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Tissue-Based Multiomic Exploratory Analysis of the Urokinase Plasminogen Activator/uPAR System and Matrix Metalloproteinases in Stroma AReactive Invasion Front Areas-Positive Gastrointestinal Cancers

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Keywords
Cancer · Colon cancer · Gastrointestinal cancer · Gene expression profiling · Lipid metabolism · Pathology · Surgical pathology

Abstract
Introduction: We recently proposed SARIFA (Stroma AReactive Invasion Front Areas), defined as direct tumour-adipocyte interaction, as an H&E-based histopathologic biomarker in gastrointestinal cancers, particularly gastric cancer (GC) and colorectal cancer (CRC). Despite SARIFA's well-validated prognostic value, its mechanistic underpinnings remain unclear. We hypothesized that extracellular matrix remodelling, specifically the plasmin/plasminogen activator system, may contribute to SARIFA formation. ***Methods:*** To test this, we compared the prognostic value of H&E-based SARIFA status with enzyme-linked immunosorbent assay (ELISA)-based protein levels of the serine proteases urokinase-type plasminogen activator (uPA, encoded by *PLAU*) and plasminogen activator inhibitor-1 (PAI-1, encoded by *SERPINE1*) in CRC. We further examined associations between

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SARIFA status and the plasmin/plasminogen activator system as well as downstream metalloproteinases using both protein (ELISA, immunohistochemistry) and bulk gene expression data (TCGA-COAD/READ and TCGA-STAD), as well as spatial gene expression profiling in CRC (n = 8) and GC (n = 12). ***Results:*** Our findings show that high expression of the plasmin/plasminogen activator system and downstream metalloproteinases correlates with SARIFA positivity. Digital spatial profiling revealed *PLAU* upregulation in tumour cells and *PLAUR* (encoding uPAR) upregulation in adjacent stromal cells at SARIFAs, suggesting a potential receptor-ligand interaction. Notably, SARIFA-positive tumours showed significantly higher numbers of tumour buds. ***Conclusion:*** These results provide new insights into the biological basis of SARIFAs and suggest therapeutic vulnerabilities related to the plasmin/plasminogen activator system.

Plain Language Summary

Cancer cells constantly interact with their surrounding tissue, and some of these interactions may help tumours to grow faster and be more aggressive. At the edge of some cancers, tumour cells grow directly into adipose tissue without intervening stromal reaction. We as pathologists call these areas SARIFA (Stroma AReactive Invasion Front Areas). SARIFA positivity has been shown to be an adverse risk factor in gastrointestinal cancers. However, it is unclear why and how it arises. We studied now whether genes and proteins that help tumours break down their surrounding tissue are linked to SARIFA. Tumours with SARIFA positivity more often showed higher levels of the plasmin/plasminogen activator system and its associated downstream metalloproteinases. Spatial data indicated more *PLAU* expression in tumour cells and more *PLAUR* expression in nearby stromal cells, suggesting a close interaction between them. Moreover, SARIFA-positive tumours had more small clusters of invasive cells, so-called tumour buds, which are also characteristic for an aggressive behaviour. Overall, our results help better understand the biology behind SARIFA and highlight a pathway that might be targetable.

Introduction

Colorectal cancer (CRC) as well as gastric cancer (GC) make up around 15% of all new cancer diagnosis every year [1]. Late-stage disease is in both entities associated with very poor patient outcomes, whereas early-stage GCs and CRCs show a favourable prognosis. Given their molecular and clinical heterogeneity, identifying high-risk cases needing tailored treatment is crucial [2–6].

An optimal biomarker in this scenario is reliable, easy to evaluate, reproducible and inexpensive. To come up with such a solution, we developed Stroma AReactive Invasion Front Areas as solely H&E-based histopathologic biomarker that is defined by the direct contact between tumour cells and adipocytes at the invasion front. Interestingly, such SARIFAs occur in both CRCs as well as GCs, and we and others could show that SARIFA-positive CRCs and GCs are characterized by a poor prognosis in several cohorts comprising more than 5,000 cases [7–15]. Furthermore, we could show that SARIFA positivity is closely linked to an upregulated lipid metabolism [10, 12, 13, 16], which could convey tumour-promoting properties. Moreover, we could link SARIFA positivity to a more stromal phenotype and immune alterations [7, 12, 13, 16, 17].

However, the exact underlying mechanism behind the formation of SARIFAs remains unclear to date. In our pathway analysis based on bulk gene expression data, we observed an upregulation of processes involved in extracellular matrix (ECM) remodelling [12, 13]. One key player in the tumour-matrix interaction is the plasmin/plasminogen system. This system consists mainly of the urokinase plasminogen activator (uPA), plasminogen activator urokinase receptor (PLAUR), and its inhibitors, namely, plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2). For the practice of oncology, uPA and PAI-1 have already been implemented in the routine care in some settings to predict the behaviour of node-negative breast cancers [18]. Previously, we could show that uPA and PAI-1 protein levels are also associated with poorer outcomes in CRC and correlate with a higher extent of tumour budding [19, 20], which is believed to reflect epithelial-mesenchymal transition on a histomorphological level [21]. Now, we hypothesize that formation of SARIFAs could rely on a degradation of ECM at the invasion front, which could eventually lead up to a direct tumour-adipocyte interaction (Fig. 1). As there is increasing evidence that a tumour-adipocyte interaction, which is a main feature of SARIFA-positive GI cancers, promotes tumour growth and aggressiveness [22–24], targeting the plasmin/plasminogen system could be a potential new treatment option in this subgroup of aggressive cancers.

