Cancer Biomarkers -1 (2018) 1–9
DOI 10.3233/CBM-181683
IOS Press

High serum Hsp70 level predicts poor survival in colorectal cancer: Results obtained in an independent validation cohort

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Abstract.
BACKGROUND: Hsp70 plays important role in the development and progression of cancer. Previously we described the association between serum Hsp70 levels and mortality of colorectal cancer.
OBJECTIVE: In this new prospective study we aimed to confirm and extend our previous findings in a larger cohort of patients, based on a longer follow-up period.
METHODS: Two hundred and thirty-two patients diagnosed with colorectal cancer were enrolled in the study. Baseline serum Hsp70 level and classical biomarker levels were measured. Patients were treated according to stage of the tumor and follow-up lasted for a median 46.4 months.
RESULTS: We found that serum Hsp70 concentrations increase significantly with stage of the disease (1.79; 2.23 and 3.21 ng/ml in stage I+II, III and IV respectively, $p = 0.012$ and 0.002, Mann-Whitney test) and with other known biomarkers of the disease. We managed to confirm our previous finding that high baseline serum Hsp70 level ($>$ 1.64 ng/ml) predicted poor 5-year survival (risk of death HR: 1.94 CI: 1.294–2.909; univariate; HR: 2.418 CI: 1.373–4.258; multivariate Cox regression analysis) in the whole patient population and also in subgroups of stage IV and stage III disease. The strongest association was observed in women under age of 70 (HR: 8.12, CI: 2.02–35.84; $p = 0.004$; multivariate Cox regression). The power of this colorectal cancer prognostic model could be amplified by combining Hsp70 levels and inflammatory markers. Patients with high Hsp70, CRP and high baseline WBC or platelet count had 5-times higher risk of death (HR: 5.07 CI: 2.74–9.39, $p < 0.0001$; and HR: 4.98 CI: 3.08–8.06, $p < 0.0001$ respectively).
CONCLUSIONS: These results confirm and validate our previous findings that serum Hsp70 is a useful biomarker of colorectal cancer.

Keywords: Hsp70, colorectal cancer, prognostic model, CRP, survival

1. Introduction

Heat shock proteins (Hsp) are a family of evolutionary conserved proteins. Hsps are molecular chaperones with a wide array of functions, including protein folding, transport, and also the repair and degradation of damaged proteins. Hsps have a regulatory role in programmed cell death and apoptosis [1]. A prominent member of the family is Hsp70, probably the most extensively investigated heat shock protein. Hsp70 plays a key role in carcinogenesis. It is overexpressed in most human cancers to promote cancer cell survival, prolif-

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eration and to evade apoptosis and other forms of cancer cell death [2]. In the absence of Hsp70, tumor cells become senescent, a state of irreversible growth arrest with specific cell morphology. Senescent cells are unable to proliferate and are eventually eliminated by the innate immune system (in [3,4]). On the other hand, high levels of intracellular Hsp70-1 correlate with tumor burden, advanced stage and worse prognosis in non-small cell lung cancer [5]; breast, endometrial, and uterine cervical carcinoma [6]. In a study of 81 primary human colorectal tissues the expression of Hsp70 and Hsp110 highly correlated with advanced clinical stages and lymph node involvement [7]. Hsp70 expression was associated with poor prognosis, decreased overall survival in patients suffering from rectal cancer and squamous cell lung cancer [8] and resistance to oncotherapy in some cancer patient groups [9].

