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RESEARCH ARTICLE

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Secreted Protein Acidic and Rich in Cysteine (SPARC) and Chemokine's mRNA expression in Endometrial Cancer versus Normal Endometrium: A Possible Pattern of Haematogenous and Lymphovascular Metastases

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ABSTRACT

Objectives: Secreted Protein Acidic and Rich in Cysteine (SPARC) is a multifunctional glycoprotein, participating in tissue remodeling, morphogenesis and bone mineralization. Furthermore, SPARC controls important mechanisms involved in cancer progression, including angiogenesis regulation. However, in some studies SPARC was found to show tumor suppression while in other a protumorigenic and prometastatic action. In tumor microenvironment some chemokines and their receptors, thanks to their ability to modulate cancer cells migration and proliferation, are involved in the angiogenetic and metastatic process. In this study we compared, in human endometrial cancer tissue (EC) vs normal endometrium counterpart (NE), SPARC with CXCL12, CXCL11, CXCL8, and CXCR7 mRNA expression.

Material and Methods: Fresh specimens from 15 patients with EC and corresponding NE were stored at –80°. One mcg of mRNA was reverse-transcribed in cDNA. A Real-Time PCR determined relative cDNA levels of targeted gene mRNA.

Results: In EC vs NE, we observed down-regulation of SPARC mRNA in 91% (P<.05), down regulation of CXCL12 mRNA in 91% (P<.001), and down-regulation of CXCR7 mRNA in 91% (P<.001). In EC, SPARC mRNA down-regulation was directly related in 100% of samples to CXCL12 and CXCR7 (P<.001) and in 73% of samples to VEGF (P<.03).

Conclusion: In endometrial cancer, under expression of SPARC is directly related to CXCL12 and CXCR7 and this result might be consistent with a SPARC function on tumor progression and invasion mediated by CXCL12 and CXCR7 on blood and lymphatic spread, respectively.

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KEYWORDS

Endometrial cancer, SPARC mRNA, CXCL12 mRNA, CXCL8 mRNA, CXCR7 mRNA, CXCL11 mRNA

Abbreviations

SPARC: Secreted Protein Acidic and Rich in Cysteine, VEGF: Vascular Endothelial Growth Factor; EC: Endometrial cancer; NE: Normal endometrium; FIGO: International Federation of Gynecologic Oncology.

Introduction

Secreted Protein Acidic and Rich in Cysteine (SPARC), is a multifunctional calcium-binding matricellular glycoprotein, secreted by different osteoblasts, fibroblasts, endothelial cells, and platelets participating in tissue remodeling, morphogenesis, and bone mineralization [1-4]. Furthermore, SPARC is involved in cancer progression, including the regulation of angiogenesis affecting metastases dissemination [5]. However, the function of SPARC in cancer is controversial and not completely assessed, as both a suppressor and a prometastatic effect on tumor cells have been reported in the Literature in different human malignancies [6-14]. Conversely, SPARC has been found to reduce the activity of several growth factors, including VEGF, suggesting its direct participation in tumor progression and invasion via VEGF modulation [15]. In the tumoral microenvironment, some chemokines and their receptors are reported to modulate angiogenic development [16-17]. In fact, the CXC chemokine's family include members acting as inhibitors (ELR- motif) or as promoters (ELR+ motif) of angiogenesis [18]. Nevertheless, it seems that one ELR- chemokine, CXCL12 (and its receptor CXCR4) have been showed unexpectedly to promote angiogenesis and playing a principal role in carcinogenesis and metastases [19]. More in particular, in the tumor microenvironment of many cancers the CXCR4–CXCL12–CXCR7 pathway represent the critical point to direct the cancer cells towards specific metastatic sites where the over expression of CXCR4/CXCL12 is related to distant recurrence while the over expression of CXCR7 is mainly related to lymph nodal metastases [20-23]. Another chemokine related to neo-angiogenesis and invasiveness in cancer via VEGF is CXCL8 [24-26]. CXCL11 is, instead, a CXC chemokine with ELR- motif provided of angiostatic activity in response to VEGF [17-18]. Instead, in multiple myeloma, CXCL11 is mainly produced

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by macrophages and act inducing natural killer lymphocytes chemotaxis, stimulating tumor progression, invasion and metastases [27].

No reports in Literature assessed SPARC in human endometrial cancer nor in relation with

chemokines. In this study we decided to analyze, in human endometrial cancer versus normal endometrial counterpart, the mRNA gene expression of SPARC, VEGF, CXCL12, CXCR7, CXCL11 and CXCL8 and their correlation in the peritumoral microenvironment.

Methods

Immediately after surgery, fresh samples of endometrial cancer (EC) and their normal endometrial counterpart (NE) were obtained from patients submitted to primary surgery for endometrial cancer at RCCS Humanitas Clinical Institute in Milan (Italy). Parts of the samples were used for the histology diagnosis and other parts were immediately treated with RNA later (Ambion) for 24-36 h at 4°C, and subsequently dried and stored at -80°.