Therefore, we provide here, based on our previous studies, a comprehensive analysis of alterations in the plasmin/plasminogen activator system in SARIFA-positive

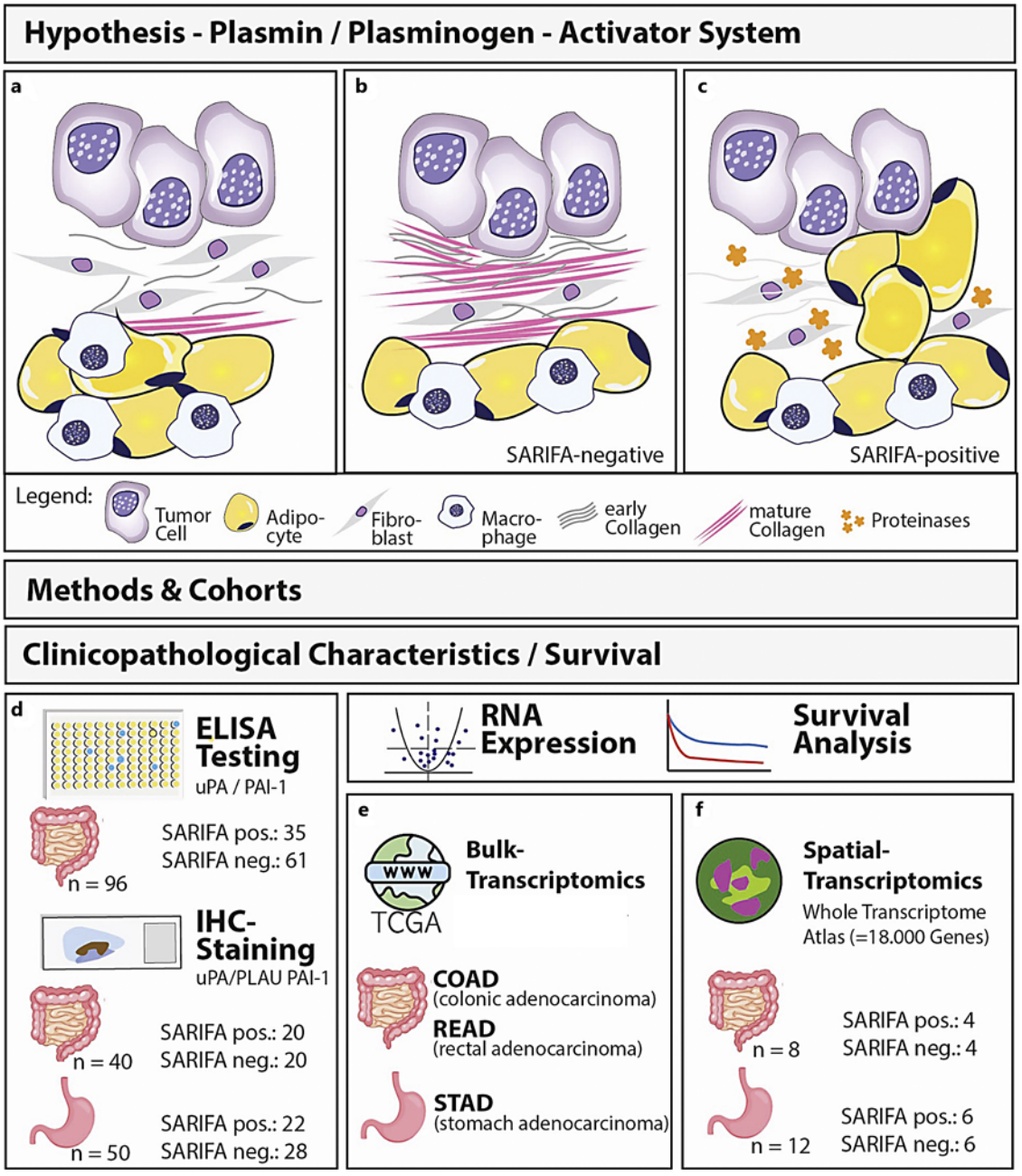

Fig. 1. Hypothesis and study design. **a–c** We hypothesize that formation of SARIFAs, which we established as negative prognostic biomarker, could rely on a degradation of ECM via proteinases of the plasmin/plasminogen activator system at the invasion front, which could eventually lead up to a direct tumour-adipocyte interaction (SARIFA-positive in **c**). If this matrix degradation does not occur and the tumour cells induce a desmoplastic reaction, histopathologic evaluation will lead to SARIFA-negative status (displayed in **b**). **d–f** By comprehensively analysing different data layers (clinicopathological characteristics, survival, ELISA-based protein levels, IHC protein expression, bulk and spatial transcriptomic data) of GC and CRC patients, we tested if SARIFA positivity is associated with an upregulation of the plasmin/plasminogen activator system. CRC, colorectal cancer; GC, gastric cancer; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor-1; SARIFA, Stroma AReactive Invasion Front Areas.

CRCs and GCs by investigating updated survival data, enzyme-linked immunosorbent assay (ELISA)-based protein, bulk as well as spatial gene expression data, and immunohistochemical (IHC) studies. The study design is summarized in Figure 1.