Beyond its intracellular occurrence Hsp70 can also be found in the plasma membrane of many solid tumors, while this is not true for normal tissues [10,11]. Membrane-bound Hsp70 is not only a biomarker in aggressive tumors, but can serve as a potential target of antitumor therapies [12]. Moreover it can be released from the cell (the mechanism of this process is still not exactly clarified) and appear in the circulation in the form of soluble Hsp70 (sHSP70), both in healthy individuals [13,14] and in various pathologic conditions. Circulating Hsp70 has been extensively investigated in a multitude of physiologic (pregnancy, aging) and non-physiologic (hearth failure, diabetes, liver disease, asthma, obesity) conditions (reviewed in [2]), on the other hand, it has been studied to a lesser extent in malignancies. According to Gehrmann and co-workers, Hsp70 serum levels were significantly increased in patients with hepatocellular carcinoma (HCC) compared to healthy controls and subjects with chronic hepatitis [15]. Another group found a significant correlation between sHsp70 and gross tumor volume in adeno- and squamous cell carcinoma of the lung [16]. Previously we reported on strong association between serum Hsp70 levels and stage, as well as unfavourable prognosis of small cell lung cancer [17].

In 2010 we published preliminary data on the correlation between elevated serum Hsp70 levels and high mortality in a cohort of early stage colorectal cancer patients [18]. The present investigation is a confirmatory work, aimed to reproduce previous findings on a larger cohort of prospectively followed CRC patients, with a longer follow-up period. We intended to prove that high serum Hsp70 level is a poor prognostic factor and propose a powerful prognostic model combining Hsp70 with easily accessible traditional biomarkers.

Table 1
Baseline demographic and tumor characteristics of patients with colorectal cancer

Variable	Number (percent)
Gender	
Male	138
Female	94
Age at diagnosis (year, mean, SD)	66.82 (11.41)
TNM stage	
I	9 (3.9)
II	101 (43.5)
III	73 (31.5)
IV	49 (21.1)
Tumor localization	
Right colon	43 (18.5)
Colon transversum	16 (6.9)
Left colon	89 (38.4)
Rectum	83 (35.8)
Unknown	1 (0.5)
Tumor grade	
1	51 (22.0)
2	113 (48.7)
3	44 (18.9)
Unknown	24 (10.4)
Surgery	
Definitive or palliative surgery	210 (90.5)
No surgery	22 (9.5)

2. Methods

2.1. Patients, controls and sample collection

Two hundred and thirty two patients diagnosed with colorectal cancer and 110 controls were involved in the study between January 2011 and June 2013 in the oncology ward of 3rd Department of Internal Medicine, Semmelweis University. After confirmation of invasive colorectal cancer with any stage, patients were consented consecutively and clinical data and blood samples were collected before starting anticancer therapy. Baseline demographic and clinical characteristics of patients are summarized in Table 1. Mean age of patients was 66.8 years, with a male/female ratio of 138/94. After diagnosis and adequate surgery patients were treated and followed by the oncology ward according to the stage of their disease and to the actual national and European [19] guidelines. Patients with rectal cancer received radiochemotherapy before definitive surgery from cT3 or N+ disease. Twenty-two patients who had unresectable and/or metastatic disease received upfront primary systemic treatment without definitive surgery. During a follow-up period that lasted for maximum 5 years (median 46.42 months), progression free survival and overall survival data were collected.

The control group consisted of 110 healthy individuals (mean age 64.5 years, male/female ratio 43/67),

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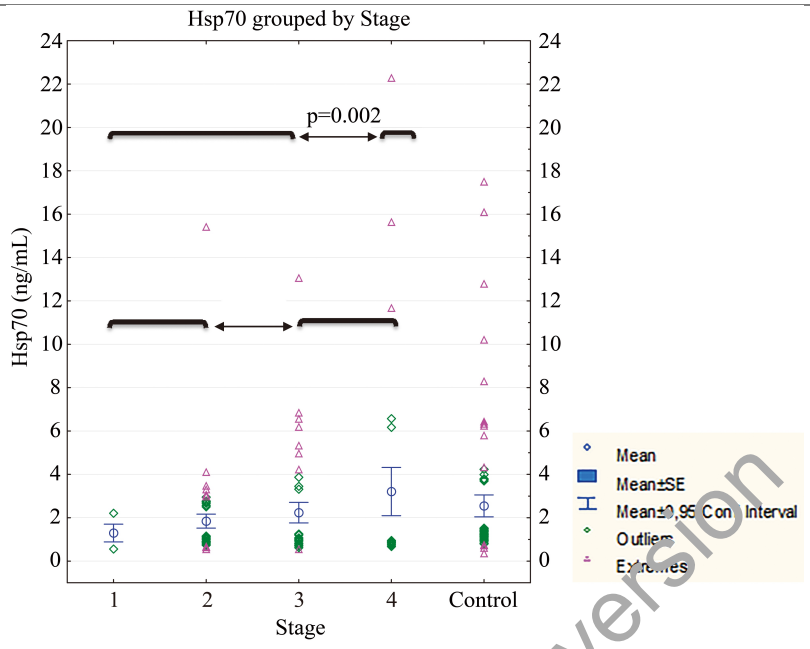

