Proteoglycan Research

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Comprehensive Investigation of Proteoglycan Gene Expression in Breast Cancer: Discovery of a Unique Proteoglycan Gene Signature Linked to the Malignant Phenotype

Simone Buraschi[1] | Gabriel Pascal[1] | Federico Liberatore[2] | Renato V. Iozzo[1]

[1]Department of Pathology and Genomic Medicine, and the Translational Cellular Oncology Program, Sidney Kimmel Cancer Center, Sidney Kimmel Medical College at Thomas Jefferson University, Philadelphia, Pennsylvania, USA | [2]School of Computer Science and Informatics, Cardiff University, Cardiff, UK

Correspondence: Simone Buraschi (simone.buraschi@jefferson.edu) | Renato V. Iozzo (renato.iozzo@jefferson.edu)

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ABSTRACT

Solid tumors present a formidable challenge in oncology, necessitating innovative approaches to improve therapeutic outcomes. Proteoglycans, multifaceted molecules within the tumor microenvironment, have garnered attention due to their diverse roles in cancer progression. Their unique ability to interact with specific membrane receptors, growth factors, and cytokines provides a promising avenue for the development of recombinant proteoglycan-based therapies that could enhance the precision and efficacy of cancer treatment. In this study, we performed a comprehensive analysis of the proteoglycan gene landscape in human breast carcinomas. Leveraging the available wealth of genomic and clinical data regarding gene expression in breast carcinoma and using a machine learning model, we identified a unique gene expression signature composed of five proteoglycans differentially modulated in the tumor tissue: Syndecan-1 and asporin (upregulated) and decorin, PRELP and podocan (downregulated). Additional query of the breast carcinoma data revealed that serglycin, previously shown to be increased in breast carcinoma patients and mouse models and to correlate with a poor prognosis, was indeed decreased in the vast majority of breast cancer patients and its levels inversely correlated with tumor progression and invasion. This proteoglycan gene signature could provide novel diagnostic capabilities in breast cancer biology and highlights the need for further utilization of publicly available datasets for the clinical validation of preclinical experimental results.

1 | Introduction

Proteoglycans are a highly heterogeneous class of glycanated proteins primarily found to be secreted in the extracellular matrix (ECM) of the connective tissues and in the pericellular and basement membrane spaces [1–4]. Forty-three proteoglycans have been described so far, of which 33 appear to be glycanated with one or more glycosaminoglycan (GAG) chain

Abbreviations: AUROC, area under the receiver operating characteristic curve; CAS, ECM, extracellular matrix; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats; EGF, epidermal growth factor; EGFR, EGF receptor; EMT, epithelial mesenchymal transition; GTEx, genotype-tissue expression; HGF, hepatocyte growth factor; HGFR, HGF receptor also known as Met receptor; IGF-IR, insulin-like growth factor receptor I; LOOCV, leave-one-out cross-validation; PROTAC, proteolysis targeting chimera; RFE, recursive feature elimination procedure; RTK, receptor tyrosine kinase; SLRP, small leucine-rich proteoglycan; TARGET, therapeutically applicable research to generate effective treatments; TCGA, The Cancer Genome Atlas; TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

Simone Buraschi and Gabriel Pascal contributed equally to this study.

covalently linked to their individual protein cores. The GAG chains are long, linear polysaccharides composed of repeating disaccharide units such as chondroitin sulfate, dermatan sulfate, heparan sulfate, keratan sulfate, or the unsulfated hyaluronan [5–7]. Proteoglycans play crucial roles in the ECM, contributing to various biological functions such as structural support, cell adhesion and migration, tissue osmotic balance, and regulation of diverse biological processes through interaction with growth factors, cytokines, and membrane receptors [8–10]. Moreover, proteolytic fragments of proteoglycan protein cores affect tumor angiogenesis [11–14] by interacting with and suppressing the action of vascular receptors [15–17]. Because of their critical roles in regulating cellular behavior and interactions across ECM molecules and cells, proteoglycans have been described as master regulators of cancer progression, invasion, and metastatic spreading [18]. Changes in the expression levels of some proteoglycans are associated with clinical outcomes of various cancers. High expression of specific proteoglycans often correlates with poor prognosis, increased metastatic potential, and resistance to therapy. Therefore, proteoglycans represent potential biomarkers for cancer diagnosis, prognosis, and therapeutic targeting [5]. In this study we discovered a novel proteoglycan gene signature in breast cancer leveraging available data from the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) databases.

The main goal of our research was to delineate the overall impact of 43 proteoglycan gene expression profiles in breast cancer diagnosis and prognosis, focusing on statistically significant changes in their mRNA expression levels in the breast tumor tissue compared with the normal, noncancerous, tissue. The applicability of our analysis lies in the generation of the scientific basis for future studies focused on proteoglycan roles in breast cancer pathophysiology including initiation, progression, and metastasis.

The utility of this study is two-fold, such that our analysis of a large, publicly available data set provides unbiased validation or criticism of experimentally determined trends of expression for several proteoglycans, as well as providing insights into the expression of several proteoglycans in breast cancer that have previously been unexplored. TCGA is a cancer genomics program led by the National Cancer Institute which includes an immense repository of transcriptomic data compiled from several thousand patient tissue samples collected since 2006. This vast array of patient data collected over nearly two decades serves as a unique and ever-growing public resource to members of the cancer research community by which we may glean novel insights into the genomic origins of cancer pathophysiology. Searching through this robust and unbiased body of data has provided us with a broader perspective of overarching trends of expression for many proteoglycans within the context of breast carcinoma, and illuminated opportunities for future expansion of investigative focus within the field of proteoglycan science.

The technological advancements of biomedical analysis tools in the past two decades have produced exciting new ways for us to understand these genomic origins and the evolution of machine learning and artificial intelligence is at the forefront of the contemporary technological revolution. We have sought to compound the potential of TCGA's robust body of data with cutting-edge machine learning techniques to drive the advancement of proteoglycan-based diagnostic knowledge so that we may continue to advance the field of proteoglycan science and provide new directions for future investigation and ultimately develop innovative proteoglycan centered therapeutic approaches in the future. This analysis aids in prioritizing proteoglycan candidates for further investigation with a data-driven approach to identify promising undiscovered therapeutic avenues. The integration of the proteoglycan expression profiles, their biological functions, and clinical outcomes has the potential to guide the development of precision therapies that may enhance the prognosis of solid tumor patients.

2 | Methods

2.1 | Data Set

The data set employed in this study consists of a combined cohort derived from TCGA, Therapeutically Applicable Research to Generate Effective Treatments (TARGET), and Genotype-Tissue Expression (GTEx) projects, accessible via the UCSC Xena Project [19], and publicly available at https://xenabrowser.net/datapages/?cohort=TCGA%20TARGET%20GTEx. Specifically, this research utilizes the gene expression RNASeq data, encompassing a total of 19,120 samples and providing the log-transformed expression values for 58,581 genes.

2.2 | Preprocessing

The data set is refined based on phenotype information sourced from the UCSC Xena Project. A filter is implemented, which keeps only the samples identified with a primary site of "Breast" and categorized under the sample types "Normal Tissue" or "Primary Tumor." Additionally, the analysis is restricted to the gene expression profiles of proteoglycans. Consequently, the refined data set encompasses expression data for 43 genes across 1271 samples. The data set is divided into 179 normal tissue samples, all sourced from GTEx, and 1092 tumor tissue samples, all derived from TCGA. Furthermore, the tumor samples are augmented with clinical data detailing the pathologic stage of the tumor tissue samples. Table 1 displays the distribution of samples across different stages.

TABLE 1 | Number of samples per pathological stage.

NA	Stage I	Stage II	Stage III	Stage IV	Stage X
12	182	617	248	20	13

2.3 | Gene Expression Analysis

Here, we present an analysis that involves detailed gene expression profiling and logistic regression modeling to identify and validate a gene signature capable of distinguishing between normal and tumor breast tissues, using advanced statistical methods and machine learning techniques. This analysis was developed and performed with initial criteria in place to select only the most pertinent genes from the data set. Genes were only considered for downstream analysis if (1) the $\log_2$ fold-change of the gene in the breast carcinoma group was > 2 or < -2, and (2) the p value of this comparison was > 0.001.