The study was approved by the Ethical Committee of Humanitas Research Institute and informed, written consent was obtained for all patients. All the clinical and surgical data were recorded on a data base. The total RNA was isolated both from endometrial cancer and normal endometrial specimen using TRI Reagent (Ambion). RNA was quantified by Nanodrop spectrophotometer ND-1000and its quality was examined by 1.5% agarose gel electrophoresis. According to the manufacturer's instructions, 1mcg of total RNA was reverse-transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems), treated with DNase I, quantified and reverse – transcribed into cDNA using random primers. A real-time quantitative polymerase chain reaction, using Syber Green I (Applied Biosystem) as detection dye, was used to determine the relative cDNA levels of genes in each sample. The amplification protocol was used as following: 2 min at 50°C to activate uracil-DNA glycosylase, 10 min at 94.5°C (activation), 40 cycles of denaturation al 97°C for 30 s and annealing and extension at 59.7°C for 1 min. The relative amount of each target gene mRNA to the housekeeping gene (18S) was calculated as $2^{(-DCt)}$, where DCt=Ct gene-Ct housekeeping gene. The fold-change of each target gene mRNA to the corresponding normal tissue was calculated as $2^{(-DDCt)}$, where DDCt=DCt target gene in tumor tissue – DCt target gene in normal tissue. The threshold cycle Ct was automatically given by the SDS2.2 software package (Applied Biosystems). The gene sequences were for:

18S Fw 5'-CGCCGCTAGAGGTGAAATTC-3',

18S Rev 5'-CTTTCGCTCTGGTCCGTCTT-3',

SPARC Fw 5'-TGACCTGGACAATGACAAGT-3';

SPARC Rev 5'-CTAGTCCCAAAACCATCCTT-3',

VEGF Fw 5'-CTCAGAGCGGAGAAAGCATTTG-3'

VEGF rev 5'-TTAACTCAAGCTGCCTCGCCT-3';

CXCL12 Fw 5'-CAGAGTCAACGTCCAGCATCT-3',

CXCL12 rev 5'-CCTGAATCCACTTTAGCTTCGG-3',

CXCL11 Fw 5'-GAGTGTGAAGGGCATGGCTA-3',

CXCL11 rev 5'-ATGCAAAGACAGCGTCCTCT-3',

CXCL8 Fw 5'-CCAGGAAGAAACCACCGGA-3',

CXCL8 rev 5'-GAAATCAGGAAGGCTGCCAAG-3',

CXCR7 Fw 5'-TCACCTACTTCACCAGCACC-3',

CXCR7 rev 5'-ACATGGCTCTGGCGAGCAGG-3',

We analyzed SPARC, VEGF, CXCL12, CXCL11, CXCL8 and CXCR7 gene expression in EC versus NE.

Statistical analysis

Statistical significance was determined by χ-test and considered significant at a P value of ≤ 0.05.

Results

We collected tissue samples from endometrial cancer (EC) and from normal corresponding endometrium (NE) in 15 patients with endometrial cancer FIGO stage I-IIIC. All patients were submitted to primary laparoscopic total hysterectomy and bilateral salpingectomy with pelvic lymphadenectomy. Four patients dropped out from the study: two because the endometrial sample was damaged during the storage making it impossible to process, and two because no residual tumor was found in the samples, despite an initial histological diagnosis by endometrial biopsy. Tables 1 and 2 describe the clinical characteristics of the study population. Three patients (27%) underwent adjuvant chemotherapy and pelvic radiotherapy and one patient (9%) underwent adjuvant pelvic radiotherapy (Table 2). At a median 3 years follow-up, the median disease-free survival was 25 months (range 18–36). Only one patient with clear cell adenocarcinoma FIGO stage IIIA and no residual disease after surgery relapsed at 18 months (Table 2). In endometrial cancer versus normal counterpart, we observed mRNA gene expression as follows: SPARC was down-regulated in 91 % of samples (Figure 1, P<.05), VEGF was down regulated in 73% of samples (Figure 2, P=NS), CXCL12 was down-regulated in 91% of samples (Figure 3, P < .001), CXCR7 was down-regulated in 91% of samples (Figure 4, P<.001), CXCL8 was over-expressed in 64% of samples (Figure 5, P=NS) and CXCL11 was down-regulated in 54% of samples (Fig 6, P=NS). In endometrial cancer samples, we found that SPARC mRNA down-regulation was statistically significantly directly related to VEGF mRNA down-regulation (Figure 7, P=.03).

Table 1: Evaluable Patients' clinical characteristics.

No. of patients	11
Median Age	63 (range 53-81)
Mediana BMI (Kg/m^2)	28 (range 25-31)
FIGO stage I	8 (73%)
IA	7 (64%)
IB	1 (9%)
FIGO stage III	3 (27%)
III A	2 (18%)
III C	1 (9%)
Hystotype	
Endometrioid	7 (64%)
Clear Cell	2 (18%)
Villoglandular	1 (9%)
Endometrioid with squamous differentiation	1 (9%)

BMI: Body Mass Index.

