fold cross-validation and selecting the lambda with the smallest cross-validation error. To evaluate the stability of the selected variables, we fitted LASSO regularized Cox Regressions to 1,000 bootstrapped samples constructed from our Immpro dataset. Variables were then classified as "higher risk" if they had positive coefficients in more than 50% of the models selecting the variable or "lower risk" if they had negative coefficients. Finally, variable selection frequencies were reported along with their classification. LASSO regularized Cox Regressions were performed using the R package "glmnet"[23] and variable selected frequencies were plotted with the "ggplot2" package.

Results

Profiling of plasma complement proteins levels in ccRCC

We and others have previously identified that the expression of several complement genes in ccRCC associates with patient prognosis.[24,25] To gain a more comprehensive understanding on the overall status of the complement system in ccRCC patient's plasma, we explored the concentration of complement proteins in two different cohorts: a discovery cohort "ExhauCRF" (n = 25) and a validation cohort "ImmPro" (n = 75). We evidenced a significant increase in intact C2, intact C5, FD, and properdin in both ccRCC cohorts compared to healthy controls. However, this was not the case for intact C3, intact C4, FH, and FI which did not show significant differences in both cohorts (Figure 1a–s). Inflammatory marker calprotectin was reported to be elevated in serum from RCC patients.[26] We observed a robust positive correlation between complement proteins (except for MASP2, FH and FI), as well as a positive correlation between C1q, intact C2, intact C3, intact C4, intact C5 and calprotectin in both cohorts (Figure 1t–u). Of note, no such correlation was evidenced with CRP.

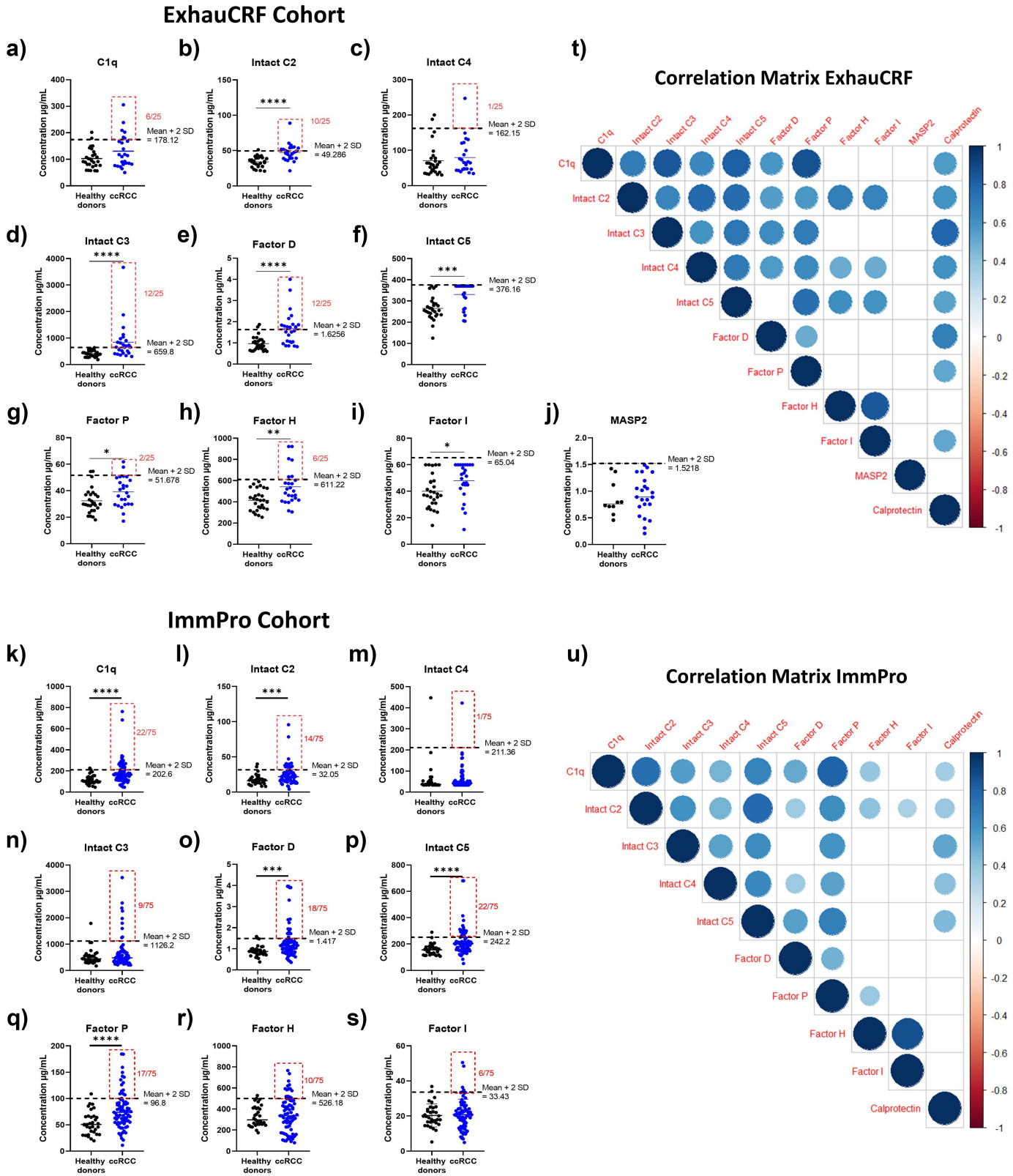


Figure 1. Humoral complementomics of ccRCC: plasma complement protein levels and association with inflammation. Plasma complement protein levels in (a–j) the ExhauCRF ccRCC discovery cohort ($n = 25$, except for MASP2 $n = 23$) vs healthy controls ($n = 28$, except MASP2 $n = 10$), and K-S) the ImmPro ccRCC validation cohort ($n = 75$) vs healthy controls ($n = 33$). Correlation of plasma calprotectin and complement levels among T) ExhauCRF ccRCC patients, and U) ImmPro ccRCC patients; circles represent the spearman correlation coefficient and only correlations with adjusted p-value >0,05 (Benjamini-Hochberg) are shown. For the comparison of plasma complement levels between ccRCC vs healthy: * p-value ≤0,05; ** p-value <0,01; *** < 0,001; **** p-value <0,0001. Mann–Whitney test. The cutoff of the normal range is calculated as mean ±2SD, patients above this limit are boxed by a red square, and the number of patients concerned is marked beside.

