

Clinical Group

# International Journal of Clinical Endocrinology and Metabolism

DOI: <http://dx.doi.org/10.17352/ijcem>

ISSN: 2640-7582

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**Dates: Received:** 29 July, 2017; **Accepted:** 19 September, 2017; **Published:** 19 September, 2017

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## Research Article

# Protein S100A8/A9: A Potential New Biomarker for Pancreatic Diseases

## Abstract

**Objectives:** S100A8/A9 expression has been linked to carcinogenesis and inflammation. We hypothesized that S100A8/A9 protein serum levels are a useful stratification marker for patients with pancreatic ductal adenocarcinoma (PDAC), intraductal papillary mucinous neoplasm (IPMN) or chronic pancreatitis (CP).

**Methods:** S100A8/A9 serum levels were analysed in PDAC, CP and IPMN patients and compared to S100A8/A9 healthy donor controls (HD) using ELISA. S100A8/A9 levels and clinical data were statistically analysed.

**Results:** Out of 134 patients included, 84 were diagnosed with PDAC (46%), 30 patients with IPMN (16%) and 20 patients with CP (11%), 50 patients were HD (27%). When compared to HD (343.3 ng/ml), S100A8/A9 serum concentration was elevated in PDAC (402.0 ng/ml,  $p = 0.001$ ) and CP patients (426.51 ng/ml  $p < 0.001$ ). Also, S100A8/A9 levels were elevated in PDAC compared to IPMN group (369.0 ng/ml  $p = 0.026$ ) and in CP compared to IPMN group ( $p = 0.001$ ). A multivariate model including age, gender, leukocyte levels, C-reactive protein (CrP), S100A8/A9 and CA 19-9 concentrations reported a diagnostic sensitivity of 74.8%.

**Conclusion:** S100A8/A9 serum levels are increased in patients with PDAC, CP, and IPMN and might be useful to distinguish malignant and inflammatory diseases from normal and non-malignant pathological conditions.

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal cancers worldwide and is still substantiated by insufficient diagnostic tools and therapeutic options. The overall 5-year survival rate among PDAC patients is less than 5%, which is partially due to an almost symptomless progression. Today, PDAC is ranked as the 4<sup>th</sup> leading cause of cancer related death worldwide.

The S100 protein family is the largest subgroup of the Ca<sup>2+</sup>-binding EF-hand (helix E-loop-helix F) proteins [1]. First being identified by Moore in 1965 [2], S100 proteins are expressed in a wide range of tissues featuring multiple cellular functions such as contraction, motility, cell growth, differentiation, and secretion [1,3]. As a member of the S100 family, S100A8/A9 is composed of two subunits, S100A8 and S100A9, both forming heterogenous multimers. The S100A8/A9 protein expression is primarily found in cells of the innate immune response [4-6], as well as in inflammatory endothelial and epithelial cells [7,8]. Several autoimmune diseases are associated with S100A8/A9 expression such as rheumatoid arthritis, cystic fibrosis, or inflammatory bowel disease [5,9,10].

Initially, the S100A8/A9 protein has been described in neutrophils and macrophages and was shown to be involved in the regulation of innate immunity and inflammation [11]. Known S100A8/A9 functions include regulation of phagocyte transmigration and extravasation [9-13], arachidonic acid transport in neutrophils [14], regulation of the NADPH oxidase complex [10,15,16] and NO transport [17]. Consequently, it was identified in serum of patients with acute pancreatitis [18].

More recently, an association of S100A8/A9 protein expression with adenocarcinoma in human has emerged [19-21]. Immunohistochemical investigations have shown that the protein is overexpressed in gastric [22,23], ovarian [24], colorectal [25-27], thyroid [28], bladder [29], hepatocellular [30], prostate cancer [31] and invasive ductal carcinomas of the breast [32,33]. In these tumors, elevated S100A8/A9 expression was correlated with poor differentiation or prognosis, respectively. In patients with ovarian carcinomas, S100A8/A9 was found to be enriched in cystic fluid and serum [24]. Consequently, S100A8/A9 has been examined in regard of its suitability as a possible biomarker for cancer diagnosis. Genomic profiling studies revealed overexpression

of the protein in pancreatic cancer tissue, microdissections, pancreatic cyst fluid, and pancreatic juice [34–39]. To the best of our knowledge, sera of patients with neoplastic or inflammatory pancreatic lesions have not been screened for S100A8/A9 so far. Therefore, this study investigates S100A8/A9 protein serum levels in PDAC patients, intraductal papillary mucinous neoplasia (IPMN) patients and chronic pancreatitis (CP) patients in comparison to serum levels from healthy donors (HD) in order to evaluate its role as a potential biomarker.

