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The Prognostic Value of Plasma Small Extracellular Vesicles' Phenotype in Patients With Gastrointestinal Stromal Tumor

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Abstract. *Background/Aim: For patients with local gastrointestinal stromal tumor (GIST), risk stratification is used to assess the prognosis and identify patients to offer adjuvant treatment. For patients with advanced or metastatic GIST, no such risk stratification exists. This study aimed to investigate the prognostic value of 31 different plasma small extracellular vesicles' (SEVs) surface proteins in GIST patients. Materials and Methods: GIST patients from the two sarcoma centers in Denmark were included. Patients were divided into three groups; group 1: patients undergoing radical surgery; group 2: patients with local, locally advanced, or metastatic GIST; and group 3: patients without evidence of disease after radical surgery. Protein microarray technology was used for the analysis of plasma SEVs. The median plasma SEV marker level was used when comparing groups of patients. The primary endpoint was the progression of GIST. Iterative statistical modeling was used to identify a SEV marker profile/model with a prognostic value. Results: A total of 157 patients were included, with a median follow-up time of 2.05 years. In group 2, a high level of carcinoembryonic antigen (CEA) and a low level of glucose transporter 1 (GLUT-1) were found to be poor prognostic factors [univariate analysis; GLUT-1: hazard ratio (HR)=0.47, 95% confidence interval (CI)=0.22-0.98; CEA: HR=2.12, 95%CI=1.02-4.44]. Composing a model consisting of CEA and GLUT-1 adjusted for age at inclusion was found to have a prognostic value (HR=4.93, 95%CI=2.30-10.57, p<0.0001). Conclusion: Plasma SEVs in GIST showed that CEA and GLUT-1 might be of prognostic value. However, external validation is needed.*

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Key Words: Small extracellular vesicles, gastrointestinal stromal tumor, biomarker, EV array.

Gastrointestinal stromal tumor (GIST) is a mesenchymal tumor with an annual incidence of 10-15 per million inhabitants (1). The primary treatment for GIST patients is surgical resection and adjuvant treatment with imatinib, a tyrosine kinase inhibitor (TKI), for most patients (2, 3). GIST cells often harbor a mutation located in the tyrosine-protein kinase (*KIT*) or platelet-derived growth factor receptor A (*PDGFRA*) genes (4). After surgery, well-established risk stratification systems based on tumor size, mitotic rate, location (5), and surgery-related factors are prognostic with regard to the risk of relapse. The risk stratification is used to identify patients with local GIST eligible for adjuvant treatment (3). The first-line treatment for patients with recurrent or metastatic GIST is imatinib, followed by other TKIs at progression (2, 3). However, there is no risk stratification for patients with advanced or metastatic GIST. Furthermore, no soluble biomarkers exist for GIST that can monitor disease activity, help clinical decision-making, and identify patients with poor prognosis.

One interesting biomarker in oncology is small extracellular vesicles (SEVs). SEVs, often termed exosomes, are lipid bilayers containing mRNA, proteins, DNA fragments, and surface proteins reflecting the cells from which they arise (6)

and are released into several different types of fluid such as blood (7), bronchoalveolar lavage fluid (8), ascites (9), and cerebrospinal fluid (10). SEVs are identified by surface proteins expressed independently of the cell of origin, such as tetraspanins (CD63, CD9, and CD81) (11) (Figure 1).

It is suggested that SEVs are responsible for removing excess components from the cells and intercellular communication (6). In cancer, SEVs are proposed to influence tumor growth, microenvironment, resistance to oncological treatment (12), immune suppression, and promote tumor cell invasion and metastasis (13). Possible clinical implications of SEVs are diagnostics, prognostics, and assessing the treatment effect (12).

One of the only studies of SEVs in GIST patients was reported by Atay *et al*. in 2018 (7) and showed that patients with GIST had twice as many SEVs in the blood compared to age-matched healthy controls. Furthermore, compared to primary localized GIST, a significantly higher number of SEVs was found in patients with metastatic disease. However, the surface composition of the individual SEVs in GIST patients has not been described.

This study aimed to investigate the surface composition (phenotype) of the individual plasma SEVs and the prognostic value of SEV surface markers in patients with GIST.

Materials and Methods

This is a prospective, non-randomized, non-interventional, explorative study investigating the prognostic value of plasma SEV phenotypes in patients with GIST.

The study protocol was approved by the Regional Ethics Committee (H-18029854) and the Head of the Knowledge Centre on Data Protection Compliance (P-2019-706). The study was performed with the Good Clinical Practice standard, according to the latest revised Helsinki declaration, and according to national laws. All patients provided signed informed consent before inclusion.

Patients. Patients were included at the Department of Oncology at Herlev & Gentofte Hospital, Department of Oncology at Aarhus University Hospital, and Department of Surgery and Transplantation at Rigshospitalet from January 2019 to December 2021. Patients planned for surgery for local disease with a GIST of ≥2 cm had blood samples collected pre-operative and one day post-operative. Patients diagnosed with GIST were included independent of disease or treatment status in the oncological departments with the following exceptions: patients who stopped adjuvant treatment more than two years ago were excluded, only patients starting on adjuvant treatment or having a maximum of six months left of the adjuvant treatment were enrolled. Blood samples were collected at inclusion and synchronized with every control scan, usually every third month.

The patients were allocated into three groups (Figure 2). Group 1 included patients undergoing radical surgery, group 2 included patients with local, locally advanced, or metastatic disease, and group 3 included patients with no sign of disease (patients in adjuvant treatment after radical surgery or in control after ended adjuvant treatment). Group 1A included the pre-operative blood samples from patients in group 1, and group 1B included the post-operative blood samples in group 1.

Blood sampling. Blood was collected from the patients in 3.5 ml sodium citrate tubes. All blood samples were handled by the Danish CancerBiobank, Bio- and GenomeBank, Denmark. The maximum time from blood sampling to centrifugation was 4 h; subsequently, the time to storage was 1 to 2 h. Plasma was isolated through centrifugation at 2,000 or 2,500 g for 10 min. After centrifugation, plasma was transferred to another tube and mixed lightly. The plasma was stored at –80˚C until use.

EV array. The analysis for SEVs was performed using the extracellular vesicle (EV) array, based on protein microarray technology (14). The method is used to determine the phenotype of unpurified plasma SEVs or other EVs. In this study, the phenotype is defined as the protein composition of the individual SEV.

Production of antibody microarray. The antibody microarrays were produced on epoxy-coated slides (75.6×25.0 mm; SCHOTT Nexterion), and the printing of the antibodies was performed with a sciFLEXARRAYER S12 micro-array printer installed with a piezo dispense capillary (PDC) size 60 with coating type 3 (Scienion AG, Berlin, Germany). Printing buffer consisted of 50 mM trehalose in phosphate-buffered saline (PBS) throughout the experiment. As positive controls (Figure 2), 10 or 20 μg/ml biotinylated goat anti-mouse IgG (H+L) antibody (Novus Biologicals, Centennial, CO, USA) was printed, and a printing buffer was used as a negative control. The 34 anti-human antibodies used are listed in Table I and were printed at 200 μg/ml. See Figure 3 to visualize the print.

Catching and visualization of SEVs. The EV Array analysis was performed as described by Jørgensen *et al*. (15) with modifications. In short, the printed microarray slides were initially blocked (50 mM ethanolamine, 100 mM Tris, 0.1% SDS, pH 9.0) before incubation with a 15 μl plasma sample diluted to 100 μl in wash-buffer (0.05% Tween20 in PBS). The same volume of plasma was used from each patient. The incubation was performed in Multi-Well Hybridization Cassettes (ArrayIt Corporation, Sunnyvale, CA, USA) at room temperature for 2 h, followed by overnight incubation at 4˚C. After the 31 cancer-specific antibodies coated on the microarray slides caught the SEVs, biotinylated detection antibodies (antihuman-CD9, -CD63, and -CD81, LifeSpan BioSciences, Seattle, WA, USA) diluted 1:1,500 in a wash-buffer followed by 30 min incubation with Cy5-labelled streptavidin (Life Technologies, ThermoFisher Scientific, Waltham, MA USA) diluted 1:1,500 were used for visualization. Before scanning, the slides were washed in wash buffer, then in ultrapure/deionized water, and finally dried using a Microarray High-Speed Centrifuge (ArrayIt Corporation).