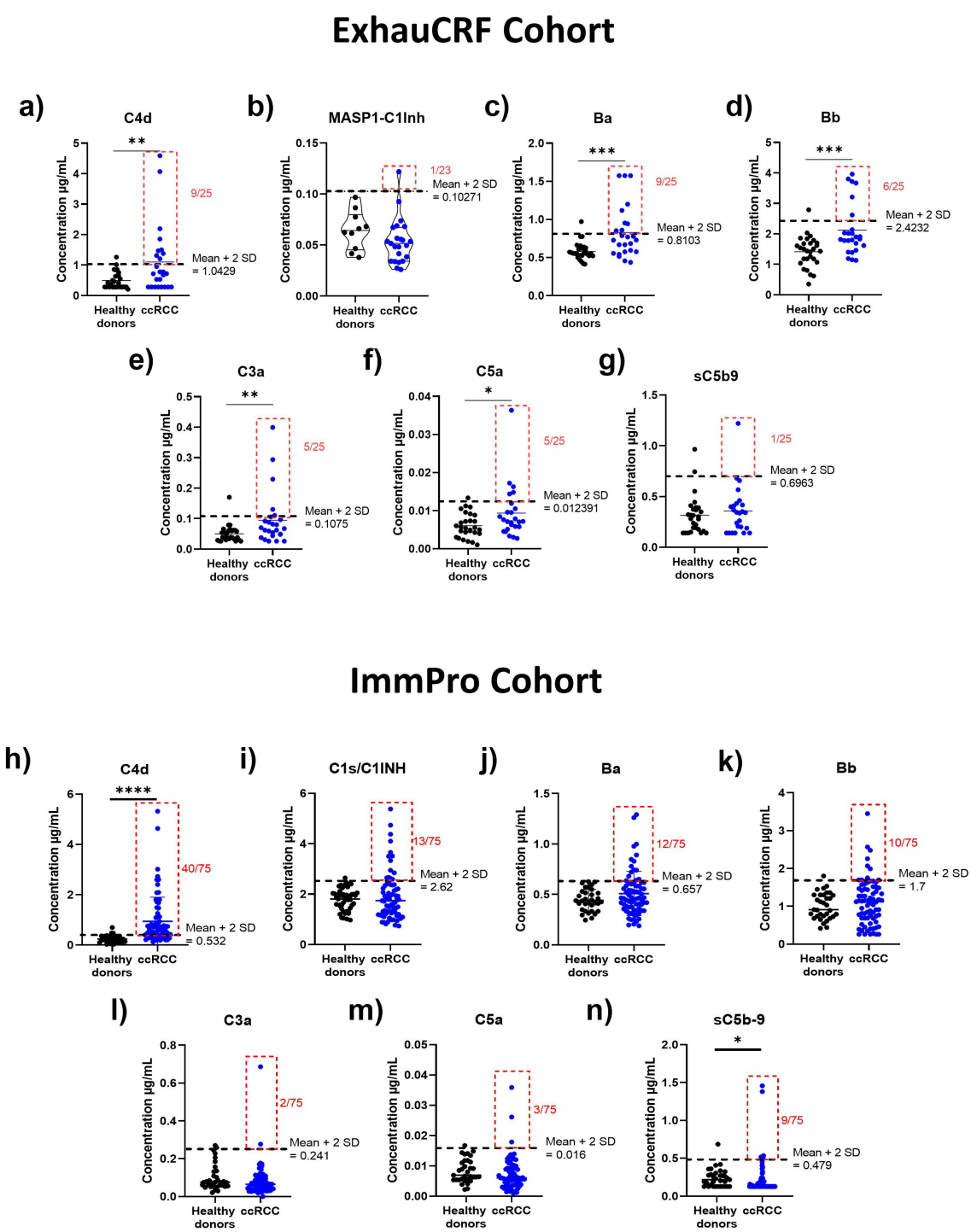


Figure 2. Humoral complementomics of ccRCC: plasma complement activation fragments. (a–g) comparison of plasma complement activation fragment levels in the ExhauCRF ccRCC discovery cohort ($n = 25$, except for MASP1/C1INH $n = 23$) vs healthy controls ($n = 28$, except MASP1/C1INH $n = 10$) (h–n) comparison of plasma complement activation fragment levels in the ImmPro ccRCC validation cohort ($n = 75$) vs healthy controls ($n = 33$). * p-value ≤0,05; ** p-value <0,01; *** < 0,001; **** p-value <0,0001. Mann–Whitney test. The cutoff of the normal range is calculated as mean ±2SD, patients above this limit are boxed by a red square, and the number of patients concerned is marked beside.

Profiling of plasma complement activation in ccRCC

Here, we observed a significant increase of Ba, Bb, C3a and C5a compared to normal controls in our discovery cohort ExhauCRF. Additionally, we observed elevated C4d levels without a concurrent increase in MASP1/C1INH, suggesting that not only the alternative pathway was active in ccRCC, but also the classical pathway (Figure 2a–g). In agreement with the ExhauCRF cohort, C4d was significantly increased when compared to healthy controls (Figure 2h) in the validation cohort ImmPro. The activation products C3a, C5a, and sC5b-9 were within the normal range (defined as mean ± 2SD of the healthy control group), while for the C1s/C1INH complexes, Ba and Bb, at least 10 samples exhibited values above the normal ranges, even if not significantly different from controls

(Figure 2i–n). sC5b-9 was significantly lower because, for most patients' samples, the concentration was below the detection limit, even though there were 9 at the limit or above the normal range (Figure 2n).

Classical and alternative pathway activation fragments and regulators correlated between each other in both cohorts, but no consistent associations between activation fragments and calprotectin were detected (Suplementary Figure S1). Classical pathway markers C1s/C1INH complexes and C4d correlated as did the alternative pathway markers Ba and Bb. No correlation was observed between C4d and MASP1-C1INH levels.

Interestingly, Fuhrman grade appeared to be associated with increased levels of the complement activation fragments Ba and Bb, which may explain the observed differences between cohorts. Patients of the ImmPro cohort with Fuhrman Grade 3 or above presented significantly higher levels of these fragments compared to controls (Suplementary Figure S2). Notably ExhauCRF cohort is composed of patients with higher Fuhrman grade than the ImmPro cohort (Suplementary Figure S2G). No statistically meaningful association between Fuhrman Grade and alternative pathway activation could be stablished in the ExhauCRF cohort due to the limited number of tumors with low grade.