Methods

Cohort and Ethical Approval

The analyses carried out in the study are covered by an ethic approval of the Ludwig-Maximilians University in Munich (project No. 22-0120). The part of the study focusing on ELISA-based uPA/PAI-1 protein expression was approved by the Internal Review Board of the Klinikum Augsburg based on the recommendation by the Ethical Committee of Landesärztekammer Bayern (for further details, please refer to our previous publications [19, 20]). The need for written informed consent was waived by the Ethics Committee of the Ludwig-Maximilians University Munich.

The CRC cohort with available uPA/PAI-1 data consisted in total of 96 CRCs (SARIFA-negative: n = 61, SARIFA-positive: n = 35). By using the reverse Kaplan-Meier method [25], the median follow-up for the whole cohort was 168 months (95% confidence interval, CI: 113–185 months). The estimated follow-ups did not differ between SARIFA-positive and SARIFA-negative CRCs (log-rank, p value: 0.6; SARIFA-negative: 168 months [95% CI: 112–184 months], SARIFA-positive: 149 months [112–NA months]). For the clinicopathological characteristics of CRC and GC cases, please refer to online supplementary Tables S1 and S2 (for all online suppl. material, see https://doi.org/10.1159/000549919). An overview of the cohort size and investigations performed on them is displayed in Figure 1.

Assessment of SARIFA Status

SARIFA status was retrospectively assessed according to our previous publications [7–13, 16, 17, 26] on routine H&E-stained histopathologic slides. SARIFA positivity is defined as the direct contact between at least 5 tumour cells or at least 1 tumour gland and adipocytes at the tumour invasion front.

ELISA-Based uPA/PAI Protein Expression

For uPA/PAI-1 protein quantification as part of earlier work [19, 20], a commercially available ELISA kit for uPA and PAI-1 that is certified for the usage in breast cancer was used (Femtelle test [EF 899], Sekisui Diagnostics, Stamford, CT, USA). The recommended standard protocol was applied. The levels of uPA and PAI-1 are given in nanograms per milligram (ng/mg) of tumour protein. Based on the evaluation of the test set [19, 20], the cutoff values for uPA and PAI-1 were ≥4 ng/mg and ≥40 ng/mg, respectively, to discriminate between low- and high-grade levels. Details are described in our previous work [19, 20].

Bulk and Spatial Transcriptomics Data

As part of previous studies [12, 13], we determined SARIFA status (positive vs. negative vs. not assessable) for cases in the publicly available The Cancer Genome Atlas (TCGA) cohorts STAD (stomach adenocarcinoma), COAD (colonic adenocarcinoma), and READ (rectal adenocarcinoma) [2, 6, 27, 28]. We used the bulk RNA data here to study SARIFA-dependent gene expression differences of *SERPINE1* (encoding for PAI-1), *SERPINB2* (encoding for PAI-2), *PLAU* (encoding for uPA), and *PLAUR* (encoding for the urokinase receptor), as well as metalloproteinases activated by uPA (*MMP2, MMP9, MMP14*) [29, 30]. Differential gene expression analysis was performed with *DESeq2* [31] (for details, please refer to our previous publications [12, 13]). Consensus molecular subtypes (CMSs) of CRC samples within TCGA were performed with CMScaller v2.0.1 [32].

For spatial transcriptomic analyses, we, on the one hand, deployed previously published NanoString GeoMx digital spatial profiling (DSP [33]) data of 12 GCs (six SARIFA-positive, six SARIFA-negative) [10, 12] and, on the other hand, performed DSP on eight additional CRCs (four SARIFA-positive, four SARIFA-negative, all pT3). Based on the corresponding H&E morphology and on fluorescence imaging, regions of interest were chosen for multiplex profiling using a whole transcriptome atlas (covering around 18,000 genes) (see online supplementary Figure S1). Segmentation was performed into tumour cells (PanCK-positive) and stroma (PanCK-negative). Sequencing libraries were generated according to the standard protocol [34]. The R packages in particular used for the analysis of the DSP data were *NanoStringNCTools*, *GeomxTools*, *GeoMxWorkflows*, as well as the GeoMx DSP analysis suite, and standard quality control, preprocessing, normalization, and analysis was performed in line with recommended standard end-to-end *GeoMx-NGS* gene expression analysis workflows: https://bioconductor.org/packages/devel/workflows/vignettes/GeoMxWorkflows/inst/doc/GeomxTools_RNA-NGS_Analysis.html. In GeoMx spatial transcriptomic CRC

data, *SERPINB2* probes did not meet quality control thresholds and therefore could not be included in the spatial analysis.

Immunohistochemistry

Immunohistochemistry was performed on a representative subset of cases of the cohort (for CRC: $n = 40$, for GC: $n = 49$). IHC staining was performed on 2-µm whole slide sections using primary antibodies for PAI-1 (Invitrogen, Waltham, MA, USA; PAI-1 monoclonal antibody, clone 1D5, dilution 1:800 with CC1 32 min) and uPA/PLAU (OriGene, Rockville, MD, USA; uPA/PLAU monoclonal antibody, clone OTI5H4, dilution 1:150 with CC1 32 min) in a fully automated manner on a Ventana BenchMark ULTRA platform with an iVIEW DAB detection system (Roche, Mannheim, Germany).

For each case, an H-score was determined separately for the tumour cells and the stroma at the tumour centre and the invasion front. The H-score was calculated conventionally as follows: (1 × percentage of weak staining) + (2 × percentage of moderate staining) + (3 × percentage of strong staining) within the target region, therefore ranging from 0 to 300.