Slides were scanned in an InnoScan 710 AL microarray scanner (Innopsys, Carbonne, France) with the following settings: 532 nm at 10 V, PTM at 100%, and 5 μm resolution. The spots were visualized in Mapix (microarray analysis software, Innopsys) (Figure 3). For analyzing the total intensity at a spot, a GenePix Array List (GAL) file containing the data was used with a constant diameter (Ø135 μm). Through manual examination, contaminated spots were identified and deleted.

Data normalization. Quality control of raw data ensured that the intensity of the triplicate of a protein marker was within a reasonable range. The mean intensity for each protein marker for each sample was calculated. If the relation between the positive control (K20) and the negative control (blank) was >0.97, the sample was considered acceptable.

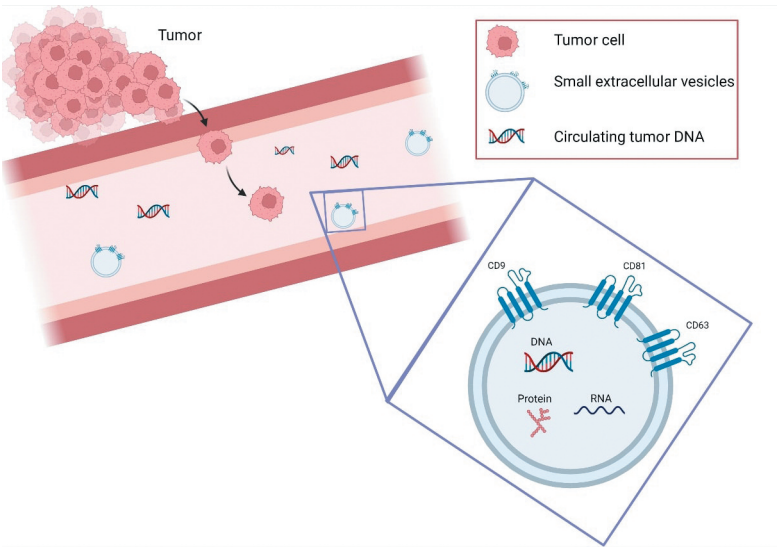

Figure 1. *An illustration of a tumor and a blood vessel containing several biomarkers: tumor cells, circulating tumor DNA, and small extracellular vesicles. The figure was created with BioRender.com.*

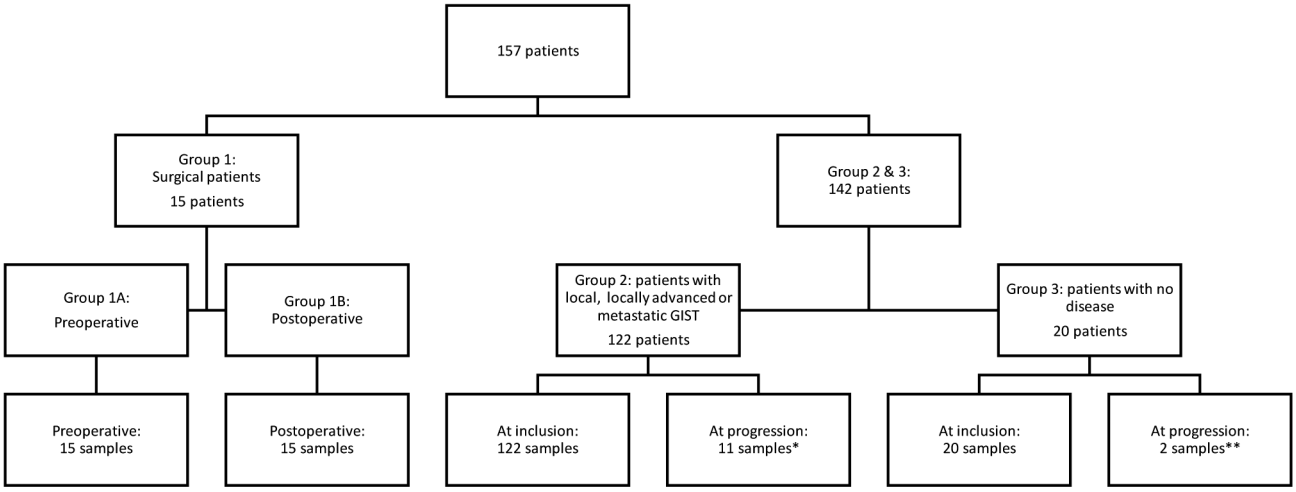

Figure 2. *Flow chart of included patients. Group 1: patients undergoing radical surgery; group 2: patients with local, locally advanced, or metastatic disease; and group 3: patients with no sign of disease (patients in adjuvant treatment after radical surgery or in control after ended adjuvant treatment). *Thirty-one patients progressed, but only progression samples from 11 patients were available. **Three patients progressed, but only progression samples from two patients were available.*

The total intensity of a protein marker was calculated as follows: the patient sample's intensity at a specific protein marker minus the blank well's intensity on the slide divided by the patient's background intensity at the negative control in the well (blank spot). Intensity values lower than 1, meaning that the signal for a protein marker was lower than the background signal for a patient, were removed from the dataset. Subsequently, the data were log2 transformed. Since CD81, CD9, and CD63 were used to identify the SEVs, data regarding these SEV markers are not reported. The plasma SEV levels of the different SEV markers were normalized to CD9 and CD81 by dividing each SEV marker level with the geometric mean of CD9 and CD81. Performing a *t*-test showed no difference in the mean of CD9 (p=0.56) and CD81 (0.47) when comparing group 1B and group 2.

Statistical analyses. The primary endpoint was the progression of GIST as per Response Evaluation Criteria in Solid Tumours 1.1. (16) and GIST-related death. The data analysis cut-off date was 30 March 2022.

Table I. *The function of the protein markers used in the analysis of the small extracellular vesicles.*

Protein marker (antigens)	Family	Function in cancer	Antibody supplier	Antibody
Alix (AIP1, WDR1)	WD repeat family of proteins (38)	Alix is involved in the formation of multivesicular bodies and intraluminal vesicles by being associated with the ESCRT (39). Alix is also involved in apoptotic signaling (40). The expression of Alix on SEVs is increased in patients with pancreatic cancer (41).	Biolegend	Alix (3A9)
Annexin V (Annexin A5)	Annexin (42)	Annexin V is a marker of apoptosis (43). Phosphatidylserine becomes more exposed at the cell membrane in apoptotic cells and Annexin V binds phosphatidylserine. This increases the immune response against the cells (44).	R & D Systems	Annexin V
AREG	EGF (45)	AREG increases the migration of tumor cells in several types of cancer, for example, osteosarcoma (46).	Santa Cruz Bio	AREG (S-13, Santa Cruz Biotechnological)
CD105 (Endoglin)	Zona Pellucida (47)	Endoglin is highly expressed on the surface of endothelial cells of blood vessels undergoing angiogenesis (48). High expression of endoglin is associated with poor survival for patients with Ewing sarcoma (49).	LS Bio	CD105 (LifeSpan Biologicals)
CD151	Tetraspanins (50)	CD151 exists on most epithelial, endothelial, and fibroblastic cells (51). CD151 aids in the regulation of cell- proliferation, - adhesion, and motility (52) but also contributes to tumor progression through inducing migration, invasion, and neovascularisation (53).	R & D Systems	CD151 (210127)
CD163	Scavenger receptor cysteine-rich receptors (29)	CD163 expression is associated with tumor cell proliferation and progression in sarcomas through the activation of macrophages (30). CD163 is associated with a poorer disease-free survival in patients with breast cancer (54).	R&D Systems	CD163
CD197 (CCR7)	G-protein coupled receptors (55)	CD197 is a chemokine receptor expressed on immune cells and cancer cells, leading to the migration of these cells to the lymph nodes where the ligands are expressed (55).	BD Biosciences	CD197 (3D12, BD Biosciences)
CD276 (B7-H3)	B7 (56)	CD276 inhibits T cell proliferation. Expression of CD276 is increased in several cancer types and plays a role in, for example cell proliferation and metastasis (57).	R&D Systems	CD276
CD31 (PECAM-1)	Immunoglobulin superfamily (58)	PECAM-1 is expressed in the following components of the blood: platelets, monocytes, neutrophils, T-cells (58), immature B-cells, endothelial cells to aid in adhesion, and some cancer cell types (59). PECAM 1inhibit apoptosis (59, 60).	R & D Systems	CD31