Patterns of tumor tissue staining in ccRCC

In ccRCC, there are three types of staining for the complement proteins: positivity of the cells from the tumor microenvironment (immune cells, fibroblasts, endothelial cells), deposits at the membrane of the tumor cells and intracellular staining of the tumor cells concordant with local production or internalization.[27] These patterns are illustrated in Figure 3. C1q was present as expected in the infiltrating immune cells (macrophages) and as deposits (Figure 3a–b). Strikingly, we found in some patients the presence of intracellular C1q staining in tumor cells, which was rarer in our previous experience (Figure 3c). C4d-positive staining (recognizing C4d but also C4b and full-length C4) was present mostly as deposits and as intracellular patterns in tumor cells (Figure 3d–e). Furthermore, plasma C4d correlated to the intratumoral C4d+ deposits in ImmPro cohort for which data is available (Figure 3f).

Using multiplex imaging, we found colocalization of IgG, C1q, C1s, and C4 activation fragments on the tumor cells, suggesting classical complement pathway activation on IgG+ immune complexes (Figure 3g). There was also a colocalization between activation fragments of C3 and FB (Figure 3h) or C3 and FH (Figure 3i) from the alternative pathway. Occasionally the classical and alternative pathway components co-inside on the surface of the same cells, illustrating the action of the alternative pathway amplification loop (Figure 3j). Factor H had, similarly to C4 and C3, a membranous deposit pattern but also intracellular staining of the tumor cells (Figure 3k–l). Finally, we did not observe C5b-9 staining in ccRCC patients when compared to a lupus nephritis-positive control (Figure 3m–n). Together, these results indicate complement activation and regulation at the surface of ccRCC tumor cells.

Anti-complement proteins autoantibodies in ccRCC

Since anti-complement protein autoantibodies were evidenced in diseases with kidney complement deposits, and anti-reduced FH autoantibodies were described in lung cancer, we set up a detection system for exploration of anti-FH autoantibodies against the native or the reduced form, as well as against native C4bp, FB and C3, of IgG but also IgM and IgA isotypes.

In the ExhauCRF discovery cohort, we did not detect significant differences between ccRCC patients and normal controls, albeit few patients presented IgA anti-FB, anti-FH, anti-reduced FH and anti-C4bp above normal range (Suplementary Figure S3). In ImmPro cohort, we detected IgA and IgM against reduced FH, and IgA anti-C3, anti-FH and anti-FB, with at least 10 patients above the normal range (mean +2SD of values obtained from healthy donors), while the remaining antibodies did not show any significant differences (Figure 4). The difference in IgM anti-C3 was significant but likely irrelevant as patients had titers lower than the normal controls (Figure 4f). There was an increase for anti-FB and anti-FH IgG (Figure 4i–k) with 14 and 16 patients, respectively, exceeding the normal range without reaching significance. No correlations were observed between autoantibodies and calprotectin, but some correlations within each antibody isotype were detected (Supplementary Figure S4). Additionally, we did not observe correlations between complement features in plasma and tissues and the autoantibodies.

Association between humoral complement signatures and clinical features

One advantage of Humoral Complementomics is that it offers the benefit of enabling the unbiased exploration of connections between complement features and clinical parameters. In order to profile complement signatures among ccRCC patients, we performed unsupervised hierarchical clustering to interrogate the subjacent structure of the data. Within the ExhauCRF cohort, we identified a "complement-rich" subgroup, comprising approximately 60% of the patients, which displayed high levels of complement proteins and their activation fragments (Supplementary Figure S5A). Similarly, we also observed a subgroup of patients in the validation cohort characterized by elevated complement biomarker levels (Figure 5a). No evident subgroups were observed when patients were clustered according to the anti-complement antibodies (Supplementary Figure S6).

In ExhauCRF cohort, the "complement-rich" subgroup showed a trend toward higher TNM stages, Fuhrman Grade and shorter Progression-Free Survival (PFS) (Supplementary Figure S5B-D). Within the ImmPro cohort, patients from this subgroup displayed a trend toward shorter PFS, along with associations with TNM stage and Fuhrman Grade (Figure 5b–d).

Association between complement biomarkers and autoantibody titers with patients' prognosis

To further dissect the complement features associated with PFS, we stratified patients based on the optimal cutoff as