Statistical Analyses

Continuous variables were compared using the Mann-Whitney U test. We compared estimates of Kaplan-Meier survival probabilities using log-rank tests and established a univariate Cox proportional hazard model using the "coxph" function. p values <0.05 were considered statistically significant. The median follow-up was calculated using the reverse Kaplan-Meier method [25]. For gene expression data, adjusting for multiple comparisons using Benjamin-Hochberg procedure was performed. For statistical analysis, R, version 4.2.2 was used. R packages used for data processing, visualisation, and analysis were survival, survminer, ggplot2, knitr, dplyr, tidyverse, qs, scales, ggforce, patchwork, data.table, Seurat, DESeq2, NanoStringNCTools, GeomxTools, GeoMxWorkflows, and Matrix.

Results

SARIFA Status Outperforms uPA and PAI-1 Protein Expression as Prognostic Marker in CRC

In a previous study [20], we could show data that high ELISA-based PAI-1 protein expression is associated with a trend toward adverse outcomes in CRC. We now used updated survival data of 96 CRCs and performed Kaplan-Meier analysis, which is displayed in Figure 2. Even though we could again see a clear trend toward poorer survival of PAI-1-high CRC patients (hazard ratio [HR] and 95% confidence interval [CI]: 1.486 [0.87–2.54], $p = 0.14$; in 2017: $p = 0.255$ [20]), PAI-1 as well as uPA protein measurements via ELISA failed to significantly stratify CRC patients (for uPA: HR [95% CI]: 1.22 [0.72–2.069], $p = 0.46$). In contrast, we could observe significantly poorer outcomes of SARIFA-positive CRCs compared to SARIFA-negative CRCs (HR [95% CI]: 1.76 [1.056–2.98], $p = 0.027$), showing that solely H&E-based SARIFA is prognostically relevant. The strong independent prognostic value of SARIFA status in CRC and GC has been extensively validated in larger cohorts through multivariate analyses, as referenced here [7–15].

High uPA and PAI-1 Protein Expression Are Linked to SARIFA Positivity in CRC

We have previously demonstrated a correlation between high tumour budding and uPA/PAI-1 protein levels [20] and could link SARIFA to higher budding [11]. As absolute tumour buds per ×20 magnification were established for a subset of our cohort for our previous work, we could now validate that SARIFA-positive CRCs show indeed higher number of absolute tumour buds ($p = 0.011$). Interestingly, SARIFA-positive CRCs are characterized by a higher uPA protein expression ($p = 0.027$) and showed a slight numeric trend toward higher PAI-1 expression ($p = 0.21$). Results are visualised in Figure 3.

Bulk as well as Spatial Transcriptomics Data Indicate Upregulation of Plasmin/Plasminogen System in SARIFA-Positive GI Cancers

Next, we investigated our previously published bulk mRNA differential gene expression analysis [12, 13] of the TCGA cohorts COAD and READ (both taken together as CRC cohort), and STAD with focus on the plasmin/plasminogen system. In CRC, we observed a significant upregulation of *PLAU* and *SERPINE1* (both adjusted p values at least 0.001) in SARIFA-positive cases. *PLAUR* and *SERPINB2* both showed a trend toward upregulation in SARIFA-positive CRCs (*PLAUR*: adjusted p value = 0.089, *SERPINB2*: adjusted p value = 0.11). In GC, *SERPINE1* was upregulated in SARIFA-positive cases (adjusted p value <0.05). When considering CMS in CRC, *PLAU*, *PLAUR*, *SERPINE1* and *SERPINB2* expression seems to be associated to CMS1 and CMS4, quite like SARIFA positivity (online suppl. Fig. S3).

