

# Elevated CSF1 serum concentration predicts poor overall survival in women with early breast cancer

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## Abstract

Colony-stimulating factor 1 (CSF1) is a key regulator of mammary gland development, and a modulator of tissue macrophages. Expression of the CSF1 receptor gene *C-FMS* (*CSF1R*) is strongly associated with poor outcome in breast cancer and results in tumor cell invasiveness and pro-metastatic behavior *in vitro*. However, CSF1's role as a predictive factor in breast cancer remains unclear. We have prospectively measured circulating CSF1 using ELISA in 572 women with early breast cancer and in 688 women with benign breast lesions, and correlated these concentrations with overall survival (OS), nodal status, and other clinical and histological parameters. Serum CSF1 concentrations were significantly elevated in patients with early breast cancer when compared with those with benign tumors ( $P < 0.0001$ ). Within breast cancer patients, CSF1 was higher in women with axillary lymph nodes ( $P = 0.03$ ). Serum CSF1 correlated with tumor size ( $P = 0.002$ ), age ( $P < 0.001$ ), and Ki67 expression ( $P = 0.006$ ). Log CSF1 serum concentrations were predictive of poor survival in both univariate (hazard ratio (HR): 3.77, 95% CI: 1.65–8.65,  $P = 0.002$ ) and multivariate analyses (HR: 3.1, 95% CI: 1.03–9.33,  $P = 0.04$ ). Post- but not premenopausal women with CSF1 serum concentrations  $> 873$  pg/ml experienced a significantly poorer outcome ( $P = 0.004$  log-rank test). Serum CSF1 concentrations are elevated in women with malignant breast tumors. In early breast cancer, elevated serum CSF1 is associated with nodal involvement, and in postmenopausal women also with poor OS.

## Key Words

- ▶ breast cancer
- ▶ diagnosis
- ▶ CSF1
- ▶ survival
- ▶ lymph node metastasis

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## Introduction

Colony-stimulating factor 1 (CSF1) is produced by several cell lineages, and signaling through its proto-oncoprotein receptor *c-fms* (CSF1 receptor (CSF1R)) promotes the differentiation of myeloid progenitors into heterogeneous populations of monocytes, macrophages, dendritic cells, and bone-resorbing osteoclasts (Hume & MacDonald 2012). The cytokine regulates the function and survival of macrophages, which act at multiple levels within the innate and adaptive immune systems (Ryan *et al.* 2001,

Pixley & Stanley 2004, Hume & MacDonald 2012). CSF1 also appears to have an important physiological role in mammary gland development as it is synthesized in ductal epithelium, and macrophages that are recruited by CSF1 promote both mammary ductal invasion during puberty and lobulo-alveolar differentiation during pregnancy (Pollard & Hennighausen 1994, Gouon-Evans *et al.* 2000, Ryan *et al.* 2001). CSF1 is overexpressed in the mammary gland during pregnancy and lactation, suggesting the

existence of a paracrine mode of action in the normal mammary gland (Sapi 2004).

In addition to its role in physiological breast development and function, enhanced recruitment of macrophages to mammary tumors (Liotta & Kohn 2001, Kacinski 2002) and the poor prognosis associated with elevated number of tumor-associated macrophages (Bingle *et al.* 2002) also suggest a role of CSF1 in breast cancer (Pixley & Stanley 2004). This assumption is supported by the observation that metastatic progression of mammary gland tumors is profoundly reduced in CSF1-deficient mice (Lin *et al.* 2001). Our own work points to the benefit of small interfering RNAs and antibodies directed against CSF1 in suppressing the growth and reversing chemoresistance of human mammary tumor xenografts in immunodeficient mice (Aharinejad *et al.* 2004, Paulus *et al.* 2006).

In the clinical setting, serum CSF1 concentrations are higher in breast, ovarian, and endometrial cancer patients when compared with healthy individuals (Kacinski *et al.* 1991, Kacinski 1995). A pilot study by Scholl *et al.* (1996) found that CSF1 was detectable in the serum of both early and metastatic breast cancer, and that increased CSF1 serum levels in women with early breast cancer were associated with a significantly shorter disease-free interval.