Fig. 1. Baseline serum Hsp70 level of healthy subjects ($N = 110$) and colorectal cancer patients according to stage of the disease. Differences between groups were calculated with Mann-Whitney test. Significant differences are shown between stages (Stage I+II vs III+IV and Stage I–III vs IV). Non significant difference was observed between controls and patients with advanced (Stage IV) disease (not shown in the figure). Explanation for stages: Stage I: T1-2 N0; Stage II: T3-4b N0; Stage III (IIIa-b): any T N1-2; Stage IV: any T any N M1 (according to 7th edition of TNM staging system).

who underwent screening colonoscopy in the preceding 2 months and were free of colorectal cancer or premalignant lesions and whose history was negative for colorectal cancer or other malignancies. The study was approved by the Medical Research Council Scientific and Research Committee. Serum samples were aliquoted and stored at −70 degrees of Celsius for Hsp70 analysis.

2.2. *Serum Hsp70 analysis*

Soluble Hsp70 level was measured by using R&D Systems (Minneapolis, MN, USA, Catalogue No. DYC1663E) enzyme-linked immunosorbent assay kit. Ninety-six-well microtitre plates were coated with mouse anti-human Hsp70 capture antibody (100 μl; 2 μg/ml) in carbonate buffer (pH 9.5) overnight at 4°C. Plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 three times and nonspecific binding sites blocked by incubation with 200 μl of PBS containing 0.5% gelatinec and Tween 20 for 1 h at room temperature. After washing, 100 μl of the reference preparation (recombinant human Hsp70, 0–10 μg/ml) or samples (1:1) were added and the plates were incubated for 2 h at room temperature. Plates were subsequently washed and Hsp70 binding was determined using biotinylated rabbit anti-human antibody (100 μl; 0.5 μg/ml) in PBS gelatine. After 1.5 h at room temperature, plates were washed and incubated with streptavidin-horseradish-peroxidase (1:200) in PBS gelatine for 20 min at room temperature. Plates were washed and 100 μl of o-phenylene-diamine (Sigma, St Louis, MO, USA) in citrate buffer was added. The optical density was measured at $\lambda = 490$ nm (reference at $\lambda = 620$ nm). The detection range of the assay was 0.05–10 ng/ml, the intra/inter-assay variability $< 10/< 16\%$, respectively.

2.3. *Tumor marker and other prognostic biomarker analysis*

Determination of the additional laboratory parameters including complete blood counts, clinical chemistry and tumor markers were performed by Roche Integra 800 analyzer, by Cell-Dyn 3500 hematology analyzer at the time of study entry of each patient.

2.4. *Statistical analysis*

For descriptive purposes data are given as mean ± standard deviation (SD) or median and interquar-

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Table 2
Correlation between serum Hsp70 level and the known biomarkers of colorectal cancer. Spearman's rank correlation test was used