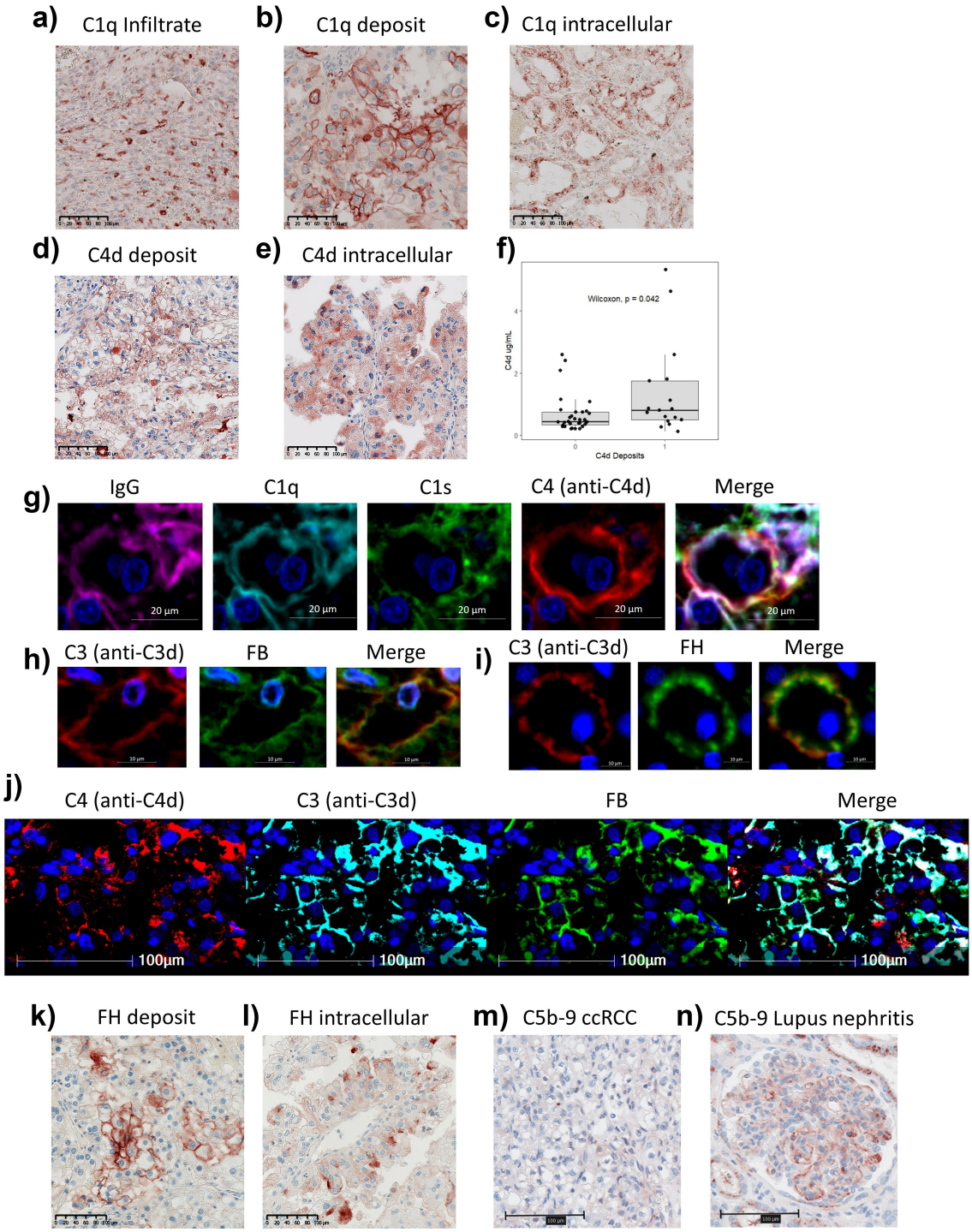


Figure 3. Complement proteins are found at the tumor cell membrane and intracellularly. immunohistochemistry against C1q highlighted staining (a) in immune cells, (b) at the tumor cell membrane, and (c) intracellularly in tumor cells. Immunohistochemistry against C4d highlighted staining (d) at the tumor cell membrane, and (e) intracellularly in tumor cells. (f) association between the concentration of C4d in plasma and the presence of C4d at the surface of tumor cells intratumorally determined by Wilcoxon rank sum test. (g) immunofluorescence multiplex showed colocalization of IgG, C1q, C1s, and C4d at the surface of tumor cells. Immunofluorescence multiplex showed co-localization of (h) C3 and FB, and (i) C3 and FH at the surface of tumor cells. (j) immunofluorescence multiplex showed colocalization of C4, C3 and FB. Immunohistochemistry against FH highlighted staining (k) at the tumor cell membrane, and (l) intracellularly in tumor cells. Immunohistochemistry against C5b9 in (m) ccRCC primary tumor, and (n) lupus nephritis glomerulus.

proposed by Reese et al.[15] Subsequently, we evaluated the importance of each complement variable in ccRCC prognosis by performing LASSO regularized Cox Regression. This technique allows for automatic selection of key variables and mitigates overfitting of the model to the data (detailed LASSO explanation can be found in the Methods section). The stability of the variable selection was evaluated by fitting LASSO regularized Cox Regressions to 1,000 bootstrapped samples constructed from the ImmPro cohort. We observed that Bb and C4d were the two complement variables influencing the most of the patient prognosis, as they were the most frequently selected with non-zero coefficients in more than 75% of the