A nested case-control study within the Nurses' Health Study which included 726 breast cancer patients provided the first evidence that elevated CSF1 concentrations are associated with a 33% increase in the risk of breast cancer in postmenopausal women, while it is associated with an 85% reduced risk in premenopausal women (Tamimi *et al.* 2008). The said retrospective study was performed in a serum bank obtained from women who had developed breast cancer during 1992 and 1998, and the results were published in 2008. We prospectively evaluated serum CSF1 concentrations, clinical, and histo-pathological parameters and 5-year overall survival (OS) in 1260 women with malignant and benign breast tumors between 2004 and 2009. Our results point to yet unknown roles of CSF1 in breast cancer.

## Subjects and methods

### Patients

Serum samples were prospectively obtained from 572 consecutive women with early breast cancer and in 688 consecutive women with benign breast tumors between 2004 and 2009. Cases had either been identified by opportunistic screening which was implemented on a

national level during these years (benign and malignant breast tumors), or had been identified by women who specifically requested the removal of a radiologically un-suspicious breast lesion (benign breast tumors). Benign breast lesions included fibroadenomas, papillomas, complex sclerosing lesions, and pre-neoplastic lesions such as atypical ductal hyperplasia. None of the breast cancer patients had undergone local or systemic anti-cancer treatment before serum collection. This study has been approved by the Institutional Review Board of the Medical University of Vienna. All patients had given written informed consent to be enrolled in the study before blood sampling. The clinical and histopathological data were obtained from the patient's chart and OS was evaluated by the National Austrian Statistics Institute (Statistik Austria). All the data were coded and the key was broken at the end of the study.

### ELISA

Serum samples were shock frozen in liquid N<sub>2</sub> and stored at -80 °C until analysis. CSF1 ELISA assays (Quantikine, R&D Systems, Minneapolis, MN, USA) were performed according to the manufacturer's protocol. In brief, 100 µl assay diluent was added to the microplate wells, which were precoated with the monoclonal anti CSF1 antibody followed by the addition of patient's serum. The wells were then incubated for 2 h at room temperature, aspirated, and washed. CSF1 antibody conjugated to HRP (200 µl) was added, and another 2 h incubation and three washes followed. Then, 200 µl substrate solution (hydrogen peroxide, chromogen, and tetramethylbenzidine) was added to each well. The reaction was stopped after 30 min incubation by adding 50 µl 1 M sulfuric acid. The optical density was measured at 450 nm using an automated microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

### Statistical analysis

All parameters were compared between patient groups by  $\chi^2$ -test, *t*-test, ANOVA (one-way ANOVA), and *post hoc* Tukey's test according to the scale of the variable. In case of skewed data, a nonparametric test (Mann-Whitney *U* test) or one-way ANOVA; Tukey's test accomplished with log transformation were applied. To assess the correlation between CSF1 and other parameters, Spearman's rank correlation coefficients (*r*<sub>s</sub>) were computed. Survival rates were estimated using the Kaplan-Meier method. The prognostic value of CSF1 serum concentrations was

studied using univariate and multiple Cox models. All *P* values are shown as the results of two-sided tests.  $P < 0.05$  was considered statistically significant. All statistical analyses were performed by M Salama using SPSS software version 15.0 (SPSS, Inc.).

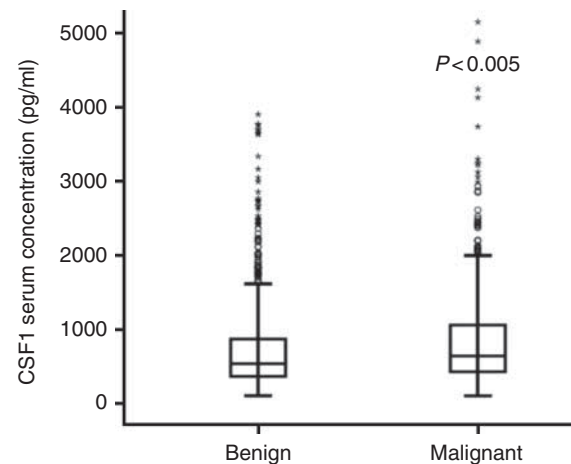
## Results

### Clinical characteristics of the study cohort

In this prospective study, a total of 1260 patients, 572 with breast cancer and 688 with a benign breast mass were included. Clinical and histopathological parameters are shown in Table 1. The median age of patients with breast cancer was 59 years and was significantly higher than the