Biomarker correlated with serum Hsp70	All patients r	Significance (p)	Stage IV r	Significance (p)
WBC	0.060	0.365	0.363	**0.010**
CRP	0.066	0.323	0.362	**0.010**
LDH	0.234	$<$ **0.001**	0.367	**0.009**
SAP	0.168	**0.015**	0.377	**0.012**
THR	0.069	0.224	0.267	**0.073**
CEA	0.186	**0.005**	0.347	**0.012**
CA 19-9	0.214	**0.002**	0.500	**0.001**

tile range (IQR) if data were not Gaussian distributed. The differences between groups were evaluated with the Mann-Whitney test. Correlations between the variables were expressed using the non-parametric Spearman's correlation coefficients. The association of serum protein levels on survival was analysed with Cox regression. Survival was calculated according to the Kaplan-Meier method. The curves were compared for statistical significance using long-rank testing. Receiver operating characteristic (ROC) curve analysis was used to determine the optimal cut-off value of Hsp70. Cut-off value of other biomarkers and tumour markers were selected according to the upper level of normal range used by the local laboratory. All tests were two-tailed, p values of < 0.05 were accepted as statistically significant.

Statistical analysis was performed using the GraphPad Prism v6.01 (GraphPad Software Inc, San Diego, CA, USA, www.graphpad.com) and SPSS v22 (SPSS Inc., Chicago, IL, USA) software.

3. Results

3.1. Serum Hsp70 concentration in patients with colorectal cancer according to stage of the disease and in healthy subjects

We studied whether baseline serum concentration of soluble Hsp70 is different between patients with CRC and controls (Fig. 1). Circulating Hsp70 level was in the same range in the whole patient population and controls (2.21 (SD: 2.36) versus 2.55 (SD: 2.66) ng/ml, NS). However, within colorectal cancer patients Hsp70 levels increased along with the stage of the disease. In early stage CRC (Stage I and II) mean Hsp70 level was 1.79 ng/ml (SD: 1.53), in stage III it was 2.23 (SD: 1.93) and in metastatic, stage IV disease we measured 3.21 ng/ml (SD: 3.87). The difference was statistically

