

# Exercise training reduces high mobility group box-1 protein levels in women with breast cancer: findings from the DIANA-5 study

## *Il training fisico riduce i livelli di high mobility group box-1 protein in donne con cancro alla mammella: risultati dallo studio DIANA-5*

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**ABSTRACT:** *Exercise training reduces high mobility group box-1 protein levels in women with breast cancer: findings from the DIANA-5 study. F. Giallauria, M. Gentile, P. Chiodini, F. Berrino, A. Mattiello, L. Maresca, A. Vitelli, M. Mancini, A. Grieco, A. Russo, R. Lucci, G. Torella, S. Panico, C. Vigorito.*

**Purpose:** To determine whether exercise training might exert anti-inflammatory effect by reducing HMGB1 levels in women with breast cancer (BC).

**Methods:** We analyzed monocentric data from the DIANA (DIET AND ANDROGENS)-5 PROJECT. Study population consisted of 94 patients randomized into two groups: 61 patients (53±8 yrs, training group) were assigned to a structured exercise training intervention (3 times/week for the first 3 months, and once /week for the following 9 months); whereas 33 patients (52±7 yrs, control group) followed only the general indications to adhere to the life-style intervention suggestions of the DIANA protocol. At study entry and after 12 months, all patients underwent cardiopulmonary exercise testing, biochemical assessment [HMGB1, high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6)]; and lipid and glycemic profile.

**Results:** There were no significant differences between groups in baseline clinical and inflammatory profile. Among the training group, only 19/61 patients had high adherence to the exercise intervention. After stratifying the study population according to the level of adherence to the exercise intervention, 1-year HMGB1 levels were lower among patients more adherent to exercise (p for trend=0.001). Further adjusting for age, body mass index and baseline values, 1-year HMGB1 levels remained significantly and inversely associated to the level of adherence to the exercise intervention (B=-0.97, SE=0.43, p=0.01).

**Conclusions:** Moderate intensity exercise training in BC survivors is associated with reduced HMGB1 levels that are proportional to the level of adherence to the exercise intervention, independently from other classical inflammatory molecules, suggesting an exercise-induced anti-inflammatory effect mediated by HMGB1.

**Keywords:** cancer, breast cancer, high mobility group box 1 protein, exercise training, cardiopulmonary exercise testing.

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## Introduction

Breast cancer (BC) is the most common cancer in women worldwide. It is also the principle cause of death from cancer among women globally. Studies on lifestyle and occurrence of BC indicate a key role for diet and physical activity; evidence is available on the potential influence of dietary modification on BC risk [1]. Data from the large European Investigation into Cancer and Nutrition (EPIC Study), which include Italian sub-cohorts are consistent with these main findings [2-7]; moreover the

observational analysis on BC risk from the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR) suggested that dietary recommendations was associated with reduced BC incidence in those individuals mostly adherent to the recommendations [8].

Epidemiological studies have also investigated and quite consistently reported a possible effect of physical activity on the incidence of primary BC [10-14]. In secondary prevention after BC diagnosis, observational studies consistently showed that the level of physical activity of BC women was cor-

related to an improvement in several cancer-related outcomes. Several studies including physical intervention have also suggested that a formal program of exercise training is also able to improve several cancer-related outcomes in BC survivors [15-18].

Inflammation plays a crucial role in the development and evolution of cancer. Among several markers of inflammation, the high-mobility group box 1 protein (HMGB1) is emerging as a potential important mediator. HMGB1 is a highly conserved nuclear protein, acting as a chromatin-binding factor that binds DNA and promotes access to transcriptional protein assemblies on specific DNA targets [19, 20]. Besides its nuclear role, HMGB1 also functions as an extracellular signaling molecule during inflammation, cell differentiation, cell migration, and tumor metastasis [19-22]. HMGB1 has been implicated in disease states, including sepsis [23], ischemia-reperfusion [24, 25], arthritis [26], meningitis [27], neurodegeneration [28], aging [29], and cancer [30, 31]. In cancer, overexpression of HMGB1 is associated with each of the hallmarks of cancer [32]. Increased HMGB1 protein expression is found in primary human breast carcinomas compared with normal tissues, with HMGB1 mRNA present during mouse mammary development and with low amounts measured during periods of apoptotic activity [33].