Table 2: Clinic Characteristics of 11 evaluable patients.

Pt.	age	FIGO stage	LVS	N	G	Hystotype	ADJ	DFS mths
1	66	IA	-	-	G3	AE	FU*	30
2	65	IA	-	-	G3	ACC	PAC + RT	32
3	75	IA	-	-	G1	AV	FU	36
4	63	IA	-	-	G2	AE	FU	35
5	58	IA	-	-	G2	AE	FU	23
6	68	IA	-	-	G2	AE	FU	24
7	61	IA	-	-	G2	AE	FU	36
8	81	IB	-	-	G2	AE	FU	22
9	53	IIIA	-	-	G2	AS	PAC + RT	25
10	81	IIIA	+	-	G2	ACC	CT +RT	18°
11	63	IIIC	+	+	G2	AE	RT	23

LVS: Lymphvascular space

N: Lymph nodes; **G**: Histological grade; **ADJ**: Adjuvant therapy; **DFS**: Disease free survival in months; **PAC**: Cisplatin, Paclitaxel **CT**: Carbo Taxol; **RT**: Radio Therapy; **AE**: Endometrioid Adenocarcinoma **ACC:** Clear Cell Adenocarcinoma **AV**: Villoglandular adenocarcinoma **AS**: Squamous Adenocarcinoma.

*Patient refused RT

° abdomino-pelvic relapse after 18 months

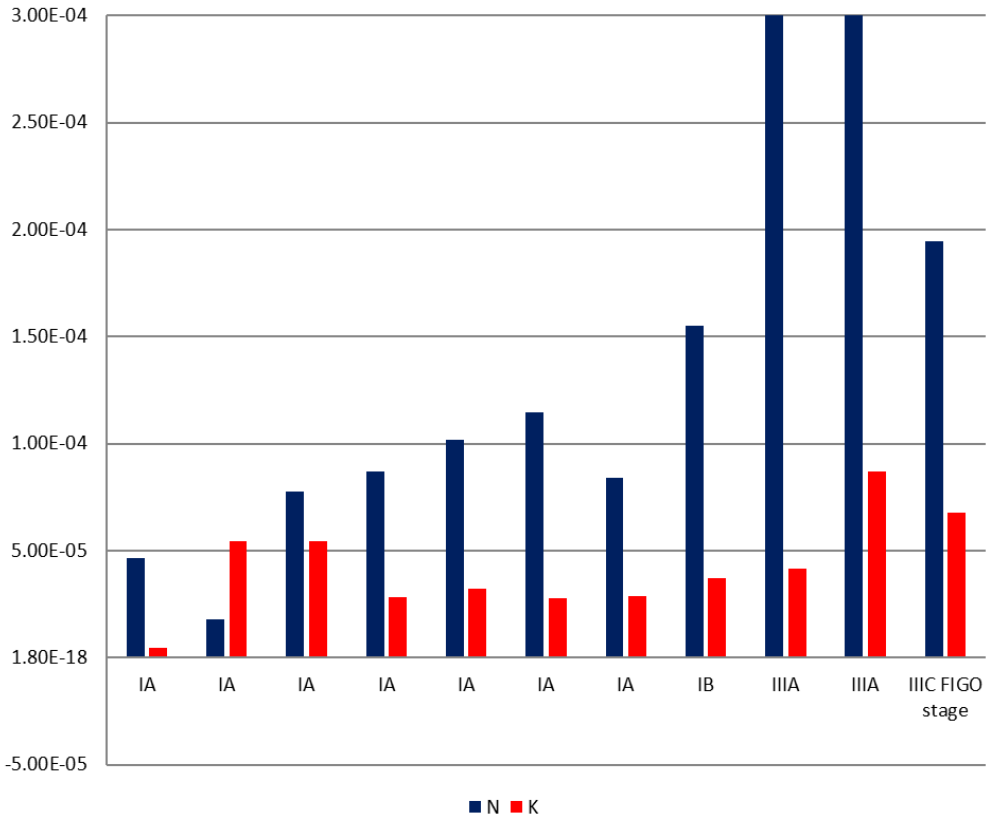

Figure 1: Secreted Protein Acidic and Rich in Cysteine (**SPARC)** mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K). In 91% Endometrial Cancer (K) versus normal endometrium counterparts (N), SPARC mRNA gene expression was down regulated (P<.05).

In endometrial cancer samples, we also observed that SPARC mRNA down-regulation was directly related in 100% of cases to CXCL12 mRNA down-regulation (Figure 8, P<.001) and to CXCR7 mRNA down-regulation (Figure 9, P<.001). No statistically significant correlation were found in endometrial cancer samples between SPARC and CXCL8 or CXCL11 mRNA expression.

