

## Serum from morbidly obese patients affects melanoma cell behavior *in vitro*

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Here we examined whether serum from obese patients could create a growth-enhancing microenvironment that alters gene expression in *BRAF*- and *NRAS*-mutated melanoma cell lines. SK-Mel-28 (*BRAF*-mutated) and Sk-Mel-147 (*NRAS*-mutated) cells were treated with pooled serum from 10 severely obese patients (BMI > 40 kg/m<sup>2</sup>), pooled serum from 6 healthy lean individuals (BMI = 18.5–24.9 kg/m<sup>2</sup>), or recombinant TNF- $\alpha$ . We found that obese patient serum enhanced migration capacity and increased *NRAS* expression levels in both *BRAF*- and *NRAS*-mutated melanoma cells. Although TNF- $\alpha$  is the major obesity-related cytokine and TNF- $\alpha$  levels were found to be increased in the serum of obese individuals, this cytokine made only a modest contribution to the migration capacity of melanoma cells. These results indicate that other components present in the serum of severely obese patients may be responsible for enhancing the migration capacity of melanoma cells. As TNF- $\alpha$  alone did not seem to significantly affect tumor cell behavior, anti-tumor strategies aimed at blocking TNF- $\alpha$  should be considered with caution in future studies, particularly when *in vitro* models are used as screening platforms for antitumor activity.

**Keywords:** Melanoma. Tumor progression. Inflammation. Morbidly obesity.

### INTRODUCTION

Malignant melanoma is a heterogeneous disease with complex molecular mechanisms involving genetic alterations that control the proliferation, differentiation, and survival of tumor cells (Palmieri *et al.*, 2015). A central mechanism of melanoma cell survival is activation of the MAPK pathway, which is responsible for mediating cellular responses to extracellular mitotic stimuli. This pathway is controlled by phosphorylation of the intracellular proteins RAS (H-RAS, K-RAS, and N-RAS), RAF (A-RAF, B-RAF, and C-RAF), MEK, and ERK (Palmieri *et al.*, 2015; Davies *et al.*, 2002). MAPK

pathway alterations have been reported in about 20% of all cancers (Castellano, Downward, 2011). More than 60% of cutaneous melanoma patients carry the B-RAF<sup>V600E</sup> mutation (Davies *et al.*, 2002) and about 20% carry the N-RAS<sup>Q61R</sup> mutation (Ribas, Flaherty, 2011; Uguen *et al.*, 2015; Sheen *et al.*, 2016).

Inflammation plays an important role in melanoma cell progression, invasion, and metastasis (Fan, Mao, Yang, 2013; Quail, Joyce, 2013). The link between inflammation and melanoma development is established by intrinsic and extrinsic pathways. The former involves activation of oncogenes, including *BRAF* and *NRAS*, both of which encode members of the MAPK signaling pathway, and inactivation of tumor suppressors such as p53 (Hocker, Singh, Tsao, 2008; Madan, Lear, Szeimies, 2010; Hanahan, Weinberg, 2011). These alterations lead to inflammation by stimulating the production of inflammatory mediators,

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such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1), in the tumor microenvironment (Mantovani *et al.*, 2002; Mantovani *et al.*, 2008; Sica *et al.*, 2006). The association between tumor progression and inflammation was first observed in 1863, when researchers discovered macrophage infiltration in neoplastic tissues, suggesting a strong correlation between tumor survival and chronic inflammation (Balkwill, Mantovani, 2001).

The tumor microenvironment is composed of a variety of cells, including fibroblasts and immune cells, mostly macrophages. Tumor-associated macrophages may represent up to 50% of solid tumors, and their activities are usually pro-tumorigenic, enhancing cancer cell survival, proliferation, and dissemination (Mantovani, Sica, Locati, 2005; Sica *et al.*, 2006; Qian, Pollard, 2010).

A similar inflammatory condition observed in obese individuals is low-grade inflammation (Maachi *et al.*, 2004; Klein-Platat *et al.*, 2005; Apovian *et al.*, 2008). White adipose tissue secretes hormones, cytokines or adipokines, and growth factors (e.g., leptin, transforming growth factor- $\beta$ , IL-1 $\beta$ , IL-6, adiponectin, resistin, and the pleiotropic cytokine TNF- $\alpha$ ) (Ahima, Flier, 2000; Labrie *et al.*, 2000). These inflammatory mediators induce macrophage infiltration, causing local inflammation and enhancing the production of angiogenic factors, an example of which is vascular endothelial growth factor (Vona-Davis, Rose, 2009). Obesity is a risk factor for tumor development and the cause of about 20% of cancer cases (Wolin, Colditz, 2008; De Pergola, Silvestris, 2013). A previous study suggested that UV radiation may promote inflammatory responses in obese subjects, increasing the levels of hormones and cytokines, mainly leptin, and thereby stimulating cutaneous carcinogenesis (Sharma, Katiyar, 2010). A previous meta-analysis showed that overweight or obese males are at a higher risk for melanoma and that adipose tissue inflammation might be responsible for the proliferation of melanoma cells in these subjects (Sergentanis *et al.*, 2013). It has been shown that tumor cells become more aggressive and proliferate faster in the presence of white adipose tissue (Zhang *et al.*, 2009). Adipose tissue-derived inflammatory cytokines involved in obesity (such as TNF- $\alpha$ ) have a significant

role in tumorigenesis, angiogenesis, and invasion (Mantovani *et al.*, 2008; Moore *et al.*, 1999; Balkwill, 2006; Mueller, 2006).