To the best of our knowledge, the serum levels of HMGB1 and their changes after exercise interventions in women with BC have never been reported. Therefore, this study tested the hypothesis that exercise training might exert anti-inflammatory effect by reducing HMGB1 levels in BC women belonging to the DIANA-5 cohort.

## Methods

### *Study population*

The DIANA (Diet and Androgens)-5 study is a multicentre randomized controlled trial of the effectiveness of a diet based on Mediterranean and macrobiotic recipes and principles, associated with moderate physical activity, in reducing additional breast cancer (BC) events in women with early stage invasive BC at high risk of recurrence because of metabolic or endocrine milieu [34]. The overall design of the study has been described elsewhere [34]. We analyzed monocentric data (University of Naples "Federico II") consisting of 94 patients randomized into two groups: 61 patients (53±8 yrs, training group) were assigned to a structured exercise training intervention (3 times/week for the first 3 months, and once /week for the following 9 months); whereas 33 patients (52±7 yrs, control group) received only general recommendations to adhere to the life-style intervention suggestions of the DIANA protocol. The study was conducted according to the guidelines of the Declaration of Helsinki, and our institutional ethical committee approved the protocol. The purpose of the protocol was explained to the patients and written informed consent was obtained from each patient before inclusion.

### *Study protocol*

At the study enrollment and after 12 months, all patients underwent anthropometrical and biochem-

ical assessment, cardiovascular clinical examination, and cardiopulmonary exercise stress testing. Before randomization, all women received a leaflet illustrating the WCRF/AICR recommendations for cancer prevention [35]. Training group patients were administered both the dietary and exercise training program as detailed below. After randomization, only the intervention group (training group, 61 patients) underwent, in addition to exercise training sessions, a counseling dietary program supported by cooking classes, reinforcing meetings and print materials. The counseling program aimed at increasing leisure-time physical activity level, controlling weight, and promoting a healthy and low calorie diet. Control group (33 patients) received only general recommendations on life-style.

### *Anthropometrical and biochemical assessment*

Body mass index (BMI) was used as a general measure of obesity and was calculated at the final visit as weight (kilograms) divided by height (meters squared). Waist circumference (WC), an index of abdominal obesity, was measured midway between the bottom of the rib cage and the top of the iliac crest. Blood pressure was measured twice after 5 min rest using a mercury sphygmomanometer. Anthropometric measurements were made with the subjects in indoor clothing and without shoes. Blood specimens were collected after 12- to 14-h fast, from 08:00 to 09:30 hours, to reduce the influence of circadian variation. Total cholesterol, triglyceride and high-density lipoprotein (HDL) cholesterol levels were measured using standard enzymatic methods [36]. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula. Fasting glucose levels were enzymatically determined by the peroxidase method. Fasting insulin levels were determined by enzyme immunoassay (Ultrasensitive Insulin Elisa, Mercodia, Sweden). Apolipoprotein B and high sensitivity C-reactive protein (hs-CRP) were measured with turbidimetric assay, using automated methods (Pentra 400 ABX Horiba, Italy). The error of the method was evaluated daily by analyzing a plasma pool and was <5%. The homeostatic assessment model (HOMA) index was used to estimate insulin resistance and calculated as fasting serum insulin (mU/L) x fasting serum glucose (mM)/22.5, as previously described [37]. Fasting HMGB1 levels were determined by enzyme immunoassay (HMGB1 Elisa, IBL International, Germany). Fasting high sensitivity IL-6 levels were determined by enzyme immunoassay (IL-6 Elisa, Diaclone, France). The inter-assay as well as the intra-assay coefficient was <10%.