For each gene, the $\log_2$-fold change is calculated as the logarithmically normalized difference between the mean expression values of cases (TCGA) and controls (GTEx). Following the DESeq. 2 analytical framework [20], p values were derived using a Wald test [21] applied within a simple linear regression model. Then, these p values were adjusted to control for multiple testing errors using the Benjamini–Hochberg procedure [22]. We note that while DESeq. 2 was used as an analytical framework, this package was not used to perform the analysis. Data retrieved from the UCSD Xena Project is log-transformed upon download and reverting these transformed data to raw counts required DESeq. 2 compatibility, thereby introducing error and consequently decrease the precision of downstream analysis. The code was developed in R (https://www.r-project.org/). We used the Wald test function, *waldtest()*, provided in the package *lmtest* [23]. p values adjustment was carried out through the function *p.adjust()*, included in the base package *stats*, specifying the parameter *method* = "BH" in the function call.

Of the 43 proteoglycans evaluated in this analysis, only 11 proteoglycans meet these stringent criteria, namely *GPC3, DCN, PTPRZ1, EPYC, ACAN, PODN, SDC1, TGFBR3, OGN, ASPN*, and *PRELP*. Notably, *ACAN*, which is predominantly expressed in cartilaginous tissues and plays a key role in the structural integrity of cartilage, also appears in this list. Given its usual tissue-specific expression and the focus of this study on breast cancer, this finding was unexpected. To refine our analysis further, we applied a mean-based filter [24] excluding genes with a mean log-counts across all samples of 4.45435 or less. This threshold, considering the data is log-transformed as $X' = log_2(X+1)$, corresponds to a minimum average gene count of approximately 85, effectively excluding ACAN from the data set. After applying this filter, we narrowed the focus of our study to the following proteoglycans: *GPC3, DCN, PODN, SDC1, TGFBR3, OGN, ASPN, PRELP*, which became the subject of further investigation.

2.4 | Gene Signature for Breast Cancer Detection

The objective of this analysis was to identify a gene signature for breast cancer that would effectively differentiate between normal (class 0) and tumor (class 1) tissues. To achieve this, we implemented a Recursive Feature Elimination (RFE) procedure [25] to isolate the minimal set of proteoglycans that yields the highest classification accuracy. This approach involved evaluating all possible combinations of the eight previously identified proteoglycans. For each combination, we used a Logistic Regression Model, a binary classification method that models the probability of an outcome using predictor variables, producing interpretable estimates for risk prediction. This model was then fitted for each combination and the accuracy of each model was assessed using Leave-One-Out Cross-Validation (LOOCV). LOOCV is a model evaluation method where each data point is used once for testing, with the rest used for training. This maximizes data use and provides an unbiased performance estimate. The optimal model demonstrated a validation accuracy of 0.9984 and a training accuracy of 1.0. This model includes the following five proteoglycans: *ASPN, PODN, SDC1, PRELP, and DCN*. The coefficients of these proteoglycans, which contribute to their discriminative power in the logistic regression model, are detailed in Table 2.

The exceptionally high accuracy achieved underscores the efficacy of these five proteoglycans in distinguishing between normal and tumor tissues. Consequently, this gene signature holds promise as a reliable tool for breast cancer detection. The positive value of the intercepts indicates a bias of the model towards class 1 (tumor tissue). This bias is anticipated, given the unbalanced nature of the data set, where class 1 is approximately six times more prevalent than class 0.

2.5 | Cancer Stage Analysis

Given the promising findings from the previous analysis, it has been extended to explore the influence of cancer stage on proteoglycan expression. Unlike the initial analysis which focused on distinguishing between normal and tumor tissues, here the cancer stage serves as the dependent variable. Therefore, samples from normal tissues and those lacking a stage assignment were excluded. Additionally, owing to the scant number of samples in Stage IV and Stage X (Table 1), we have arbitrarily pooled these samples into Stage III group. Despite the success of previous models, the Ordered Logistic models fitted through the RFE only achieved an Area Under the Receiver Operating Characteristic curve (AUROC) of 0.5035587 during training, suggesting performance akin to a trivial classifier that assigns all samples to the most prevalent class. Consequently, this analysis suggests that there are no significant variations in the expression of proteoglycans across different tumor stages and a gene signature for the severity of breast cancer could not be derived.

TABLE 2 | Coefficients of logistic regression for selected proteoglycans. Negative coefficients indicate downregulation of the corresponding gene in tumor tissues relative to normal tissues, while positive coefficients indicate upregulation.

Intercept	ASPN	PODN	SDC1	PRELP	DCN
121.42	59.27	−49.12	57.50	−22.01	−23.27

2.6 | Cancer Type Analysis

Given the heterogeneity of breast cancer, tumor type must be a primary consideration in studies of gene expression and disease progression. We therefore conducted a stratified analysis of gene expression profiles, gene signatures, and cancer stage across the most prevalent breast tumor subtypes. In our data set, the most represented tumor types were infiltrating duct carcinoma (774 samples) and lobular carcinoma (201 samples); the remaining types presented limited sample sizes (with the largest being 28 and most others fewer than 9), rendering them unsuitable for robust analysis.

2.6.1 | Infiltrating Duct Carcinoma

The data set includes a total of 953 samples for this subtype. Applying initial filtering criteria based on $\log_2$ fold-change and the *p*-value thresholds, we retained the following genes: *GPC3, DCN, PODN, SDC1, TGFBR3, OGN, ASPN, and PRELP*—which is identical to the gene set identified when analyzing all tumor types collectively. This overlap probably is because the infiltrating duct carcinoma is the most prevalent type of tumor in the data set.

For infiltrating duct carcinoma, a refined gene signature comprising *ASPN, PODN, and DCN* was identified, achieving both validation and training accuracies of 1.0. This result indicates an optimal separation of tumor and normal tissues, suggesting these genes may serve as reliable markers for this carcinoma subtype. Interestingly, this signature is a subset of the broader data set's signature, suggesting that a minimal yet distinct gene set can retain high discriminatory power within this subtype. Regression coefficients for this signature (Table 3) exhibit the same directional influence (positive or negative) as those from the full data set signature, highlighting regulatory consistency across tumor contexts.

Stage analysis, however, reveals that proteoglycan expression alone does not effectively distinguish between cancer stages for this subtype; the AUROC for stage classification remains low at 0.502, indicating performance no better than random chance. This finding suggests that while these genes are effective for tumor detection, they are limited in capturing the complexity of tumor progression in infiltrating duct carcinoma.

2.6.2 | Lobular Carcinoma

For lobular carcinoma, the data set contains 380 samples. Following similar filtering criteria, we retained a gene set comprising *GPC3, EPYC, CHAD, SDC1, TGFBR3, ASPN, PRELP, and BGN*. This set introduces three additional genes—*EPYC, CHAD, and BGN*—while excluding *DCN, PODN, and OGN*. These differences highlight potential subtype-specific expression patterns, reflecting the molecular diversity within breast cancer and possibly indicating unique pathobiological roles for these genes in lobular carcinoma.

The gene signature specific to lobular carcinoma includes *GPC3, ASPN, PRELP, SDC1, and EPYC*, achieving validation and training accuracies of 0.982 and 1.0, respectively. This high performance suggests a robust separation capability between tumor and normal tissues, supporting the potential utility of these genes as subtype-specific biomarkers. Of note, three genes—ASPN, PRELP, and SDC1—appear in both the lobular carcinoma and full data set signatures, with consistent regression coefficient signs (Table 3), reinforcing their importance across multiple breast cancer contexts (Table 4).

Stage classification for lobular carcinoma achieved an AUROC of 0.587, which, while superior to that of the full data set and infiltrating duct carcinoma, remains insufficient as a reliable stage classifier.