Other obesity-related factors may also influence the tumor microenvironment. Interleukins and protein-induced cytokines, for instance, stimulate the proliferation of tumor cells (Rundhaug, Fischer, 2010). Serum amyloid A (SAA), an acute-phase apolipoprotein associated with high-density lipoprotein (HDL), is produced mainly by the liver during regulation of chronic and acute inflammation (Yamada, 2006; Yamada, 1999). However, SAA is also released by hypertrophic adipocytes associated with enhanced lipolysis and insulin resistance (Filippin-Monteiro *et al.*, 2011). Research has demonstrated that SAA induces the secretion of TNF- $\alpha$  and other cytokines by mononuclear cells (Cai *et al.*, 2007; Furlaneto, Campa, 2000) and is a potential serum biomarker in human cancer patients (Chan *et al.*, 2007).

Although many studies and epidemiological data have confirmed the association between cancer and obesity (Chan *et al.*, 2007; Bhaskaran *et al.*, 2014), the relationship between high body mass index (BMI) and malignant melanoma has been little investigated, with reports showing modest or inconsistent associations (Calle *et al.*, 2003; Renehan *et al.*, 2008; Roberts, Dive, Renehan, 2010). In the current study, we demonstrate that serum from subjects with a BMI higher than 40 kg/m<sup>2</sup> promotes an increase in the migration capacity of SK-Mel-28 and SK-Mel-147 bearing the oncogenes *BRAF*<sup>V600E</sup> (Davies *et al.*, 2002; Daveri *et al.*, 2015; Gorden *et al.*, 2003) and *NRAS*<sup>Q61R</sup> (Nissan *et al.*, 2014), respectively.

## MATERIAL AND METHODS

### Reagents

Recombinant human TNF- $\alpha$  was purchased from BD Biosciences (San Jose, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Grand Island, USA). Penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Cultilab (Campinas, Brazil). All other reagents were from Merck (Darmstadt, Germany) unless stated otherwise.

## Subjects and sample collection

This study used serum from morbidly obese patients and lean individuals. All 20- to 65-year-old patients scheduled to undergo Roux-en-Y gastric bypass surgery at the Professor Polydoro Ernani de São Thiago University Hospital (Florianópolis, Brazil) were eligible for inclusion if their BMI was greater than 40 kg/m<sup>2</sup> before surgery. Exclusion criteria were cancers, acquired immunodeficiencies (such as HIV infection), and acute disorders. The lean control group was composed of healthy volunteers with a BMI between 18.5 and 24.9 kg/m<sup>2</sup>. Exclusion criteria were chronic or acute diseases and continuous medication. Anthropometric parameters were determined for all participants. This study was approved by the ethics committee of the Federal University of Santa Catarina, Florianópolis, Brazil (protocol no. 24279013.7.0000.0121). Written informed consent was obtained from all subjects before participation.

Blood was collected in tubes without anticoagulant and centrifuged at 1500 × *g* for 10 min. After centrifugation, the serum was withdrawn and inactivated for 30 min at 56 °C. Samples from obese (*n* = 10) and lean (*n* = 6) patients were pooled separately and diluted to a final concentration of 10% (v/v) in DMEM containing 100 U/mL penicillin and 100 µg/mL streptomycin.

## Serum assays

Total cholesterol, triglycerides, HDL cholesterol, and glucose were measured by enzymatic analysis (Dimension RXL Max, Siemens, Berlin, Germany). Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald equation. Serum concentrations of TNF-α and MCP-1 were determined using a commercial quantitative sandwich immunoassay kit (DuoSet ELISA, R&D Systems, Minneapolis, USA) with a sensitivity of 2.0 pg/mL for TNF-α and 1.0 pg/mL for MCP-1. Serum concentrations of SAA were quantified using a commercially available kit (hSAA ELISA kit, Sigma–Aldrich, St. Louis, USA) with a sensitivity of 4.0 ng/mL.

## Cell culture

SK-Mel-28, a *BRAF*-mutated human melanoma cell line, was obtained from the Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil) and SK-Mel-147, an *NRAS*-mutated human melanoma cell line, was kindly provided by Professor Ana Campa (São Paulo University, São Paulo, Brazil). Cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 10 mM HEPES at 37 °C and 5% CO<sub>2</sub>.

### Migration (scratch) assay

Cells were seeded at a density of 3 × 10<sup>5</sup> cells/well in 12-well plates in triplicate. On the following day, a path was scratched in each well with a 200 µL pipette tip, old culture medium was replaced by 10% pooled serum or 50 pg/mL TNF-α in FBS-free DMEM, and plates were incubated for 24 h. Microphotographs were taken at 0 and 24 h after treatment. Cell migration was analyzed using ImageJ® software (Rueden *et al.*, 2017) according to Grada *et al.* (2017), with some modifications. The difference in cell layer width was calculated as the mean distance between the borders of the scratch path at 0 and 24 h after incubation. Results were compared with those of untreated cells (control, 100% gap closure). Calculation parameters are provided as Supplementary material (Figure 1S).