## Materials and Methods

### Patient samples

Patient serum samples were obtained before surgery with approval of the institutional review board and after informed written consent. In total, 84 Patients were diagnosed with PDAC, 30 patients with IPMN or 20 patients CP (n=20) as well as 50 HD were included. Samples were immediately processed and stored at  $-80^{\circ}\text{C}$ . Serum marker C-reactive protein (CrP), Carbohydrate-Antigen 19-9 (CA 19-9) and leukocyte serum levels were determined before surgery. Patients with neoadjuvant chemo - or radiotherapy were excluded.

All patients were treated at the Department of General, Visceral, and Thoracic Surgery, University Medical Center Hamburg-Eppendorf between 2008 and 2013. Tissue diagnosis was reviewed by an experienced hepato-pancreatico-biliary pathologist.

### S100A8/A9 ELISA

S100A8/A9 (serum) protein levels were measured using Calprotectin Enzyme Linked Immunosorbent Assay (ELISA) kit (Hycultec; Beutelsbach, Germany) in accordance to the manufacturer's protocol. The Tracer was incubated overnight at  $4^{\circ}\text{C}$ .

### Statistical analysis

Metric variables were tested for normality via Kolmogorov-Smirnov and Shapiro-Wilk tests. Their distributions were also assessed via histograms and boxplots. Means, medians and 1<sup>st</sup> and 3<sup>rd</sup> quartiles (interquartile range; IQR) of metric variables were reported. The variables CrP and CA 19-9, which displayed heavily right-skewed distributions, were ln-transformed ( $\ln[x+1]$ ) prior to further analyses. The means of variable S100A8/A9 were compared across diagnostic groups using student's t-test. Variables CA 19-9, CrP and leukocytes had about 27%, 8% and 6% missing values, respectively. Multiple imputations were therefore performed using the monotone method for data having a monotone pattern of missing and the Markov chain Monte Carlo method for data with non-monotone pattern of missing, thus yielding 10 imputed data sets. Diagnosis, as a dependent nominally scaled variable consisting of 3 categories (0=PDAC 1=IPMN and 2=CP), was then subjected to multinomial logistic regression modelling, using the variables  $\ln(\text{CA } 19-9+1)$ , S100A8/A9,  $\ln(\text{CrP}+1)$ , leukocytes, age and gender of the 10 imputed data sets as independent variables (covariates). All independent variables were tested in univariate models as well as in multivariate models. The

multivariate approach started with an initial model containing all main effect terms of the independent variables plus all their two-way interaction terms. Non-significant terms were then removed, based on the p-values of likelihood-ratio tests ( $\alpha = 0.05$ ), following a stepwise hierarchical backward elimination procedure sensu Kleinbaum & Klein [40]. However, the main effect terms of all independent variables were kept in the final multivariate model, even if they were not significant. Model-predicted diagnosis was cross-tabulated versus observed diagnosis for comparison of models and Nagelkerke's pseudo  $R^2$  was computed to indicate strength of association and model fit. All statistical analyses were done using SPSS version 22.

## Results

Patient demographics are summarized in tables 1,2. A total of 184 individuals were included in the study. Of these, 81 males (44%) and 90 females (49%). In 13 patients (7%) no gender information was available. Mean age at operation was 66.0 years, with median age of 67.0 years and a range between 40 to 83 years of age. Out of 134 patients, 84 were histopathologically diagnosed with PDAC (63%), 30 patients featured IPMN (22%), 20 patients had CP (15%). Fifty healthy controls (HD) were included.