Discussion

This is the first report in Literature investigating SPARC mRNA expression in endometrial cancer fresh tissue and comparing it with VEGF mRNA expression and cytokines. We chose the mRNA gene expression evaluation because during the transcription process, from cellular DNA to the final product, many mechanisms can interfere. Therefore we decided to evaluate the mRNA expression level of the examined genes, as mRNA expression is the primary index of gene activity. We can consider our patients cohort (see table 2) at low prognostic risk, due to the histotypes (mainly type I endometrial cancer), the FIGO stage (72% early stage of disease), the histological grade (81% Grade1-2) and the lymphvascular space involvement (81% negative). Since SPARC, VEGF, CXCL12 and CXCR7 have been previously described as protumorigenic and prometastatic mediators in other types of cancer, our results seems to confirm this data in endometrial cancer type I [6-14]. The down regulation of SPARC, VEGF, CXCL12 and CXCR7

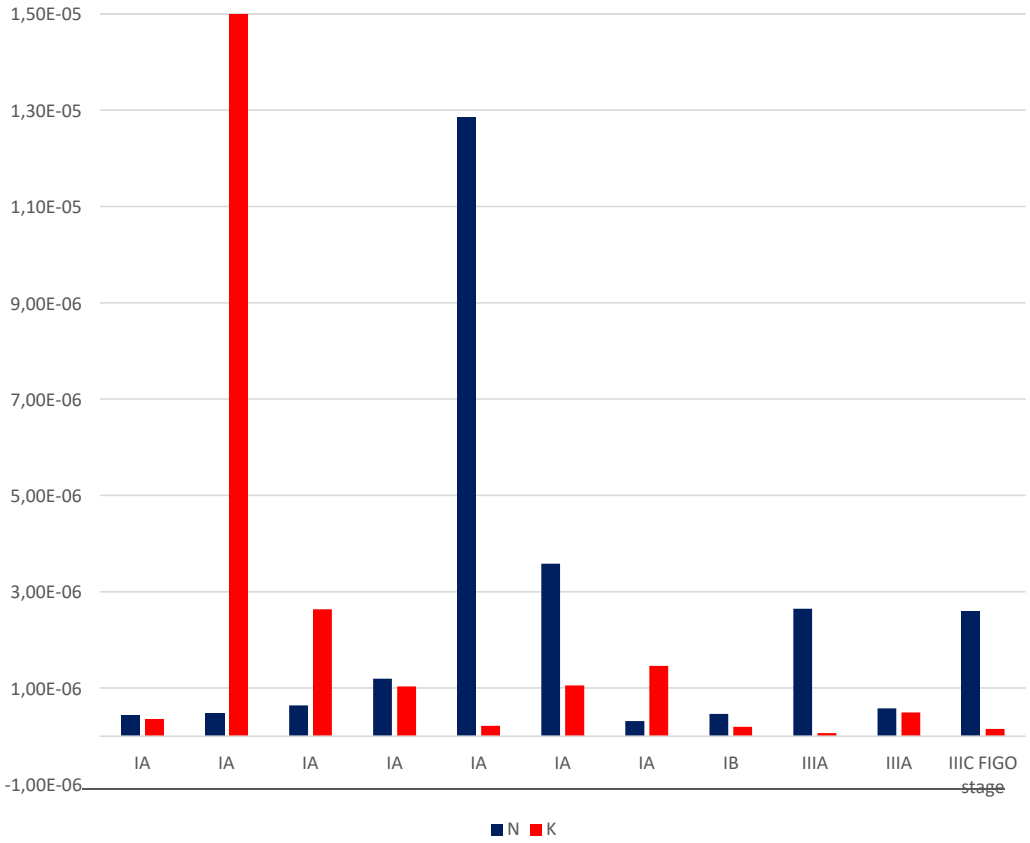

Figure 2: Vascular Endothelial Growth Factor (**VEGF**) mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K). In 73% endometrial cancer versus normal endometrium counterparts, VEGF mRNA gene expression was down- regulated (P=NS).