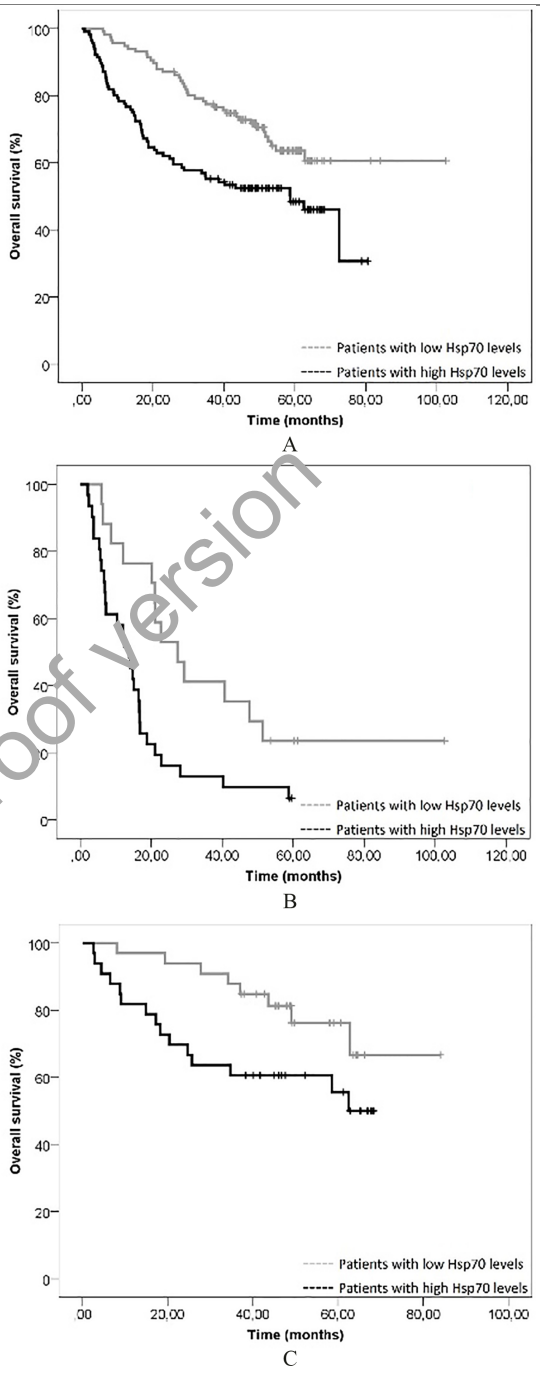

Fig. 2. Survival (Kaplan-Meier) of colorectal cancer patients according to high (black curves) or low (grey curves) serum Hsp70 level. A: all patients ($n = 232$); B: patients with metastatic stage IV disease ($n = 49$); C: patients with stage III disease ($n = 73$). Log Rank overall comparison showed significant difference in survival between patients with high (> 1.64 ng/ml) versus low ($\leqslant 1.64$ ng/ml) baseline serum Hsp70 level: 10.66; $p = 0.001$; 6.84; $p = 0.009$ and 3.53; $p = 0.06$.

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Table 3
Univariate and multivariate Cox-regression analysis: Association between baseline clinical parameters and serum biomarker levels and colorectal cancer patient's 5 year survival

	Univariate Cox regression HR (95% CI)	Significance (p)	Multivariate Cox regression HR (95% CI)	Significance (p)
Age at diagnosis (> 68 year)	**2.118 (1.394–3.216)**	**< 0.001**	**2.223 (1.231–4.014)**	**0.008**
Gender (male versus female)	1.070 (0.712–1.608)	0.745	NA	
TNM Stage (stage IV versus stage I–III)	**6.615 (4.312–10.150)**	**< 0.001**	**6.516 (3.689–11.510)**	**< 0.001**
Tumor grade (grade 2/3 versus grade 1)			NA	NA
Grade 2	**1.638 (0.885–3.033)**	**0.116**		
Grade 3	**2.038 (1.006–4.131)**	**0.048**		
Tumor localization (right versus left colon)	1.430 (0.930–2.201)	0.103	NA	NA
Hsp70 (> 1.64 ng/ml)	**1.940 (1.294–2.909)**	**0.001**	**2.418 (1.373–4.258)**	**0.002**
WBC (> 10 800 /ul)	**2.368 (1.477–3.796)**	**< 0.001**	**2.123 (1.076–4.186)**	**0.030**
CRP (> 5 mg/l)	**2.569 (1.634–4.040)**	**< 0.001**	NA	NA
LDH (> 248 U/l)	**1.750 (1.146–2.671)**	**0.010**	NA	NA
SAP (> 120 U/l)	**3.175 (1.993–5.040)**	**< 0.001**	NA	NA
THR (> 300 /ul)	**1.611 (1.078–2.407)**	**0.020**	NA	NA
CEA (> 4 ng/ml)	**3.141 (2.093–4.714)**	**< 0.001**	NA	NA
CA 19-9 (> 39 ng/ml)	**4.077 (2.559–6.509)**	**< 0.001**	NA	NA

The cut-off value for serum biomarkers were the upper limit of their normal range (shown in column (1)). Cut-off value of serum Hsp70 level (> 1.64 ng/ml) was calculated by ROC curve analysis. The same variables were included in multivariate analyses (column (4)) as in the univariate analysis (column (2)), and the best adjusted set of significant variables were highlighted.

significant between early and advanced stage disease (stage I+II versus III+IV, $p = 0.012$) or between non metastatic and metastatic (stage I–III versus stage IV, $p = 0.002$) disease.

Presence or absence of a primary tumor at sample collection (i.e. sample collection before or after operation) was not associated with altered serum Hsp70 levels. Similarly, we did not find a significant difference in Hsp70 levels between right ($n = 59$, Hsp70 = 2.03 ng/ml) and left-sided ($n = 173$, Hsp70 = 2.27 ng/ml) colorectal tumors.

3.2. Correlation of soluble Hsp70 level with other biomarkers

Hsp70 levels showed significant but weak positive correlation with tumor markers and other biomarkers that are known prognostic factors of CRC. These correlations were more pronounced (however also weak) in metastatic disease. In this subgroup we found positive association of Hsp70 level with LDH, SAP, CRP, baseline platelet and white blood cell count as well as CA19.9 and CEA (Table 2).