The limited predictive capacity of proteoglycans for stage, despite high accuracy in tumor detection, confirms that additional factors or markers beyond proteoglycan expression may be needed to capture the intricacies of tumor progression in lobular carcinoma, as well as in infiltrating duct carcinoma. Further studies incorporating larger data sets and additional biomarkers could improve stage differentiation for this subtype.

TABLE 3 | Coefficients of logistic regression for "Infiltrating duct carcinoma." Negative coefficients indicate downregulation of the corresponding gene in tumor tissues relative to normal tissues, while positive coefficients indicate upregulation.

Intercept	ASPN	PODN	DCN
148.78	126.15	−120.97	−69.84

2.7 | Protein Crystal Structure Prediction and Generation

To provide visual characterization of several proteoglycans included in our breast carcinoma signature, we utilized previously published knowledge and state-of-the-art artificial intelligence prediction tools to create crystal structures for the SLRPs. While the structure of several SLRPs have been previously determined experimentally, it was necessary to predict the structures of others, which we accomplished using the AlphaFold Database driven by Google's DeepMind Artificial Intelligence. This tool was created as part of the European Molecular Biology Laboratory's European Bioinformatics Institute [26, 27]. We were able to generate crystal structures highlighting primary and secondary structures of our SLRPs of interest using the computerized crystal structure generation software PyMol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger LLC).

3 | Results and Discussion

3.1 | Gene Expression Analysis

Using the previously described data from TCGA and GTEx, facilitated by the UCSB Xena platform, mRNA levels of 11 proteoglycans were found to be differentially expressed by a $\log_2$ fold-change factor > 2 in breast carcinoma when compared with

TABLE 4 | Coefficients of logistic regression for lobular carcinoma. Negative coefficients indicate downregulation of the corresponding gene in tumor tissues relative to normal tissues, while positive coefficients indicate upregulation.

Intercept	GPC3	ASPN	PRELP	SDC1	EPYC
2.56	−3.50	4.76	−3.51	6.84	3.31

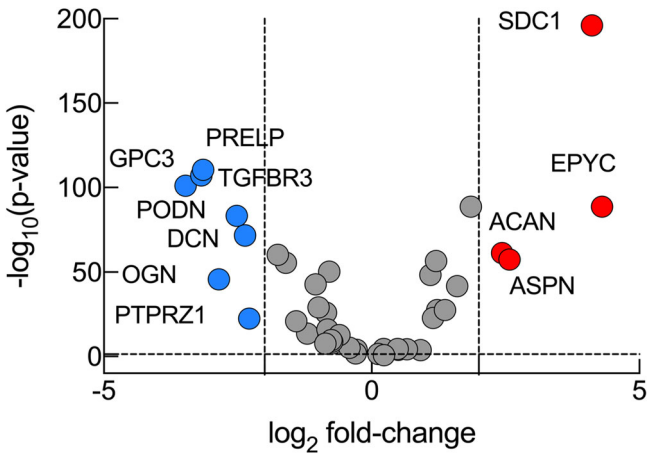

FIGURE 1 | Differential expression of proteoglycans in breast carcinoma. Gene expression data for 42 proteoglycans was compared between normal breast tissue samples obtained from GTEx and breast carcinoma samples obtained from TCGA. Proteoglycans with a $\log_2$ fold-change > 2 and a $p < 0.001$ are named in the volcano plot, with downregulated genes shown in blue, upregulated genes shown in red, and all genes that did not meet our criteria for significance going unnamed with gray icons. Dashed lines represent intersecting limits of $\log_2$ fold-change $= |2|$ and $p = 0.001$.

healthy controls. We determined that *PTPRZ1, OGN, DCN, PODN, GPC3, TGFBR3, and PRELP* were all significantly downregulated in breast carcinoma, whereas *ASPN, ACAN, EPYC, and SDC1* were all found to be upregulated (Figure 1).

The 11 proteoglycans identified in our differential expression analysis were further scrutinized to determine their feasibility and possible utility as prognostic markers for breast carcinoma. Through comparison of basal mRNA expression levels of each of these proteoglycans with their expression levels in breast carcinoma, we determined that 10 of the identified proteoglycans were fit for further evaluation. *ACAN* was excluded from downstream analysis on account of its extremely low levels of expression in both healthy breast tissue, and breast carcinoma, which cast doubt on both its biological validity and reliability as a prognostic marker. From the 10 remaining proteoglycans, a novel machine learning model generated a genetic signature from a combination of 5 of these which was able to accurately predict the presence or absence of breast carcinoma. Prediction of carcinoma, which has a success rate greater than 99%, is based on the combinatorial upregulation or downregulation of *ASPN, PODN, SDC1, DCN, and PRELP* as described in Table 2. Interestingly, the trends of upregulation and downregulation associated with each of these respective proteoglycans align with overarching trends observed when investigating each of them individually, with upregulation of *ASPN* and *SDC1* (Figure 2A) and downregulation of *PODN, PRELP*, and *DCN* (Figure 2B) helping to predict disease. We must note, however, that this may not always be the case, and we cannot conclude it to be the case for other proteoglycans not named in this model.

4 | Machine Learning Model and Differentiation of Tumor Stages

While the machine learning model that was employed to construct the combinatorial proteoglycan signature of *SDC1, ASPN, DCN, PRELP*, and *PODN* to identify difference between normal breast tissue and cancerous tissue was able to do so with an accuracy of over 99%, this model struggled to make distinctions between samples of breast carcinoma when they were evaluated on the basis of diagnostic grade of disease. With an AUROC of 0.5035587 during training, the model was unable to differentiate between stages of disease with much more accuracy than flipping a coin. Therefore, we interrogated the TCGA data set to explore the possibilities of why the model could be so effective in preliminary diagnostic determination, but ineffective with a more granular task. We evaluated breast carcinoma samples that had been labeled within the TCGA data set as stage I, stage II, or stage III. We found that there was a significant difference in mRNA expression of all five proteoglycans used by the machine learning model compared with normal breast tissue samples.

We further discovered that within the breast carcinoma samples available through TCGA, the expression of our five proteoglycans of interest was not significantly different between stages of disease for four out of our five candidates. *SDC1* and *ASPN* mRNA were significantly upregulated in each stage of disease in samples of breast carcinoma graded as stages I–III, however no differences were present between different stages of disease (Figure 3A). While *DCN* and *PRELP* mRNA were significantly downregulated in each stage of breast carcinoma when compared with normal tissue samples, no differences were detected between stages of disease (Figure 3B). *PODN* expression was significantly different between stage I and stage II, and stage II and stage III breast carcinoma respectively, however, we did not determine a significant difference in *PODN* expression between stage I and stage III within the data set (Figure 3B). This suggests that the model's inability to differentiate disease stages could be due to the lack of perceptible differences in mRNA expression of the proteoglycans used. Therefore, it would be beneficial to return to this model as the TCGA repository of breast carcinoma samples continues to grow, and the robustness of the data set continues to increase.