In the PDAC group, the 30-day mortality rate was 4.8% (4/84). Median overall survival in the PDAC group (n=84) was 6.9 months (31 to 1189 days). Most tumors showed moderate (n=54) or poor (n=21) histological differentiation. Only a minority (n=2) featured a well-differentiated histological phenotype. M-status was available for 21 patients; 14 patients featured M1 status and 7 patients M0 status. 24/84 patients were treated with a palliative approach only, due to advanced stage of disease. For seven PDAC patients, no histological data were available.

In 73% (61/84) of the investigated PDAC patients, the pT

**Table 1:** Serum parameter for patients resected for PDAC, CP, IPMN, and healthy donors PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary mucinous neoplasm of the pancreas; CP, chronic pancreatitis; CA 19-9, carbohydrate antigen 19-9.

Parameter	PDAC n= 84	IPMN n= 30	CP n= 20	HD n= 50
CA 19-9 (U/ml)				
n	69	18	11	n/a
Median (1 <sup>st</sup> & 3 <sup>rd</sup> quartile)	311.6 (44.0 & 1182.5)	11.2 (4.0 & 35.9)	11.2 (8.60 & 83.7)	
Mean	1452.3	20.8	52.5	
CrP (mg/l)				
n	76	30	17	n/a
Median (1 <sup>st</sup> & 3 <sup>rd</sup> quartile)	6.5 (0 & 18)	0 (0 & 6)	0 (0 & 19)	
Mean	19.2	6.3	34.3	
S100A8/A9 (ng/ml)				
n	84	30	20	50
Median (1 <sup>st</sup> & 3 <sup>rd</sup> quartile)	428.5 (351.6 & 471.7)	380.1 (342.2 & 414.2)	430.1 (397.9 & 470.1)	361.5 (266.1 & 417.8)
Mean	402.0	369.0	426.5	343.3

stage was recorded and the majority of the PDAC patients featured a pT3 stage tumor (85%). Lymph node metastases were present in 81% (50/62) of PDAC patients. A positive resection and circumferential surgical margin status was present in 59% (33/56). None of the PDAC patients received neo-adjuvant chemo-/radiotherapy.

CA19-9 serum concentrations are shown in table 1. CA19-9 data were heavily right-skewed. In 77% (53/69) of the PDAC patients, pre-operative serum CA19-9 values were increased (> 37 U/mL; (Table 1). Median CA 19-9 was 311.60 U/mL (1<sup>st</sup> and 3<sup>rd</sup> quartile: 40 and 1182.5 U/mL, respectively). 15 of the 84 (18%) PDAC patients did not feature pre-operative CA 19-9 data.

In the IPMN patient group, median pre-operative CA 19-9 levels were 11.15 U/mL (3.98 and 35.90 U/mL). In total, 39% (7/18) of IPMN patients had increased pre-operative CA 19-9 levels.

In the CP patients group, CA 19-9 data were available for 55% (11/20) of the CP patients with a median value of 11.20 U/ml (8.60 and 83.70 U/mL).

In 184 individual serum probes, we analyzed and quantified S100A8/A9 protein levels using ELISA. S100A8/A9 data were fairly normally distributed. Mean values for serum levels of S100A8/A9 were 402.02 (1<sup>st</sup> and 3<sup>rd</sup> quartile: 351.55 and 471.69, respectively) ng/mL in PDAC patients, 369.00 (342.23 and 414.16) ng/mL in IPMN patients, 426.51 (397.91 and 470.10) ng/mL in CP patients and 343.32 (266.09 and 417.76) in healthy donors, respectively (Table 1, Figure 1).

Interestingly, we found significantly increased S100A8/A9 protein serum levels in PDAC and CP patients, compared to IPMN patients and HD ( $p < 0.01$ ;  $P=0.026$  for PDAC vs. IPMN; table 3).

No difference was detected in both PDAC and CP patient serum levels. Also, there was no significant difference between HD and IPMN S100A8/A9 serum levels (Table 1,3).

Additionally, we investigated a possible relation between serum S100A8/A9 protein levels and histological grading, TNM-staging, survival time, serum CA 19-9 levels, leukocytes and CrP as well as patients' age and gender.

We found that leukocytes and CrP serum levels correlate with S100A8/A9 protein serum levels on a statistically significant level ( $p < 0.01$ , data not shown).