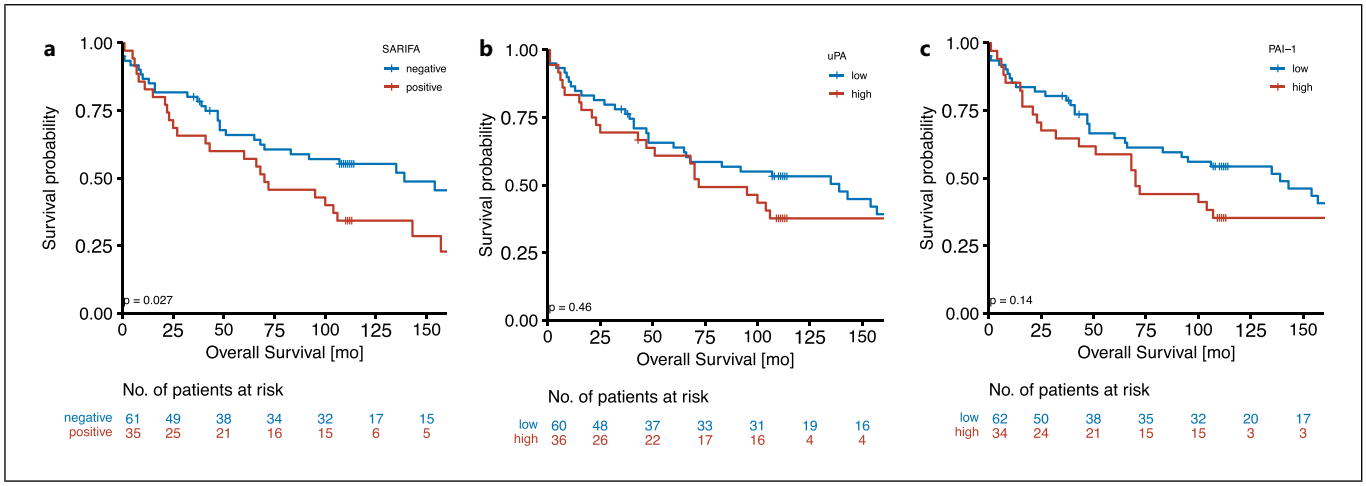

Fig. 2. Kaplan-Meier curves for overall survival in months. **a** SARIFA-positive CRC patients display a significantly shorter overall survival than SARIFA-negative CRC patients (p = 0.027). **b**, **c** No clear survival differences can be observed for uPA-low vs. uPA-high as well as for PAI-1-low vs. PAI-1-high CRC patients (p = 0.46 and p = 0.14). CRC, colorectal cancer; mo, months; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor-1; SARIFA, Stroma AReactive Invasion Front Areas.

By using DSP, we could then investigate more deeply if gene expression of the plasmin/plasminogen system shows SARIFA-dependent differences at the invasion front in tumour (PanCK-positive) and stromal cells (PanCK-negative), as SARIFA is a spatially restricted process localized at the invasion front. Here, many genes of the plasmin/plasminogen system are upregulated specifically in tumour cells in CRC as well as GC. Interestingly, in CRC, *PLAU* is highly upregulated in the tumour cells at the invasion front (LFC: 2.58, p adjusted <0.001), whereas *PLAUR* is particularly upregulated in the stroma cells (LFC: 1.076, p adjusted <0.01), indicating a potential ligand-receptor interaction. Notably, in GC, *SERPINB2* was significantly upregulated in tumour cells at the invasion front (LFC: 1.96, p adjusted <0.001). The findings of bulk and spatial transcriptomics are visualized in Figure 4 and online supplementary Figures S2 and S3 and summarized in online supplementary Tables S3 and S4.

Given the known functional link between uPA activity and downstream activation of matrix metalloproteinases (MMPs) [29, 30], we further examined the expression of *MMP2*, *MMP9*, and *MMP14* in the TCGA bulk mRNA data and in our spatial transcriptomics dataset. In CRC, SARIFA-positive tumours showed significantly higher expression of *MMP2* compared to SARIFA-negative tumours (p adjusted = 0.002), whereas *MMP9* and *MMP14* demonstrated a nonsignificant trend toward upregulation (*MMP9*: p adjusted = 0.11, *MMP14*: p adjusted = 0.13) on bulk level. Spatial transcriptomics localized these differences predominantly to tumour cells, with *MMP14* showing the strongest enrichment in SARIFA-positive regions (LFC: 1.06, p adjusted <0.001). In GC bulk and spatial data, there was no significant upregulation of MMPs in SARIFA-positive GCs. These findings are illustrated in the Figure 4 and detailed in online supplementary Tables S5 and S6.

IHC Analysis of PAI-1 and uPA/PLAU Expression

Currently, there is little and conflicting evidence on the IHC expression of PAI-1 and uPA/PLAU in GI cancers [35]. As we observed a clear association between SARIFA positivity and the plasmin-plasminogen activator system on gene expression level and as some data suggest that IHC upregulation of PAI-1 is associated with advanced/nodal-positive GC [36], we performed PAI-1 and uPA/PLAU immunohistochemistry and evaluated the expression at the tumour centre and the invasion front as well as in the tumour cells and the stroma. Here, no statistically significant differences between PAI-1 and uPA/PLAU immunoexpression based on SARIFA status could be observed – neither at the tumour centre nor at the invasion front (all p values above 0.05). The expression of both markers showed relevant inter- and intratumoural heterogeneity. The results stratified for GC and CRC are displayed in online supplementary Figure S4, and the IHC stains are visualized in online supplementary Figure S5.