**Table 1** Patient and tumor characteristics.

Characteristic	Benign, n=688	Malignant, n=572	P value
Age			
Median (years) (range)	45 (15–83)	59 (28–91)	<0.0001
Tumor size			
Median (cm) (range)	1.1 (0.4–2.5)	1.5 (0.1–11)	0.19
Menopausal status			
Premenopausal	504 (73.3%)	166 (29.0%)	
Postmenopausal	184 (26.7%)	406 (71.0%)	<0.0001
Nodal status			
Negative		332 (58.0%)	
Positive		240 (42.0%)	
Tumor grade			
G1		123 (21.5%)	
G2		268 (46.9%)	
G3		179 (31.3%)	
Unknown		2 (0.3%)	
Estrogen receptor			
Negative		130 (22.7%)	
Positive		416 (72.7%)	
Unknown		26 (4.6%)	
Progesterone receptor			
Negative		266 (46.5%)	
Positive		280 (49.0%)	
Unknown		26 (4.5%)	
HER2			
Negative		464 (81.1%)	
High		81 (14.2%)	
Unknown		27 (4.7%)	
Ki67, n=511			
<20		237 (46.4%)	
≥20		274 (53.6%)	
p53, n=536			
Negative		411 (76.7%)	
Positive		125 (23.3%)	
Adjuvant therapy			
Endocrine therapy		304 (53.1%)	
Chemotherapy		208 (36.4%)	
Local irradiation		403 (70.5%)	



**Figure 1**

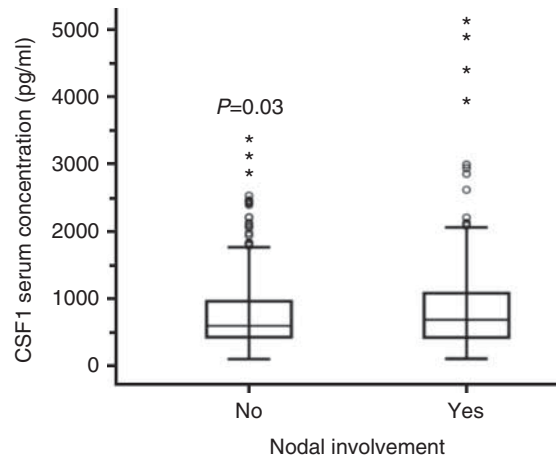
Box blot depicts CSF1 serum concentrations in patients with benign breast tumors and with early breast cancer.

median age of 45 years in patients with a benign breast mass ( $P < 0.0001$ ). Consequently, breast cancer in our patient cohort was more frequently associated with postmenopausal status ( $P < 0.0001$ ), while benign breast tumors were more common in premenopausal women ( $P < 0.0001$ ). Of those patients who were diagnosed with breast cancer, 53% received endocrine therapy, 36% had chemotherapy, and more than 70% were irradiated (Table 1).

### CSF1 serum concentrations in benign and malignant tumors

The mean circulating CSF1 concentration was  $889.0 \pm 801$  pg/ml in patients with breast cancer and was significantly higher than in those patients with benign breast lesions ( $772.7 \pm 677$  pg/ml) ( $P < 0.005$ ; Fig. 1). No difference was observed in CSF1 serum levels when benign breast tumors (i.e., fibroasnomas and papillomas) were compared with other benign conditions such as complex sclerosing lesions or atypical ductal hyperplasia (data not shown).

Within the 572 patients with invasive breast cancer and concomitant lymph node metastasis, the mean serum CSF1 concentrations were  $956.2 \pm 983$  pg/ml and significantly higher than in those patients without lymph node involvement ( $805.9 \pm 609$ ,  $P = 0.03$ ; Fig. 2). Also patients with N3 lymph node status had significantly higher serum CSF1 concentrations when compared with those with N0 ( $P = 0.03$ ) and N1 ( $P = 0.04$ ; data not shown).

**Figure 2**

Box blot depicts CSF1 serum concentrations in patients without (no) or with (yes) nodal involvement.