### Clonogenic assay

Colony formation was assessed by plating 300 cells/well in triplicate in a six-well plate one day before cell treatment with TNF-α (50 pg/mL) in DMEM medium. After 7 days of incubation, colonies were fixed with 10% formalin, stained with Giemsa, and counted.

### Cell cycle analysis

Cells (2 × 10<sup>5</sup> cells/well) in DMEM supplemented with 10% FBS were plated in triplicate in a 24-well plate and incubated for 24 h. After this period, the medium was replaced by DMEM without FBS for cell cycle

synchronization for 24 h. Then, cells were treated with FBS-free DMEM (control) or TNF- $\alpha$  (50 pg/mL) in FBS-free DMEM and incubated for a further 24 h. Cells were washed in PBS, fixed in 70% ethanol for 30 min at  $-20^{\circ}\text{C}$ , and washed and resuspended in PBS containing 10  $\mu\text{g/mL}$  RNase. Propidium iodide (10  $\mu\text{g/mL}$ ) was added for flow cytometric analysis (FACSCanto™ II system, BD Biosciences, San Jose, USA), performed at the Multiuser Laboratory for Biological Studies of the Federal University of Santa Catarina (LAMEB/UFSC). A total of 10 000 events were acquired for each measurement, and cell cycle data were analyzed using Flowing software version 2 (University of Turku, Turku, Finland).

### **BRAF and NRAS expression in SK-Mel-28 and -147 cells**

Cells were treated with 10% obese or lean patient serum in FBS-free DMEM. After 48 h of incubation, total RNA was extracted with TRIzol® (Gibco, Grand Island, USA). cDNA was then synthesized using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, New Jersey, USA). For quantitative real-time PCR, the following primers were used: *NRAS*, 5'-ACAAACTGGTGGTGGTTGGA-3' and 5'-ATTGTCAGTGCCTTTTC-3'; *BRAF*, 5'-CTCCTTGAATCGGGCTGGTT-3' and 5'-GCCTGGATGGGTGTTTG-3'; and *GAPDH* (constitutive housekeeping gene), 5'-CAATTGACCCCTTCATTGACC-3' and 5'-GACAAGCTTCCCGTTCG-3'. Each amplification reaction was performed in duplicate together with a negative control (absence of cDNA) using SYBR<sup>®</sup> Green Master Mix (Applied Biosystems, New Jersey, USA). Reaction conditions were as follows: 95  $^{\circ}\text{C}$  for 10 min, 45 cycles of 95  $^{\circ}\text{C}$  for 10 s (melting), and 57  $^{\circ}\text{C}$  for 1 min (annealing and elongation). Melting curve analyses from 76 to 84  $^{\circ}\text{C}$  were performed at the end of each run as quality control. The cycle threshold (Ct) within the log

phase was set at 0.1. Results are expressed as arbitrary units relative to the housekeeping gene.

### **Statistical analysis**

Demographic and serum data from obese and lean patients are presented as median values, and groups were compared by the Mann–Whitney *U*-test. Cell assay data are presented as the mean and standard deviation of three to five independent experiments. Comparisons were performed by Student's *t*-test and correlations were investigated by Spearman's rank correlation coefficient ( $r_s$ ) using Graph Pad Prism version 4 (Graph Pad Software, Inc., San Diego, USA). The level of significance was set at  $p < 0.05$ .

## **RESULTS**

### **Demographic characteristics and serum parameters**

Ten obese and six lean subjects were recruited. Most participants were female (80%) and all were White. Differences in BMI, gender, age, and biochemical parameters related to obesity are shown in Table I. The median BMI of obese and lean subjects was 47.3 and 21.7  $\text{kg/m}^2$ , respectively ( $p = 0.0002$ ). Serum levels of obesity-related inflammatory proteins (TNF- $\alpha$ , SAA, and MCP-1) were determined. No differences in MCP-1 levels were found between groups ( $p = 0.6354$ ). The levels of TNF- $\alpha$  ( $p = 0.0226$ ) and SAA ( $p = 0.0002$ ), however, differed between obese and lean subjects. Obese individuals had higher serum levels of triglycerides ( $p = 0.0016$ ), LDL cholesterol ( $p = 0.0120$ ), and glucose ( $p = 0.0002$ ) than lean subjects. Moderate correlations were found between BMI and inflammatory cytokines SAA ( $p = 0.0029$ ,  $r_s = 0.6941$ ) and TNF- $\alpha$  ( $p = 0.0303$ ,  $r_s = 0.5416$ ) and between BMI and triglycerides ( $p = 0.0058$ ,  $r_s = 0.6750$ ), LDL cholesterol ( $p = 0.0445$ ,  $r_s = 0.5250$ ), and glucose ( $p = 0.0051$ ,  $r_s = 0.6637$ ) (Supplementary material, Table IS and IIS, Figure 2S).