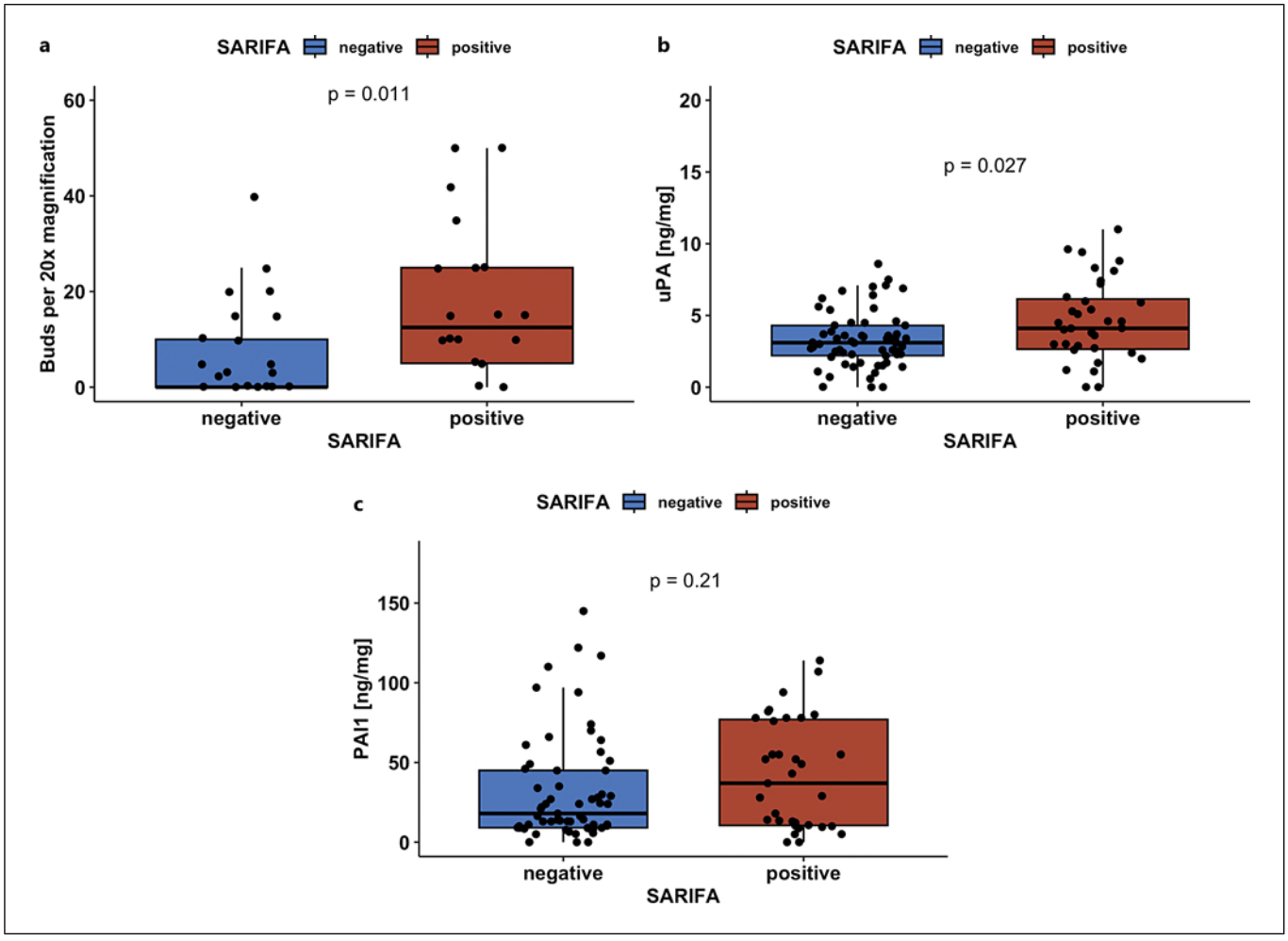

Fig. 3. Boxplots for absolute tumour buds, uPA and PAI-1 protein expression (ELISA) based on SARIFA status. **a** SARIFA-positive CRCs show a higher number of absolute tumour buds per ×20 magnification compared to SARIFA-negative CRCs: SARIFA-positive (n = 21), mean ± SD: 26.05 ± 42.86; SARIFA-negative (n = 30), mean ± SD: 11.10 ± 21.70, p = 0.011 (two outliers not depicted in the boxplot: SARIFA-positive [n = 1] with 200, and SARIFA-negative [n = 1] with 100 buds per ×20 magnification). **b** SARIFA-positive CRCs are characterized by higher uPA protein levels (measured by ELISA in ng/mg): SARIFA-positive (n = 35), mean ± SD: 4.56 ± 2.87; SARIFA-negative (n = 61), mean ± SD: 3.31 ± 1.95, p = 0.027. **c** SARIFA-positive CRCs potentially tend to show higher PAI-1 protein levels (measured by ELISA in ng/mg) – however, without reaching statistical significance: SARIFA-positive (n = 35), mean ± SD: 41.57 ± 34.35; SARIFA-negative (n = 61), mean ± SD: 32.12 ± 33.42, p = 0.21. Boxplots represent the interquartile range (Q1–Q3), with the line in the middle depicting the median. CRC, colorectal cancer; ELISA, enzyme-linked immunosorbent assay; PAI-1, plasminogen activator inhibitor-1; SARIFA, Stroma AReactive Invasion Front Areas; SD, standard deviation; uPA, urokinase-type plasminogen activator.

Discussion

In previous studies, we and others have described and validated the strong prognostic value of SARIFA as novel H&E-based biomarker for GC and CRC [7–15], which can be easily integrated into a routine pathology workflow as it relies solely on H&E histology and shows a high interobserver agreement [10, 11]. In our current study, we provide evidence that the prognostic power of SARIFA status surpasses that of ELISA-based uPA and PAI-1 protein expression in CRC – two biomarkers that have historically reached clinically application in breast cancer [18] and have been discussed as biomarkers for CRC for a long time now [37–39]. Nevertheless, it is known that a high expression of uPA and PAI-1 seems to be associated