### *Cardiopulmonary exercise training testing*

Respiratory gas exchange measurements were obtained breath by breath with the use of a computerized metabolic cart (Vmax 29C, Sormedics, Yorba Linda, CA, USA) as detailed elsewhere [24]. Briefly, after a 1-min warm-up period at 0 Watts workload, a ramp protocol of 20 W/min was started and continued until exhaustion. The pedaling was kept constant at 55-65 revolutions/min. A 12-lead electrocardiogram (ECG) was monitored continuously during the test, and cuff blood pressure was

manually recorded every 2 min.  $VO_{2peak}$  was recorded as the mean value of  $VO_2$  during the last 20s of the test and was expressed in milliliters per kilogram per minute. Predicted  $VO_{2peak}$  was determined by use of a sex-, age-, height- and weight-adjusted and protocol-specific formula as previously detailed [24]. The ventilatory anaerobic threshold (VAT) was detected by use of the V-slope method. The VE versus  $VCO_2$  relationship was measured by plotting ventilation (VE) against carbon dioxide production ( $VCO_2$ ) obtained every 10s of exercise (VE/ $VCO_2$ slope): both VE and  $VCO_2$  were measured in liters per minute. The VE/ $VCO_2$ slope was calculated as a linear regression function, excluding the non-linear part of the relationship after the onset of acidotic drive to ventilation [24].

### ***Exercise training program and cooking session***

Training group patients were enrolled in an in-hospital structured exercise training program. The frequency goals of in-hospital exercise training sessions was 3 times/week for the first 3 months, and once /week for the following 9 months. Each session consisted of cycle or treadmill 30 min exercise, preceded by 5 min of warming and followed by 5 min of cooling down, at a work load of 75% of baseline peak  $VO_2$ . This structured exercise training was on top of the general leisure-time physical activity recommendations or prescription of the general DIANA study (questionnaires, caloric control, etc, see above). From the 4th month to the 12th month, in-hospital exercise training sessions were reduced to one session/week, integrated with general leisure-time physical activity sessions, according to recommendations and prescription of the general DIANA study [34]. Training group was subdivided into 3 subgroups according to the adherence to the in-hospital exercise training sessions: no sessions (group 1); 1 to 45 sessions per year (group 2); 46 to 130 sessions per year (group 3). During cooking sessions, patients were invited cooking and eating some dishes prepared according to the WCRF/AICR recommendations and inspired by a macrobiotic Mediterranean diet [35].

### **Statistical Methods**

Continuous variables were reported as either mean and standard deviation (SD) or median and range according to their distribution, as assessed by the Shapiro-Wilk test. Categorical variables were reported as percentage. Differences in characteristics of patients among groups were tested by means of one-way ANOVA and Pearson chi-square test for continuous and categorical variables, respectively. A modified version of regression analysis, called TOBIT model was used to analyze HMGB1 values, where the normality assumption was not met because of the left-truncation for subjects with zero values [38]. Linear regression model and TOBIT (for HMGB1 values) were used to analyze one-year values including in the model physical category, age, BMI and baseline value. Trend test was also calculated where appropriate. Data were analyzed using SAS version 9.2 (SAS Inc, Cary, NC). All statistical tests were two-sided and p-values<0.05 were considered significant.

### **Results**

Baseline characteristics of the study population are shown in Table 1. There were no significant differences between groups in baseline anthropometrical, BC characteristics, metabolic and inflammatory profile (Table 1).

The study period was similar in both groups. In training group, the average exercise intensity was  $74.4\pm 2.3\%$  of baseline  $VO_{2peak}$ . No adverse events took place during any of training sessions in BC patients undergoing exercise training sessions.

Among the training group, 24 patients had very low adherence to the exercise intervention sessions, whereas 19 patients had high adherence. After stratifying the study population according to the level of adherence to the exercise intervention sessions, 1-year HMGB1 levels were lower among patients more adherent (p for trend=0.001) (Table 2). After adjusting for age, body mass index and baseline values, 1-year HMGB1 levels were significantly and inversely associated to the level of adherence to the exercise intervention (B=-0.97, SE=0.43, p=0.01) (Table 3). No significant correlations between exercise intervention and the other inflammatory mediators (hs-CRP and IL-6) were found (Table 3).