### Serum CSF1 and clinically relevant parameters

In Spearman's  $\rho$  test, circulating CSF1 correlated weakly but significantly with tumor size ( $r=0.48$ ,  $P=0.002$ ), age ( $r=0.157$ ,  $P<0.0001$ ), and Ki67 expression levels ( $r=0.119$ ,  $P=0.006$ ). There was, however, no statistically significant correlation with grading or nodal status (Table 2). Tumor size correlated weakly with nodal status ( $r=0.122$ ,  $P=0.015$ ) and Ki67 expression level ( $r=0.176$ ,  $P=0.001$ ). There was also a significant correlation between age and grading ( $r=0.097$ ,  $P=0.025$ ), and between grading and Ki67 expression ( $r=0.564$ ,  $P<0.0001$ ).

### CSF1 serum concentrations and OS

To evaluate the effect of CSF1 serum concentrations on survival, patients were dichotomized according to their median serum CSF1 concentration. The median serum concentration in breast cancer patients was 873 pg/ml and

was selected as a cutoff because it classified half of the breast cancer patients into high and low CSF1 concentration groups. At a median follow-up of 5.2 years, 62 of 572 (12%) of patients had died (33 patients with low CSF1 (<873 pg/ml) and 29 with high CSF1 (>873 pg/ml) serum concentrations). Age (hazard ratio (HR) for death, 1.03; 95% CI: 1.01–1.05,  $P=0.007$ ), tumor size (HR for death, 1.02; 95% CI: 1.01–1.04,  $P=0.001$ ), nodal status (HR for death 1.91; 95% CI: 1.08–3.40,  $P=0.02$ ), tumor grade (HR for death 1.97; 95% CI: 1.32–2.93,  $P=0.001$ ), and logCSF1 serum concentrations (HR for death 3.77; 95% CI: 1.65–8.65,  $P=0.002$ ) were significantly associated with breast cancer-specific survival in univariate analysis as well. The independent effect of CSF1 serum concentration on OS was assessed by multivariate Cox proportional hazard models adjusted for age, tumor size, nodal status, and tumor grade. In these multivariate analyses, logCSF1 serum concentrations remained significantly associated with OS (adjusted HR for relapse 3.10; 95% CI: 1.03–9.33) (Table 3). Kaplan–Meier estimates for OS showed a significantly poorer outcome if CSF1 concentrations were higher than the median CSF1 concentration of 873 pg/ml in our study population ( $P=0.02$ , log-rank test; Fig. 3). When including the menopausal status into the analyses, it turned out that the poorer outcome in patients with CSF1 concentrations >873 pg/ml was confined to postmenopausal women ( $P=0.004$ ), while no such effect was observed in premenopausal women with early breast cancer ( $P=0.61$ ) (Fig. 4).

### Discussion

We have measured CSF1 serum concentrations in 1260 prospectively collected samples from women with benign and malignant breast lesions and correlated them with established clinically relevant parameters and OS. To our knowledge, this is the first prospective study, which

**Table 2** Correlation between CSF1 serum concentrations and tumor parameters (Spearman's  $\rho$  test).

	Tumor size (cm)	Age	Grading	Nodal status	Ki67
CSF1 (pg/ml)	$r=0.148$ $P=0.002$	$r=0.157$ $P<0.0001$	NS	NS	$r=-0.119$ $P=0.006$
Tumor size	NS	NS	$r=0.193$ $P<0.0001$	$r=0.122$ $P=0.015$	$r=0.176$ $P=0.001$
Age	NS	NS	$r=-0.097$ $P=0.025$	NS	NS
Grading	NS	NS	NS	NS	$r=0.564$ $P<0.0001$
Nodal status	NS	NS	NS	NS	NS

**Table 3** Uni- and multivariate Cox proportional hazard models for overall survival.

Variable	Univariate			Multivariate		
	HR for overall survival	95% CI	P value	HR for overall survival	95% CI	P value
Age	1.03	1.01–1.05	0.007	1.03	0.99–1.06	0.07
Tumor size	1.02	1.01–1.04	0.001	1.02	1.01–1.04	0.004
Nodal status	1.91	1.08–3.4	0.02	1.27	0.64–2.49	0.49
Tumor grade	1.97	1.32–2.93	0.001	1.96	1.21–3.16	0.006
Log. CSF1	3.77	1.65–8.65	0.002	3.1	1.03–9.33	0.04