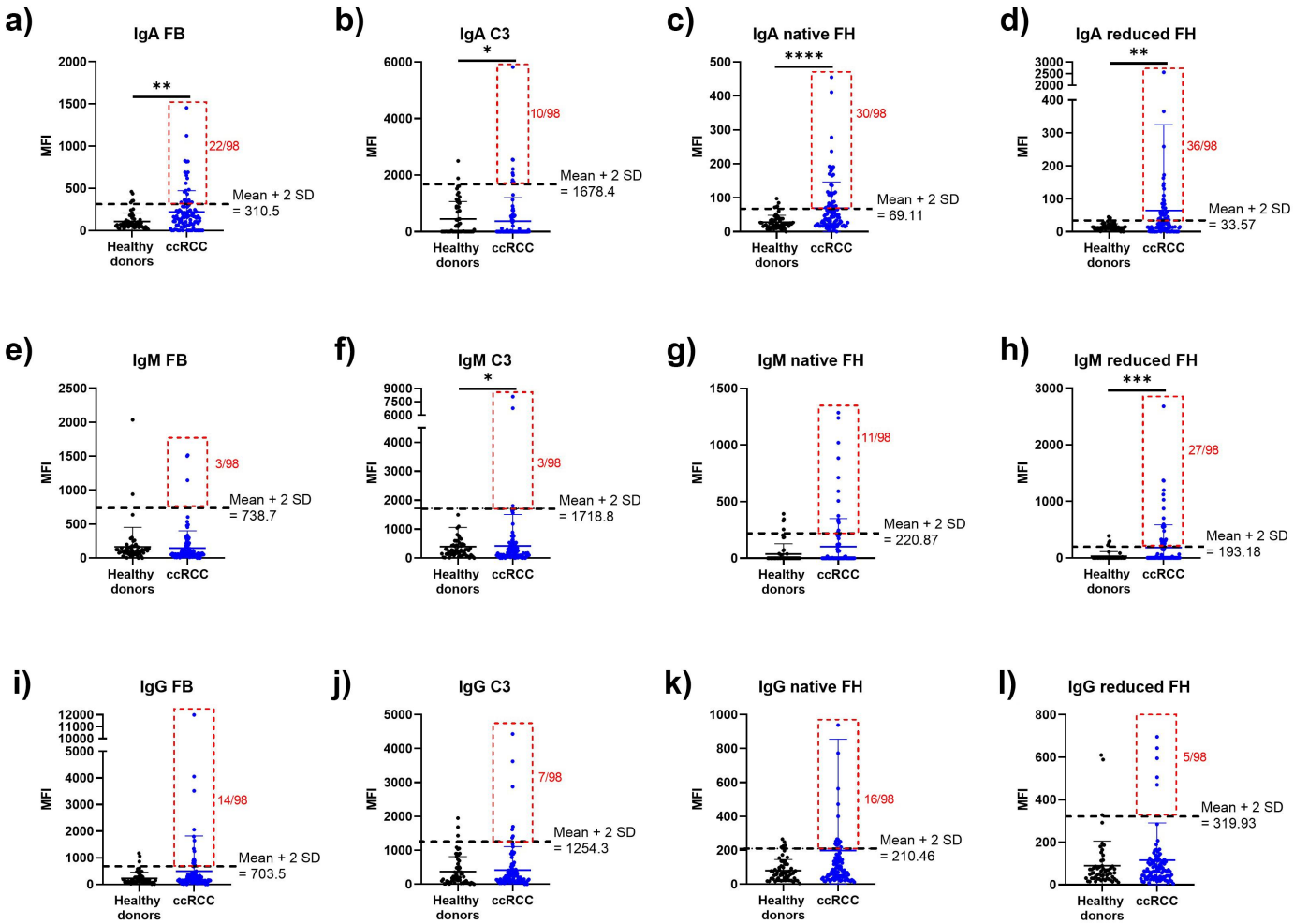


Figure 4. Humoral complementomics of ccRCC: autoantibody profiles in ImmPro cohort vs healthy controls. IgA autoantibodies against (a) FB, (b) C3, (c) native FH, and (d) reduced FH in ccRCC patients compared to healthy controls. IgM autoantibodies against (e) FB, (f) C3, (g) native FH, and (h) reduced FH. IgG autoantibodies against (i) FB, (j) C3, (k) native FH, and (l) reduced FH in ccRCC patients compared to healthy controls. 98 ccRCC patients and 59 healthy controls were analyzed. * p-value ≤0,05; ** p-value <0,01. Mann–Whitney test. The cutoff of the normal range is calculated as mean ±2SD.

models (Figure 6a). Notably, multivariate cox regression analysis of the two most selected Lasso features demonstrated that the prognosis value of C4d and Bb was significant even after adjustment for Fuhrman Grade, TNM stage, age, and sex (Figure 6b–c).

The selection of the C4d biomarker is consistent with our previous work where we reported an association between C4d activation fragments and PFS[5] (Figure 6d). Here, we also identified an association between C1s/C1INH complexes levels and PFS (Figure 6e), and a correlation between the two markers, indicating that C1s activation could be involved in C4d generation. Importantly, we also observed an association between alternative pathway activation fragments and poor patient prognosis in both discovery and validation cohorts (Figure 6f–g).

Patient stratification according to autoantibody levels revealed that high anti-reduced FH and anti-C3 IgG antibodies (which are not significantly higher compared to controls; likely because of positivity in the normal controls) were associated with a favorable outcome (Figure 6h–i). In cox analysis, high anti-reduced FH and anti-C3 IgG were associated with lack of tumor progression (Figure 6j) even after adjustment for age, sex, Fuhrman Grade and TNM stage (Figure 6k). We also observed a weak association between anti-C3 IgM levels and patient prognosis, which was not significant after adjustment by clinical parameters. In the ExhauCRF cohort, neither the anti-reduced FH or anti-C3 IgG autoantibodies correlated with PFS.

Discussion

Here, we present the Humoral Complementomics approach, measuring multiple-complement proteins, activation fragments, and autoantibodies targeting complement proteins in human plasma that we applied to renal cancer. Intact complement components were elevated in plasma correlating with calprotectin levels, but not with plasma CRP or the intratumoral complement production or deposition or with patients' survival. A subset of patients

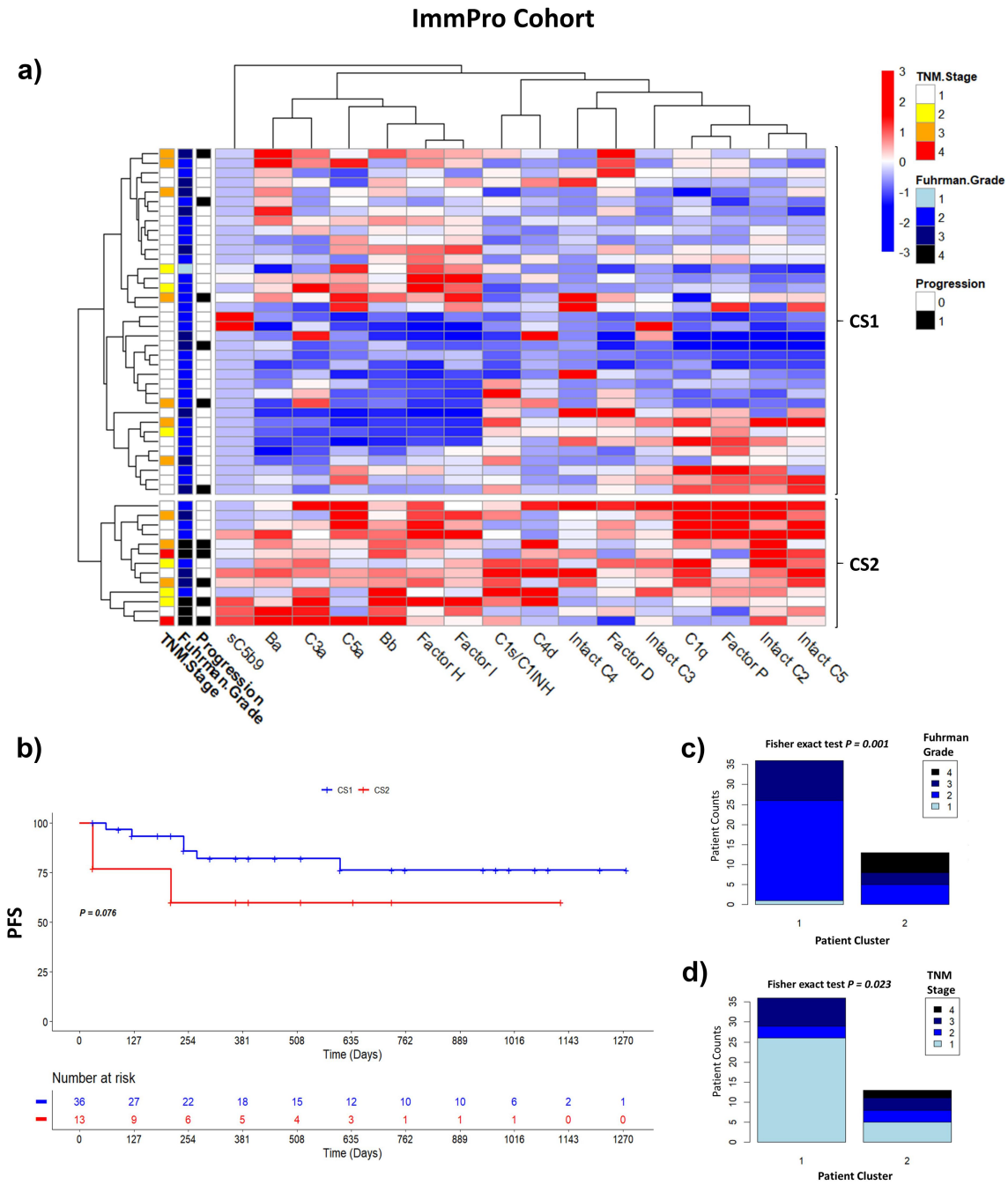
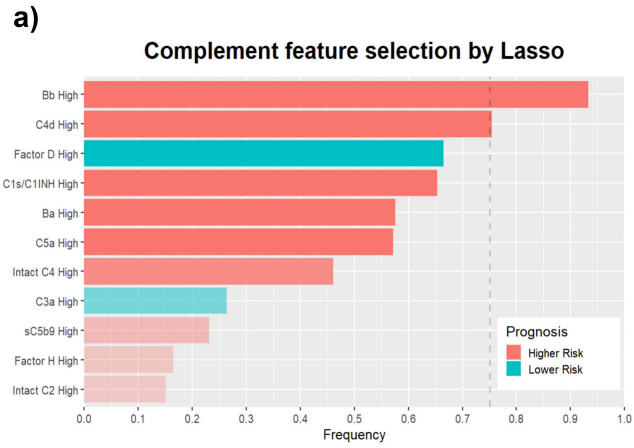