3.3. The relationship of Hsp70 and other biomarkers with survival

Using the ROC curve analysis the cut-off value of Hsp70 was 1.64 ng/ml. Values $\leqslant$ 1.64 ng/ml were regarded to be favorable and values $>$ 1.64 unfavorable prognostic markers. According to the Kaplan-Meier survival estimate it is clearly demonstrated that high Hsp70 levels correlate with poor survival in the whole patient population, as well as in the subgroups of stage IV (metastatic) and stage III disease (Fig. 2; p values are 0.001; 0.009 and 0.06 respectively). The risk of death within 5 years was two-fold higher with high initial Hsp70 level, according to univariate (HR: 1.94 CI: 1.29–2.91) and multivariate (HR: 2.42 CI: 1.37–4.26) Cox regression analysis. In addition to Hsp70 level age, tumor stage, grade, high WBC and platelet count, high CRP, LDH, SAP and tumor marker proved to be predictive factors of 5-year survival (Table 3). With the multiple Cox regression analysis age, Hsp70, tumor stage and high baseline white blood cell count were independent factors of death in the entire patient population. As in our pivotal publication [18] we observed the strongest relationship in the subgroup of women under the age of 70. Using the same set of variables beside advanced stage of the disease (HR: 6.6, CI: 2.08–21.48; $p = 0.001$), high Hsp70 level (HR: 8.12, CI: 2.02–35.84; $p = 0.004$, white blood cell number (HR: 6.8, CI: 1.56–29.79; $p = 0.011$) and high baseline CRP level (HR: 6.6, CI: 1.84–24.22; $p = 0.011$) proved to be the strongest independent predictors of death by multiple Cox regression analysis.

3.4. Combined prognostic model of survival

Next we determined whether our earlier model [20] that proposed the aggregate prognostic effect of high Hsp70 levels and high acute phase protein levels like

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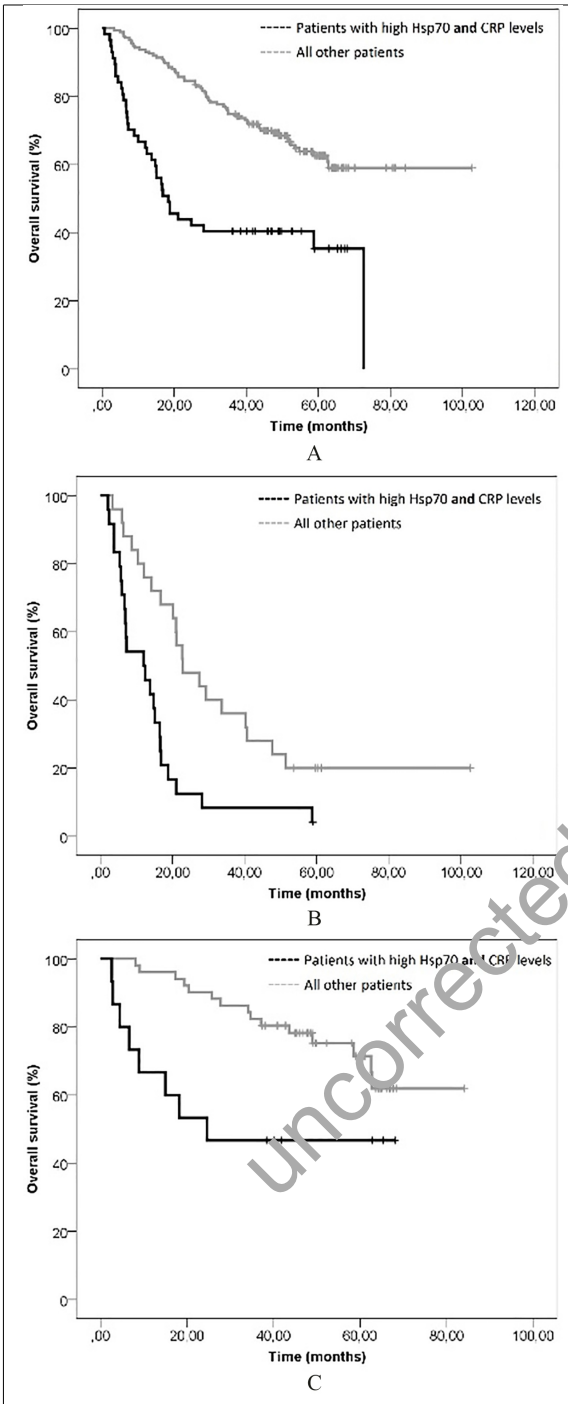