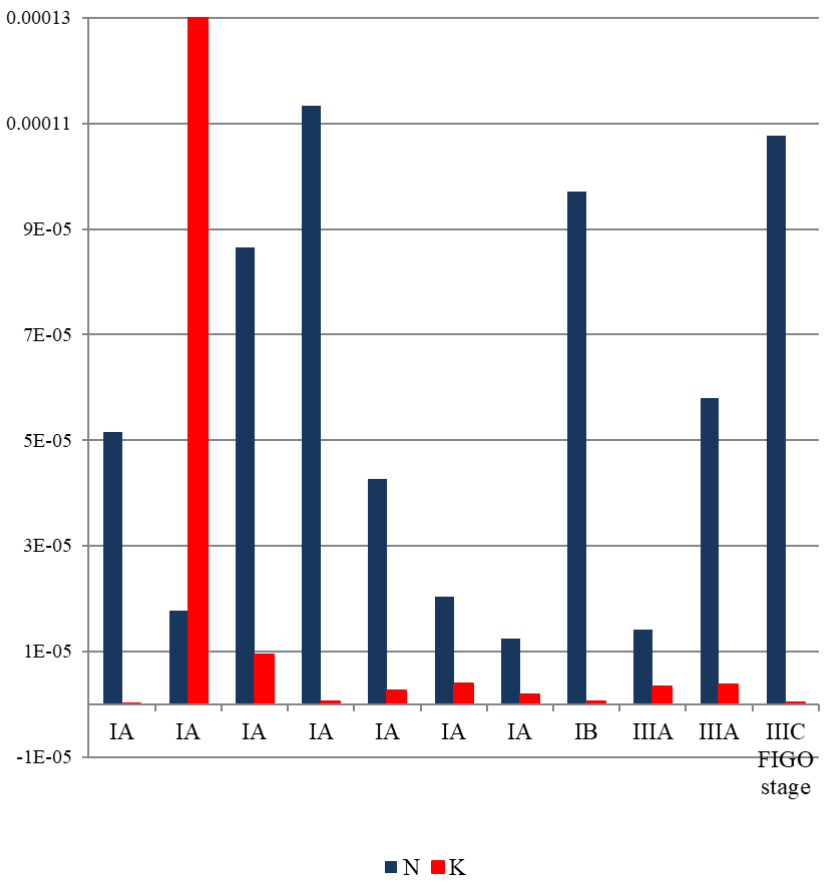

Figure 3: CXCL12 mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K). In endometrial cancer samples, versus normal endometrium counterpart, CXCL12 is under- expressed in 91% of samples (P<.001).

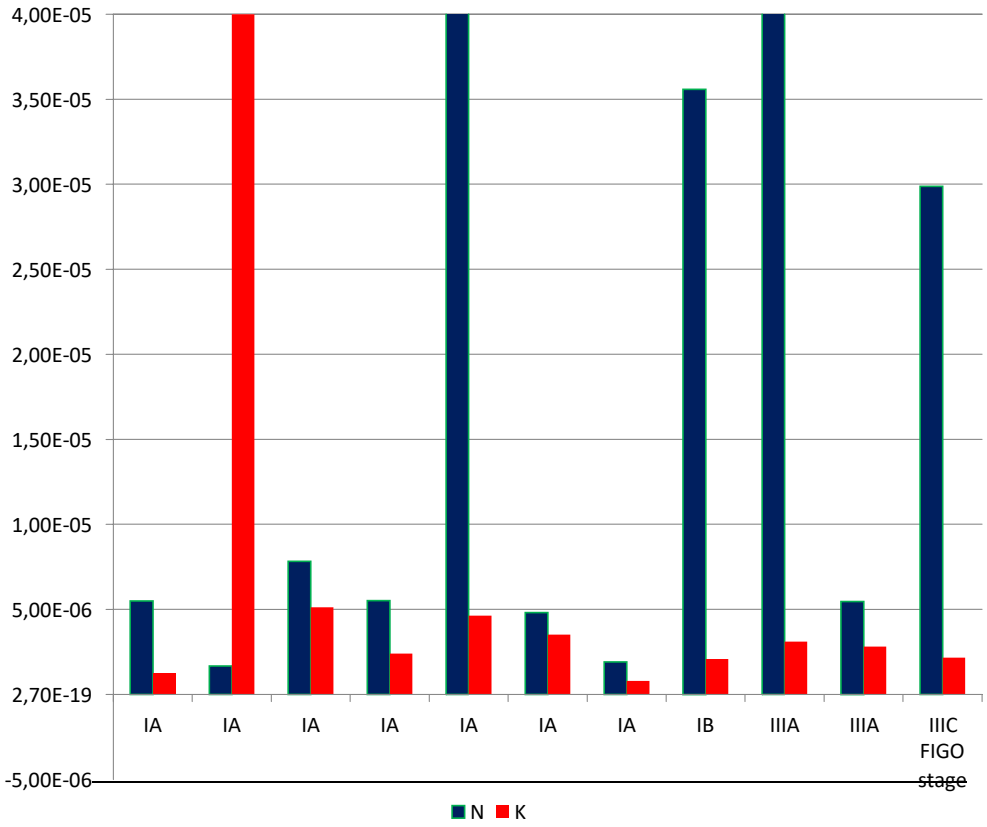

Figure 4: **CXCR7** mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K).
In endometrial cancer samples, versus normal endometrium counterpart, CXCR7 is under- expressed in 91% of samples (P <.001).