**TABLE 1** - Demographic characteristics and serum parameters of obese and lean subjects included in the study

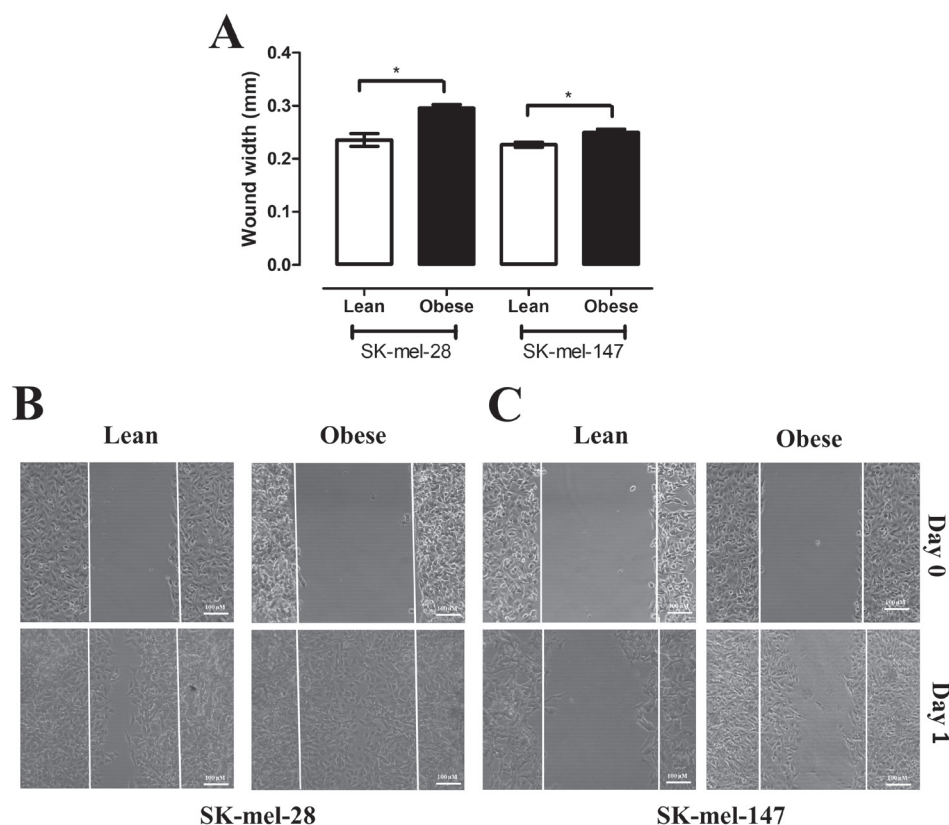
Parameter	Obese patients	Lean subjects	p-value	p-value for BMI correlation	rs value for BMI correlation
Subjects (n)	10	6	-		
Age (years)	47.5 (31–61)	29.5 (23–40)	0.0506	-	-
Gender (N)	F (8) M (2)	F (4) M (2)		-	-
Weight (kg)	131.3 (105–175)	63.1 (58–72)	0.0010*	-	-
BMI (kg/m <sup>2</sup> )	47.3 (40–5.9)	21.7 (19.3–24.3)	0.0002*	-	-
MCP-1 (pg/mL)	21.9 (9.5–59.8)	18.38 (8.74–41.61)	0.6354	0.4246	0.2147
SAA (ng/mL)	368.15 (15.6–1882.3)	10.2 (6.6–13.22)	0.0002*	0.0029*	0.6941
TNF- $\alpha$ (pg/mL)	11.15 (3–39.8)	3.0 (2.0–10)	0.0226*	0.0303*	0.5416
TG (mmol/L)	2.13 (0.98–4.77)	0.96 (0.57–2.01)	0.0016*	0.0058*	0.6750
LDL-C (mmol/L)	2.69 (1.45–4.79)	0.96 (0.57–1.08)	0.0120*	0.0445*	0.5250
HDL-C (mmol/L)	0.9324 (0.82–1.08)	0.84 (0.45–1.35)	0.6889	0.6384	0.1323
Glucose (mmol/L)	5.35 (4.7–7.0)	4.1 (3.8–4.4)	0.0002*	0.0051*	0.6637

BMI, body mass index; F: Female; M: Male; MCP-1, monocyte chemoattractant protein-1; SAA, serum amyloid A; TNF- $\alpha$ : *tumor necrosis factor-alpha*; TG, *triglycerides*; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. Values are the median, followed by the range in parentheses. Differences were analyzed by the Mann–Whitney U-test. Significant differences are denoted by asterisk (\*  $p < 0.05$ ). Serum reference values are as follows: TG  $\geq 8.3$  mmol/L, LDL-C  $\leq 7.2$  mmol/L, HDL-C  $\geq 3.3$  mmol/L, glucose = 3.9–5.8 mmol/L

### Effects of obese patient serum on melanoma cell migration

The scratch assay was performed using *BRAF*<sup>V600E</sup> (SK-Mel-28) and *NRAS*<sup>Q61R</sup> (SK-Mel-147) melanoma cells in DMEM medium supplemented with pooled serum from obese or lean subjects (Supplementary Material,

Figures 3S and 4S). As shown in Figure 1, SK-Mel-28 and -147 showed different migration patterns: SK-Mel-28 exhibited a more aggressive behavior than SK-Mel-147. When cells were exposed to pooled serum from obese or lean subjects, SK-Mel-147 ( $p = 0.0408$ ) and SK-Mel-28 ( $p = 0.0111$ ) showed increased cell migration after 24 h of treatment.