5 | Syndecan-1

Syndecan-1 (*SDC1*) with its ~33 kDa protein core, is the first of its name amongst the syndecan family of four homologous type I transmembrane proteoglycans. Its structure is characterized

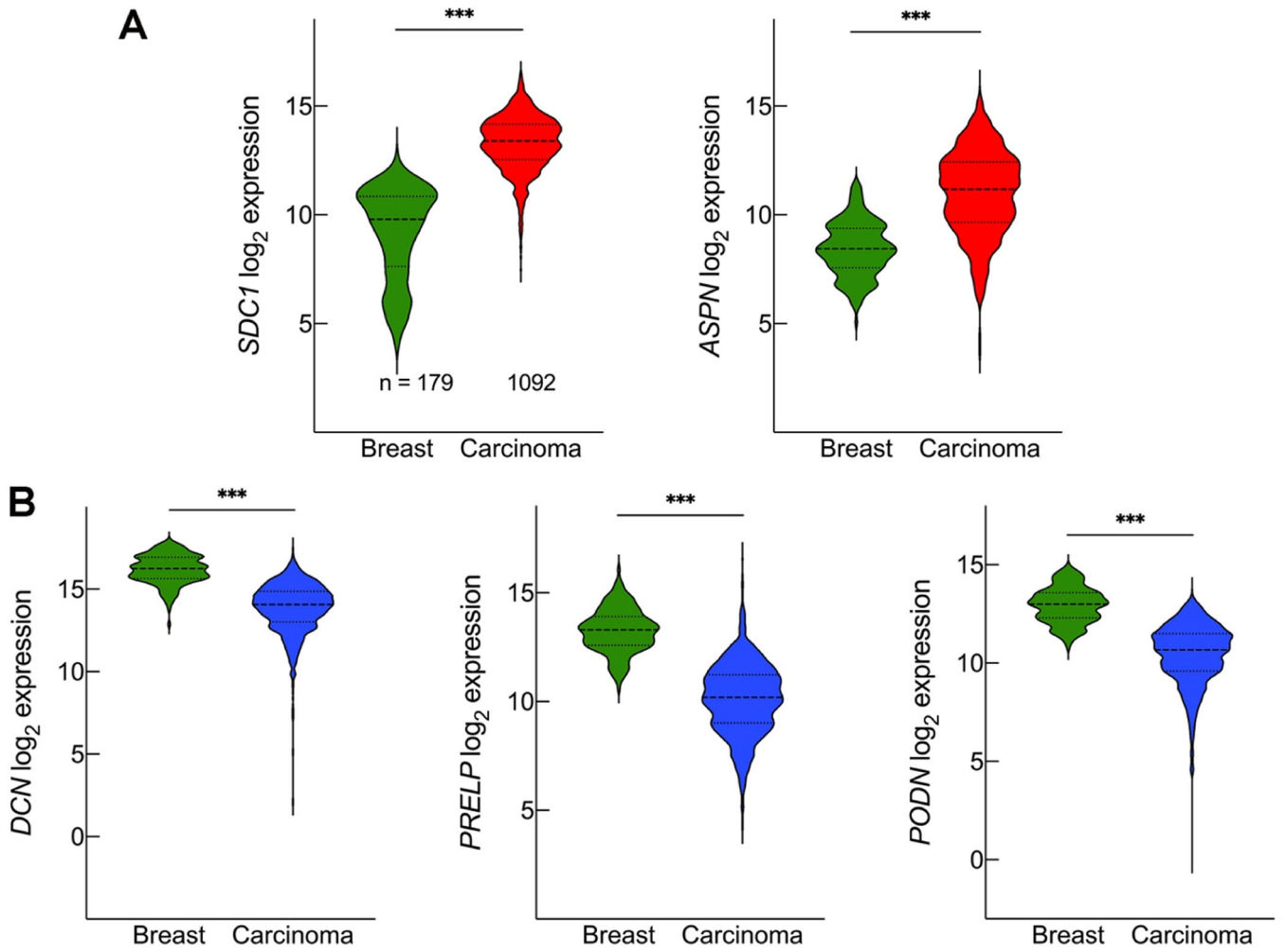

FIGURE 2 | Differential expression of machine learning identified proteoglycans in breast carcinoma. (A) *ASPN and SDC1* were found to be upregulated in breast carcinoma samples obtained from TCGA compared with normal tissue samples obtained from GTEx. (B) *PODN, PRELP*, and *DCN* were found to be downregulated in breast carcinoma samples obtained from TCGA compared with normal breast tissue samples from GTEx.

by an ectodomain with distal heparan sulfate (HS) chains and chondroitin sulfate (CS) chains located nearer to the plasma membrane [28]. It has a hydrophobic transmembrane domain and a C-terminal cytoplasmic domain containing two conserved domains interspersed by a variable region. SDC1 has been identified as having various roles in cell–cell and cell-matrix interactions, and within the context of breast cancer, its complex amalgam of effects have been shown to influence cell adhesion and migration, tumor growth and progression, tumor angiogenesis, and prognosis [5, 29, 30].

The ectodomain of SDC1 regulates matrix-dependent signaling in breast carcinoma through interaction with $\alpha_v\beta_3$ integrin, such that an increased presence of cell-surface SDC1 is associated with increased epithelial cell invasion during early tumorigenesis [31–33]. SDC1 has been implicated in multiple signaling pathways related to cell adhesion and migration, including the Wnt/FGF pathway. In multiple breast cancer cell types, SDC1 knockdown significantly inhibited various gene products of Wnt and Fibroblast Growth Factor (FGF) signaling, suggesting that SDC1 may mediate tumor cell migration through these pathways [34]. The PI3K/Akt pathway has also been implicated in SDC1 behavior, whereby cleavage of SDC1 was shown to enhance breast carcinoma cell proliferation via increased SUMOylation of Akt [35]. Acting as a membrane bound co-receptor for FGF Receptor, SDC1 activates FGF2 mediated MAPK signaling through induction of MAPK phosphorylation, ultimately leading to breast cancer cell proliferation. Unsurprisingly, *SDC1* knockdown inhibits MAPK signaling [36]. Additionally, *SDC1* plays a role in IL6/JAK/STAT3 signaling, such that *SDC1* silencing inhibits IL-6 signaling, suggesting that overexpression of SDC1 activates this important pathway for breast cancer cell proliferation and migration [37].

Despite the ever-growing arsenal of knowledge regarding the role of *SDC1* in breast cancer, it is not unilaterally agreed upon whether increased or decreased SDC1 expression indicates a more positive prognosis. Some studies have shown increased *SDC1* protein expression to be associated with worse patient outcomes and a more aggressive disease phenotype [38–40]. Others indicate the opposite, stating that decreased SDC1 expression is associated with poorer outcomes [37, 41]. However, more recent large scale, bioinformatic analyses of large-scale public datasets in triple-negative breast cancer has given support for the former assertion that high SDC1 expression is