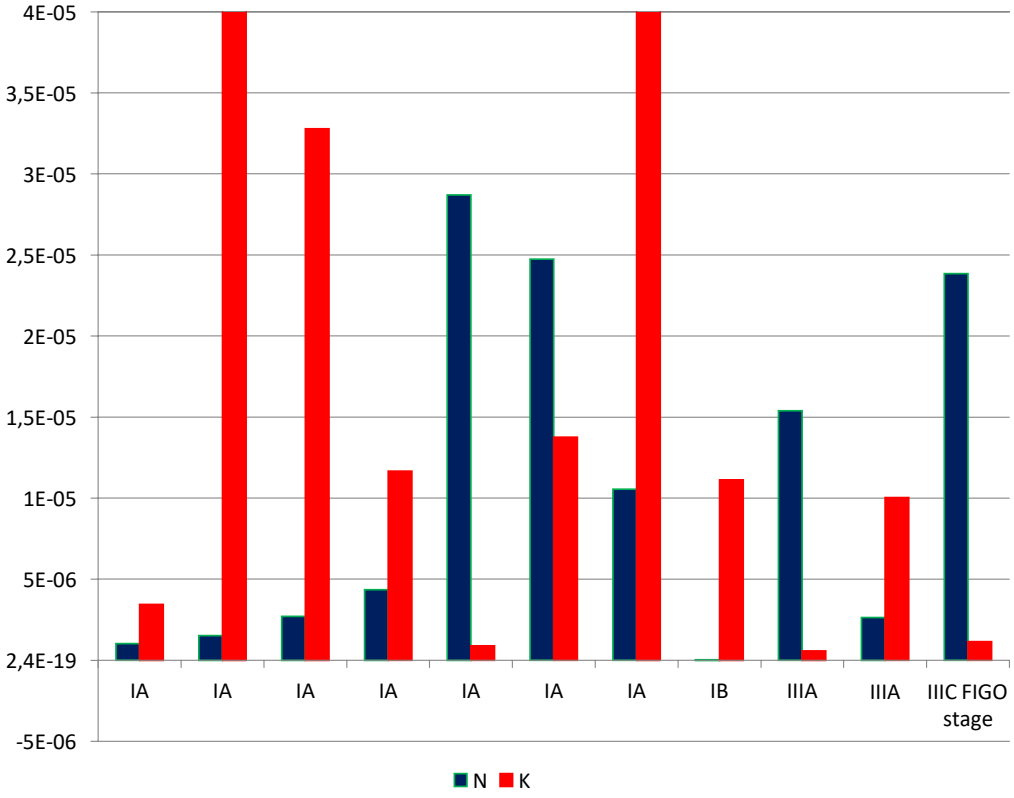

Figure 5: **CXCL8** mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K).
In endometrial cancer samples, versus normal endometrium counterpart, CXCL8 is over-expressed in 64% of samples (P =NS).

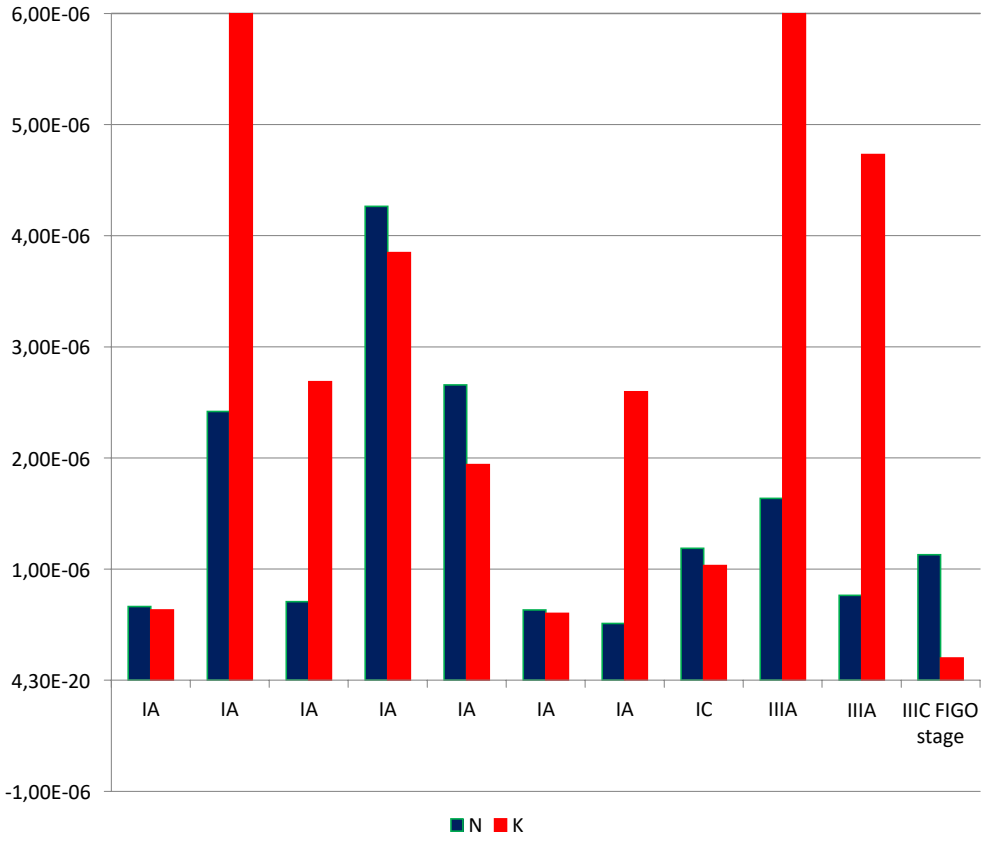

Figure 6: **CXCL11** mRNA gene expression in normal endometrium (N) versus endometrial cancer samples counterpart (K).
In endometrial cancer samples, versus normal endometrium counterpart, CXCL11 is under- expressed in only in 54% of samples (P =NS).