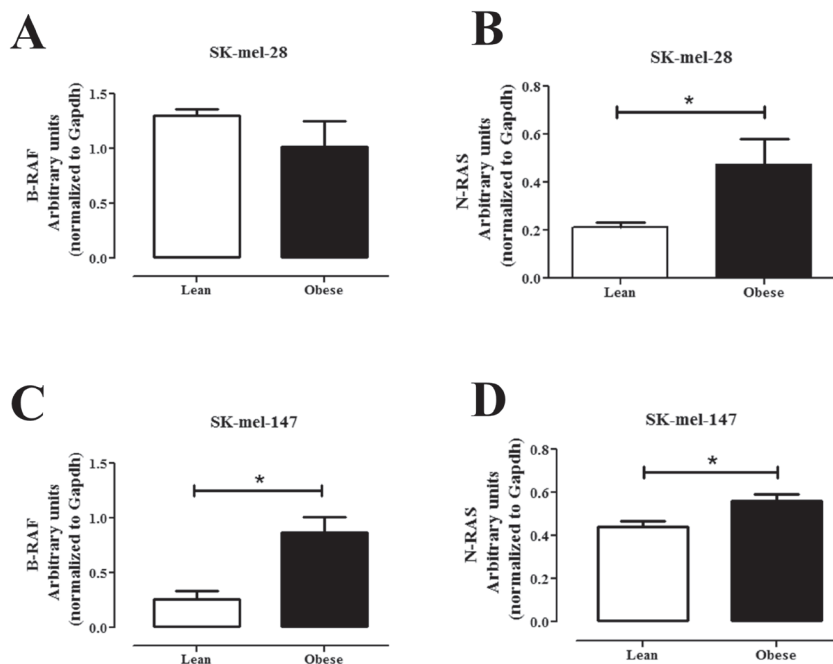


**FIGURE 1 - A:** Migration of *BRAF*- (SK-Mel-28) and *NRAS*-mutated (SK-Mel-147) melanoma cells treated with pooled serum from morbidly obese or lean individuals for 24 h. Results are expressed as wound width in mm. Data are the mean  $\pm$  standard error of five independent experiments. \* $p=0.0408$  for SK-mel-147 cells and \* $p=0.0111$  for SK-mel-28 (unpaired one-tailed *t*-test). **B and C:** Representative micrographs of the migration assay showing (B) SK-Mel-28 and (C) SK-Mel-147 cell monolayers (100 $\times$  magnification) before and after treatment with pooled serum from morbidly obese or lean individuals.

### Effect of obese patient serum on *BRAF* and *NRAS* expression

SK-Mel-28 and SK-Mel-147 melanoma cells were exposed to pooled serum from obese or lean subjects, and the expression of *BRAF* and *NRAS* was assessed after 24 h of treatment. Exposure to obese or lean subject serum ( $p = 0.2802$ ) did not influence *BRAF* expression in SK-

Mel-28 cells, as shown in Figure 2A. However, *NRAS* expression was 2-fold higher in SK-Mel-28 cells exposed to obese patient serum than cells exposed to lean subject serum ( $p = 0.0125$ , Figure 2B). Figure 2C and D shows the results for SK-Mel-147. Exposure to obese patient serum upregulated wild-type *BRAF* ( $p = 0.0024$ ) and *NRAS* ( $p = 0.0142$ ) expression compared with exposure to lean subject serum.