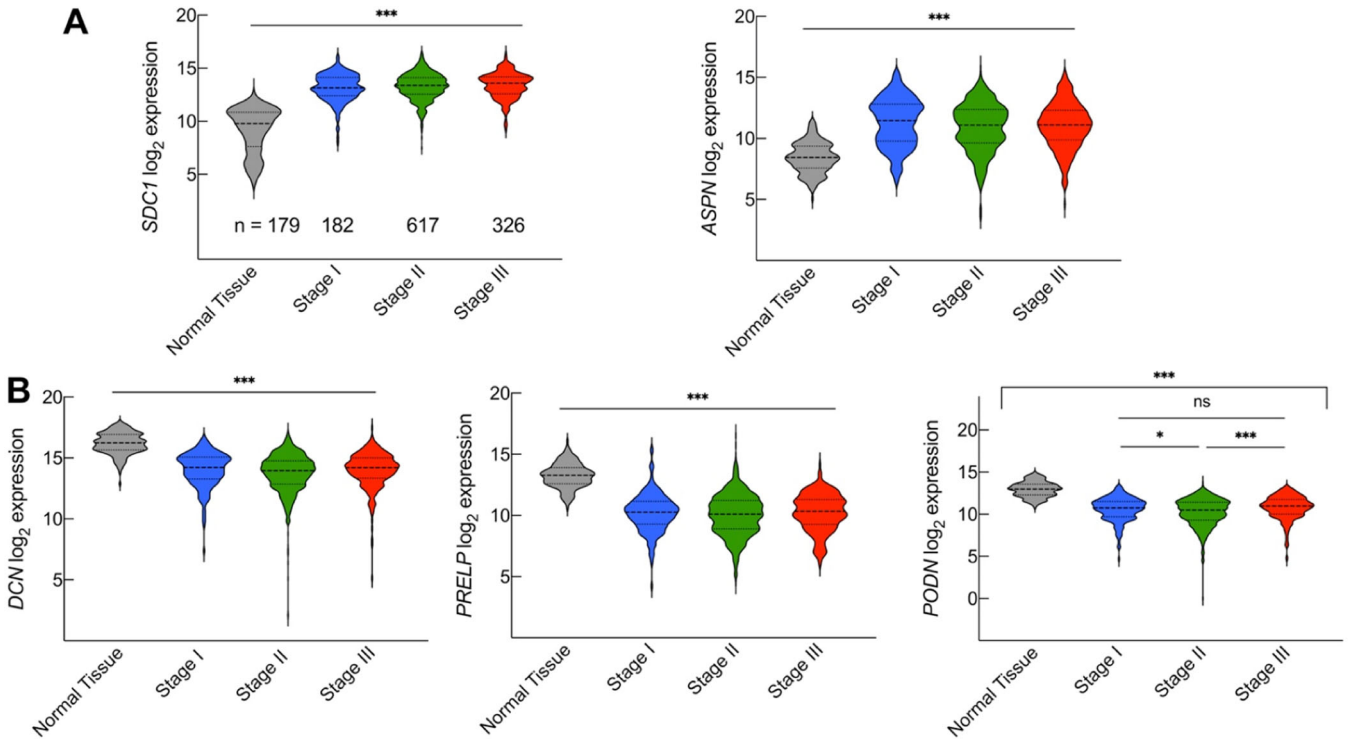

FIGURE 3 | Evaluation of differential expression of proteoglycans in breast carcinoma by stage. (A) *SDC1* and *ASPN* mRNA were found to be upregulated in all stages of breast carcinoma samples obtained from TCGA compared with healthy breast tissue samples. There were, however, no significant differences in expression of either of these mRNA transcripts between samples graded by stage of disease. (B) *DCN, PRELP*, and *PODN* mRNA were found to be downregulated in all stages of breast carcinoma samples obtained from TCGA compared with healthy breast tissue samples. When breast carcinoma samples were evaluated by stage of disease, there was no differential expression determined for *DCN or PRELP* mRNA, however, we identified differences in *PODN* expression between stage I and stage II carcinoma and between stage II and stage III carcinoma. *Significance is defined by $p < 0.05$. Furthermore, **significance is defined as $p < 0.01$ and ***significance is defined by $p < 0.001$.

associated with poorer rates of both overall survival and disease-free survival [42, 43]. These recent findings are corroborated in this study, in which we also propose a novel prognostic utility for *SDC1*.

6 | Asporin

Asporin, originally purified from human articular cartilage [44], is a class I SLRP [45, 46] as it is closely related to decorin and biglycan. In addition of containing the conserved N-terminal C-X_3-C-X-C-X_6-C pattern typical of decorin and biglycan [47], asporin harbors a unique N-terminal stretch of aspartic acid residues thus its eponym [44, 48] (Figure 4). Unlike decorin and biglycan, asporin contains no consensus glycosaminoglycan attachment sites (Ser-Gly) at its amino terminus. However, it is possible that the polyaspartate region due to its polyanionic nature would modulate the interaction of asporin with growth factors and collagen as shown for other SLRPs, with a function analogous to that of the glycosaminoglycan chains of decorin and biglycan, the closest family members [44]. Indeed, asporin competes with decorin in collagen binding and promotes osteoblast collagen mineralization [49]. Due to its ubiquitous expression, asporin has been implicated as an activator of invasion in schirrhous gastric carcinomas [50], as well as growth and migration of gastric cancers cells [51, 52]. Moreover, stromal-derived asporin is a biomarker associated with prostate cancer progression [53], bladder cancer where asporin levels correlate with the degree of malignancy [54]. Prostate tumor allografts in $Aspn^{-/-}$ mice show a decreased propensity toward pulmonary metastases [55] suggesting that asporin could promote metastatic progression. A recent report has convincingly corroborated these findings by discovering a reciprocal interplay between asporin and decorin, with asporin acting as a gastric cancer promoting gene and decorin as a tumor repressor [56]. The proposed mechanism of action involves and asporin/TGFβ interaction suggesting that preventing this interaction, together with overexpression of decorin would counteract the gastric cancer growth and invasion [56]. In line with these results, is the finding that asporin can repress gastric cancer apoptosis by activating LEF1-mediated gene transcription independent of β-catenin [57], suggesting that asporin could act as a prognostic marker in this malignancy [56]. Additional evidence for broad protumorigenic activity of asporin derives from its ability to reprogram gastric cancer cells to acquire resistance to oxidative stress [58], and its interaction with HER2 in promoting thyroid cancer metastases [59].

The multifaceted roles of asporin are challenging and somewhat controversial as asporin levels have also been linked to anti-tumorigenic activity [60, 61]. While asporin acts as an oncogene in pancreatic, colorectal, gastric, and prostate cancers, and some types of breast cancer, there are reports that asporin can act as a tumor suppressor gene in triple-negative breast cancer [62]. Intriguingly, high asporin expression associates with a significantly better relapse free survival rate in patients with

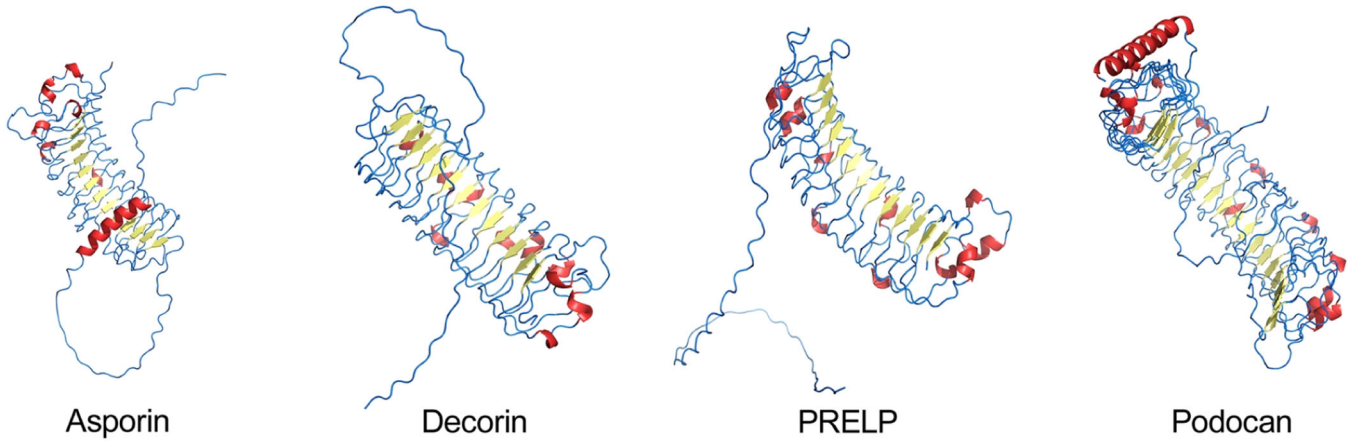

FIGURE 4 | Structure of the four SLRPs linked to the breast carcinoma signature. Using PyMol, published knowledge regarding proteoglycan structure, and the AlphaFold structure predictor driven by Google's DeepMind artificial intelligence tool, we created crystal structures for each of the four SLRPs included in our machine learning breast carcinoma signature. The primary structure of each SLRP is shown in blue, with secondary structures delineated by alternate colors: α helices are marked in red and yellow arrows are used to identify β-pleated sheets.

low-grade tumors but is significantly worse in breast cancer patients with stage 3 tumors [63]. Our results show in an unbiased way that asporin gene expression correlates with a pro-malignant role in breast cancer independently of the stage of the tumor (Figure 3A). If confirmed at the protein level, upregulated syndecan-1 and asporin protein levels could be therapeutically challenged by proteolysis targeting chimera (PROTAC) protein degraders [64]. This novel approach in cancer treatment harnesses the body's natural protein degradation machinery to target specific proteins for destruction. As heterobifunctional molecules, PROTACs function by recruiting and binding the protein of interest and E3 ubiquitin ligase, hence inducing ubiquitylation of the targeted protein and its subsequent degradation by the ubiquitin–proteasome system [64]. Syndecan-1 and asporin, as a transmembrane and secreted proteoglycan respectively, could represent ideal candidates for PROTAC therapy as exposed amino acidic domains could be easily identified as targets of this innovative therapeutic modality.