### **Discussion**

To the best of our knowledge, this is the first study demonstrating an exercise training-induced reduction of HMGB1 levels in BC women. Notably, the reduction of HMGB1 levels is strongly correlated to the level of adherence to exercise program and is more sensitive than that of other systemic inflammatory mediators such as hs-CRP and IL-6.

In cancer, overexpression of HMGB1 is associated with each of the hallmarks of cancer including unlimited replicative potential, angiogenesis, reduction of apoptosis, self-sufficiency in growth signals, and insensitivity to inhibitors of growth, inflammation, tissue invasion and metastasis [30]. In primary human breast carcinomas, increased HMGB1 protein expression has been found compared with normal tissues, with HMGB1 mRNA present during mouse mammary development with low amounts measured during periods of apoptotic activity [33].

The role of the immune system during cancer development is complex, involving extensive reciprocal interactions between genetically altered cells, adaptive and innate immune cells, their soluble mediators and structural components present within the tumor microenvironment. Immune system strictly regulates each stage of cancer development in most adult tumors. Whereas full activation of adaptive immune cells at the overt invasive tumor stage may result in eradication of malignant cells, chronic activation of innate immune cells at sites of premalignant growth may actually enhance tumor development. In addition, the balance between desirable antitumor immune responses and undesirable pro-tumor chronic inflammatory responses largely depends on the context in which a malignancy is developing [39]. HMGB1 alerts the innate immune system to stress and excessive or unregulated cell death [40, 41].

Table 1. - Anthropometrical and clinical characteristics of the study population

	Training Group (n=61)	Control Group (n=33)	P value
<b>Anthropometrics</b>			
Age (yrs)	53.5 ± 8.6	52.3 ± 7.0	0.505
Height (cm)	160.0 ± 5.4	158.9 ± 4.9	0.313
Weight (Kg)	71.6 ± 14.1	67.1 ± 12.1	0.120
Waist Circumference (cm)	96.0 ± 14.9	90.3 ± 12.3	0.065
Body Mass Index (Kg/m <sup>2</sup> )	28.01 ± 5.52	26.57 ± 4.60	0.203
<b>Breast Cancer Characteristics</b>			
Presence of invasive carcinoma (%)	61/61 (100)	33/33 (100)	–
Node positivity (%)	49 (n=30/61)	55 (n=18/33)	0.624
Estrogen positivity (%)	84 (n=51/61)	82 (n=27/33)	0.828
<b>Metabolic and Hormonal Profile</b>			
Glycemia (mg/dL)	93.5 ± 12.5	92.2 ± 15.5	0.669
Insulin (ng/mL)	7.76 ± 4.03	8.80 ± 5.00	0.278
HOMA index	1.836 ± 1.192	2.042 ± 1.250	0.435
Total Cholesterol (mg/dL)	202.3 ± 35.7	203.4 ± 32.0	0.885
LDL-Cholesterol (mg/dL)	128.1 ± 35.1	120.1 ± 29.4	0.264
HDL-Cholesterol (mg/dL)	54.6 ± 11.59	55.8 ± 13.7	0.659
Triglycerides (mg/dL)	100.6 ± 46.9	119.6 ± 72.0	0.126
Testosterone (nmol/L)	0.361 ± 0.362	0.383 ± 0.259	0.756
<b>Inflammatory Markers</b>			
hsCRP (mg/dL) [n=82]	1.3 ± 1.7	0.9 ± 1.1	0.492
IL-6 (pg/mL) [n=82]	1.3 ± 1.3	1.4 ± 1.4	0.254
HMGB1 (ng/mL)	0.9 ± 1.4	1.3 ± 1.5	0.337

**Captions:** HOMA, homeostatic assessment model; LDL, low-density lipoprotein; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin-6; HMGB1, high mobility group box-1.