Table I. *Continued*

Table I. *Continued*

Protein marker (antigens)	Family	Function in cancer	Antibody supplier	Antibody
CD34	CD34 (61)	The function of CD34 is not clear. However, CD34 is often used as a marker for hematopoietic stem cells and hematopoietic progenitor cells (62). GISTs are CD34 positive in about 50-100% of the patients and are associated with the tumor location in the gastrointestinal tract (63).	R&D Systems	CD34 (756510)
CD42a (GP9)	Leucine-rich repeat family (34)	CD42a is a part of the GPIb-IX-V complex, which is expressed on the platelet surface, binds the von Willebrand factor, and aids in the adhesion of platelets to endothelial cells (34). Platelets play a role in tumor growth and metastasis (36).	LS Bio	CD42a
CD56 (NCAM)	Immunoglobulin superfamily (31)	CD56 is expressed primarily on natural killer (NK) cells, which aid in the adhesion to target cells (32). CD56 expression is correlated to poorer survival in several cancer types, such as renal cell carcinoma (31) and non-small-cell lung cancer (33).	BD Biosciences	CD56 (3G8)
CD63	Tetraspanins (51)	Together with other tetraspanins, CD63 aid in the regulation of several cellular activities such as cell- proliferation, - adhesion, and motility (52). CD63 is one of the main markers of SEVs (64). CD63 can potentially be a prognostic marker for early adenocarcinomas of the lung (52) and gastric cancer (65) for example.	Biorad	CD63 (MEM-259, BioRad)
CD81	Tetraspanins (51)	Together with other tetraspanins, CD81 aid in the regulation of several cellular activities such as cell- proliferation, - adhesion, and motility (52). CD81 is a frequent component of SEVs (11).	Ancell	CD81 (1.3.3.22, Ancell)
CD9	Tetraspanins (51)	Together with other tetraspanins, CD9 aid in the regulation of several cellular activities such as cell-proliferation, - adhesion, and motility (52). CD9 expression is of positive prognostic value in cancer patients (66). CD9 is one of the main markers of SEVs (64).	Ancell	CD9 (SN4/C3-3A2)
CEA (CD66e)	CEA (25)	CEA overexpression in colon cancer increases the adhesion of the cancer cells through selectins and thereby also the metastatic process (26).	R & D Systems	CEA (487609)
CTLA4 (CD152)	Immunoglobulin superfamily (67)	When CTLA-4 bind to ligands of the T-cells (CD80 and CD86) it leads to a decreased activation of these cells (68). Treatment with anti-CTLA-4 monoclonal antibodies leads to an increased anti-tumor response (69).	LS Bio	CTLA4 (AN152.2/8H5)
Fas ligand (CD95 ligand)	TNF (70)	The Fas ligand induces apoptosis when it binds to its receptor (70). Cancer cells are quite resistant to this way of inducing apoptosis (71). The Fas ligand and Fas receptor interaction can lead to tumor growth and invasion (71).	R&D Systems	Fas Ligand

Table I. *Continued*

Table I. *Continued*

Protein marker (antigens)	Family	Function in cancer	Antibody supplier	Antibody
Flotillin	Flotillins (72)	Flotillins are associated with membranes and participate in several cellular functions, such as signal transduction and endocytosis (73). Up-regulation of flotillins is associated with poor prognosis in several types of cancer, including sarcomas, probably due to increased tumor invasion and metastasis (74).	Abcam	Flotillin-1
GLUT-1	GLUT (27)	GLUT-1 is a glucose transporter often overexpressed in cancer cells (27). An overexpression of GLUT-1 is associated with poorer survival in patients with solid tumors (28).	Abcam	GLUT-1 (Abcam)
Hsp90	Hsp90 (75)	Hsp90 is overexpressed in several types of cancer (76). Hsp90 activity in stressed cells can lead to avoidance of apoptosis when stressed (77).	Abcam	Hsp90 (IGF1)
Integrin β6	Integrins (78)	Integrins ensure cell-cell, and cell-extracellular matrix adhesion (78). The β6 subunit of integrins forms a heterodimer with the αv subunit (78). Integrin αvβ6 is expressed on epithelial cells only (79). The Integrin αvβ6 is overexpressed on tumor cells in several different types of cancer such as colon cancer. The integrin αvβ6 on tumor cells enhances the metastatic process and is often associated with a poorer prognosis (79).	Santa Cruz	Integrin β6 (C-19)
KIT (CD117)	Type III receptor tyrosine kinase (80)	When activated, CD117 regulates different cellular activities such as proliferation, apoptosis, and adhesion (80). In >95% of patients with GIST, the tumor expresses CD117 (80).	R&D Systems	KIT (47233, R&D Systems)
L1CAM (CD171 or NCAM-L1)	Immunoglobulin superfamily (81)	L1CAM is a neural adhesion molecule. In cancer, it is believed to increase motility, invasion, and metastasis of tumor cells (82). L1CAM is expressed in several types of cancer (82). Overexpression of L1CAM has been shown to be related to a poorer prognosis in several types of cancer such as gastric and colorectal cancer (82).	R&D Systems	L1CAM (84321)
NY-ESO-1	Cancer testis antigen (83)	NY-ESO-1 activates the humoral and cellular immune response (84). Expression of NY-ESO-1 in solid tumors is associated with poor survival (83).	Santa Cruz Bio	NY-ESO-1 (E978)
Osteopontin	SIBLING (85)	Osteopontin functions in bone remodeling as well as in modulating the immune response. Overexpression of osteopontin in cancer cells contributes to metastasis through increased proliferation, migration, and adhesion (86).	Novus	Osteopontin (Novus Biologicals)
p53	p53 (87)	TP53 is a tumor suppressor gene encoding the protein p53 (88) and harbours the most common mutations in human malignancies (89). P53 is activated through cellular stress and can protect the genome through, for example DNA repair and apoptosis (88).	Abcam	p53 (pAb240)

Table I. *Continued*

Table I. *Continued*

Protein marker (antigens)	Family	Function in cancer	Antibody supplier	Antibody
PD-L1 (B7-H1)	B7 (90)	Binding of PD-L1 to PD-1 lead to inhibition of T- and B-cell functions (90).	Sino Biological	PD-L1 (Sino Biological)
PLAP	Alkaline Phosphatase (91)	PLAP is primarily expressed in germ cell tumors (92) such as ovarian cancer and testicular seminoma (93). The function is, however not clear (92).	Santa Cruz Bio	PLAP (8B6)
TGFβ1	TGFβ (94)	TGFβ1 is a cytokine aiding in sustaining cellular homeostasis (94). An overexpression of TGFβ1 is associated with tumor progression (94).	BD Pharmingen	TGFβ1 (BD Pharmingen)
TRAIL	TNF (95)	TRAIL is a cytokine that induces apoptosis. The induction of apoptosis is greater in cancer cells than normal cells, but cancer cells can be relatively resistant to TRAIL (95).	R&D Systems	TRAIL (75411)
TSG101 protein	Ubiquitin E2 variant family (96)	TSG101 belongs to the ESCRT (97) and is up-regulated in some types of cancer, such as papillary thyroid carcinoma (97).	Abnova	TSG101 (Abnova)
VEGFR2	VEGFR (98)	VEGFR2 is located in the endothelium of the vasculature (99). The expression of VEGFR2 on endothelial cells in the vasculature of the tumor (99) promote angiogenesis (100).	Biolegend	VEGFR2 (7D4-6, Biolegend)
Vimentin (VIM)	Type III intermediate filaments (101)	Vimentin is primarily located in the cytoplasm but can also be located in the cell membrane. In cancer, vimentin is a marker for epithelial-mesenchymal transition and is often correlated with a poor prognosis. Overexpression of vimentin is correlated with increased invasiveness of the tumor cells (101). Vimentin regulates cellular adhesion, migration, and cell signaling (102).	Avivasysbio	Vimentin (VI-RE/1, Avivasysbio)

AIP1: Actin-interacting protein 1; WDR1: WD-repeat protein 1; AREG: amphiregulin; EGF: epidermal growth factor; ESCRT: endosomal sorting complex required for transport; CD: cluster of differentiation; GIST: gastrointestinal stromal tumor; CCR7: CC-chemokine receptor type 7; PECAM-1: platelet endothelial cell adhesion molecule-1; GP: glycoprotein; NCAM: neural cell adhesion molecule; CEA: carcinoembryonic antigen; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; GLUT: glucose transporter; Hsp90: heat shock protein 90; L1CAM: L1 cell adhesion molecule; NCAM-L1: neural cell adhesion molecule L1; NY-ESO-1: New York esophageal squamous cell carcinoma 1; SIBLING: small integrin-binding ligand N-linked glycoprotein; PD-L1: programmed death-ligand 1; PD-1: programmed death 1; PLAP: placental alkaline phosphatase; TGF: transforming growth factor; TRAIL: TNF-related apoptosis-inducing ligand; TNF: tumor necrosis factor; TSG101: tumor susceptibility gene 101; VEGFR: vascular endothelial growth factor receptor.

