Association of C-Reactive Protein (CRP) Gene Polymorphisms, Serum CRP Levels and Cervical Cancer Prognosis

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Abstract. *Background: C-reactive protein (CRP) is the prototypical biomarker of inflammation. Genetic variations within the CRP gene have been shown to be associated with alterations of CRP expression and prognosis in cancer patients. The purpose of this study was to evaluate the association between four polymorphisms of the CRP gene, CRP serum levels, clinicopathological parameters of cervical cancer and survival in patients with cervical cancer. Materials and Methods: The four most common single nucleotide gene polymorphisms CRP1919 (rs1417938), CRP2667 (rs1800947), CRP3872 (rs1205), and CRP5237 (rs2808630) were evaluated in 178 patients with cervical cancer. DNA was extracted from blood samples and CRP gene polymorphisms were investigated, using pyrosequencing. Findings were correlated with CRP serum levels, clinico-pathological parameters of cervical cancer, and disease-free and overall survival. Furthermore, the association between haplotype combinations and survival was investigated. Results: Presence of the CRP gene polymorphism CRP5237A>G was associated with lower CRP serum levels (p=0.04). Univariate survival analysis revealed that CRP1919T>A polymorphism (p=0.02), International Federation of Gynecology and Obstetrics stage (p<0.001), lymph node involvement (p=0.004), histological grade (p=0.01), and serum CRP levels (p<0.001) correlate with overall survival. In the multivariable Cox regression model, CRP1919T>A (p=0.02), tumor stage (p<0.001), lymph node involvement (p=0.03), patients' age (p=0.02), and serum CRP levels (p<0.001) were found to be independently associated with overall survival. None of the haplotype combinations were associated with prognosis of patients with cervical cancer. Conclusion: Presence of the CRP1919T>A polymorphism was associated with impaired overall survival in patients with cervical cancer. The CRP gene polymorphism CRP5237A>G was associated with decreased serum CRP levels.*

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C-Reactive protein (CRP) is widely used as an inflammation-sensitive marker (1). An association between high serum CRP levels and poor prognosis in cancer patients was reported in recent studies. It was shown that rising CRP levels correlate with advanced tumor stage and impaired survival in women with ovarian, endometrial, and breast cancer (2-4). Recently, our study group showed that high serum CRP levels are independently associated with impaired disease-free and overall survival in patients with cervical cancer (5).

The *CRP* gene is located on locus 1q21-q23 of chromosome 1. Several single-nucleotide polymorphisms (SNPs) in this gene have been described in the literature (6). Among these, the four most common polymorphisms, *i.e. CRP1919T>A, CRP2667G>C, CRP3872G>A,* and *CRP5237A>G* – known to be associated with changes of CRP production *in vivo* – have been investigated in a number of human conditions and diseases, such as myocardial infarction, chronic heart failure, and cancer (6-12). The location of these four polymorphisms on the *CRP* gene is shown in Figure 1. A recent population-based study, including 46,618 participants from Denmark, found an association between *CRP* polymorphisms and cancer risk (9). The modifying effect of *CRP* gene polymorphisms on the risk of endometrial cancer was reported as part of the

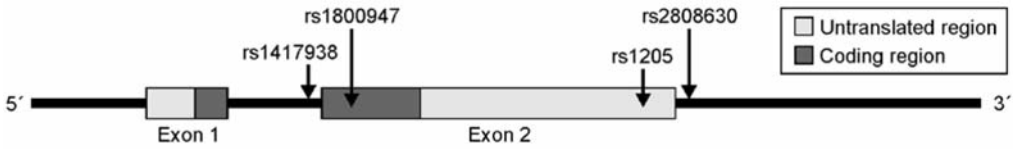

Figure 1. *Location of the investigated polymorphisms on the CRP gene on chromosome 1.*

Rotterdam Study (13). In a number of studies, *CRP* gene polymorphisms were associated with a higher rate of metastasis and impaired survival in cancer patients (10, 14).

The objective of this study was to investigate the association between the four most common *CRP* gene polymorphisms *CRP1919* (rs1417938), *CRP2667* (rs1800947), *CRP3872* (rs1205), and *CRP5237* (rs2808630), serum CRP levels, clinicopathological parameters of cervical cancer, and prognosis in patients with cervical cancer. Furthermore, we intended to establish a critical combination of *CRP* haplotypes as an independent prognosticator for patients with cervical cancer.