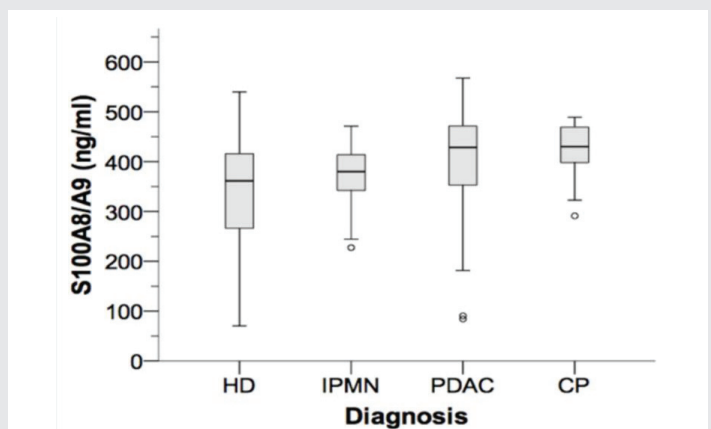
However, no statistically significant difference was found between well (G1-G2) and poorly (G3) differentiated cancers as well as between T3 and T4 staging and lymph node status.

Statistical analyses and correlation of S100A8/A9 protein levels with survival time, CA 19-9, patients' age or gender revealed no correlation at all.

To determine whether S100A8/A9 protein levels might be suitable as a possible stratification marker for pancreatic cancer, we chose a panel of four serum markers (Table 4).

**Table 2:** Staging parameters of PDAC patients. n, total number of patients with available data <sup>a</sup> Staging based on AJCC TNM Staging System, 7th Edition, 2010.

Gender	n=
Male (%)	39 (46.4)
Female (%)	45 (53.6)
missing	0
Age, y	
Median (range)	67 (46-83)
Mean	66
missing	0
Staging parameters <sup>a</sup>	
Differentiation	n=77
G0	0
G1	2
G2	54
G3	21
R (Resection margin)	n= 56
0	23
1	28
2	5
T (Tumor size)	n= 61
0	0
1	1
2	0
3	52
4	8
N (Lymph node status)	n= 62
0	12
1	50



**Figure 1:** S100A8/A9 serum levels. S100A8/A9 distribution in sera of healthy donors (HD), patients with intraductal papillary mucinous neoplasia (IPMN), pancreatic ductal adenocarcinoma (PDAC), and chronic pancreatitis (CP).

A multivariate multinomial logistic regression model was developed using leukocytes,  $\ln(\text{CrP} + 1)$ ,  $\ln(\text{CA19-9} + 1)$  and S100A8/A9 serum levels as covariates combined with the variables gender and age.

The final multivariate model after removal of redundant

variables yielded 74.8% correctly classified diagnoses. In comparison, a univariate model based on  $\ln(\text{CA19-9} + 1)$  alone yielded 61.3% correctly classified cases (Table 4). Nagelkerke's pseudo- $R^2$  indicated that only the multivariate model fits the data relatively well.

## Discussion

The current study reports for the first time serum protein levels of S100A8/A9 in PDAC and IPMN patients. Members of the S100 protein family are frequently up regulated in various types of autoimmune diseases such as rheumatoid arthritis, psoriasis or inflammatory bowel disease, but also in numerous cancer types [5,9,10,33].

**Table 3:** significance levels of S100A8/A9 serum levels in different patient cohorts (t-test) Highly significant group comparisons are marked with \*. PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary mucinous neoplasm of the pancreas; CP, chronic pancreatitis; HD, healthy donors.

Test	p =
PDAC vs. HD*	0.001
HD vs. IPMN	0.158
HD vs. CP*	0.000
PDAC vs. CP	0.076
PDAC vs. IPMN	0.030
CP vs. IPMN*	0.001

**Table 4:** Number of observed diagnoses vs. diagnoses estimated by multinomial logistic regression modelling (means of analyses of 10 imputed data sets). All independent variables were tested in univariate models and in a multivariate model. The multivariate approach yields a 74.8% correct classifications.