The Kruskal–Wallis H test was used to compare continuous variables through the one-way analysis of variance between groups. The *t*-test was used to compare categorical variables between groups. The Wilcoxon matched pairs signed rank test was used to compare categorical variables at repeated measurements for patients since the difference in means between the measurements were not normally distributed.

Univariate and multivariate analyses were conducted using the Cox regression model. The multivariate analyses included the individual SEV markers, age at inclusion (continuous variable), and sex (categorical variable) were incorporated. For each SEV profile, for which the development is described below, different prognostic models were tested against each other.

The signal intensity of each SEV marker was categorized into a low and high value based on the median value (intensity of the spot) in the group investigated. A poor prognosis was assigned a value of 1, and a good prognosis was assigned a value of 0. The sum of the assigned values for each SEV included in the profile was calculated, and the

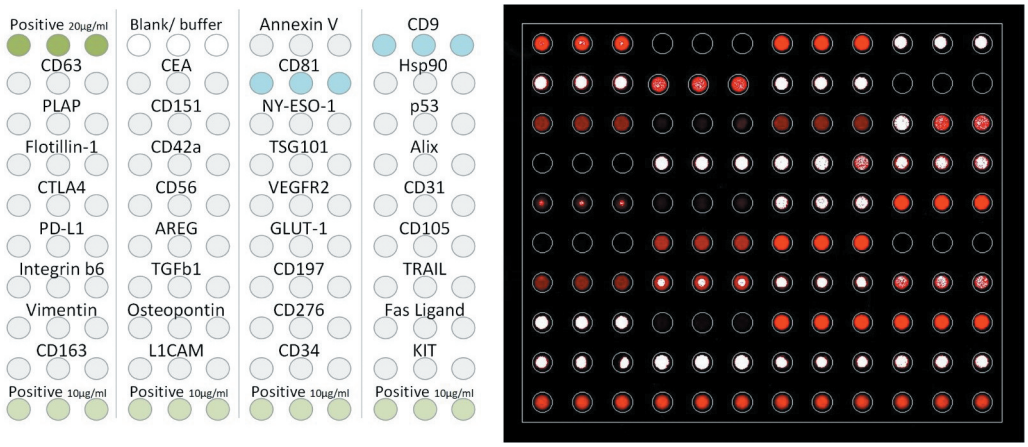

Figure 3. *Visualization of the extracellular vesicle array print. (A) The antibodies or controls were printed in triplicates in each well. The positive controls K10 and K20 (green) consist of goat anti-Mouse IgG (H+L) secondary antibody (biotin) (NB7537) from Novus with known concentrations. The blank well of the print was the negative control. CD9 and CD81 (blue) are the SEV markers used to normalize the data. (B) An example of the visualization of the spots after the scan. PBS: Phosphate-buffered saline; CD: cluster of differentiation; CEA: carcinoembryonic antigen; Hsp90: heat shock protein 90; PLAP: placental alkaline phosphatase; NY-ESO-1: New York esophageal squamous cell carcinoma 1; TSG101: tumor susceptibility gene 101; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; VEGFR: vascular endothelial growth factor receptor; PD-L1: programmed death-ligand 1; AREG: amphiregulin; GLUT: glucose transporter; TGF: transforming growth factor; TRAIL: TNF-related apoptosis-inducing ligand; L1CAM: L1 cell adhesion molecule.*

Table II. *Patient characteristics at the time of inclusion for patients undergoing radical surgery (group 1), patients with active gastrointestinal stromal tumors (GIST) (group 2), and patients without evidence of disease (group 3).*

Patient characteristics	Patients undergoing radical surgery (group 1)[a], n=15 (%)	Patients with active GIST (group 2)[b], n=122 (%)	Patients without evidence of disease (group 3)[c], n=20 (%)	*p*-Value
Sex				
Male	6 (40.0)	65 (53.3)	7 (35.0)	0.23
Female	9 (60.0)	57 (46.7)	13 (65.0)	
Age				
Median[d]	73	69	66	0.22
Range	44-92	20-87	32-81	
Disease status at inclusion				
No evidence of disease	0 (0.0)	0 (0.0)	20 (100.0)	
Local or locally advanced disease	15 (100.0)	38 (31.1)	0 (0.0)	
Metastatic disease[e]	0 (0.0)	84 (68.9)	0 (0.0)	
Treatment at inclusion				
No treatment	15 (100.0)	≤3 (<2.5)	8 (40.0)	
Adjuvant	0 (0.0)	0 (0.0)	12 (60.0)	
Neoadjuvant	0 (0.0)	18 (14.8)	0 (0.0)	
Lifelong	0 (0.0)	102 (83.6)	0 (0.0)	

[a]Patients undergoing radical resection of GIST. [b]Patients with local, locally advanced, or metastatic disease. [c]Patients without evidence of disease in adjuvant treatment after radical resection or in control after radical resection of GIST. [d]Median: the median is the middle value in a sorted dataset. [e]Patients with micro- or macro-metastatic GIST.

profile was then dichotomized. Only SEV markers significant in the univariate analysis were incorporated in a prognostic profile.

In the prognostic models confounding variables, age at inclusion and sex were included along with the SEV profile. We used Harrell's C statistics, Akaike information criterion (AIC), Bayesian information criterion (BIC), and the likelihood ratio to find the best prognostic model. The total number of events (n=31) restricted the maximum number of variables (n=3) incorporated in the model testing analysis.

Since we performed multiple testing, a significance level of 0.0015 would be preferable (0.05 divided by 31 SEV markers used in the study). However, since this is an explorative and hypothesis-generating study, we accepted 0.05 as a significance level.

Table III. *Univariate and multivariate analyses of the small extracellular vesicle (SEV) markers at the time of inclusion for patients with gastrointestinal stromal tumors (group 2).*

SEV marker	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Alix	1.08	0.53-2.18	0.83	1.08	0.52-2.22	0.84
AnnexinV	1.27	0.62-2.59	0.52	1.27	0.62-2.58	0.52
AREG	1.08	0.54-2.19	0.82	1.03	0.50-2.10	0.94
CD105	1.75	0.85-3.61	0.13	1.74	0.83-3.65	0.14
CD151	1.32	0.65-2.68	0.44	1.42	0.69-2.90	0.34
CD163	1.27	0.62-2.57	0.51	1.28	0.63-2.62	0.50
CD197	1.04	0.52-2.11	0.91	1.06	0.53-2.15	0.88
CD276	1.20	0.59-2.44	0.62	1.12	0.55-2.30	0.75
CD31	1.28	0.63-2.61	0.494	1.30	0.64-2.64	0.47
CD34	1.04	0.51-2.10	0.92	1.10	0.54-2.24	0.80
CD42a	1.36	0.67-2.77	0.39	1.28	0.62-2.63	0.50
CD56	1.03	0.51-2.09	0.93	1.02	0.50-2.06	0.96
CEA	2.12	1.02-4.44	**0.045**	2.14	1.02-4.47	**0.044**
CTLA4	0.63	0.22-1.80	0.39	0.65	0.22-1.87	0.42
FasLigand	1.33	0.66-2.71	0.43	1.33	0.65-2.70	0.43
Flotillin	1.89	0.91-3.90	0.086	2.05	0.96-4.39	0.064
GLUT-1	0.47	0.22-0.98	**0.043**	0.39	0.18-0.85	**0.018**
Hsp90	1.25	0.62-2.55	0.53	1.31	0.63-2.70	0.47
Integrin β6	1.30	0.56-3.03	0.54	1.29	0.55-3.06	0.56
KIT	1.34	0.66-2.73	0.42	1.35	0.65-2.80	0.42
L1CAM	1.31	0.65-2.67	0.45	1.25	0.61-2.55	0.55
NY-ESO-1	0.88	0.38-2.04	0.76	0.89	0.38-2.08	0.79
Osteopontin	1.13	0.56-2.30	0.73	1.02	0.49-2.11	0.96
p53	1.04	0.50-2.17	0.92	1.08	0.51-2.28	0.84
PDL1	1.61	0.79-3.30	0.19	1.67	0.80-3.48	0.17
PLAP	1.14	0.51-2.55	0.75	1.14	0.50-2.58	0.76
TGFb1	1.51	0.73-3.12	0.26	1.65	0.79-3.44	0.18
TRAIL	1.20	0.59-2.43	0.61	1.17	0.58-2.38	0.67
TSG101	1.06	0.52-2.14	0.88	1.21	0.58-2.51	0.62
VEGFR2	0.91	0.44-1.88	0.81	0.95	0.46-1.99	0.90
Vimentin	1.08	0.53-2.19	0.83	1.06	0.52-2.16	0.87