Patients and Methods

Patients. In the present study 178 consecutive patients with invasive cervical cancer, treated at the Department of General Gynecology and Gynecologic Oncology, Comprehensive Cancer Center, Medical University of Vienna, between January 2000 and December 2009, were enrolled. The Institutional Review Board of the Medical University of Vienna approved the present study (IRB approval number: 366/2003). All of the patients signed a human subject Institutional Review Board informed consent form prior to study inclusion.

Clinical management. Patients were clinically staged and treated according to International Federation of Gynecology and Obstetrics (FIGO) guidelines. Patients' clinical data and survival data were assessed semi-annually and documented using an electronic database.

CRP measurement. Blood samples for evaluation of serum CRP levels were obtained by a single peripheral venous puncture 24-48 h prior to therapy during routine preoperative assessment. For the measurement of serum CRP levels, a commercially available immuno-turbidimetric test (Olympus, CRP Latex, Olympus Life and Material Science Europe, Hamburg, Germany) was used, as described previously (5). After assessment by a physician specialized in internal medicine prior to therapy, patients with presence of acute infection were not included.

DNA extraction. DNA was isolated from centrifuged blood clots by a modified DNAzol procedure, from EDTA-blood using the QIAamp DNA Blood Midi Kit (Qiagen, Inc., Hilden, Germany). Extracted DNA was stored at 4˚C until analyzed.

Polymerase chain reaction (PCR). After searching the SNP database from the National Center for Biotechnology Information (dbSNP), the four most common and clinically relevant functional SNPs [*CRP1919* (rs1417938), *CRP2667* (rs1800947), *CRP3872* (rs1205), and *CRP5237* (rs2808630)] within the *CRP* gene were chosen for analysis (6, 9-12).

The primers CRP1919 SE 5'-TCTCTCATGCTTTTGGCC AGAC, CRP1919 AS 5'-ACCATGAAGGATGCTCCACTGT, CRP2667 SE 5'-GTGAACATGTGGGACTTTGTGC, CRP-2667 AS 5'-GCCCGCC AAGATAGATGGT, CRP3872 SE 5'-TGCTGGATTTCCAAGC TGAG, CRP3872 AS 5'-TTGGC TCCTCCACTTCCAGTT, CRP5237 SE 5'-TTATAGAAGGCCATC CCAACTCC, and CRP5237 AS 5'-ATTAAGGCCAGAGGCTG TCTACCA, were used to amplify fragments of the *CRP* gene. Antisense primers were biotinylated. PCR was carried out in a total volume of 25 μL including 25 ng template, 5 pmol of each sense and antisense primer and puReTaq Ready-To-Go PCR beads (Amersham Biosciences, Little Chalfont, UK), which contain 2.5 units of puReTaq DNA polymerase, 10 mmol/L Tris-HCl (pH 9.0 at room temperature), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dATP, dCTP, dGTP, and dTTP, and stabilizers, including bovine serum albumin. PCR was carried out on a Perkin-Elmer GeneAmp PCR system 9600 with 40 cycles at 95˚C for 30 s, 62˚C (*CRP1919*) or 60˚C (*CRP2667, CRP3872, CRP5237*) for 30 s, and 72˚C for 30 s. The reaction was preceeded by a primary denaturation step at 95˚C for 1 min. A final extension was performed at 72˚C for 7 min.

Detection of SNPs by pyrosequencing. The four SNPs were detected using Pyrosequencer PSQ 96 and the PSQ 96 SNP reagent kit (Pyrosequencing AB, Uppsala, Sweden). Twenty-five microliters of PCR product were used for pyrosequencing according to the manufacturer's instructions. Five picomoles of the sequencing primers CRP1919 SEQ 5'-CCC ATACCTCAGATCAAA, CRP2667 SEQ 5'-GGTGTTAATCTCATCTGG, CRP3872 SEQ 5'-GCCACATGGA GAGAGAC, and CRP5237 SEQ 5'-CAGTTGCTTGCATCTTACTA were applied to detect the SNPs.

Statistics. After testing for normality using the Kolmogorov-Smirnov test, values were given as mean (standard deviation [SD]) or median (interquartile range [IQR]) where appropriate.