Table 2. - One-year metabolic and inflammatory parameters according to the level of adhesion to the exercise intervention (3=highest)

Physical Category	0	1	2	3	P value	P value
	(N = 33)	(N = 24)	(N = 18)	(N = 19)	(between groups) trend test	
Triglycerides (mg/dL)	104.00 ± 44.03	105.83 ± 41.00	93.61 ± 31.11	80.16 ± 33.02	0.122	0.029
Total Cholesterol (mg/dL)	205.09 ± 36.64	193.67 ± 36.85	202.00 ± 28.79	194.84 ± 39.03	0.607	0.423
HDL Cholesterol (mg/dL)	56.61 ± 16.21	51.04 ± 16.28	53.89 ± 12.94	58.37 ± 11.87	0.373	0.741
LDL Cholesterol (mg/dL)	127.67 ± 34.92	119.88 ± 38.25	129.44 ± 30.59	120.37 ± 32.39	0.719	0.648
BMI (Kg/m <sup>2</sup> )	26.31 ± 4.41	29.67 ± 7.08	27.25 ± 3.83	25.41 ± 4.07	0.034	0.526
HMGB-1 (ng/mL)*	0.50 [0.00 - 3.60]	1.15 [0.00 - 4.10]	0.00 § [0.00 - 3.10]	0.00 § [0.00 - 0.60]	0.001	0.001
hsCRP (mg/dL)	0.78 ± 1.12	1.63 ± 1.76	1.67 ± 1.89	0.99 ± 1.05	0.145	0.668
IL-6 (pg/mL)	1.66 ± 1.68	1.53 ± 0.81	1.78 ± 2.34	0.95 ± 0.66	0.328	0.209

Values are mean and standard deviation; \* values were median and range, p-value from Tobit model.; § 0 = not detectable

**Captions:** LDL, low-density lipoprotein; HDL, high-density lipoprotein; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin-6; HMGB1, high mobility group box-1.

Table 3. - One-year  $\beta$ -estimates and Standard Errors (SE) adjusted for age, BMI and baseline values according to the level of adherence to the exercise intervention (3=highest)

Physical Category	0 (Reference)	1		2		3		P value	P value (trend test)
		$\beta$	SE	$\beta$	SE	$\beta$	SE		
Hs-CRP (mg/dL)	0	0.59	0.36	0.53	0.38	0.13	0.37	0.306	0.755
IL-6 (pg/mL)	0	-0.34	0.47	0.11	0.49	-0.68	0.48	0.594	0.289
HMGB-1 (ng/mL)*	0	0.42	0.37	-0.71	0.42	-0.97	0.43	0.008	0.010

\*estimated from Tobit model.

**Captions:** hsCRP, high sensitivity C-reactive protein; IL-6, interleukin-6; HMGB1, high mobility group box-1.

HMGB1 mediates a number of important functions including stimulating the release of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , and other inflammatory products, endothelial cell activation, recruitment and activation of innate immune cells, and also dendritic cell maturation that, in the setting of cancer, lead to a chronic inflammatory response [21].

Epidemiological studies have shown that the level of physical exercise is associated to improved outcome in BC patients, included cancer related and total mortality [11-14]. Moderate intensity exercise interventions in postmenopausal breast cancer (i.e. 60%  $VO_{2peak}$ ) are associated with several favorable effects in several cancer-related outcomes, such as physical and psychological function and quality of life [42, 43]. Several factors mediate the relationship between exercise, inflammation and immune system. The individual characteristics, type and intensity of exercise, as well as the timing of cancer diagnosis and stage may have an interplay role. Both the incidence of cancer, chronic inflammatory status and the impairment of the immune system also show negative age-related changes that can partially be counteracted with exercise [44]. Weight loss, reduced levels of insulin, glucose, and sex hormones, decreased inflammation and immunostimulation might also explain the beneficial effects of exercise on cancer outcomes [15-18, 45, 46]. However, these effects mainly depend on type and duration of exercise; and the clinically relevant cut-off points of the exercise-induced anti-inflammatory response are still largely unknown within the context of cancer. In fact, it should be noted that reduced inflammatory status, enhanced immune function and low susceptibility to cancer occur with regular moderate exercise, whereas sedentary habits and exhaustive exercise suppress immune system inducing a pro-inflammatory state and higher oxidative DNA damage, both of which are hypothesized to be markers of cancer recurrence [47]. The present study showed that the reduction of HMGB1 is proportional to the level of adherence to 1-year exercise intervention of moderate intensity; whereas no effect was found on other classical inflammatory molecules such as hs-CRP or IL-6. It could be hypothesized that exercise training may exert an early anti-inflammatory effect mediated by HMGB1 at nuclear level. Whether exercise-induced reduction of HMGB1 might have prognostic impact on BC survivors should be investigated.