HR: Hazard ratio; CI: confidence interval; p5: the 5th percentile; p95: the 95th percentile; AREG: amphiregulin; CD: cluster of differentiation; CEA: carcinoembryonic antigen; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; GLUT-1: glucose transporter; Hsp90: heat shock protein 90; L1CAM: L1 cell adhesion molecule; NY-ESO-1: New York esophageal squamous cell carcinoma 1; PD-L1: programmed death-ligand 1; PLAP: placental alkaline phosphatase; TGF: transforming growth factor; TRAIL: TNF-Related apoptosis-inducing ligand; TSG101: tumor susceptibility gene 101; VEGFR: vascular endothelial growth factor receptor. Bold value(s) denote significance.

Stata v. 17 (StataCorp LLC, College Station, TX, USA) was used for the data analysis, and Graphpad Prism v. 9 (San Diego, CA, USA) was used for ROC curve analysis.

Results

Patient characteristics. A total of 157 patients were included in this study, with a median follow-up time of 2.05 years.

Patient and disease characteristics at the inclusion time are summarized in Table II. For patients with either local, locally advanced, or metastatic disease (group 2), 31 progressed or died during the follow-up. In group 3, three patients progressed during the follow-up, while no patient progressed in group 1.

SEVs. Univariate and multivariate analyses of risk for progression were performed for each protein investigated (Table III). Glucose transporter 1 (GLUT-1) and carcinoembryonic antigen (CEA) were the only SEV markers found significant on a 0.05 level in the univariate- [GLUT-1; hazard ratio (HR)=0.47, 95% confidence interval (CI)=0.22-0.98, p=0.043, CEA; HR=2.12, 95%CI=1.02-4.44, p=0.045] and multivariate analysis (GLUT-1; HR=0.39, 95%CI=0.18-0.85, p=0.018, CEA; HR=2.14, 95%CI=1.02-4.47, p=0.044). A low level of GLUT-1 and a high level of CEA was related to poor prognosis in the uni- and multivariate analyses. The Kaplan–Meier plots of CEA low/high and GLUT-1 low/high are shown in Figure 4. There

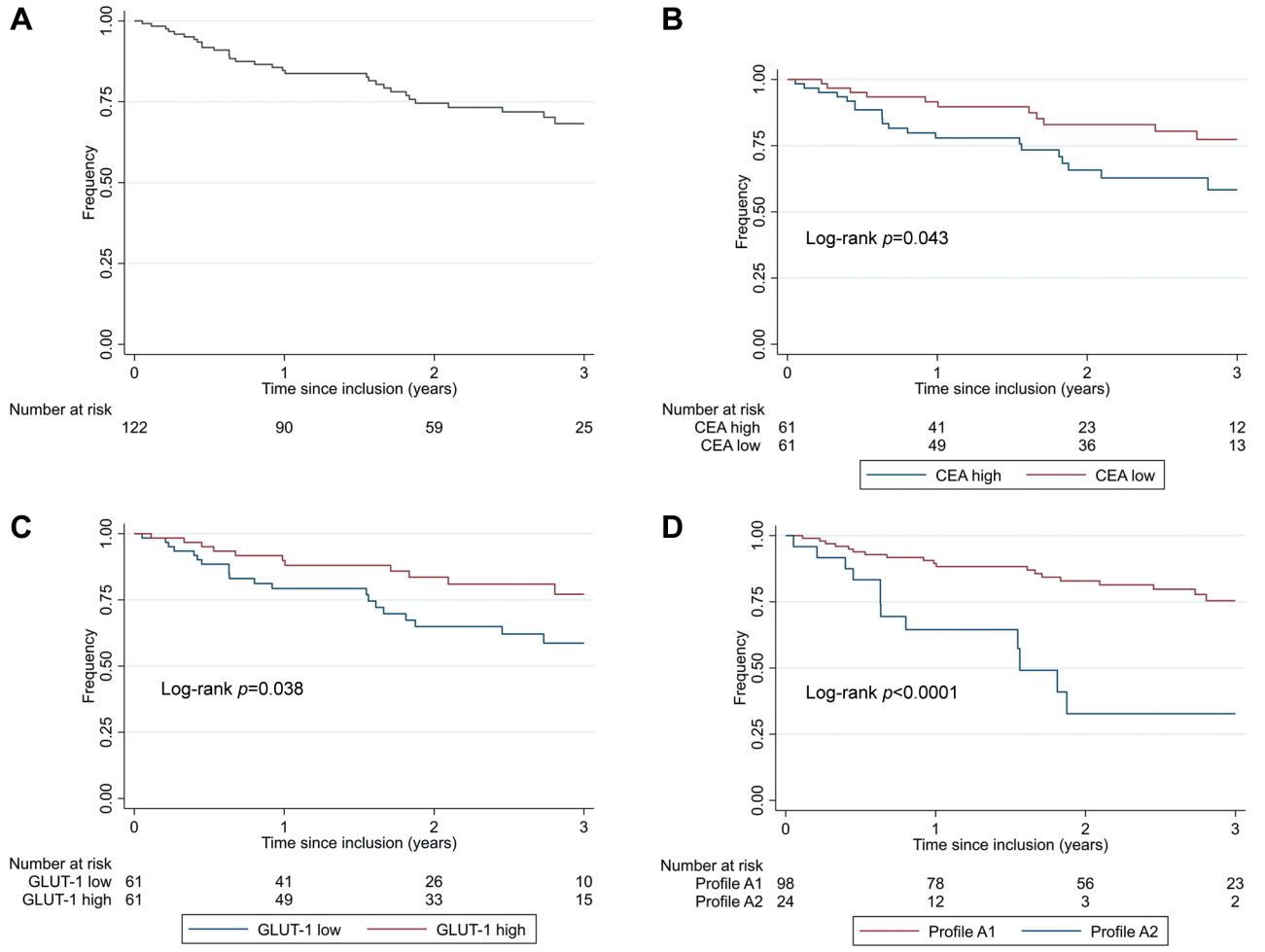

Figure 4. *Kaplan–Meier plots for time to progression from the time of inclusion for patients in group 2. (A) All patients in group 2. (B) The patients were stratified into CEA low (↓) and CEA high (↑). (C) The patients were stratified into GLUT-1 low (↓) and GLUT-1 high (↑). (D) Profile A1 (good prognosis): one of the following scenarios is present; the GLUT-1 was high (GLUT-1↑) and the CEA was low (CEA↓), or GLUT-1 was high (GLUT-1↑) and the CEA was high (CEA↑), or the GLUT-1 was low (GLUT-1↓) and the CEA was low (CEA↓). Profile A2 (poor prognosis): GLUT-1 was low (GLUT-1↓), and the CEA was high (CEA↑). *Low (↓): The SEV marker value was lower or equal to the median value of the SEV marker in group 2. High (↑): The SEV marker value was higher than the median value of the SEV marker in group 2.*

was no relation between the value of GLUT-1 or CEA and patient age at study inclusion.