The homozygous wild-type genotype (wt/wt) was compared to the heterozygous (wt/mut), and homozygous mutant (mut/mut) genotypes. Values regarding the linkage disequilibrium were expressed as Lewontin's D' and associated *p*-values. The highest possible Lewontin's D' value is 1, indicating maximum linkage between the respective gene polymorphisms; a Lewontin's D' value of 0 indicates no linkage. Chi-square tests were used to compare frequencies of CRP genotypes between groups defined by clinical–pathological parameters and results were given as *p*-values and odds ratio (95% confidence interval, CI). Continuous variables were compared between groups using Kruskal–Wallis non-parametric analysis of variance. Student's *t*-tests were used to compare mean CRP serum levels between groups (wt/wt *vs*. wt/mut and mut/mut) for the four SNPs in order to detect a modifying effect of the genetic variations on CRP expression.

Median follow-up time was computed using the method of Schemper and Smith (14). Overall cancer-related survival time was defined as time from diagnosis to cancer-related death or progression

of disease. Observations on patients with no evidence of disease, with stable disease, and on patients having died of non-cancer-related events were censored with the last follow-up date or date of death. Disease-free survival time was defined as time from diagnosis to recurrence of disease. Observations on patients with no evidence of disease and on patients who died without evidence of disease were censored at the last follow-up date or date of death, respectively. Disease-free and overall survival probabilities were calculated by the product limit method of Kaplan and Meier and resulting survival curves were compared using the log-rank test (15). In order to evaluate independent predictors of disease-free and overall survival, a multivariate Cox regression model was estimated comprising the parameters FIGO stage (III-IV *vs*. II *vs*. IB *vs*. IA), lymph node involvement (positive *vs*. negative), tumor grade (G3 *vs*. G2 *vs*. G1), histopathological type (adenocarcinoma *vs*. squamous cell carcinoma), patients' age at diagnosis (per decade), and serum CRP levels (16). The SNPs (*CRP1919T>A*, *CRP2667G>C*, *CRP3872G>A*, and *CRP5237A>G*) were simultaneously included as independent variables (wt/wt *vs*. wt/mut and mut/mut) in order to evaluate their potential additive effects.

Association between haplotypes and cervical cancer survival was assessed by Cox regression models. Haplotypes could not be uniquely determined in all patients. Therefore, we used SAS/Genetics software to compute, for each haplotype, its probability of being present in a patient. The probability scores on the haplotypes for each patient were then included in Cox regression as independent continuous variables (17).

The proportional hazards assumption of the Cox regression model was visually checked by plotting Schoenfeld residuals of each variable against time and tested by evaluating the statistical significance of each variable's interaction with log time (18-19).

The SAS System (version 9.1, SAS Institute, Inc., Cary, NC, USA) and the PASW software system (PASW 18.0, PASW, Inc., Chicago, IL, USA) were used for statistical computations. Two-sided *p*-values <0.05 were considered statistically significant.

Results

Patients' characteristics are provided in Table I. Genotype frequencies were 46.9% (TT), 38.3% (TA), and 14.9% (AA) for *CRP1919T>A*; 86.0% (CC), 12.9% (CG), and 1.1% (GG) for *CRP2667G>C*; 42.3% (GG), 46.9% (GA), and 10.8% (AA) for *CRP3872G>A*; 55.6% (AA), 38.2% (AG), and 6.2% (GG) for *CRP5237A>G*; all genotype frequencies were in Hardy-Weinberg equilibrium (p=0.06, p=0.3, p=0.6, and p=0.9, respectively) and were similar to the genotype frequencies reported in the National Cancer Institute SNP500 database within the study population. All four SNPs of the *CRP* gene were in strong linkage disequilibrium (*CRP1919T>A* and *CRP2667G>C*: Lewontin's D'=0.82; p=0.028; *CRP2667G>C* and *CRP3872G>A*: D'=0.93; p<0.0001; *CRP3872G>A* and *CRP5237A>G*: D'=0.93; p<0.0001) within the study population.

Median serum CRP levels at the time of DNA sampling were 0.5 (IQR=0.4-0.9) mg/dL. In our study population, the presence of *CRP5237A>G* was associated with lower serum CRP levels (p=0.04). None of the other investigated SNPs were associated with alterations in serum CRP levels (p=0.5, p=0.4, and p=0.5 for *CRP1919T>A, CRP2667G>C*, and *CRP3872G>A*, respectively).