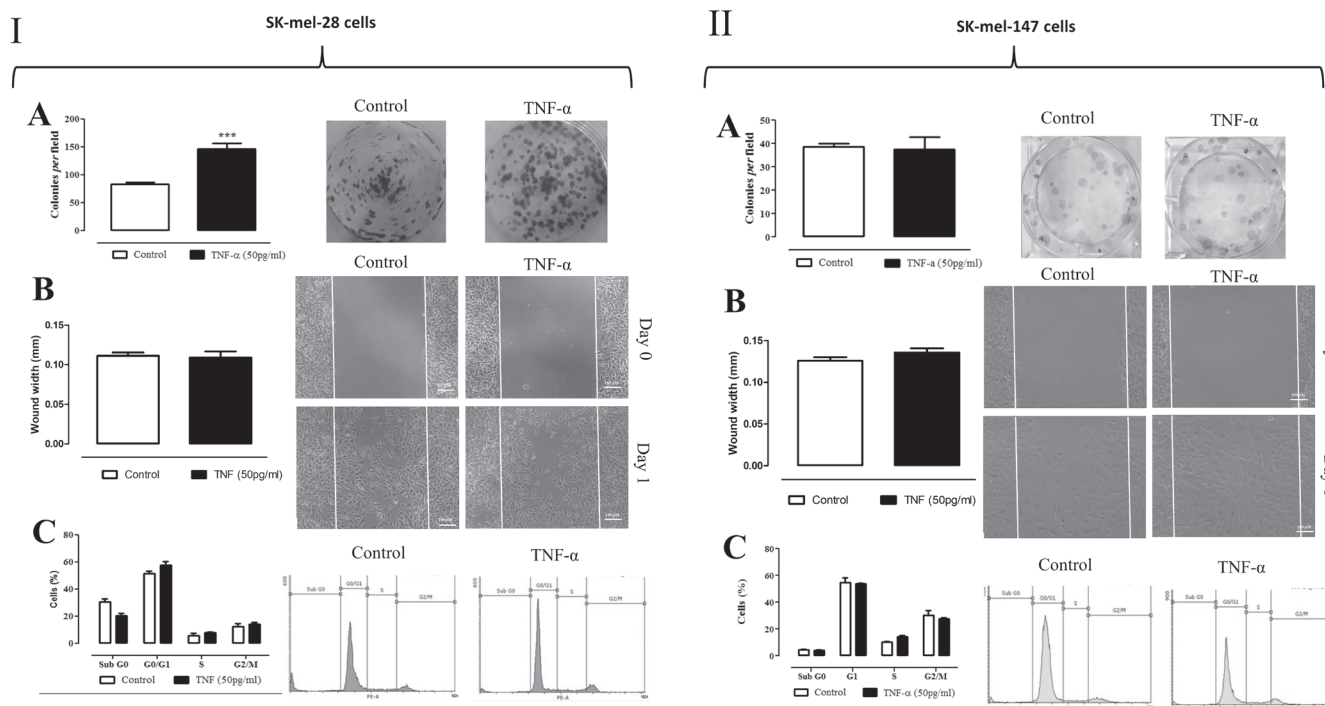


**FIGURE 2** - *BRAF* and *NRAS* gene expression in (A and B) SK-Mel-28 and (C and D) SK-Mel-147 melanoma cells treated with pooled serum from morbidly obese or lean subjects. Results are the mean of four independent experiments and are presented as relative expression ( $2^{-\Delta\Delta Ct} \times 100$ ) normalized to a housekeeping gene (*GAPDH*). \*  $p < 0.05$  (unpaired one-tailed *t*-test).

### Mitogenic effects enhanced by TNF- $\alpha$ in melanoma cells

Because TNF- $\alpha$  has been associated with obesity-related low-grade inflammation and metastatic melanoma, we assessed the impact of TNF- $\alpha$  on the clonogenic growth, migration, and cell cycle of *BRAF*- and *NRAS*-mutated melanoma cell lines (Figure

3). Exposure to TNF- $\alpha$  did not affect the migration capacity of SK-Mel-28 ( $p = 0.3419$ ) or SK-Mel-147 ( $p = 0.0665$ ) (Figure 3IB and 3IIB, respectively) or the proportion of cells in each cell cycle phase (Figure 3IC and 3IIC). However, the cytokine increased the clonogenicity (Figure 3IA) of *BRAF*-mutated cells ( $p = 0.0005$ ). *NRAS*-mutated cells were not affected by TNF- $\alpha$ .



**FIGURE 3** - Effects of TNF- $\alpha$  on the migration, clonogenic, and mitogenic capacity of **(I)** SK-Mel-28 and **(II)** SK-Mel-147 melanoma cells. Values are the mean and standard deviation of **(I)** four or **(II)** three independent experiments. **A:** Colony-forming unit (CFU) count of TNF- $\alpha$ -treated (50 pg/mL) and untreated (control) cells and representative images of cells fixed with 10% formalin and stained with Giemsa. \*\*\*  $p = 0.0005$  (unpaired one-tailed  $t$ -test). **B:** Cell migration of TNF- $\alpha$ -treated and control cells after 24 h of incubation. Representative micrographs of melanoma cell monolayers (100 $\times$  magnification). **C:** Cell cycle analysis of TNF- $\alpha$ -treated and control cells.

## DISCUSSION

The presence of pro-inflammatory mediators derived or not from adipose tissue appears to contribute to the *in vitro* progression and aggressiveness of melanoma cells. Our results demonstrated the effects of obese patient serum on the migration of mutated melanoma cell lines. The migration capacity and expression of two major oncogenes related to tumor aggressiveness and poor prognosis (Heptt *et al.*, 2017) were enhanced in *BRAF*- and *NRAS*-mutated melanoma cells. We also tested the effect of recombinant TNF- $\alpha$ , as the cytokine is associated with tumor progression and aggressiveness for its pro-inflammatory properties in the tumor microenvironment (Rossi *et al.*, 2018). However, the results showed that TNF- $\alpha$ , the major obesity-related cytokine (Hotamisligil, Shargill,

Spiegelman, 1993), had only a mild influence on the aggressiveness of tumor cells.

The experiments were performed aiming to establish a link between circulating obesity-related cytokines (which are often pro-inflammatory) and melanoma progression (Ahima, Flier, 2000). Ten obese subjects were recruited, and we hypothesized that their serum contained pro-inflammatory factors that might cause alterations in melanoma cells.

SK-Mel-28 and SK-Mel-147 are extremely aggressive melanoma cell lines with a high capacity to metastasize and a poor response to anti-tumor treatments. In this study, SK-Mel-147 cells bearing *NRAS*<sup>Q61R</sup> and SK-Mel-28 bearing *BRAF*<sup>V600E</sup> were exposed to serum from obese or lean individuals. Obese patient serum increased the migration capacity of both cell lines. This effect might be related to the presence of pro-inflammatory factors in the serum. Previous studies reported similar *in vitro*



results. Price and colleagues (2012) showed that serum from obese mice containing high concentrations of IL-6, vascular endothelial growth factor, and leptin was able to increase prostate cancer cell proliferation, invasion, migration, and matrix metalloproteinase activity. Similar results were also reported by Lamarre et al. (2007), who found that serum from obese rats significantly increased the mitogenic response of prostate cancer cells *in vitro*.