Fig. 3. Survival (Kaplan-Meier) of colorectal cancer patients according to high serum Hsp70 and high CRP levels (black curves) versus all other patients (gray curves). A: all patients ($n = 232$; χ^2: 36.025; $p < 0.0001$)); B: patients with metastatic stage IV disease ($n = 49$; χ^2: 7.443; $p = 0.006$); C: patients with stage III disease ($n = 73$; χ^2: 12.181; $p < 0.0001$). Cut off value for Hsp70: 1.64 ng/ml, CRP: 5 mg/l.

CRP could be validated in this new cohort. We found that the combined effect of Hsp70 and CRP levels are additive and exceeds that of Hsp70 alone in the whole patient population (χ^2: 36.025; $p < 0.0001$) as well as in the different subgroups (χ^2: 7.443; $p = 0.006$; 5.536; $p = 0.019$ and 12.181; $p < 0.0001$ in stage IV, stage I–III and stage III groups respectively) (Fig. 3).

The power of this double model could be improved by adding other inflammatory parameters like WBC or platelet count. In the triple model patients with high Hsp70, CRP and either high baseline WBC or platelet count had a 5-times higher risk of death (HR: 5.07, CI: 2.74–9.39, $p < 0.0001$; and HR: 4.98, CI: 3.08–8.06, $p < 0.0001$ respectively; χ^2: 33.166; $p < 0.0001$ and 52.528; $p < 0.0001$ respectively).

4. Discussion

In this prospective follow-up study we confirmed that baseline serum Hsp70 levels correlate with the stage of the disease and with many well established biomarkers of CRC. The most important result of the present study is the validation of the original observations that Hsp70 is an independent, potent prognostic factor in colorectal cancer [18]. The risk of death with high serum Hsp70 level was very similar to what we described in the pivotal study. Also the highest risk was observed in women under 70 years of age, similarly to our earlier results. With the combination of two or three independent inflammatory/immune related prognostic factors (Hsp70, CRP and WBC or platelet count) we could establish a more potent prognostic model, supporting our previous results too. We believe that these results are strongly valid, based on concordant reproduction in an independent patient cohort, therefore the possibility of fals conclusion is very low.

In 1993 Ciocca and co-workers found highly elevated Hsp70 expression in breast cancer. They also observed that in cases without regional metastases at the time of diagnoses, 70% of patients with low levels of Hsp70 expression survived for 5 years, comparing with 30% survival of patients with high levels of Hsp70 [21]. This was the first implication of Hsp70 as a prognostic marker in cancer. In the following more than two decades extensive research was done in the field, and in addition to intracellular Hsp70, extracellular (circulating) Hsp70 is also emerging as a biomarker of potential prognostic value in different types of cancer.

Our recent results are in line with our previous observations [17,22] that high serum Hsp70 levels sig-

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nificantly correlate with poor outcome and predict a shorter than expected overall survival.