The SEV markers from the univariate analysis with a *p*-value ≤0.05 were incorporated into a profile. Patients with active GIST (group 2) were divided into a good (profile A1) and poor (profile A2) prognosis profile. A comparison of profiles A1 and A2 showed that the profile is of prognostic value (HR=4.17, 95%CI=1.99-8.74, $p<0.0001$). The accompanying Kaplan–Meier plot illustrating profiles A1 and A2 is shown in Figure 4.

Of the 122 patients in group 2, 98 patients belonged to profile A1, and 19 of these had disease progression after inclusion into the study (19.38%). Of the 24 patients belonging to profile A2, 12 progressed (50%).

A profile B containing the five SEV markers with a $p\leq0.2$ from the univariate analysis: CEA, GLUT-1, Flotillin-1, PD-L1, and CD105 was created. We divided patients into a poor (profile B1) and a good (profile B2) prognosis profile (Table IV). A comparison of profiles B1 and B2 showed that the profile is of prognostic value (HR=0.37, 95%CI=0.18-0.76, $p=0.006$).

To find the best possible model based on profiles A and B, different models were tested against each other, and model E was the best obtainable prognostic model. Age at inclusion was not a significant factor in univariate analysis ($p=0.16$), but in a multivariate analysis in model E, age at inclusion was of significant prognostic value ($p=0.036$). Sex was not an independent prognostic factor in univariate analysis ($p=0.86$).

Table IV. *Comparison of prognostic profiles/models A-E for patients with gastrointestinal stromal tumors (group 2).*

Profile/model	Content of profile/model	Univariate analysis			Harrel's C	AIC	BIC	Likelihood ratio, *p*-Value
		HR	95%CI	*p*-Value				
A[#]	A	4.17	1.99-8.74	<0.0001	0.63	266.27	269.07	
B[$]	B	0.37	0.18-0.76	0.006	0.63	271.34	274.14	
		Multivariate analysis						
		HR	95%CI	*p*-Value				
C	A + age[&] + sex	5.48	2.46-12.20	<0.0001	0.70	265.42	273.83	0.09*
D	B + age[&] + sex	0.35	0.17-0.73	0.005	0.66	272.89	281.30	0.29**
E	A + age[&]	4.93	2.30-10.57	<0.0001	0.71	264.30	269.91	0.05***
F	B + age[&]	0.36	0.18-0.73	0.005	0.66	270.98	276.59	0.12****

[#]Profile A includes carcinoembryonic antigen (CEA) and glucose transporter 1 (GLUT-1). Profile A1 (good prognosis): one of the following scenarios is present; the GLUT-1 value was high (GLUT-1↑) and the CEA was low (CEA↓), or GLUT-1 was high (GLUT-1↑) and the CEA was high (CEA↑), or the GLUT-1 was low (GLUT-1↓) and the CEA was low (CEA↓). Profile A2 (poor prognosis): GLUT-1 was low (GLUT-1↓), and the CEA value was high (CEA↑). [$]Profile B includes CEA, GLUT-1, Flotillin, programmed death-ligand 1 (PD-L1), and cluster of differentiation 105 (CD105). Profile B1 (poor prognosis): ≥4 SEV markers were on the unfavorable side of the median value for each SEV marker. Profile B2 (good prognosis): <4 SEV markers were on the unfavorable side of the median value of the individual SEV marker. [&]Age at inclusion. *Comparing profile A and model C. **Comparing profile B and model D. ***Comparing profile A and model E. ****Comparing profiles B and model F. Low (↓): The SEV marker value was lower or equal to the median value for the SEV marker in group 2. High (↑): The SEV marker value was higher than the median value of SEV marker in group 2. HR: Hazard ratio; CI: confidence interval; AIC: Akaike information criterion; BIC: Bayesian information criterion.

The CD163 intensity on SEVs was found to be significantly lower at progression than at the time of inclusion (p=0.032) for patients with matched samples at the time of inclusion and at tumor progression (13 samples) (Table V). Two SEV markers, CD163 (p=0.027) and CD56 (p=0.029), were found to have significantly lower intensity post-operative than preoperative in patients undergoing radical surgery for GIST (n=15) (Table V).

The median SEV marker levels in group 1B were compared with those in patients with active GIST (group 2) (Table VI). The median intensity of CD42a in SEVs from patients with active GIST was significantly higher than that in patients without evidence of disease (p=0.008) (Figure 5). The receiver operating characteristics (ROC) curve for CD42a is shown in Figure 6, where the area under the curve was 0.70 (95%CI=0.58-0.82, p=0.011), indicating that it is a moderate marker.

Discussion

In this national study, we investigated the prognostic value of the phenotype of plasma SEVs in patients with GIST. Our study showed that patients with active GIST having a high CEA value and/or a low GLUT-1 have a significantly higher risk of progression or death. Age and sex, which are possible confounders, were incorporated in the multivariate analysis with the SEV markers. Disease status, another potential confounder, was not incorporated in the analysis since we already had selected the groups of interest for the analysis based on disease status. We also found a highly significant prognostic model, including CEA, GLUT-1, and age at inclusion.

In GIST, risk stratification is based on tumor size, location, mitotic count (5), and *KIT* and *PDGFRA* mutational status (17, 18), which are factors proved to have a prognostic value in patients with resectable GIST. Only *KIT* and *PDGFRA* mutational status (17, 18) is a factor with prognostic value in patients with advanced and metastatic GIST. However, no soluble biomarker is known to be of prognostic value for patients with GIST.

Most cells produce SEVs (19), and it has been proposed that cancer patients have more SEVs than healthy individuals (20). The SEVs have been shown to have clinical utility in some cancer types. For example, the National Comprehensive Cancer Network has included SEV-derived biomarkers (RNA) from urine to be considered for early detection of prostate cancer (21). SEV phenotyping has been shown to separate patients diagnosed with advanced-stage non-small cell lung cancer from matched controls with 75.3% accuracy (22). The prognostic value of SEV phenotyping has not been investigated in GIST.

The scope of this study was to investigate the prognostic potential of SEV phenotypes in patients with GIST based on a blood sample and not to gain a quantitative measure of the