Obese patient serum is characterized by high glucose and insulin levels, factors associated with obesity and diabetes that might have increased the migration of SK-Mel-28 and SK-Mel-147. Previous studies revealed that high levels of blood glucose and insulin promoted growth and proliferation in tumor cells (Han et al., 2011; Okumura et al., 2002) via stimulation of epidermal growth factor (EGF) receptors by increasing EGF mRNA expression. Glucose, insulin, and insulin-like growth factor 1 (IGF-1) were shown to enhance colon cancer cell viability and resistance to chemotherapy (Volkova et al., 2014). IGF-1 increased melanoma survival by inhibiting apoptosis of *BRAF*-mutated A375 melanoma cells (Hilmi et al., 2008).

Acute reactive protein serum levels peak 36 to 48 h after an acute event and return to baseline after 4 days, a finding that is suggestive of the role of these proteins in the restoration of homeostasis post-inflammation (Wu et al., 2015). In contrast, in low-grade inflammation, which predominates in obese individuals, the levels of pro-inflammatory SAA, IL-6, and TNF- $\alpha$  remain consistently high, potentially contributing to tumor development and aggressiveness (Kern et al., 2019).

The two most critical pro-inflammatory cytokines in obese patient sera are SAA (Gómez-Ambrosi et al., 2006; Lappalainen et al., 2008) and MCP-1 (Kim et al., 2006). In the present study, obese patient serum contained high levels of SAA, MCP-1, and TNF- $\alpha$ , corroborating the low-grade inflammation state observed in morbidly obese patients (BMI  $\geq$  40 kg/m<sup>2</sup>). Some of these pro-inflammatory factors were strongly correlated with BMI (e.g., SAA,  $r_s = 0.6941$  and  $p = 0.0029$ ), evidence of their suitability as markers of adiposity.

In the present study, *BRAF* and *NRAS* expressions were upregulated by exposure to obese patient serum, probably associated with the action of pro-inflammatory

cytokines. Future studies should investigate the expression of *p53* and characterize the relationship between *p53* mutation in SK-Mel-28 and enhanced migration capacity (Daveri et al., 2015). The oncogenic protein RAS is known to cause changes in the polymerization, organization, and contraction of actin, polymerization and stability of microtubules, and transcriptional regulation of gene expression; together, these changes contribute to enhancing the motility of tumor cells (Castellano et al., 2016).

Because of the limited number of serum samples, we focused our investigations on the effects of obesity-related factors on cell morphology, proliferation, migration capacity, and clonogenicity. Literature data show that BRAF and N-RAS proteins, which were highly expressed in this study, enhance MAPK signaling (Wellbrock, Karasarides, Marais, 2004). B-RAF<sup>V600E</sup> is the most effective mutant in activating ERK in both melanocytes and melanoma cells, as stated by Whitwam et al. (2007) and revised by Ryan et al. (2016).

On the basis of the obesity-induced effects observed on melanoma cells and in an attempt to refine results related to the mitogenic profile, we carried out several experiments to assess clonogenicity, migration ability, and cell cycle alterations in melanoma cells (SK-Mel-28 and SK-Mel-147) treated with recombinant TNF- $\alpha$ . Cells were initially treated with 25 pg/mL TNF- $\alpha$ , twice the concentration found in patients' sera, but cells were not affected (data not shown). However, when cells were treated with 50 pg/mL TNF- $\alpha$ , the clonogenicity of SK-Mel-28 was increased. In previous studies using *in vitro* models of tumor invasion and migration, TNF- $\alpha$  concentrations of 100 to 500 pg/mL produced significant effects (Katerinaki et al., 2003; Zhu et al., 2004). It was not our aim to assess the effects of different concentrations of TNF- $\alpha$  on melanoma cells, but we observed that the concentrations of TNF- $\alpha$  in the serum of obese individuals seem to be insufficient to alter the migration profile of tumor cells.

Katerinaki and colleagues (2003) showed that TNF- $\alpha$  increased the migration ability of HLB (wild type B-RAF) by 21%. Zhu et al. (2004) found that HBL melanoma cells showed increased migration capacity after TNF- $\alpha$  exposure. Likewise, Cantón et al. (2003) reported a 180% increase in

uveal melanoma cell invasion in the presence of TNF- $\alpha$ . In the present study, significant effects were only observed on *BRAF*-mutated cell clonogenicity, suggesting that other cytokines are involved in the pro-tumor properties of serum from obese individuals. Obesity-related factors secreted by hypertrophic adipocytes, such as SAA, could be involved in the enhanced migratory pattern and *NRAS* and *BRAF* expression in melanoma cells (Castellano *et al.*, 2016). SAA was associated with the migration and invasion of human and mouse breast cancer cells because of its role in stimulating the transcription of RANTES, an inflammatory chemokine, and MMP2, a critical matrix metalloproteinase involved in metastasis (Hansen *et al.*, 2015).