No significant associations between the four investigated SNPs (*CRP1919T>A, CRP2667G>C, CRP3872G>A*, and *CRP5237A>G*) and the clinicopathological parameters FIGO stage (p=0.4, p=0.4, p=0.2, and p=0.7, respectively), lymph node involvement (p=0.6, p=0.4, p=0.2, and p=0.2, respectively), histological grade (p=0.8, p=0.3, p=0.6, and p=0.7, respectively), patients' age at diagnosis (p=0.7, p=0.9, p=0.9, and p=0.5, respectively), and histopathological type (p=0.9, p=0.9, p=0.6, and p=0.3, respectively) were ascertained.

Regarding disease-free survival, an association with FIGO stage, lymph node involvement, histological grade, and pre-therapeutic serum CRP levels, but none of the four investigated

Table I. *Characteristics of 178 patients with cervical cancer.*

Parameter	N
Mean age in years at first diagnosis	49.2 (SD=13.9)
Histopathological tumor type	
Squamous cell carcinoma	148 (83.2%)
Adenocarcinoma	26 (14.6%)
Adenosquamous carcinoma	4 (2.2%)
FIGO tumor stage	
IA1	11 (6.2%)
IA2	7 (3.9%)
IB1	55 (30.9%)
IB2	16 (9.0%)
IIA	7 (3.9%)
IIB	60 (33.7%)
IIIA	2 (1.1%)
IIIB	17 (9.6%)
IVA	1 (0.6%)
IVB	2 (1.1%)
Histological grade	
G1	19 (10.7%)
G2	87 (48.9%)
G3	49 (27.5%)
Unknown	23 (12.9%)
Lymph node involvement	
Negative	96 (53.9%)
Positive	59 (33.1%)
Not evaluated	23 (12.9%)
CRP level (mg/dL), median	0.5 (IQR=0.4-0.9)
Median time of follow-up in months	46 (IQR=18-80)
Recurrence status	
Patients with recurrent disease	62 (34.8%)
Five-year cumulative probability of recurrence-free survival (95%CI)	57.7% (48.5-65.9)
Status at last observation	
Alive with no evidence of disease, or stable disease	132 (74.2%)
Progressive disease	9 (5.1%)
Tumor-related death	35 (19.7%)
Dead as a result of other causes	2 (1.1%)
Five-year cumulative probability of survival (95% CI)	71.9% (62.4-79.4)

Table II. *Univariate and multivariate survival analyses in 178 patients with cervical cancer.*

	Disease-free survival			Overall survival		
	Univariate[1,2]	Multivariate[3]		Univariate[1,2]	Multivariate[3]	
	p-Value	*p*-Value	HR (95% CI)	*p*-Value	*p*-Value	HR (95% CI)
*CRP1919*T>A (TT and TA *vs*. AA)	0.3	0.6	1.2 (0.6-2.4)	0.01	0.02	3.6 (1.2-9.5)
*CRP2667*G>C (GC and CC *vs*. GG)	0.8	0.4	0.7 (0.4-1.4)	0.9	0.2	0.5 (0.2-1.6)
*CRP3872*G>A (GA and AA *vs*. GG)	0.4	0.3	0.7 (0.4-1.5)	0.8	0.8	1.1 (0.4-2.8)
*CRP5237*A>G (AG and GG *vs*. AA)	0.3	0.4	0.8 (0.4-1.5)	0.2	0.9	0.9 (0.4-2.6)
Tumor stage (FIGO III-IV *vs*. II *vs*. IB *vs*. IA)	<0.0001	0.001	2.3 (1.4-3.6)	<0.001	<0.001	3.4 (1.8-6.6)
Lymph node involvement (positive *vs*. negative)	0.005	0.09	0.6 (0.3-1.1)	0.004	0.03	0.4 (0.1-0.9)
Histological grade (G3 *vs*. G2 *vs*. G1)	0.005	0.9	1.1 (0.6-1.8)	0.01	0.7	1.1 (0.6-2.3)
Patients' age (per decade)	0.2	0.1	0.8 (0.6-1.1)	0.6	0.02	0.6 (0.4-0.9)
Histopathological type (adenocarcinoma *vs*. squamous cell carcinoma)	0.4	0.6	1.2 (0.6-2.6)	0.8	0.9	1.0 (0.3-3.2)
Serum CPR levels	<0.001	0.03	1.2 (1.1-1.4)	<0.001	0.001	1.4 (1.2-1.8)

[1]Log rank test, [2]univariate Cox-regression analysis, [3]multivariate Cox-regression analysis; HR, hazard ratio.