Hsp70 is a versatile protein, crucial in maintaining cellular integrity and homeostasis. Cancer cells heavily depend on Hsp70 overexpression, since it protects them from exogenous (chemotherapy, irradiation, hypoxia) and endogenous (oncogene accumulation) stress. Oncogene accumulation engages senescence (OIS = oncogene induced senescence) however, cancer cells can bypass through the up-regulation of Hsp70 [23]. Membrane-bound and extracellular Hsp70 is known to interact with the innate and adaptive immune system, although this interaction is paradoxical and not fully understood yet. On one hand, Hsp70 can elicit an anti-tumor immune response, mainly by presenting antigenic peptides to APCs, which in turn activate cytotoxic T lymphocytes [24–27]. Natural killer (NK) cells were found to kill mHsp70-positive tumor cells after activation with a naturally occurring Hsp70 peptide (TKD) plus low dose IL-2 (TKD/IL-2). In their ongoing proof-of-concept study Multhoff and her team examine whether adjuvant treatment of NSCLC patients after platinum-based radiochemotherapy (RCTx) with TKD/IL-2 activated, autologous NK cells is clinically effective [11]. On the other hand there are data supporting that Hsp70 can also play a role in suppressing immune-mediated tumor-killing. Jaattela and Wissing found that Hsp70 can protect cells from monocyte cytotoxicity [28], moreover; another group reported that membrane bound Hsp70, located in exosomes, can activate myeloid-derived suppressor cells, thereby counter-regulating anti-tumor immune responses [29].

Knowing it's multitude of housekeeping functions in cancer cells, it is no wonder Hsp70 is an important target of anti-cancer drug development [30,31]. More than a dozen Hsp70 inhibitors have been reported, some of these molecules reaching early phase clinical trials. Of note is 15-deoxyspergualin, ver-155008, PES and others ([32], review in [33]). Even though the primary target of these agents is intracellular Hsp70, high concentrations of circulating Hsp70 could influence their efficacy and probably would have to be taken into account, in a future clinical scenario.

The era of immuno-oncology is on the doorstep, with novel drugs (antibodies) targeting the immune system to enhance anti-tumor immunity, mainly by inhibiting cancer immune tolerance [34]. Knowing Hsp70's interplay with the immune system it is an interesting question whether the concentration of serum Hsp70 influences the efficacy of immune-oncology treatments (i.e. PD-1 inhibitors); data are lacking in this field yet. On the other hand it is also a question, whether high circulating Hsp70 could influence pre-existing tumor-specific immune response. According to our present results it should be a negative effect, shifting the immune response toward immune tolerance.

Colo-rectal cancer is the second leading cause of cancer mortality worldwide, in 2017 more than 50000 patients are estimated to die of the disease just in the US [35]. Apart from disease stage at diagnosis, there are other prognostic factors that influence mortality in early CRC. Standard prognostic factors are grade of cancer, presence or absence of lymphatic/venous/perineural invasion and the involvement of resection margins. High serum concentrations of CEA, and to a lesser extent CA19-9, indicate a negative prognosis. Bowel obstruction and perforation are clinical traits associated with poor prognosis [36]. From an array of molecular markers some have established prognostic value (18q deletion – negative for prognosis; microsatellite instability/mismatch repair – positive for prognosis), others are still under investigation (TP53, bcl-2 expression, TGF-alpha etc.) [37]. Recent research is focusing on the immune status and immune environment of colorectal cancer. According to Gallon and co-workers it seems that immunoscore, that reflects the amount of memory and cytotoxic T cells in the tumor and tumor microenvironment is a strong prognostic factor of survival [38].

In summary, according to our recent and former very concordant results, we propose that circulating Hsp70 levels could be considered in the staging and risk assessment of colorectal cancer, either alone or in combination with CRP, platelet or WBC levels. Moreover, as Hsp70 can modulate antitumor immunity, it is possible that these findings will have relevance in the development of new immunooncology therapy modalities. Reproducibility of results hold considerable value in the era of many unreproducible observations.

Abbreviations

CEA:	carcinoembryonic antigen
CA:	19-9 cancer antigen 19-9
CRC:	colorectal cancer
CRP:	C-reactive protein
Hsp:	heat shock protein
SAP:	serum alkaline phosphatase
HCC:	hepatocellular carcinoma

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Conflict of interest

The authors declare that they have no conflict of interest.

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