7 | Decorin

Decorin is the prototype member of Class I SLRP and is composed of 12 LRRs and a single glycosaminoglycan chain at the N-terminus [65, 66] (Figure 4). Abundantly found in multiple tissues throughout the body, decorin was named after its unique ability to interact with and "decorate" collagen fibrils to maintain, regulate and organize their spacing and structure [67]. This interplay with collagen is crucial for the tensile strength and elasticity of tissues, ensuring proper tissue architecture and homeostasis, and structural integrity of the ECM [68, 69]. The role of decorin in cancer has been the subject of several studies in the past 3 decades [70–72], starting with colon carcinoma [73–75], and then extending to several solid tumors with diverse histogenetic backgrounds [76–95]. Decorin was subsequently described as an active modulator of cell behavior and function through its interactions with different growth factors and its role as a pan-receptor tyrosine kinase (RTK) inhibitor [96–98]. Indeed, decorin deficiency promotes epithelial-mesenchymal transition (EMT) and colon cancer metastasis [75]

Decorin binds to the transforming growth factor β (TGFβ) and vascular endothelial growth factor (VEGF), sequestering them from their respective receptors and modulating their bioavailability in the ECM. This regulation is crucial for maintaining tissue homeostasis and preventing excessive growth factor signaling [99–103]. One of the most studied roles of decorin is its ability to interact with TGFβ [104, 105] and to block TGFβ/Smad downstream signaling [106, 107], which in general leads to suppression of fibrosis [78, 108, 109]. Decorin has also been involved in inhibiting angiogenesis [99, 110–120], and also in promoting angiogenesis in a mouse model of corneal injury [100]. Moreover, decorin is involved in regulating inflammation [121, 122], bone homeostasis [123], biomechanical properties of cartilage [124], mitophagy [111, 125–135], and can signal through modulation of intracellular Ca^{2+} [136, 137].

Decorin interactome has expanded significantly with the discovery of several RTKs that specifically bind to decorin with nanomolar affinity, including the epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGF-IR), and Met receptor (HGFR), as well as the vascular endothelial growth factor receptors 2 and 3 (VEGFR2, VEGFR3) [138–141]. By binding to these receptors, decorin interferes with their activation and downstream signaling pathways overall offsetting their ability to sustain growth of cancer cells and stimulate angiogenesis in the tumor stroma [30].

By interfering with VEGF signaling and disrupt endothelial cell interactions with ECM components, decorin reduces endothelial cell proliferation, a critical step in angiogenesis [117, 142], and induces autophagy in endothelial cells. Autophagy, a highly conserved process involving the degradation and recycling of cellular components [143, 144], is tightly regulated by decorin. The mechanism involves decorin-mediated inhibition of Akt/mTOR signaling pathway, leading to activation of autophagy-related genes and formation of autophagosomes [130, 132, 145, 146]. Overall, decorin exemplifies the complexity of ECM proteins in regulating cellular processes through interactions with RTKs, modulation of autophagy, and influence

on angiogenesis. Its diverse interactions with the ECM, growth factors, and immune mediators highlight its importance in normal physiological processes beyond its structural role.

Decorin affects the growth of various tumors [76, 77], and we further discovered that there is cooperative action of germline mutations in $Dcn^{-/-}$ and the tumor suppressor $p53^{-/-}$ that leads to an acceleration of lymphoma tumorigenesis [147]. Administration of recombinant decorin to these lymphoma cells derived from the doubly-mutant $Dcn^{-/-};p53^{-/-}$ mice significantly retarded their growth further, supporting a potential therapeutic role for decorin in tumor suppression. In an in vivo triple-negative orthotopic breast cancer model, we found that systemic administration of recombinant decorin modulated the differential expression of 374 genes within the stromal compartment of the tumor xenograft associated with immunomodulatory responses, cellular adhesion and tumor suppressive gene properties [148]. In inflammatory breast cancer (IBC) cells overexpression of DCN markedly decreased migration and invasion, and inhibited tumor growth and metastasis in IBC xenograft mouse models through inhibition of the EGFR/ERK signaling pathway and decorin-mediated autophagic degradation of E-cadherin [79]. Moreover, the inhibition of colon carcinoma growth and migration by decorin also involves modulation of E-cadherin levels [83]. The therapeutic benefits of systemic delivery of decorin to different in vitro and in vivo tumor models dictate the need for a deeper investigation of its the expression level as they could be associated with prognosis and survival rate estimate of cancer patients [149, 150].

However, when examining decorin expression in breast cancer tissue compared with normal breast tissue, studies have shown contrasting results depending on the specific subtype of breast cancer and the stage of the disease [88]. In normal breast tissue, decorin expression is typically well-maintained and plays a crucial role in regulating the ECM and growth factor signaling, as described earlier [151]. Decreased expression of decorin in breast cancer has been associated with poorer prognosis, increased tumor growth, and enhanced metastatic potential.

8 | PRELP

A member of the Class II SLRP genes is PRELP (Proline/arginine-Rich End Leucine-rich repeat Protein), also known as prolargin, and was originally cloned from human articular chondrocytes [152] (Figure 4). Its eponym is based on its unique N-terminal domain which, in contrast to asporin, harbors several basic amino acid residues such as proline, arginine and leucine [152]. The N-terminus domain of PRELP binds heparin and heparan sulfate; as fibroblasts interact with PRELP in an heparin-dependent manner, it has been proposed that PRELP can function as linker also between the ECM and cell surface proteoglycans [153], acting as an important regulator of cell adhesion [154]. Indeed the N-terminus of PRELP is involved binding to perlecan and collagen [155], potentially functioning as a basement membrane anchor [156].

An interesting biological role of PRELP is its ability to directly inhibit all complement pathways by binding C9 and thereby preventing the formation of the complement membrane attack complex [157]. Notably, the N-terminal basic domain of PRELP functions as a cell specific inhibitor of NF-κB signaling and impairs osteoclastogenesis [158]. Specifically, a recombinant form of the basic N-terminal region of PRELP reduces osteoclast number and activity in ovariectomized mice, underlying its role in skeletal remodeling [158]. Additional functions of PRELP include its role as a natural TGFβ antagonist and inhibitor of fibrosis [159], and an interaction with IGF-IR and low-affinity nerve growth factor receptor (p75NTR) which results in growth inhibition of A549 lung carcinoma cells [160]. A recent report utilizing $Prelp^{-/-}$ mice has shown that PRELP secreted by mural cells protects the blood brain barrier by promoting the integrity of endothelial cells [161].

There are only few reports of PRELP association with various cancer types, although the majority of independent studies point to a tumor suppressor role in ovarian cancer [162], oral squamous cell carcinomas [163] and colon cancer cells [164]. Moreover, there is a positive correlation between *PRELP* expression and survival in hepatocellular [165] and pancreatic carcinoma patients [166]. A variant of PRELP was found to be uniquely expressed in chronic lymphocytic leukemia cells [167], suggesting that there might be an organ- and tissue-specific bioactivity. PRELP is an endogenous inhibitor of bladder cancer initiation and progression [168], and by regulating cell-cell adhesion and EMT inhibits retinoblastoma progression [169]. Loss of *PRELP* expression in melanomas correlates with tumor escape and enhanced aggressiveness [170]. There is also evidence that PRELP inhibits colon cancer progression by suppressing EMT and angiogenesis [171]. In contrast to these studies, PRELP has been shown to promote EMT in colon cancer and to stimulate growth and invasion of colon cancer cells [172]. So, much more research needs to be done to clarify these contrasting results.