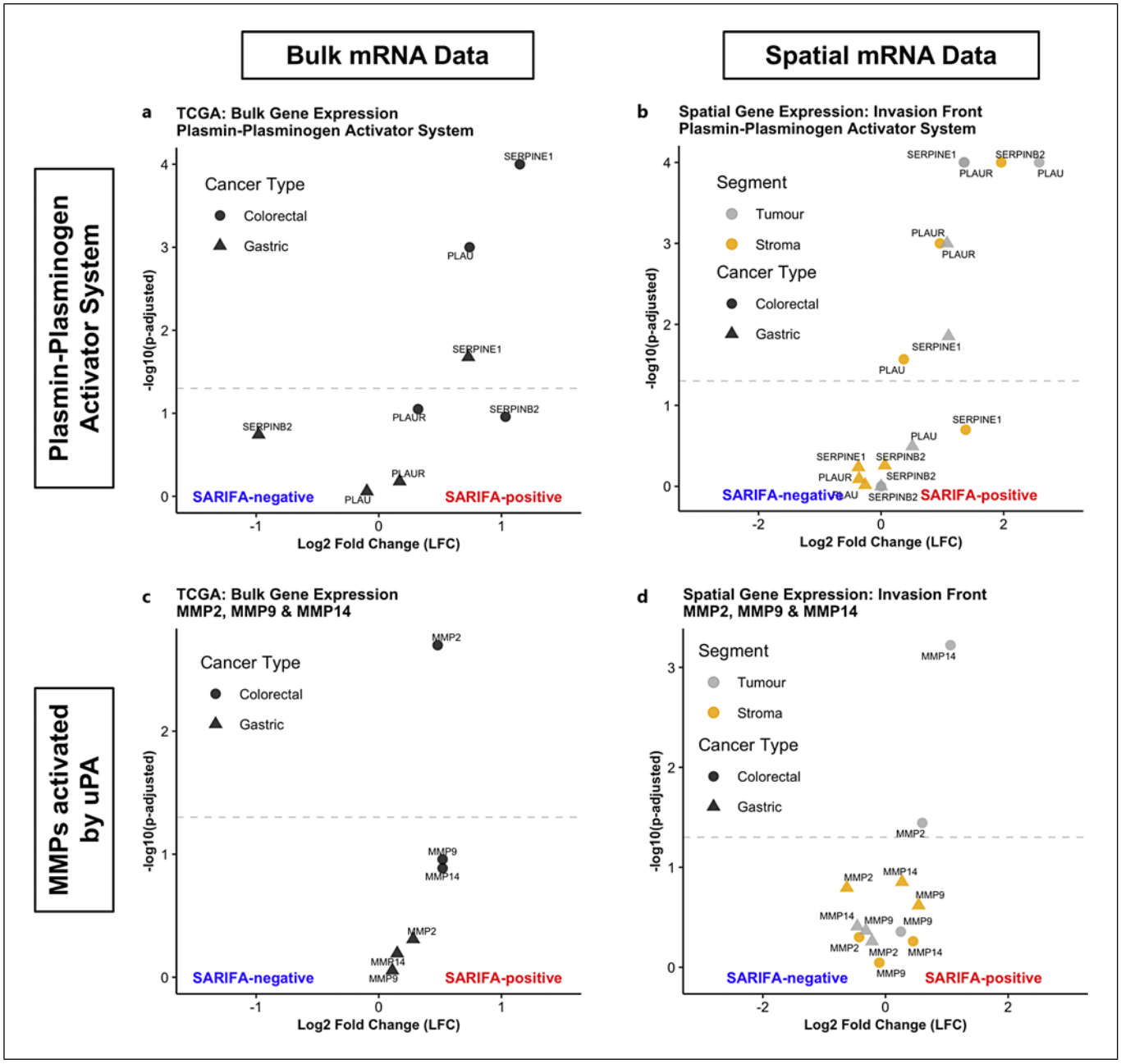

Fig. 4. Bulk as well as spatial gene expression of the urokinase plasminogen activator system and MMPs in gastric and colorectal cancers stratified by SARIFA status. **a**, **c** In the TCGA cohorts CRC (COAD/READ) and STAD, differential gene expression analysis revealed significant (dashed line at *p* adjusted = 0.05) upregulation of several players of the plasmin-plasminogen activator system in SARIFA-positive colorectal as well as gastric cancers on bulk gene expression level. **b**, **d** GeoMx DSP also indicated a significant upregulation of the plasmin-plasminogen activator system as well as *MMP14* at the invasion front of SARIFA-positive gastrointestinal cancers. None of the genes of the plasmin-plasminogen activator system was significantly upregulated in SARIFA-negative samples. *PLAU* encodes for uPA, *PLAUR* for urokinase receptor, *SERPINE1* for PAI-1 and *SERPINEB2* for PAI-2. Y-axis shows −log10 (*p* adjusted), with values <0.0001 plotted as 4. CRC, colorectal cancer; GC, gastric cancer; LFC, log fold change; MMP, matrix metalloproteinase; PAI-1/2, plasminogen activator inhibitor-1/2; SARIFA, Stroma AReactive Invasion Front Areas; uPA, urokinase-type plasminogen activator; TCGA, The Cancer Genome Atlas.

with a poor prognosis in GC as well as CRC – just like SARIFA positivity [19, 20, 36, 40].

Even though we could link SARIFA positivity to a tumour-promoting upregulation of lipid metabolism and an altered immunity in previous studies [10, 12, 13, 16, 17], little is currently known how SARIFAs arise mechanistically. Here, we provide new insights into the plasmin/plasminogen activator system's potential role in SARIFA formation. Morphologically, the "areactive" nature of SARIFA, characterized by tumour clusters that neither elicit desmoplastic reaction nor adopt a pushing growth pattern, suggests a microenvironment permissive to direct tumour-adipocyte interaction. In this context, matrix degradation through the plasmin/plasminogen activator system emerges as a plausible mechanism contributing to SARIFA formation. The significant upregulation of plasminogen activator (*PLAU*) in SARIFA-positive CRC and GC tumour cells, along with the upregulation of its receptor (*PLAUR*) in adjacent stromal cells, suggests a receptor-ligand interaction that may facilitate ECM degradation at the tumour invasion front, thus allowing for direct tumour-adipocyte interactions. This upregulation was confirmed across multiple layers of analysis, including ELISA, bulk RNA, and DSP data. Notably, DSP allowed us to spatially pinpoint this interaction, highlighting *PLAU's* localized expression in tumour cells at the invasion front and *PLAUR's* enrichment in nearby stromal cells. This spatial specificity emphasizes a biologically distinct microenvironment at the invasion front, potentially driven by ECM remodelling through the plasmin/plasminogen system, which may support tumour invasion and metastasis in SARIFA-positive cases. Indeed, several downstream MMPs known to be activated by uPA, namely, *MMP2*, *MMP9*, and *MMP14*, were upregulated in SARIFA-positive tumours. Spatial transcriptomics localized these alterations predominantly to tumour cells at the invasion front. The upregulation of *MMP14* in CK-positive tumour cells at the invasion front of SARIFA-positive CRCs is consistent with *MMP14*'s well-established role as a membrane-bound protease, expressed by tumour cells, facilitating local ECM degradation and pro-MMP2 activation at the invasive edge of tumours [41]. The mechanistic plausibility of our findings is underlined by Sier et al. [42], who found elevated PAI-1/PAI-2 and persistent uPA increase despite inhibition in CRCs, implying a net proteolytic drive that can facilitate invasion.