### Study limitations

A number of limitations of the present study should be considered. Training patients underwent moderate intensity exercise program: whether an exercise program of higher intensity might have additional clinical and prognostic value should be clarified. Moreover, exercise training attendance was good in 19 out of 61 patients, highlighting the gender specific difficulties in attending long-term exercise training programs as previously described in women with cardiovascular disease attending cardiac rehabilitation programs [48]. Despite the aforementioned limitations, this study has several unique strengths. This is the first study enrolling high-risk BC survivors (women with early stage invasive BC at high risk of recurrence because of metabolic or endocrine milieu) to 1-year exercise intervention, and the first to demonstrate a reduction of a potent inflammatory marker such as the HMGB1 protein in these high risk patients.

### Conclusions

Moderate intensity exercise training in BC survivors is associated with reduced HMGB1 levels that are proportional to the level of adherence to the exercise program. The correlation to exercise dose is more sensitive than that observed for other systemic inflammatory mediators such as hs-CRP and IL-6. These results confirm that exercise training—n favorably modulate the inflammatory status in high risk BC patients. Further studies are needed in order to evaluate whether the reduction of HMGB1 levels has prognostic value in BC women.

### Riassunto

*Scopi:* Determinare se un programma di training fisico esplica un potere antinfiammatorio riducendo i livelli di HMGB1 in donne con cancro alla mammella (CM).

*Metodi:* Sono stati analizzati dati monocentrici del progetto DIANA (DIET AND ANDROGENS)-5. La popolazione di studio consiste di 94 pazienti randomizzate in 2 gruppi: 61 pazienti (53 $\pm$ 8 anni, gruppo training) sono state assegnate ad un programma strutturato di training fisico (3 volte alla settimana per i primi 3 mesi, poi 1 volta alla settimana

per successive 9 mesi); mentre 33 pazienti (52±7 anni, gruppo controllo) hanno seguito soltanto indicazioni generiche ad aderire a corretto stile di vita in accordo al protocollo DIANA. All'arruolamento e dopo 12 mesi, tutte le pazienti hanno effettuato test cardiopolmonare, valutazione biochimica (HMGB1, proteina C-reattiva ad alta sensibilità (hsPCR), interleuchina 6 (IL-6); profilo lipidico e glicemico.

**Risultati:** Non sono state osservate differenze tra i gruppi circa il profilo clinico e infiammatorio a livello basale. Tra le donne appartenenti al gruppo training, soltanto 19/61 pazienti mostravano una alta aderenza al programma di esercizio. Dopo stratificazione per il livello di adesione al programma di esercizio, i livelli di HMGB1 ad 1 anno erano significativamente inferiori nei pazienti con una maggiore adesione al programma di esercizio ( $p$  per trend=0.001). L'analisi multivariate ha mostrato che dopo aggiustamento per età, indice di massa corporea e valori basali di HMGB1, i valori di HMGB1 ad 1 anno restavano significativamente ed inversamente associati al livello di adesione al programma di esercizio ( $B=-0.97$ ,  $SE=0.43$ ,  $p=0.01$ ).

**Conclusioni:** Un programma di training di intensità moderata in donne con CM è associato a ridotti livelli di HMGB1, in maniera proporzionale al livello di adesione al programma di esercizio, indipendentemente dai classici parametri infiammatori, suggerendo un effetto antinfiammatorio indotto dall'esercizio mediato dall'HMGB1.

**Parole chiave:** cancro; cancro alla mammella; high mobility group box 1 protein; esercizio fisico; test da sforzo cardiopolmonare.

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