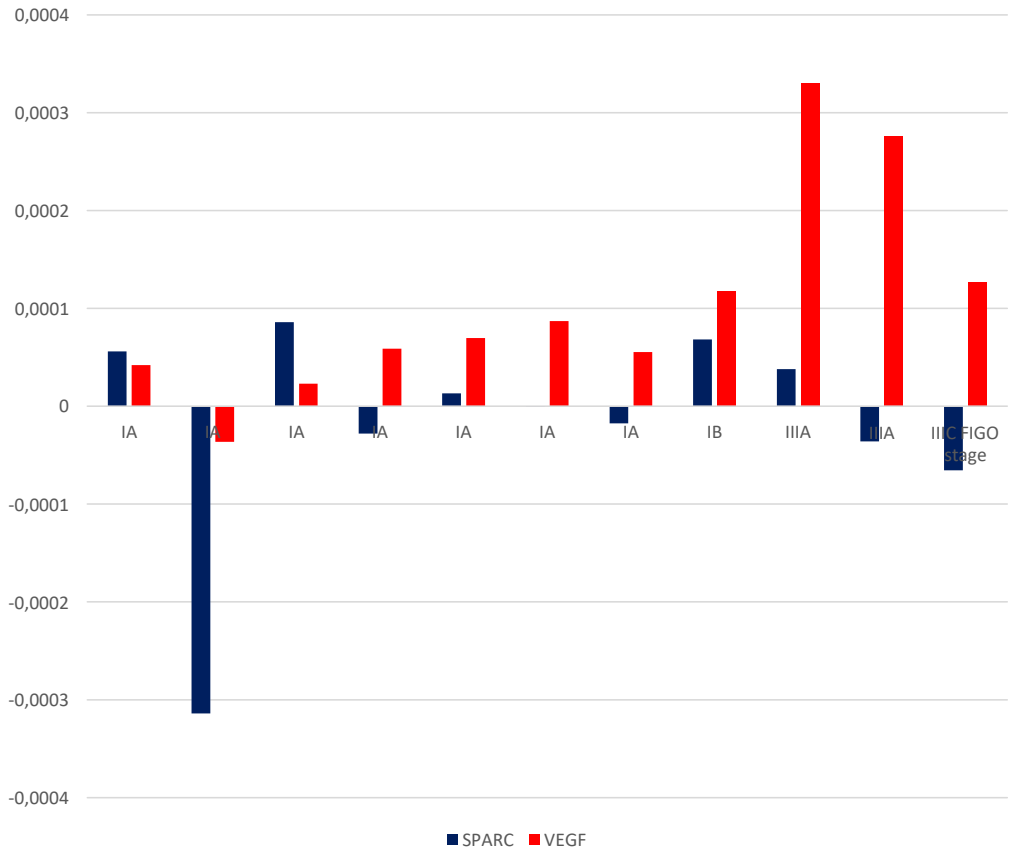

Figure 7: In endometrial cancer samples, correlation between VEGF mRNA and SPARC mRNA down-regulation.
In 73% SPARC mRNA expression was directly related to VEGF mRNA expression (P=.03).