Figure 5. Humoral complementomics of ccRCC: complement profiles at the time of nephrectomy associate with clinical features in ccRCC. (a) heatmap of hierarchically clustered ccRCC patients ($n = 49$) across the different complement features in the ImmPro cohort. Each row represents an individual subject and each column represents a complement feature. Complement measurements are scaled and centered per feature and the range was truncated ±3 SD. Higher responses are indicated in red and lower responses are indicated in blue. (b) PFS Kaplan Meier curves of ImmPro patients stratified according to hierarchical clustering, log-rank test p-value is shown. Association of hierarchical clusters with (c) Fuhrman grade, and (d) TNM stage, p-values were calculated using a two-sided Fisher's exact test. PFS: progression-free survival; CS1: cluster 1; CS2: cluster 2.

presenting elevated plasma C4d or Bb had shorter progression-free survival. IgG autoantibodies against the recently discovered tumor neoantigen reduced FH and anti-C3 IgGs were elevated in a subgroup of ccRCC patients and correlated with a favorable outcome. These results support the pathogenic role of early complement activation and are in favor of therapeutic targeting of complement in renal cancer.

Omics approaches allow unbiased evaluation of the experimental system. Here, we combined different complement screening approaches to get closer to what we define as Humoral Complementomics – simultaneous exploration of a large number of complement proteins, activation fragments, and autoantibodies against complement proteins in patient plasma. For this purpose, we employed a novel validated multiplexed ELISA[19] for quantification of complement proteins and activation fragments, combined with the quantification of C4d neoepitope, well-validated in the literature and associated with poor prognosis in different cancers and in lupus nephritis,[7,28,29]



b)

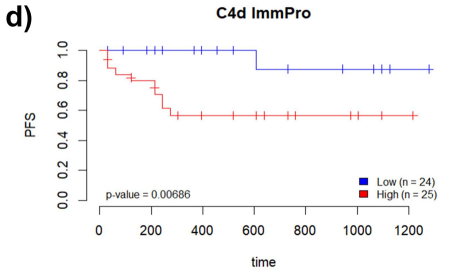
LASSO Selected C4d High vs Clinical Parameters

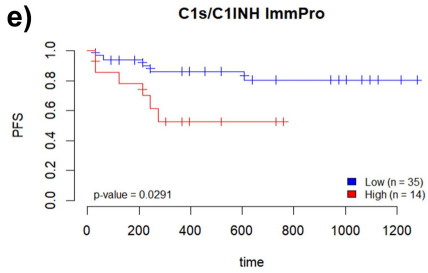
	Multivariate (C Index = 0.903 ± 0.036)	
Progression-free Survival	**HR(95% CI)**	***P***
Age	0.98 (0.93-1.03)	0.490
Sex Male vs. Female	0.81 (0.20-3.21)	0.763
Fuhrman Grade 3/4 vs. 1/2	4.42 (0.96-20.34)	0.056
TNM Stage III/IV vs. I/II	6.40 (1.41-29.14)	**0.016**
C4d High vs. Low	13.63 (1.59-116.92)	**0.017**

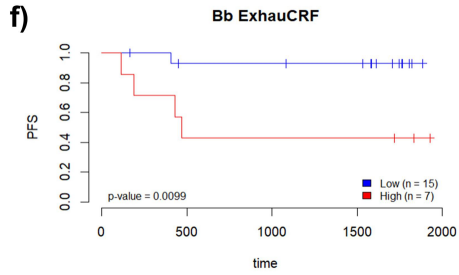
c)

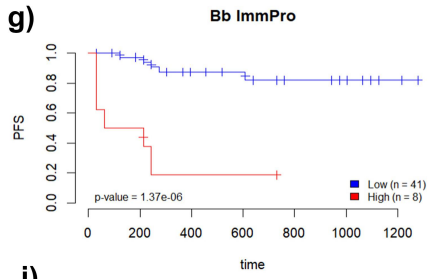
LASSO Selected Bb High vs Clinical Parameters