9 | Podocan

Podocan is a class V noncanonical SLRP that was originally identified from sclerotic glomerular lesions in experimental human immunodeficiency virus-associated nephropathy [173] (Figure 4). This SLRP has been implicated in the suppression of endothelial cell growth, proliferation, and migration, particularly in the kidney, smooth muscle, and adipose tissue. Functional analysis of podocan suggests that his proteoglycan contains N-linked oligosaccharides and that it binds to Type I collagen [174]. It has been shown that suppression of cell growth and migration by podocan is associated with changes in p21 and Rho activity [174]. Additionally, inhibition of podocan expression by MiR-3180-5p promotes human bladder smooth muscle cell proliferation [175]. Unsurprisingly, podocan is highly expressed in vascular smooth muscle cells after injury, and it has been shown that podocan-deficient mice demonstrate increased arterial lesion formation compared with their wild-type counterparts, identifying podocan as a negative regulator of smooth muscle cell proliferation [176]. Both in vivo and in vitro, podocan-deficient mice demonstrate increased smooth muscle cell proliferation through activation of the Wnt/β-catenin pathway [177]. Podocan modulation of smooth muscle cell proliferation and differentiation has been shown to be a direct result of podocan interacting with Wnt and enhancing

Wnt/b-catenin signaling [178]. Most recently, podocan has been shown to play a role in post-injury smooth muscle cell differentiation by binding with TGFβ1 thereby inhibiting its downstream signaling pathway [179].

Podocan has been characterized in pathological processes such as kidney diseases and recently colorectal cancer [180, 181]. Podocan was shown through micro-array analysis to be a significant biomarker for transplant glomerulopathy and have been previously shown to be significant to processes involving the glomerular basement membrane [173, 180]. It was also found to be correlated with diabetic nephropathy, as podocan mRNA levels negatively correlated with the urinary albumin-to-creatine ratio, a marker for glomerular injury in models of diabetic nephropathy [182]. In colorectal cancer, podocan has been identified as having location-dependent differential expression with qRT-PCR revealing decreased podocan mRNA expression in tumors compared with normal colon tissue in patient samples of left-sided colon cancer, but not right-sided colon cancer [181]. Podocan has not yet been implicated as a potential biomarker in any other cancers, and presently, no studies exist which explore podocan within the context of a transcriptomic analysis derived from a large-scale publicly available data set. This study is the first to identify a potential role of podocan as a prognostic marker in breast carcinoma.

10 | Serglycin

Serglycin (SRGN), initially discovered as a small chondroitin sulfate proteoglycan secreted by a rat yolk sac tumor, was shown to be highly enriched in Ser and Gly amino acids and to be associated with the cell surface [183]. Subsequent cloning of its protein core [184] and gene [185] showed a protein of ~10 kDa with a central region of ~50 amino acids composed of alternating Ser/Gly residues, hence its eponym [184] (Figure 5A). A number of subsequent studies have clearly shown that serglycin is the main carrier of heparin and is directly involved in packaging proteases in mast cell granules [186, 187] and regulating the maturation of mast cell granules [188, 189]. Serglycin is also expressed in embryonic stem cells [190], uterine decidua [191], neutrophils [192], myeloma cells [193, 194], and has been proposed to be a potential marker for acute myeloid leukemia [195]. Indeed, serglycin plays a critical role in the protein cargo loading of exosomes produced by multiple myeloma cells [196].

An interesting discovery was the identification of serglycin as a main product of endothelial cells where it was localized within intracellular vesicular compartments co-distributing with tissue plasminogen activator [197]. It was subsequently shown that serglycin is secreted in polarized human endothelial cells mainly in the apical region and is associated with chemokine secretion [198] and directly involved in the inflammatory response [199]. Indeed, serglycin is a major proteoglycan secreted by macrophages and is involved in regulating TNF-α secretion in response to pro-inflammatory stimuli [200]. Recently, serglycin has been implicated as a protumorigenic factor in various solid tumors including: head and neck carcinoma [201], colorectal cancer [202], hepatocellular carcinoma [203], lung adenocarcinoma [204], and glioblastomas [205, 206], including a role as a potential biomarker for glioblastoma progression [207]. Moreover, serglycin produced by gastric carcinoma cells promotes secretion of the pro-inflammatory interleukin 8 in cancer associated fibroblasts [208].

Regarding the role of serglycin in breast cancer, there are several recent scientific reports underlying and corroborating its role as a pro-tumorigenic and pro-metastatic factor [209]. An elegant study using genetic ablation of *Srgn* gene in a MMTV-PyMT-driven mouse breast carcinoma model has shown a complete suppression of lung metastases without affecting the growth of the primary tumors [210].

Serglycin is also highly expressed by infiltrating immune cells in breast cancer microenvironment [211], and is involved in epithelial-mesenchymal transition (EMT) evoked by TGFβ [211] as well as promoting a more aggressive phenotype in breast cancer [212, 213]. Mechanistically, serglycin may protect breast cancer cells from complement attack thereby supporting their survival and expansion [212]. Collectively, these data suggest that serglycin-dependent mediators may represent potential drug targets to prevent pulmonary metastases in patients with breast cancer.

Utilizing the online data exploration tool, Xena, developed by the University of California Santa Cruz, we analyzed serglycin (*SRGN*) mRNA expression in available breast carcinoma data from TCGA and GTEx. The data available to the public suggests a different conclusion than the one reported by the majority of experimental findings concerning serglycin expression on the mRNA level. These public data revealed that *SRGN* expression is lower in breast carcinoma compared with normal tissue. Additionally, *SRGN* levels decline as stage of disease progresses. Specific evaluation of infiltrating ductal carcinoma revealed decreased *SRGN* expression as stage of disease advances (Figure 5B–D).

There are at least two scientific reports corroborating our findings, showing lower levels of serglycin expression in breast carcinoma vis-à- normal tissue [211, 214]. Using the Tissue Immune Estimation Resource to explore TCGA breast carcinoma data showed lower *SRGN* levels in breast tumor tissue compared with normal tissue [214]. While this result is in opposition to the body of in vitro evidence reported elsewhere, separate analysis of breast cancer subtype data from TCGA and the OSLO2 study support the finding that *SRGN* expression is lower in tumor than normal breast tissue [211]. Furthermore, it has been shown that trends in *SRGN* expression vary between different subtypes of breast cancer based on public data and even amongst different breast cancer cell lines of variable aggressiveness studied in vitro [211, 213, 215]. Indeed, qPCR of *SRGN* expression in basal-like subtypes of variable phenotype, from epithelial to mesenchymal, revealed higher *SRGN* expression in mesenchymal subtypes compared with epithelial or partial epithelial cancer cell lines, however, single cell RNA sequencing (scRNAseq) of 18 patient samples of variable subtypes revealed very low *SRGN* expression specifically in cancer cells, but much higher expression shown in T-cells, dendritic cells, and mast cells [211]. Interestingly, SRGN immunogenically evokes IL-8/CXCR2 signaling to promote epithelial mesenchymal transition in breast cancer cells of less aggressive phenotype, to then have more *SRGN expression in* aggressive