Table V. *Small extracellular vesicle (SEV) marker levels.*

SEV marker	Pre-and postoperative samples in group 1			Samples at inclusion and at progression*		
	Pre-surgical (group 1A), n=15	Post-surgical (group 1B), n=15		At inclusion, n=13	At progression, n=13	
	Median (P5-P95)	Median (P5-P95)	*p*-Value	Median (P5-P95)	Median (P5-P95)	*p*-Value
Alix	0.0 (0.0-0.97)	0.0 (0.0-1.02)	0.89	0.034 (0.0-0.92)	0.092 (0.0-0.73)	0.54
Annexin V	0.0 (0.0-0.85)	0.0 (0.0-0.85)	0.063	0.0 (0.0-0.54)	0.0 (0.0-0.45)	0.27
AREG	0.0 (0.0-0.56)	0.0 (0.0-0.55)	0.39	0.052 (0.0-0.78)	0.0 (0.0-0.58)	0.13
CD105	0.25 (0.0-0.58)	0.28 (0.0-0.53)	0.30	0.25 (0.0-0.72)	0.19 (0.0-0.59)	0.34
CD151	0.33 (0.015-0.62)	0.38 (0.0-0.75)	0.17	0.33 (0.12-0.81)	0.31 (0.0-0.53)	0.11
CD163	0.064 (0.0-0.78)	0.0063 (0.0-0.76)	**0.027**	0.12 (0.0-0.66)	0.045 (0.0-0.50)	**0.032**
CD197	0.0 (0.0-0.71)	0.0 (0.0-0.78)	0.50	0.024 (0.0-0.56)	0.026 (0.0-0.46)	0.75
CD276	0.0 (0.0-0.89)	0.0 (0.0-0.90)	0.34	0.26 (0.0-0.69)	0.10 (0.0-0.59)	0.79
CD31	0.60 (0.21-0.88)	0.60 (0.41-0.88)	0.98	0.65 (0.43-0.80)	0.61 (0.22-0.72)	0.068
CD34	0.13 (0.0-0.87)	0.037 (0.0-0.91)	0.39	0.13 (0.0-0.81)	0.13 (0.0-0.63)	0.84
CD42a	0.54 (0.25-0.81)	0.52 (0.34-0.88)	0.39	0.68 (0.47-0.90)	0.66 (0.41-1.05)	0.27
CD56	0.089 (0.0-0.30)	0.034 (0.0-0.28)	**0.029**	0.052 (0.0-0.42)	0.028 (0.0-0.77)	0.79
CEA	0.016 (0.0-0.45)	0.0 (0.0-0.45)	0.42	0.14 (0.0-0.52)	0.22 (0.0-0.36)	0.92
CTLA4	0.0 (0.0-0.27)	0.0 (0.0-0.36)	1.00	0.0 (0.0-0.27)	0.0 (0.0-0.11)	0.50
Fas ligand	0.0 (0.0-0.87)	0.0 (0.0-0.88)	0.25	0.0 (0.0-0.64)	0.0 (0.0-0.47)	0.42
Flotillin	0.19 (0.0-0.24)	0.16 (0.0019-0.25)	0.76	0.16 (0.0-0.48)	0.14 (0.0-0.36)	0.54
GLUT-1	0.21 (0.0-0.53)	0.21 (0.0-0.51)	0.061	0.23 (0.0-1.08)	0.28 (0.0-0.96)	1.00
Hsp90	0.054 (0.0-0.38)	0.071 (0.0-0.38)	0.51	0.22 (0.0-0.65)	0.17 (0.0-0.46)	0.87
Integrin β6	0.0 (0.0-0.18)	0.0 (0.0-0.063)	1.00	0.0 (0.0-0.52)	0.0 (0.0-0.23)	0.25
KIT	0.094 (0.0-0.73)	0.089 (0.0-0.78)	0.60	0.22 (0.0-0.67)	0.29 (0.0-0.58)	0.81
L1CAM	0.032 (0.0-0.98)	0.0 (0.0-0.97)	0.27	0.074 (0.0-0.90)	0.057 (0.0-0.74)	0.29
NY-ESO-1	0.0 (0.0-0.37)	0.0 (0.0-0.33)	0.50	0.0 (0.0-0.70)	0.0 (0.0-0.30)	0.50
Osteopontin	0.16 (0.0-0.43)	0.15 (0.0-0.44)	0.069	0.034 (0.0-0.98)	0.080 (0.0-0.89)	0.55
p53	0.0 (0.0-0.85)	0.0 (0.0-0.89)	1.00	0.0 (0.0-0.81)	0.0 (0.0-0.61)	0.34
PD-L1	0.018 (0.0-0.20)	0.017 (0.0-0.15)	0.53	0.083 (0.0-0.51)	0.051 (0.0-0.32)	0.32
PLAP	0.0 (0.0-0.32)	0.0 (0.0-0.34)	0.75	0.0 (0.0-0.70)	0.0 (0.0-0.35)	0.75
TGFβ1	0.0 (0.0-0.48)	0.0 (0.0-0.55)	0.50	0.0 (0.0-0.38)	0.0 (0.0-0.29)	0.31
TRAIL	0.096 (0.0-0.46)	0.037 (0.0-0.46)	0.39	0.13 (0.0-0.53)	0.14 (0.0-0.36)	0.76
TSG101	0.35 (0.0-0.60)	0.28 (0.046-0.59)	0.76	0.30 (0.014-0.67)	0.33 (0.025-0.55)	0.89
VEGFR2	0.0 (0.0-0.73)	0.0 (0.0-0.76)	1.00	0.0 (0.0-0.67)	0.0 (0.0-0.49)	0.14
Vimentin	0.18 (0.0-0.77)	0.15 (0.0-1.01)	0.54	0.23 (0.0-0.69)	0.19 (0.0-0.53)	0.48

*Patients in group 2 having a blood sample collected at inclusion and at image-verified progression. P5: The 5th percentile; P95: the 95th percentile; AREG: amphiregulin; CD: cluster of differentiation; CEA: carcinoembryonic antigen; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; GLUT: glucose transporter; Hsp90: heat shock protein 90; L1CAM: L1 cell adhesion molecule; NY-ESO-1: New York esophageal squamous cell carcinoma 1; PD-L1: programmed death-ligand 1; PLAP: placental alkaline phosphatase; TGF: transforming growth factor; TRAIL: TNF-related apoptosis-inducing ligand; TSG101: tumor susceptibility gene 101; VEGFR: vascular endothelial growth factor receptor.

SEVs. Therefore, it was chosen to use an already established and verified technology and not to focus on the EV characteristics despite the recommendations by the minimal information for studies of extracellular vesicles guidelines (23). The protein microarray (EV Array) technology used in our study did not allow quantitative measurements of the SEVs in contrast to the Nanoparticle Tracking Analysis (NTA) used in the study by Atay *et al*. (7).

The two SEV surface proteins found in this study to have prognostic importance were CEA and GLUT-1. CEA is an unspecific biomarker, elevated in several cancer types and other conditions such as uremia, lung fibrosis, and is also associated with age (24). CEA belongs to the family with the same name, which in turn belongs to the immunoglobulin superfamily (25). CEA is widely used in the surveillance of colorectal cancer as a prognostic biomarker. However, the specificity and sensitivity are too poor to function as a diagnostic biomarker (24). In colon cancer, overexpression of CEA increases the adhesion of the cancer cells through selectins and, thereby, enhances the metastatic process (26).

GLUT-1 is a glucose transporter belonging to the family GLUT (27). Glucose transport into the cell is essential to maintain a high cell proliferation rate (27). GLUT-1 is often

Table VI. *Small extracellular vesicle (SEV) marker level at the time of inclusion for patients that had undergone radical surgery and were without evidence of disease (group 1B) vs patients with active gastrointestinal stromal tumors (GIST) (group 2).*

SEV marker	Post-operative samples from patients radically resected for GIST (group 1B)[a], n=15			Patients with active GIST (group 2)[b], n=122			
	Median[c]	P5	P95	Median	P5	P95	*p*-Value
Alix	0	0	1.02	0.007	0	0.70	0.18
Annexin V	0	0	0.85	0	0	0.69	0.55
AREG	0	0	0.55	0	0	0.56	0.98
CD105	0.28	0	0.53	0.21	0	0.65	0.18
CD151	0.38	0	0.75	0.35	0.077	0.67	0.83
CD163	0.0063	0	0.76	0.093	0	0.62	0.069
CD197	0	0	0.78	0	0	0.56	0.16
CD276	0	0	0.9	0.016	0	0.71	0.20
CD31	0.6	0.41	0.88	0.62	0.39	0.83	0.35
CD34	0.037	0	0.91	0.13	0	0.68	0.55
CD42a	0.52	0.34	0.88	0.66	0.36	0.90	**0.008**
CD56	0.034	0	0.28	0.094	0	0.42	0.34
CEA	0	0	0.45	0.058	0	0.52	0.12
CTLA4	0	0	0.36	0	0	0.26	0.62
Fas ligand	0	0	0.88	0	0	0.7	0.5
Flotillin	0.16	0.0019	0.25	0.12	0	0.48	0.56
GLUT-1	0.21	0	0.51	0.23	0	0.97	0.26
Hsp90	0.071	0	0.38	0.1	0	0.55	0.80
Integrin β6	0	0	0.063	0	0	0.31	0.92
KIT	0.089	0	0.78	0.16	0	0.64	0.19
L1CAM	0	0	0.97	0.053	0	0.67	0.52
NY-ESO-1	0	0	0.33	0	0	0.36	0.14
Osteopontin	0.15	0	0.44	0.077	0	0.85	0.73
p53	0	0	0.89	0	0	0.62	0.27
PD-L1	0.017	0	0.15	0.032	0	0.43	0.54
PLAP	0	0	0.34	0	0	0.39	0.96
TGFβ1	0	0	0.55	0	0	0.4	0.35
TRAIL	0.037	0	0.46	0.085	0	0.53	0.27
TSG101	0.28	0.046	0.59	0.28	0	0.62	0.79
VEGFR2	0	0	0.76	0	0	0.56	0.065
Vimentin	0.15	0	1.0086	0.15	0	0.69	0.93

[a]Patients radically resected for GIST. [b]Patients with local, locally advanced, or metastatic disease. [c]Median: the median is the middle value in a sorted dataset. P5: The 5th percentile; P95: the 95th percentile; AREG: amphiregulin; CD: cluster of differentiation; CEA: carcinoembryonic antigen; CTLA-4: cytotoxic T-lymphocyte-associated protein 4; GLUT: glucose transporter; Hsp90: heat shock protein 90; L1CAM: L1 cell adhesion molecule; NY-ESO-1: New York esophageal squamous cell carcinoma 1; PD-L1: programmed death-ligand 1; PLAP: placental alkaline phosphatase; TGF: transforming growth factor; TRAIL: TNF-related apoptosis-inducing ligand; TSG101: tumor susceptibility gene 101; VEGFR: vascular endothelial growth factor receptor. Bold value(s) denote significance.

overexpressed in cancer cells (27) and associated with poorer survival in patients with solid tumors (28). Examples of cancer types with overexpression of GLUT-1 are colorectal cancer, breast cancer, and lung cancer (27). The expression levels of GLUT-1 and the SEV levels of GLUT-1 cannot be compared directly.