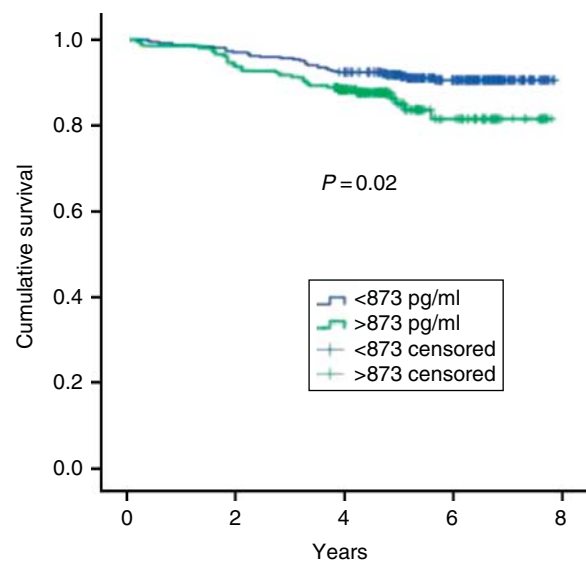
analyzes the clinical relevance of CSF1 in early breast cancer. The results of an earlier nested case–control study pointed to an inverse correlation between serum CSF1 concentrations and the subsequent risk for breast cancer in premenopausal women, while a positive correlation was found in postmenopausal women (Tamimi *et al.* 2008). Our results confirm the observations of a worse outcome in postmenopausal women with elevated serum CSF1 concentrations. The increase in systemic CSF1 levels, which we found in women with malignant breast tumors and in those with nodal positive breast cancer is, however, novel and suggests a direct involvement of CSF1 in tumor progression and malignant behavior.

At tissue level, CSF1R expression has been reported to be a strong predictor of poor outcome in nonmetastatic breast cancer and to be more frequently expressed in patients with nodal involvement (Kluger *et al.* 2004). A direct correlation between tissue array results of CSF1R and the serum concentrations of its ligand CSF1 has never been conducted (Kluger *et al.* 2004). The mode of action of CSF1 through CSF1R, however, justifies the assumption that serum concentrations of the ligand itself might have a prognostic significance in breast cancer (Hamilton 1997). The fact that tumor cells synthesize CSF1 in quantities high enough to be readily detected at tissue level and the evidence suggesting significantly elevated levels of CSF1 in serum and ascites of patients with breast, ovarian, and endometrial cancer (Chambers *et al.* 1997, Kacinski 1997, Maher *et al.* 1998) strongly suggest the functional relevance of the CSF1/CSF1R system. Finally, serum CSF1 in patients with terminal breast cancer has been reported to be more than tenfold higher than levels measured in healthy women, a finding that correlated well to increased CSF1 expression seen at sites of metastatic recurrence in another study (Kacinski *et al.* 1991, McDermott *et al.* 2002).