SNPs was associated in univariate analysis (Table II). In multivariable analysis, only FIGO stage and serum CRP level showed significant association with disease-free survival (Table II). Regarding overall survival, an association with *CRP1919T>A*, tumor stage, lymph node involvement, histological grade, and serum CRP level were ascertained in univariate analysis. Kaplan-Meier curves for overall survival according to the *CRP1919T>A* polymorphism are provided in Figure 2. In the multivariable Cox regression model, *CRP1919T>A*, FIGO stage, lymph node involvement, patients' age, and serum CRP level were found to be independently associated with overall survival (Table II). No significant violations of the proportional hazards assumption were detected for any of the variables entering the multivariable Cox regression model. The hazard ratios, confidence intervals and *p*-values resulting from the multivariable Cox proportional hazard models for disease-free and overall survival are shown in Table II.

The combined effect of the four SNPs on disease-free and overall cervical cancer survival was investigated by haplotype analysis. No significant associations between haplotype combinations and disease-free and overall survival were observed (Table III). The hazard ratios and 95% confidence intervals, estimated for each haplotype, referring to the reference haplotype TGGA (wt-wt-wt-wt) are shown in Figure 3.

Discussion

Based on the important role of chronic inflammation in cervical cancer and on previously published promising data on *CRP* polymorphisms in other gynecological malignancies, we ascertained the prognostic effect of four common functional polymorphisms within the *CRP* gene (10-12, 14). In this study, we intended to explore the individual effects of variations in the *CRP* gene and aimed at establishing a critical combination of *CRP* haplotypes as independent prognostic variables. To our knowledge, we are the first to report on the influence of *CRP* gene polymorphisms in patients with cervical cancer.

In our study, presence of the SNP *CRP5237A>G* was associated with lower serum CRP level. *CRP5237A>G*, located distal to the 3' untranslated region, has been previously reported to be associated with decreased serum CRP levels (8, 12, 20). Nevertheless, the mechanism for this influence and the functional significance remain unclear (20). For the other three investigated SNPs, we did not detect any effect on serum CRP levels. An association between the presence of these SNPs and alterations of serum CRP levels was reported in healthy women, and patients with non-malignant diseases (6, 8, 12, 20). The discordance of our findings with the previous reports might be caused by the fact that only patients with cervical cancer were included in our study and no healthy women.

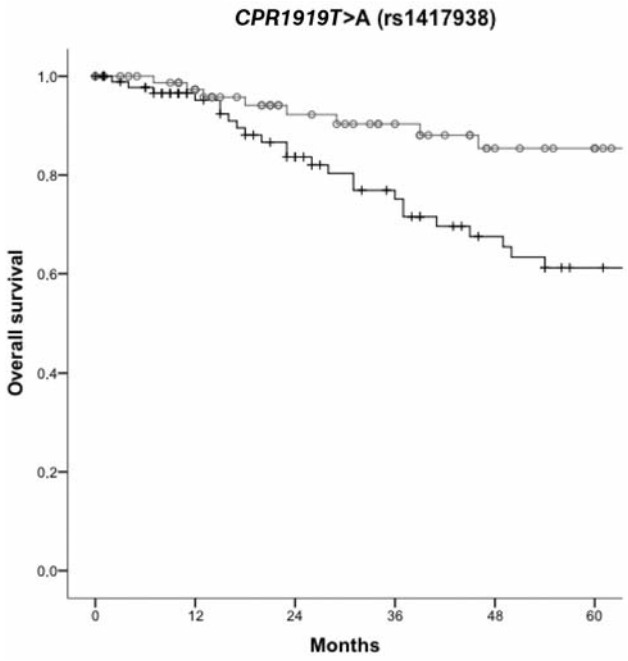

Figure 2. *Kaplan-Meier curves accounting for CRP1919T>A and overall survival in cervical cancer.*