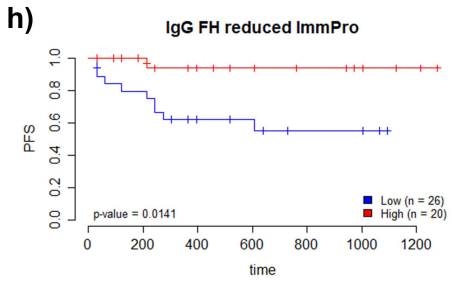
	Multivariate (C Index = 0.836 ± 0.08)	
Progression-free Survival	**HR(95% CI)**	***P***
Age	0.96 (0.91-1.02)	0.200
Sex Male vs. Female	1.82 (0.43-7.66)	0.415
Fuhrman Grade 3/4 vs. 1/2	2.32 (0.47-11.55)	0.303
TNM Stage III/IV vs. I/II	3.47 (0.73-16.47)	0.118
Bb High vs. Low	9.93 (2.06-47.99)	**0.004**

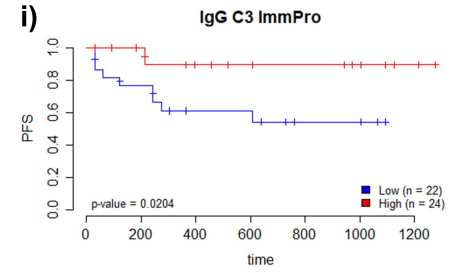












j)

Anti-complement Abs

	Univariate	
Progression-free Survival	**HR (95% CI)**	***P***
Sex Male vs. Female	1.52 (0.40-5.76)	0.536
Age	0.98 (0.93-1.03)	0.481
Fuhrman Grade 3/4 vs. 1/2	5.29 (1.40-20.03)	**0.014**
TNM Stage III/IV vs. I/II	10.12 (2.58-39.69)	**0.001**
IgG reduced FH High vs. Low	0.12 (0.02-0.92)	**0.041**
IgG C3 High vs. Low	0.20 (0.04-0.91)	**0.0384**
IgA reduced FH High vs. Low	0.41 (0.05-3.19)	0.393
IgA FH High vs. Low	1.03 (0.27-3.90)	0.960
IgG FB High vs. Low	2.42 (0.31-19.01)	0.400
IgA C3 High vs. Low	0.24 (0.05-1.19)	0.081
IgA FB High vs. Low	2.22 (0.47-10.44)	0.311
IgG FH High vs. Low	0.28 (0.06-1.30)	0.105
IgM C3 High vs. Low	0.25 (0.08-0.84)	**0.024**
IgM reduced FH High vs. Low	0.25 (0.05-1.25)	0.092
IgM FH High vs. Low	0.25 (0.05-1.25)	0.092

k)

Prognostic autoAbs vs Clinical Parameters

	Multivariate IgG FH Reduced (All clinical data)		Multivariate IgG C3 (All clinical data)	
Progression-free Survival	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age	0.99 (0.94-1.04)	0.609	0.98 (0.93-1.03)	0.459
Sex Male vs. Female	2.21 (0.56-8.81)	0.259	0.65 (0.13-3.25)	0.596
Fuhrman Grade 3/4 vs. 1/2	2.75 (0.64-11.85)	0.175	3.68 (0.81-16.83)	0.093
TNM Stage III/IV vs. I/II	6.96 (1.62-29.81)	**0.009**	6.60 (1.49-29.23)	**0.013**
IgG FH reduced High vs. Low	0.11 (0.01-0.90)	**0.040**	-	-
IgG C3 High vs. Low	-	-	0.16 (0.03-0.94)	**0.042**

Figure 6. Humoral complementomics of ccRCC: complement activation and IgG FH reduced levels in plasma predict patient prognosis in ccRCC. (a) frequency of selected complement features by multivariate Lasso regularized cox regressions fitted to 1000 bootstrapped samples constructed from the ImmPro cohort. The bar plot represents the relative number of times that each complement feature had non-zero coefficients in the models, red bars ("higher risk") indicate selection frequencies for variables with positive coefficients in more than 50% of the models selecting the variable. Blue bars are indicative of frequencies associated with variables having negative coefficients. (b–c) multivariate cox regression analysis of clinical features and Lasso selected features C4d and bb, respectively. PFS Kaplan – Meier curves according to (d) C4d, (e) C1S/C1-IHN plasma levels in the ImmPro validation cohort. PFS Kaplan – Meier curves according to bb plasma levels in the (f) ExhauCRF discovery cohort and (g) ImmPro validation cohort. PFS Kaplan – Meier curves according to (h) IgG FH reduced, and (i) IgG C3 plasma levels in the ImmPro validation cohort. (j) univariate and (k) multivariate cox regression analysis of clinical features and anti-complement autoantibody features in ImmPro cohort.

an ELISA for MASP2 and the recently described tests for quantification of the C1s/C1INH and the MASP1/C1INH complexes.[20,30] Furthermore, we developed an in-house method for simultaneous screening of plasma IgG, IgM, and IgA autoantibodies against complement proteins. In parallel 4-plex panels, detection of complement activation in tissues was performed on tumoral tissues. We applied this methodology to clear cell renal cell carcinoma, the most frequent renal cancer. These methods are transferable and applicable to any plasma sample.

Studies in lung cancer revealed the presence of IgG autoantibodies against reduced FH in patients with early-stage disease, capable to control the tumor progression.[9–11] Moreover, a therapeutic anti-FH antibody GT103 has been generated,[31] proven safe (NCT04314089) and now is being tested in a clinical trial in combination with anti-PD1 in patients with advanced lung cancer (NCT05617313). The prevalence of such antibodies in other cancers has not been studied. Here, IgG anti-reduced FH were found both in patients and in some healthy donors. Strikingly, at optimal cutoff, IgG against reduced FH correlated with favorable outcomes in the validation cohort, as reported for lung cancer, and IgG anti-reduced FH antibodies associated with protection by Cox analysis. The presence of anti-reduced FH IgG in healthy controls is consistent with previous observations[9] and suggests existence of protective autoimmunity against such tumor neoantigens. In addition to IgG anti-reduced FH, we also observed longer progression-free survival among patients with high levels of IgG targeting C3. Anti-C3 antibodies have been reported in kidney diseases involving pro-inflammatory complement activation,[15,16,32] but to our knowledge, they have not been previously studied in ccRCC. The functional consequences of these antibodies remain to be explored. IgA against different complement proteins was also elevated in ccRCC but did not correlate with progression-free survival. The specific IgA isotype was already described in a mouse model of prostate cancer to promote CD8 T cells exhaustion, through the expression of PD-L1 and the secretion of IL-10.[33,34] However, in ovarian and endometrial cancer, the immune protection is mediated in majority by IgA that sensitize tumor cells to cytolytic killing, notably through their transcytosis by tumor cells.[35,36] In ccRCC IgG and IgA, but not IgM concentrations in plasma from ccRCC patients were elevated[37] and intratumoral IgA in ccRCC was reported, especially in patients with paraneoplastic IgA nephropathy.[38] Further studies are needed to uncover the functional consequences of the autoantibodies recognizing complement C3 and the reduced FH tumor neoepitope in ccRCC and their implication in the immune surveillance. Nevertheless, our results position ccRCC as a cancer type for which the anti-reduced FH therapeutic antibody GT103 could be tested if it gives promising results in lung cancer, in patients expressing intratumoral FH.