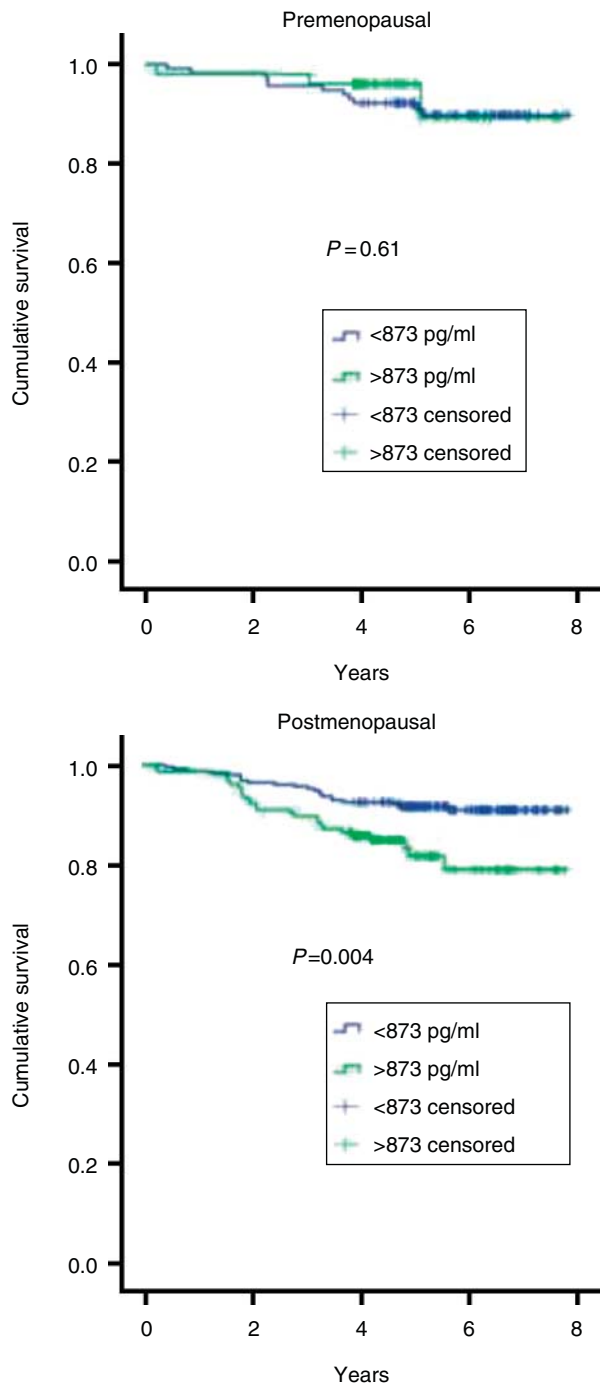
Experimental breast cancer models underscore the pathophysiological role of CSF1 in solid tumors. We have previously shown that CSF1 blockade by antisense nucleotides and small interfering RNAs suppresses growth

of human mammary tumor xenografts in mice (Aharinejad *et al.* 2004). Furthermore, the targeting of CSF1 in primary breast cancer xenografts in the MMTV PyMT transgenic mouse model results in delayed tumor progression to metastasis, while overexpression of CSF1 in xenografts enhances tumor development (Lin *et al.* 2001). Although the exact biological mechanism by which CSF1 renders tumors more aggressive remains largely unknown, we and others have shown that CSF1 contributes to tumorigenesis and angiogenesis through stimulation of the expression of the macrophage-specific matrix metalloprotease (MMP)-12 as well as the expression of MMP2, MMP9, and uPA (Wang *et al.* 1988, Talkhoud *et al.* 1991, Nowicki *et al.* 1996, Frandsen *et al.* 2001, Ha *et al.* 2001, Aharinejad *et al.* 2004, Paulus *et al.* 2006).

Another mechanism by which CSF1 might contribute to a prometastatic behavior is through the activation of the immune system. Tumor-expressed CSF1 is able to



**Figure 3** Cumulative overall survival (OS) in patients with CSF1 serum concentrations above (green) or below (blue) a cutoff of 873 pg/ml.



**Figure 4**  
Cumulative overall survival (OS) in premenopausal (top panel) and postmenopausal (bottom panel) patients with CSF1 serum concentrations above (green) or below (blue) a cutoff of 873 pg/ml.

recruit and activate macrophages that in turn release cytokines that enhance tumor cell growth *in vitro* and *in vivo* (Chambers *et al.* 1995). The existence of such a paracrine loop mechanism has already been demonstrated

for CSF1/epidermal growth factor (Wyckoff *et al.* 2004, Goswami *et al.* 2005).

Activation of the CSF1/CSF1R system results in a dramatic stimulation of cellular invasiveness via a uPA-dependent pathway *in vitro*. While such a mechanism might be essential during normal mammary gland development to allow ductal outgrowth by local matrix remodeling, the same principle would result in increased local invasion in malignant tumors. At least in ovarian cancer cell lines, the possibility of a uPA-mediated stimulation of invasion by CSF1 has already been demonstrated *in vitro* (Talkhoud *et al.* 1991).

Taken together, the results of the present prospective study show that in early breast cancer patients, elevated serum CSF1 is associated with increased tumor size, enhanced tumor cell proliferation, and nodal invasion. In postmenopausal women, CSF1 has a significant and clinically relevant role in predicting OS. Circulating CSF1 might be useful as a prognostic factor in breast cancer.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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