Model	Model-predicted diagnosis	Observed diagnosis			% correct classifications	Pseudo $R^2$ (Nagelkerke) <sup>a)</sup>
		PDAC	IPMN	CP		
Ln (CA19-9+1)	PDAC	76.7	24.6	18.5	61.3	0.162
	IPMN	7.3	5.4	1.5		
	CP	0	0	0		
S100A8A9	PDAC	82	30	20	61.2	0.053
	IPMN	2	0	0		
	CP	0	0	0		
Ln (CrP+1)	PDAC	82.9	30	20	61.9	0.087
	IPMN	1.1	0	0		
	CP	0	0	0		
Leukocytes	PDAC	83.7	29.3	20	62.5	0.037
	IPMN	0	0	0		
	CP	0.3	0.7	0		
Age	PDAC	83	29	15	65.7	0.132
	IPMN	0	0	0		
	CP	1	1	5		
Gender	PDAC	84	30	20	62.7	0.105
	IPMN	0	0	0		
	CP	0	0	0		
Multivariate model of variables above incl. interactions	PDAC	75.2	15	6.4	74.8	0.570
	IPMN	6.5	14.1	2.7		
	CP	2.3	0.9	10.9		

A plethora of various proteomic profiling studies on potentially new serum markers for PDAC diagnosis were performed within the last years [41-46]. Remarkably, various studies already identified the pro-inflammatory S100A8/A9 protein as overexpressed in PDAC tumor tissue [34-37]. Moreover, it was previously shown that human pancreatic cancer cell lines Capan1, Panc-1, MiaPaCa2, and BxPC3 express high levels of S100A8/A9 [47].

In order to evaluate the diagnostic value of S100A8/A9 in pancreatic tumors and as an easy to obtain resource in clinical practice, patient serum samples were analysed. Elevated protein levels in PDAC sera as well as in sera obtained from patients diagnosed solely with CP were detected. While there was no significant difference in S100A8/A9 between PDAC and CP, S100A8/A9 levels were elevated in PDAC, compared to IPMN patients and HD.

Chen et al. previously found an overexpression of S100A8/A9 protein levels in pancreatic main duct fluid and reported that its concentration correlates with median survival [34]. In the present study S100A8/A9 serum level was not associated with outcome. Statistically significant correlations existed between S100A8/A9 protein levels and CrP as well as leukocyte levels, however, this is not surprising since S100A8/A9 protein is known to be an inflammatory-linked protein.

In colorectal cancer, Kim et al. identified S100A8/A9 protein plasma levels to be more specific and sensitive compared to the established tumor marker carcinoembryonic antigen CEA [26]. Also, in prostate cancer, Hermani et al. reported serum levels of S100A9 to be more sensitive than PSA when discriminating between prostate cancer and benign prostate hyperplasia [31]. Likewise, S100A8 and S100A9 serum levels were recently identified as potential biomarkers for renal cell cancer early-detection [48].

Our multivariate multinomial logistic regression model (containing the biomarkers leukocytes, CrP, CA 19-9 and S100A8/A9 protein levels, gender and age), performed better in estimating different histologic subtypes of pancreatic lesions than any univariate model, yielding about 75% correctly estimated diagnoses. In contrast, the CA 19-9-based model correctly classified only about 61% of the diagnoses.

The S100A8/A9 protein has been identified to be a potent chemoattractant for myeloid-derived suppressor cells (MDSC) [49-53]. While MDSC seem to be the main source of S100A8/A9 protein in cancer patients, it remains unclear to which degree malignant cells express the S100A8/A9 protein. Immunohistochemical studies of pancreatic cancer tissue identified the S100A8/A9 protein as exclusively expressed in myeloid cells infiltrating the tumor-stroma [36], although several pancreatic cancer cell lines are already known to express S100A8/A9 [47,54,55].

In conclusion, our study identified increased S100A8/A9 expression levels in patients suffering from PDAC, CP and IPMN. Also, the correlation of S100A8/A9 protein serum levels with commonly available clinical data revealed that it could help stratification to distinguish patients with malignant and/



or inflammatory disease from normal and non-malignant pathological conditions. The combined quantification of S100A8/A9 and CA-19-9 serum levels in patients points to a higher sensitivity for diagnosis. The latter, regarding the crux of late pancreatic cancer diagnosis, might be another important and necessary step towards early cancer detection.

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