The SEV intensity of CEA and GLUT-1 was not significantly different when comparing samples obtained at the time of inclusion with samples obtained at image-verified tumor progression.

Another surface protein, CD163, was, however, found to have a significantly lower intensity at the time of progression than at the time of inclusion. Furthermore, the CD163 and the CD56 SEV intensity was significantly lower postoperatively compared to preoperatively. CD163 belongs to the scavenger receptor cysteine-rich receptors and is often expressed on macrophages (29). In a laboratory study, the CD163-induced activation of macrophages has been associated with tumor development (30) and is believed to have an immunosuppressive effect. Furthermore, a high rate of CD163 expressing tumor-associated macrophages has also been associated with poorer overall survival in sarcoma patients (30).

CD56 is a member of the immunoglobulin superfamily (31) and is primarily expressed on natural killer cells where

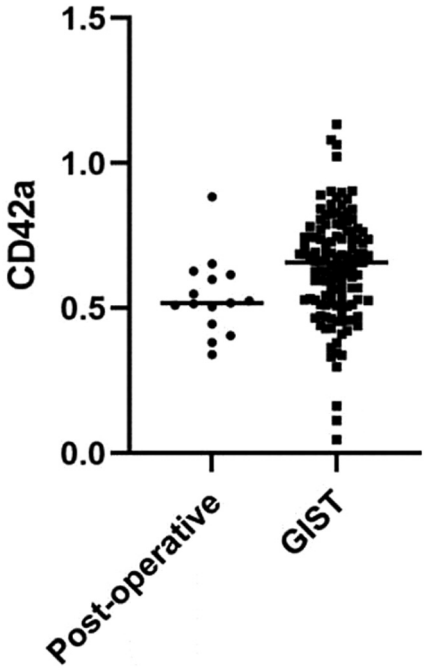

Figure 5. *The plot visualizes the CD42a values in the group of post-operative samples from patients that had undergone radical resection of gastrointestinal stromal tumor (GIST) (group 1B) and the group of patients with active GIST (group 2). The horizontal lines mark the median values in the two groups.*

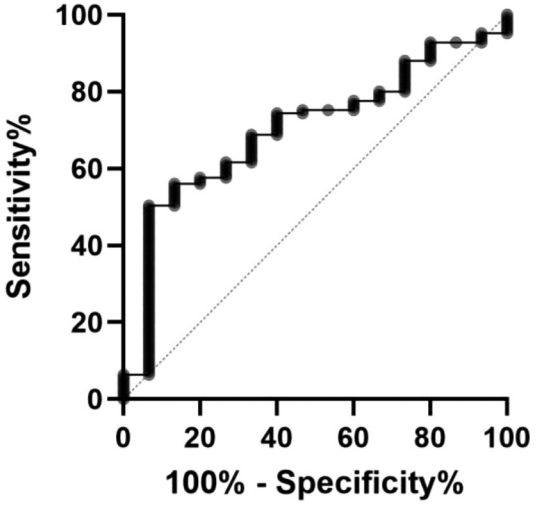

Figure 6. *The receiver operating characteristics curve for CD42a.*

it is believed to aid in the adhesion to target cells (32). CD56 expression has been correlated to a poor prognosis in patients with renal cell carcinoma (31) and non-small cell lung cancer (33). Our findings regarding SEV CD163 and CD56 intensity are non-conclusive due to the small number of patients. The relation of these SEV markers to the innate immune response (macrophages and natural killer cells) in patients with GIST should be further investigated.

Apart from the SEV markers with prognostic potential in patients with GIST, we also found that patients with active GIST had a significantly higher median level of plasma SEV CD42a than the group of postoperative samples from patients radically resected for GIST. These results could imply that a higher plasma SEV CD42a level indicates the presence of GIST cells in the body. However, no such relation was found when comparing SEV marker levels between pre- and postoperative samples, keeping the small number of patients in mind together with the early drawing of the post-operational blood samples. The antigen CD42a is also called glycoprotein IX (GPIX). Together with the GPIbα, GPIbβ, and GPV, GPIX constitutes the GPIb-IX-V complex, which is expressed on the platelet surface (34). The GPIb-IX-V complex is essential in platelet functions in adhesion, activation, and aggregation, which is essential for hemostasis (35). Platelets are reported to play a role in tumor growth and metastasis (36). In mice models, platelets are believed to promote metastases, for example, by hiding the tumor cells from the natural killer cells together with fibrin (37).

This study indicates that SEV phenotyping may have prognostic value in patients with GIST. The relatively short follow-up time (median 2.05 years) and the small number of events (31 patients with progression or death due to GIST after inclusion) affect the power of the results. The results should therefore be interpreted with caution.

Some limitations should be considered. The patient cohort is heterogenous regarding disease status, treatment status, and the time for inclusion in the disease course. The inclusion in the study at different time points in the patients' disease courses has led to a high number of included patients but complicates data interpretation. The disease status ranges from patients in adjuvant treatment after a radical surgery to patients in lifelong treatment due to metastatic GIST. We used the SEV marker levels in postoperative blood samples from patients that had undergone radical surgery and were without evidence of disease (group 1B) as a comparison to the SEV marker levels in patients with active GIST in this study. This comparison was made to investigate if the SEV marker levels could distinguish patients with GIST cells from patients not having GIST cells in the body and, thereby, if any of the SEV marker levels investigated could be a potential diagnostic biomarker. During the study's follow-up time, none of the patients undergoing surgery had a relapse of the disease. The trauma caused by surgery could, however, potentially influence the amount and the phenotype of the SEVs. Due to the lack of a healthy control group, we cannot conclude anything regarding the diagnostic potential of SEV markers in patients with GIST.

The present study has several strengths. This study is a nationwide study, including a high number of patients in a clinical setting. This is also the first study investigating the plasma SEV phenotype within patients with GIST. The study of SEVs could also help the understanding of the immunological status of GIST patients. This study suggests that the innate immune system plays a role in tumor progression and initiation. This could explain the lack of immune checkpoint inhibitor effect in GIST. We will investigate this in future studies.

Since no risk stratification is available for patients with advanced or metastatic GIST, a soluble prognostic biomarker would be of great interest to this group of patients. Our study results, however, need external validation in a larger, well-defined independent cohort to validate the prognostic role of CEA and GLUT-1 on SEVs in GIST patients; if confirmed, this could be a new prognostic marker for metastatic GIST.

Conclusion

This is the first study investigating the phenotype of plasma SEVs in GIST patients. The study showed that a high CEA and/or a low GLUT-1 is associated with a poor prognosis. We also report a highly statistically significant prognostic model containing CEA, GLUT-1, and age at inclusion in the study. However, external validation is needed.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, C.M.B., A.K.H., N.A.P., and E.H.; methodology, C.M.B., N.A.P., A.K.H., E.H., R.B. and M.M.J.; validation, C.M.B., and N.A.P.; formal analysis, C.M.B., and N.A.P.; investigation, C.M.B., N.A.P., A.K.H., B.E.E., P.B.R., P.D.H., L.P., and H.J.M.; resources, M.M.J., R.B., and H.J.M.; data curation, C.M.B., N.A.P., and A.K.H.; writing—original draft preparation, C.M.B., and N.A.P.; writing—review and editing, C.M.B., N.A.P., A.K.H., E.H., B.E.E., P.B.R., H.J.M., P.D.H., L.P., M.M.J., and R.B.; visualization, C.M.B., N.A.P., A.K.H., and E.H.; supervision, N.A.P., A.K.H., and E.H.; project administration, C.M.B., N.A.P., A.K.H., and E.H.; funding acquisition, C.M.B., and A.K.H. All Authors have read and agreed to the published version of the manuscript.

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