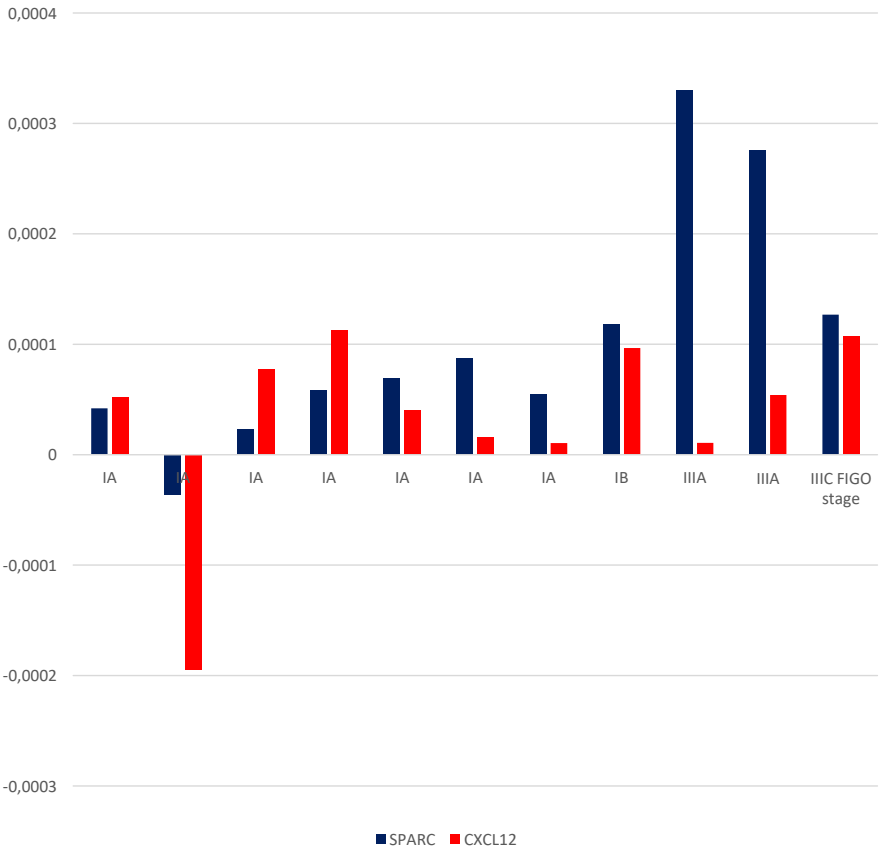

Figure 8: In endometrial cancer samples, correlation between SPARC and CXCL12 mRNA down-regulation.
In endometrial cancer samples, SPARC and CXCL12 are directly related in 100% of samples (P<.001).

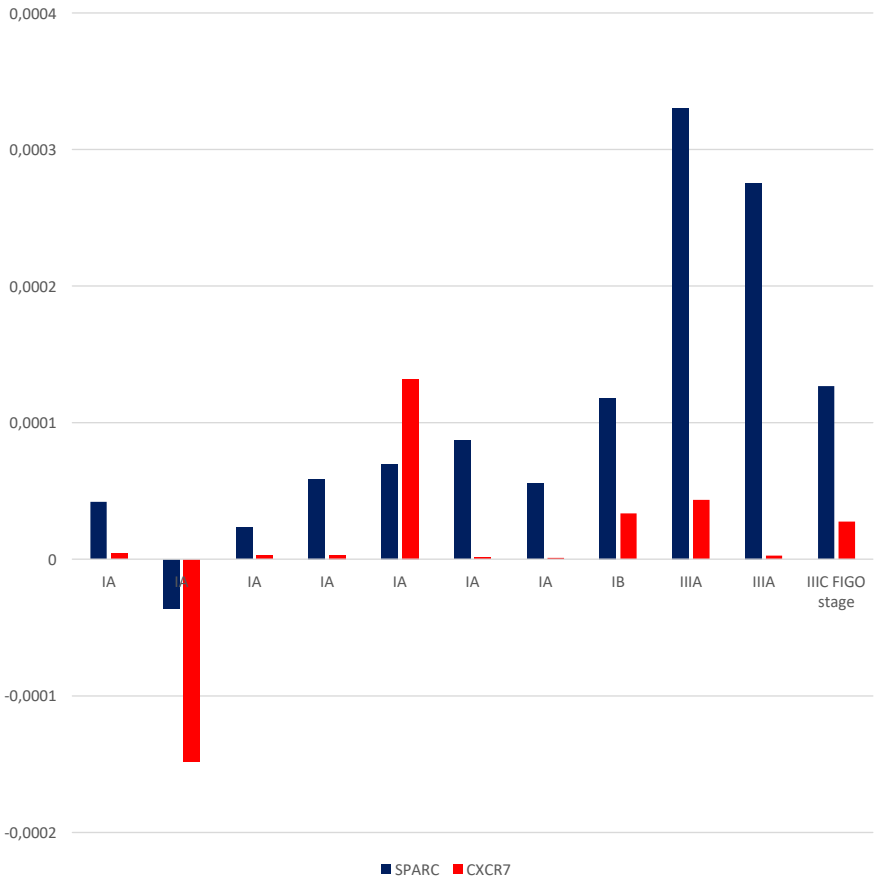

Figure 9: In endometrial cancer samples, correlation between SPARC and CXCR7 mRNA down- regulation.
In endometrial cancer samples, SPARC and CXCR7 are directly related in 100% of samples (P<.001).

in our study population at low-risk endometrial cancer, could be an expression of a low potential for blood and lymphatic metastatic route. Indeed, because the formation of new blood vessels from preexisting vascular network, is essential to tumor growth and it is well known be mediated by VEGF, we expected in our study population cohort at low risk, the down-regulation of VEGF mRNA, as we already described in an another report [28]. The statistical significant correlation between SPARC and VEGF in human endometrial microenvironment, might let us assume a precise interaction in endometrial cancer between these two mediators of tissue inflammation, repair and remodeling, as reported in Literature for gastric, breast and colon cancer [29-30]. Similarly, the statistically significant direct correlation between SPARC and CXCL12 can express their specific interaction in hematogenous metastastic spread. On another side, the statistically significant correlation between SPARC and CXCR7 down- regulation might let us also to hypothesize that SPARC can modulate lymphatic spread via CXCR7. No conclusive results are instead reported about CXCL8 and CXCL11.

Conclusion

In endometrial cancer, the under expression of SPARC, directly related to VEGF, CXCL12 and CXCR7 mRNA expression, might be consistent with a specific SPARC function on tumor progression and invasion mediated by VEGF/ CXCL12 and CXCR7 through haematologic and lymphatic spread, respectively.

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