From a biological perspective, the upregulation of PAI-1 is closely linked to the epithelial-mesenchymal transition [43]. Minoo et al. [37] could link upregulation of uPA expression in CRC to an infiltrating tumour margin, which shows some overlap with SARIFA positivity. However, SARIFA positivity seems to be characterized by a distinct aggressive tumour biology [7, 9, 12, 13, 17, 26], highlighting the need for more targeted therapies.

In a recent study by Micalet et al. [44], the authors not only demonstrated that *PLAU* (uPA) is highly expressed in invasive breast and CRC cells but also that *PLAU* gene knockout and pharmacological inhibition of uPA in 3D in vitro tumouroids can prevent matrix remodelling and degradation. By this way, cancer invasion is reduced, which provides evidence that targeting uPA could be a potential useful mechano-based cancer therapy. There is also some evidence from in vitro as well as mouse model data that anti-uPAR treatment leads to decreased invasiveness and reduced metastasis formation in colon cancer models [39]. Similar data exist for PAI-1, which may be also a suitable pharmacological target, especially in combination with immunotherapy [35].

Nevertheless, our studies have some limitations. Firstly, DSP does not enable single cell resolution, which hinders us in pinpointing the specific cellular subset, which is responsible for *PLAUR* upregulation in the stroma, potentially cancer-associated fibroblasts. Nevertheless, DSP allows the reliable identification of spatially resolved and enriched biological signals. Furthermore, we could not validate the gene expression findings on protein level by immunohistochemistry. However, we were able to detect differential protein expression using ELISA on fresh tissue. ELISA-based measurements were performed on homogenized tumour samples without spatial restriction and thus cannot capture localized proteolytic activity at the invasive front. To mitigate this, we complemented ELISA data with immunohistochemistry and spatial transcriptomics, which confirmed spatially confined expression patterns of *PLAU*, *PLAUR*, *SERPINE1*, *SERPINB2*, and *MMP14* at SARIFA areas. Secondly, mRNA expression levels do not necessarily reflect enzymatic activity, particularly for proteases requiring activation, such as uPA and MMPs. A direct functional approach, like in situ zymography on frozen sections or in vitro testing of organoids, was not possible due to the lack of suitable material.

Despite these limitations, the consistent transcriptional upregulation of the *PLAU-PLAUR* axis, PAI-2 (*SERPINB2*), and downstream MMP effectors observed by bulk and spatial mRNA data, along with differential protein expression detected by ELISA, supports a biologically relevant role of the plasminogen activator system in SARIFA-positive tumours.

In conclusion, our study reveals that SARIFA positivity, a simple H&E-based biomarker, is closely linked to the plasmin/plasminogen activator system, particularly the PLAU-PLAUR axis. These findings provide mechanistic insights into ECM remodelling in SARIFA-positive cancers, linking a distinct tumour morphology to an underlying biology and highlighting potential therapeutic targets for aggressive gastrointestinal malignancies.

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Statement of Ethics

The analyses carried out in the study are covered by an ethics application at the Ludwig-Maximilians University in Munich (project No. 22-0120). The part of the study focusing on ELISA-based uPA/PAI-1 protein expression was approved by the Internal Review Board of the Klinikum Augsburg based on the recommendation by the Ethical Committee of Landesärztekammer Bayern [19, 20]. The need for written informed consent was waived by the Ethics Committee of the Ludwig-Maximilians University Munich. Consent was waived by the Ethics Committee due to the retrospective nature of the study.

Conflict of Interest Statement

N.G.R. received compensation for travel expenses from NanoString (Bruker Company/Bruker Spatial Biology). The other authors report no disclosures.

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Author Contributions

B.G., N.G.R., and B.M. conceptualized the study and created the figures. B.G. and N.G.R. prepared the original draft. S.D., V.G., F.S., J.H., C.H., M.S., S.F., G.S., A.P., P.L., and J.W. contributed to data acquisition. B.G., N.G.R., V.G., and B.M. performed data analysis, visualization, and interpretation. All authors provided scientific input and approved the final version of the manuscript.

Data Availability Statement

The underlying raw data will be made available upon reasonable request from the first authors of this study (B.G. and N.G.R.). Some parts of the raw data are available in the following previous manuscripts [13, 16]. The additional data that support the findings of this study are not publicly available due to privacy reasons but are available from the corresponding authors upon reasonable request.

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