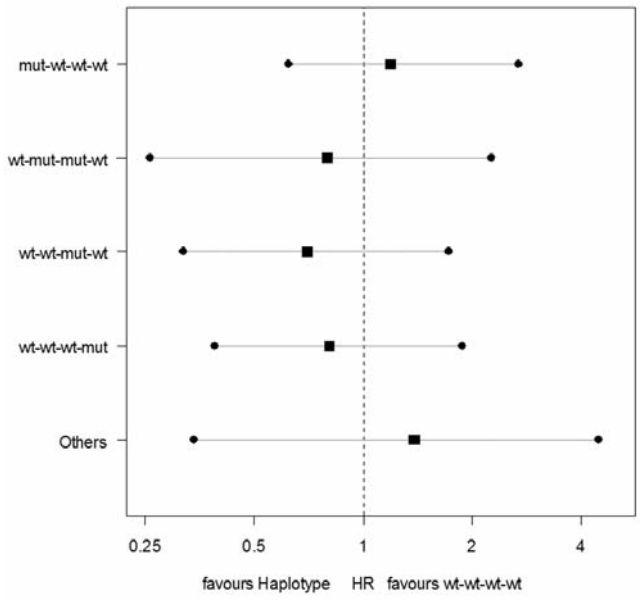

Figure 3. *Hazard ratios and 95% confidence intervals for overall survival associated with CRP gene polymorphism haplotypes.*

Table III. *Association between CRP haplotype frequencies and survival in 178 patients with cervical cancer.*

	Haplotype frequency (%)	Disease-free survival		Overall survival	
		p-Value	HR (95% CI)	*p*-Value	HR (95% CI)
TGGA†	18.7				
AGGA	21.5	0.3	1.4 (0.8-3.1)	0.3	1.2 (0.6-2.7)
TCAA	4.4	0.7	1.2 (0.5-3.1)	0.8	0.8 (0.3-2.6)
TGAA	18.0	0.9	1.0 (0.5-2.3)	0.4	0.7 (0.4-1.5)
TGGG	16.0	0.9	1.0 (0.5-2.3)	0.6	0.8 (0.4-1.9)
others	21.4	0.7	1.3 (0.3-4.1)	0.6	1.4 (0.3-4.5)

HR, hazard ratio. †The haplotype combination TGGA served as a reference variable.

Cancer patients are generally known to have increased serum levels of CRP, reflecting a state of chronic, tumor-related inflammation (5). This effect seems to be stronger than the effect of the genetic variations of the *CRP* gene. Although the literature shows that polymorphisms of the *CRP* gene, especially when located in the promoter region, can influence serum CRP levels, studies are not consistent as to this association (6-9, 12-13).

With respect to patients' prognosis, the presence of *CRP1919T>A*, known to be associated with higher CRP concentrations in healthy women, was independently associated with impaired overall survival (6). Our findings are not surprising as cervical cancer is considered to be a disease linked to chronic inflammation, and variations in serum CRP levels have been shown to be a powerful predictor of prognosis for patients with cervical cancer (5). Variations in the *CRP* gene might possibly influence the cancer-related host immune defense or have an impact on CRP expression and related mediation of inflammatory processes. Of note, the exact mechanisms of the relationship between *CRP* polymorphisms, serum CRP levels, and cancer prognosis still remain unclear.

In accordance with previous data we found a correlation between serum CRP levels and survival in patients with cervical cancer (5). This seems biologically plausible, as it is evident that a state of chronic inflammation plays a role in cervical carcinogenesis and tumor biology. CRP is known to play an anti-inflammatory role and an important part in host defense, actively increasing phagocytosis and the release of inflammatory

cytokines (1). Nevertheless, it remains unclear whether CRP only reflects the general inflammatory processes caused by cancer or is causally involved in the pathogenesis and progression of cervical cancer as an active and independent component.

Of note, the limited number of patients that were included in the analysis represents a shortcoming of our study. Our study was intended to assess the association between CRP, tumor biology, and prognosis in cervical cancer. Therefore, our results do not rule out that the investigated polymorphisms may be associated with the risk of developing cervical cancer, as we did not include any healthy controls. The choice of certain polymorphisms to be investigated as predictive or prognostic marker in a certain disease is always difficult. A relatively large number of *CRP* polymorphisms have been published in the literature up to date (6-8). To investigate the influence of all of these *CRP* polymorphisms on prognosis in a large data set would definitely be of clinical value. However, for our analysis we chose the four most promising *CRP* gene polymorphisms that were previously reported to be associated with gynecological cancer risk (8, 12). In a next step, we are planning to validate our findings in a larger, independent set of patients.

In conclusion, presence of the *CRP1919T>A* polymorphism was associated with impaired survival in patients with cervical cancer. Presence of *CRP5237A>G* was associated with decreased serum CRP levels.

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Conflict of interest statement

The Authors declare that they do not have any conflict of interest.

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