A hallmark of ccRCC is the strong intratumoral complement expression and activation. Nevertheless, it is still unclear whether the tumoral complement deposits are due to intratumoral production or reflect the overexpression/overactivation of components of the systemic complement cascade. Using two well-characterized prospective cohorts of ccRCC, we revealed that complement components in plasma were elevated at the time of surgery. These were not the liver acute-phase reactants C3 and C4 but rather properdin, FD, C2 and C5 that were elevated. Similar elevation of C5 and FD was observed in a cohort of renal cancer patients treated with checkpoint inhibitors.[24] In both cohorts here, there was no clear association between the elevated intact complement proteins with survival, even though Reese et al. found such tendencies.[24] Plasma C1q, C4 and FH concentrations did not associate with the intracellular expression of C1q, C4 and FH, suggesting that the tumoral complement expression likely does not contribute to the plasma pool. Rather, the elevation of intact complement components could reflect a particular myeloid/neutrophil-mediated inflammatory phenotype related to the presence of the tumor in agreement with the correlation observed between intact complement proteins and calprotectin (S100A8/S100A9) but not with the liver-derived CRP. The link between complement and calprotectin in ccRCC requires further investigation. FB concentration could not be measured here due to lack of validated and robust assay. Its concentration was reported in two ccRCC studies. Reese et al. found a significant decrease of intact FB in ccRCC,[24] whereas Cooley at al found elevated FB associated with presence of metastasis and metastasis dissemination in RCC.[25] Moreover, patients whose FB levels were increased under anti-angiogenic (sunitinib) treatment had faster disease progression and shortened survival compared to patients whose levels were decreased.[25] The particular case of FB requires further investigation. Nevertheless, despite the elevated concentration, measurement of the remaining intact complement proteins in ccRCC may not be an informative biomarker for the clinical practice as they did not correlate with prognosis.

Subgroups of ccRCC patients had elevated C4d and C1s/C1INH complexes, without increased levels of MASP1/C1INH or MASP2, evidencing initiation of the classical pathway rather than lectin pathway. We have previously reported that plasma C4d is elevated in the ccRCC patients[5] and further confirmed it in this work. Intact C4 levels were not modified in the plasma of ccRCC patients. C4d correlated with the intratumoral C4 activation fragments deposits at the surface of tumor cells, which are colocalized with IgG, C1q and C1s. Moreover, plasma C4d correlated with C1s/C1INH complexes, indicating that the elevated plasma C4d was related, at least partially, to the classical pathway activation occurring in the tumor microenvironment. This could not only occur on intratumoral immune complexes but also on PTX3 deposits.[4,39] In parallel, subgroups of patients had elevated Bb, which correlated with Ba, evidencing initiation of the alternative pathway. This is further supported by the observed colocalization between C3 and FB or C3 and FH indicating alternative pathway activation and regulation at the tumor site. In addition to this, we also observed the co-localization of classical and alternative pathway components on the surface of the same tumor cells. Even though there was no correlation between C4d (and C1s/C1INH complexes) levels and Bb (and Ba), nearly all Bb-high patients were also high for C4d in both cohorts, with 11 patients with high levels of both fragments, 32 patients being C4d and Bb low, 20 patients with only high levels of C4d and 3 being Bb high only (Fisher's test $p = .014$). This observation suggests that FB activation may come from the alternative pathway amplification loop, initiated after classical pathway triggering.

Finally, despite the activation of the classical and alternative pathways in ccRCC, we could not observe C5b-9 deposition, neither at the tumor site nor consistent elevation in plasma (where in the patients it was even lower compared to the healthy donors), evidencing the fine regulation of the complement activation cascade by cancer cells. This observation supports previous observations by other groups.[26,40,41]

Unsupervised hierarchical clustering identified a fraction of ccRCC patients which were enriched in plasma complement proteins and their activation fragments at the time of surgery. Interestingly, this subgroup of patients seems to be associated with higher TNM stages and Fuhrman Grade. Progression-free survival was not significantly different between patient clusters, although a trend was observed, suggesting that not all the complement features were informative of patient prognosis. A combination of a multidimensional feature selection method (LASSO) and multivariate cox regression on bootstrap samples revealed that the two most predictive complement features were related to the activation fragments Bb and C4d which were of prognostic value even after adjustment by TNM stage, Fuhrman Grade, age and sex. The association between elevated plasma C4d and shorter progression-free survival in the ImmPro is consistent with previous observations and already described for both cohorts.[5] Here, we also observed a correlation between C1s/C1INH complexes and worse patient prognosis, supporting the notion that the classical pathway is activated in ccRCC. Alternative pathway activation fragments Bb and Ba also correlated with poor prognosis in both validation and discovery cohorts and associated with Fuhrman Grade. This is in line with the observed complement deposits in the ccRCC tumors, indicating the formation of alternative pathway C3 convertase and its regulation by FH. FB and its activation fragments are promising biomarkers for ccRCC. Therefore, the activation fragments Bb and C4d (and likely FB) but not the other intact complement proteins, could be useful biomarkers to add to the work-up for ccRCC patients to predict prognosis.

Conclusion

In conclusion, here we validated a comprehensive strategy for complement exploration in plasma: Humoral Complementomics, consisting of measurement of complement proteins, activation fragments, and autoantibodies. Its application to ccRCC revealed that complement proteins are upregulated in patients due to the global inflammatory phenotype of the disease. This approach identified that the fragments C4d and Bb, reflecting the initiation of the classical and alternative pathways, could be useful to predict poor patient prognosis. Moreover, anti-C3 and anti-reduced FH autoantibodies were detected. These findings open the possibility for therapeutic targeting of complement in renal cancer.

Data availability

The raw data for complement quantification are available upon request.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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