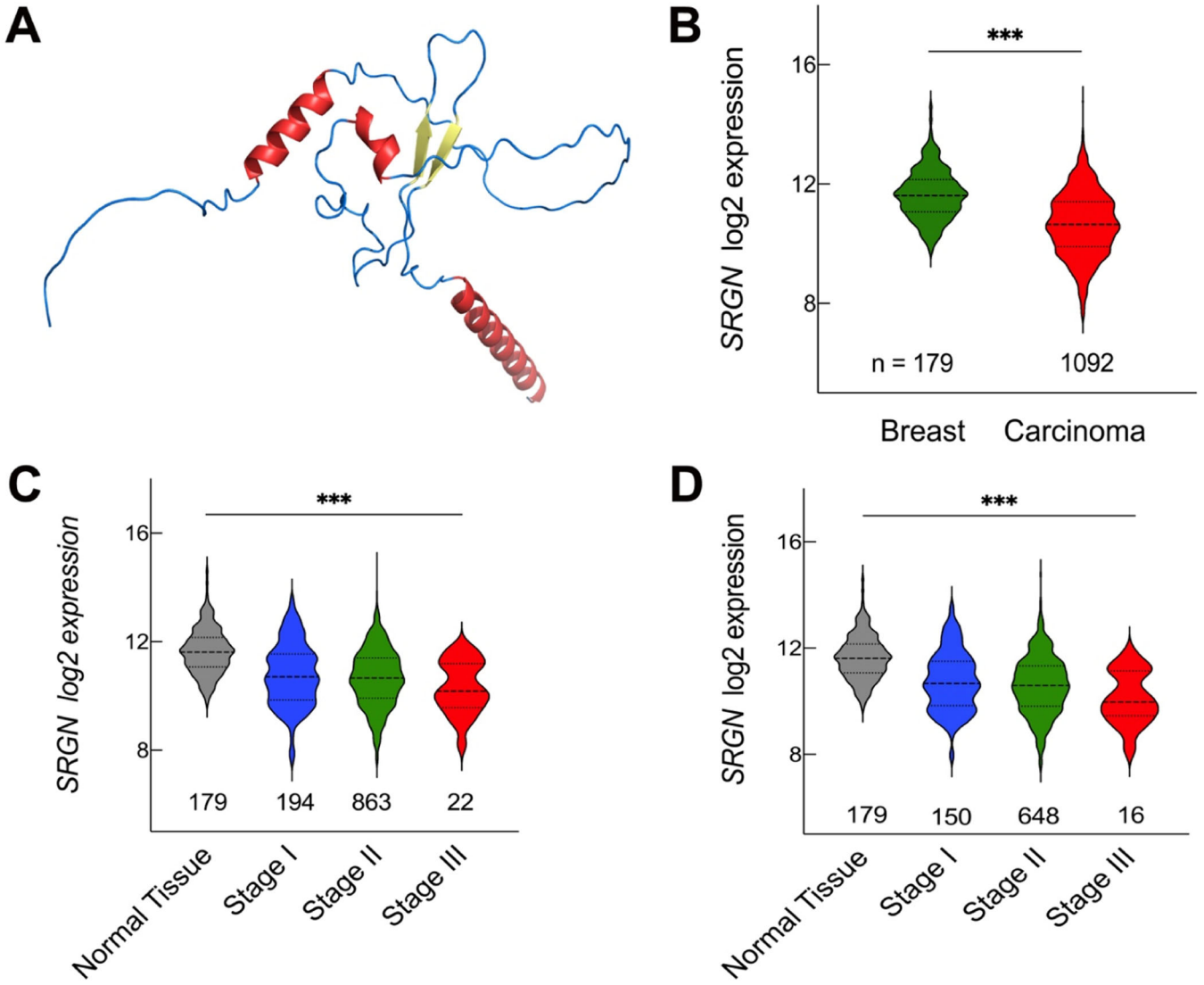

FIGURE 5 | Structure and under expression of *SRGN* in breast carcinoma malignancy. (A) Crystal structure of serglycin rendered using AlphaFold and PyMol. Primary structure is shown in blue, while α helices are shown in red and β-pleated sheets are shown with yellow arrows. (B) Breast carcinoma samples obtained from TCGA data demonstrate lower levels of *SRGN* mRNA expression compared with normal tissue samples obtained from GTEx. (C) TCGA samples categorized by diagnostic stage of disease demonstrate lower *SRGN* expression with disease progression. (D) Specifically within TCGA samples of infiltrating ductal carcinoma, relative *SRGN* levels decrease with disease progression. *** significance is defined by $p < 0.001$.

breast cancer cell-types [213]. Also interesting is immunostaining of aggressive breast tumor sections showing granular cytoplasmic localization of SRGN, suggesting localization of SRGN into secretory granules [215]. Additionally, Kaplan–Meyer analysis suggests a differential, time-based association of *SRGN* expression with overall survival, such that survival is lower in patients with low tumor *SRGN* expression up to a time point of about 3500 days, at which point, survival becomes lower for patients with high *SRGN* expression [211, 214]. This scRNAseq data along with the aforementioned in vitro findings invite questions of whether *SRGN* is truly contributing to survival through its effect on cancer cells themselves, or whether outside-in signaling in the tumor microenvironment ought to be a greater focus of its influence on disease outcomes. While large-scale datasets offer the statistical advantage of reducing bias, it is possible that the heterogeneous nature of bulk sequencing models may muddle our understanding of SRGN. Additional scRNAseq experimentation focused on *SRGN* in various breast-cancer subtypes may elucidate discrepancies between bioinformatic and in vitro approaches, since these analyses provide cell-type-specific answers that bulk RNA sequencing cannot accommodate. The variability of these results suggests that further exploration of the data directed toward disease progression, aggressiveness, and tumor microenvironment are necessary to reconcile conflicting results regarding serglycin in breast cancer. With careful consideration given to the body of experimental findings suggesting serglycin itself, or serglycin dependent mediators, to be protumorigenic, no study has thus far evaluated the publicly available data to validate these findings. The integration of proteoglycan expression profiles, their biological functions, and clinical outcomes has the potential to guide the development of

precision diagnostic and potentially therapeutic tools that may enhance the prognosis of not only breast cancer but also of other solid tumors.

11 | Conclusions

Analyzing publicly available data sets, we discovered a unique gene expression signature in breast cancer composed of five differentially modulated proteoglycans: Syndecan-1 and asporin (upregulated) and decorin, PRELP and podocan (downregulated). Although there are 45–50 proteoglycan encoding genes, our machine-learning analysis has revealed that only this small cohort or proteoglycans is meaningfully regulated in breast cancer. Notably, four out of five are SLRPs, primarily stromal derived constituents, with syndecan-1 being the only cell associated proteoglycan among the cohort. We should point out that syndecan-1 can be shed and its ectodomain is also found in the stroma [216–221]. We propose the five proteoglycan group as a new biomarker set for the diagnosis, prognosis and potential treatment of breast cancer patients.

We also performed a model literature review regarding the five proteoglycans included in the breast carcinoma gene expression signature and serglycin, a small chondroitin sulfate proteoglycan that has also been studied in the context of breast cancer. We found significant discrepancies when comparing published experimental results regarding protein and gene expression with TCGA data. While most sources in the literature have published experimentally determined findings suggesting that increased SRGN expression is associated with breast cancer development, analysis of TCGA data suggests the opposite association. This striking difference demonstrates the value of public information in efforts to validate experimentally determined data. Additionally, these results highlight the need to return to validation analyses in the future as public data sets like TCGA continue to include new patient samples and grow the statistical power of the data set. As these public datasets grow to include single cell, and eventually even single nucleus sequencing, the opportunity to validate biochemical experimental data will only grow. Great contributions can be made to our field by comparing published literature to public data for the rest of the proteoglycans that have not been assessed in the present study.

Through a therapeutic lens, our study could hold significant translational significance. For example, a potential approach would be to target the two upregulated proteoglycans, syndecan-1 and asporin, using gene editing technology such as CRIPR/Cas9. Another would be to target the protein cores using PROTACs, heterobifunctional molecules harboring two active domains and an inker that can lead to a removal specific unwanted protein. On the other hand, systemic delivery of recombinant proteoglycans or protein cores such as decorin, PRELP or podocan, could pave the way for proteoglycan-based therapies that, together with conventional chemotherapy, targeted therapies, or immunotherapy could enhance treatment efficacy and overcome drug resistance.

The continuously increasing statistical power of public datasets should not be overlooked as a tool for cost and time efficient analyses in the future. Especially as great strides are made in the field of artificial intelligence, and novel analytical methods like the machine learning model used in this project become increasingly refined, the proteoglycan research community is privy to exciting avenues for further investigation. The opportunity exists within the marriage of data and novel technology to drive inquiry towards discovering clinically relevant phenomena and developing innovative therapeutic options.

Author Contributions

Simone Buraschi: conceptualization, writing–original draft, writing–review and editing. **Gabriel Pascal:** conceptualization, writing–original draft, writing–review and editing, methodology, visualization. **Federico Liberatore:** writing–original draft, writing–review and editing, methodology. **Renato V. Iozzo:** conceptualization, funding acquisition, writing–original draft, writing–review and editing.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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