In conclusion, this study reported an increase in the migratory ability of melanoma cells exposed to serum rich in obesity-related factors. Furthermore, we demonstrated the action of inflammatory mediators in the expression of oncogenes *NRAS* and *BRAF*, associated with tumor aggressiveness. The cytokines found in obese patient serum may be responsible for the increase in *NRAS* expression in SK-Mel-28 cells. This result suggests that inflammatory proteins may serve as secondary targets in melanoma therapy. TNF- $\alpha$  made a mild contribution to melanoma progression and aggressiveness at concentrations found in the serum of obese individuals. Because of the controversial and pleiotropic roles of this cytokine, anti-tumor strategies aimed at blocking TNF- $\alpha$  should be examined with caution in future studies, particularly when using *in vitro* models to screen for antitumor activity.

## CONFLICT OF INTERESTS

All authors declare that there is no conflict of interest regarding the publication of this paper.

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## REFERENCES

- Ahima RS, Flier JS. Adipose tissue as an endocrine organ. *Trends Endocrinol Metab.* 2000;11(8):327-32.
- Apovian CM, Bigornia S, Mott M, Meyers MR, Ulloor J, Gagua M, et al. Adipose macrophage infiltration is associated with insulin resistance and vascular endothelial dysfunction in obese subjects. *Arterioscler Thromb Vasc Biol.* 2008;28(9):1654-9.
- Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet.* 2001;357(9255):539-45.
- Balkwill F. TNF-alpha in promotion and progression of cancer. *Cancer Metastasis Rev.* 2006;25(3):409-16.
- Bhaskaran K, Douglas I, Forbes H, dos-Santos-Silva I, Leon DA, Smeeth L. Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5-24 million UK adults. *Lancet.* 2014;384(9945):755-65.
- Cai H, Song C, Endoh I, Goyette J, Jessup W, Freedman SB, et al. Serum amyloid A induces monocyte tissue factor. *J Immunol.* 2007;178(3):1852-60.
- Cantón I, Eves PC, Szabo M, Vidal-Vanacloch F, Sisley K, Rennie IG, et al. Tumor necrosis factor alpha increases and alpha-melanocyte-stimulating hormone reduces uveal melanoma invasion through fibronectin. *J Invest Dermatol.* 2003;121(3):557-563.
- Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med.* 2003;348(17):1625-38.
- Castellano E, Downward J. RAS Interaction with PI3K: More Than Just Another Effector Pathway. *Genes Cancer.* 2011;2(3):261-74.
- Castellano E, Molina-Arcas M, Krygowska AA, East P, Warne P, Nicol A, et al. RAS signalling through PI3-Kinase controls cell migration via modulation of Reelin expression. *Nat Commun.* 2016;7:11245.
- Chan DC, Chen CJ, Chu HC, Chang WK, Yu JC, Chen YJ, et al. Evaluation of serum amyloid A as a biomarker for gastric cancer. *Ann Surg Oncol.* 2007;14(1):84-93.
- Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. Mutations of the BRAF gene in human cancer. *Nature.* 2002;417(6892):949-54.
- Daveri E, Valacchi G, Romagnoli R, Maellaro E, Maioli E. Antiproliferative effect of rottlerin on Sk-Mel-28 melanoma cells. *Evid Based Complement Alternat Med.* 2015;2015:1-9.

- De Pergola G, Silvestris F. Obesity as a major risk factor for cancer. *J Obes*. 2013;2013:1-11.
- Fan Y, Mao R, Yang J. NF- $\kappa$ B and STAT3 signaling pathways collaboratively link inflammation to cancer. *Protein Cell*. 2013;4(3):176-85.
- Filippin-Monteiro FB, de Oliveira EM, Sandri S, Knebel FH, Albuquerque RC, Campa A. Serum amyloid A is a growth factor for 3T3-L1 adipocytes, inhibits differentiation and promotes insulin resistance. *Int J Obes (Lond)*. 2011;36(8):1032-9.
- Furlaneto CJ, Campa A. A novel function of serum amyloid A: a potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1beta, and interleukin-8 by human blood neutrophil. *Biochem Biophys Res Commun*. 2000;268(2):405-8.
- Gómez-Ambrosi J, Salvador J, Rotellar F, Silva C, Catalán V, Rodríguez A, et al. Increased serum amyloid A concentrations in morbid obesity decrease after gastric bypass. *Obes Surg*. 2006;16(3):262-9.
- Gorden A, Osman I, Gai W, He D, Huang W, Davidson A, et al. Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues. *Cancer Res*. 2003;63(14):3955-7.
- Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research techniques made simple: analysis of collective cell migration using the wound healing assay. *J Invest Dermatol*. 2017;137(2):11-16.
- Han L, Ma Q, Li J, Liu H, Li W, Ma G, et al. High glucose promotes pancreatic cancer cell proliferation via the induction of EGF expression and transactivation of EGFR. *PLoS One*. 2011;6(11):1-7.
- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-74.
- Hansen MT, Forst B, Cremers N, Quagliata L, Ambartsumian N, Grum-Schwensen B, et al. A link between inflammation and metastasis: serum amyloid A1 and A3 induce metastasis, and are targets of metastasis-inducing S100A4. *Oncogene*. 2015;34(4):424-35.
- Heppt MV, Roesch A, Weide B, Gutzmer R, Meier F, Loquai C, et al. Prognostic Factors and Treatment Outcomes in 444 Patients With Mucosal Melanoma. *Eur J Cancer (Oxford, England : 1990)*. 2017;81:36-44.
- Hilmi C, Larribere L, Giuliano S, Bille K, Ortonne JP, Ballotti R, et al. IGF1 promotes resistance to apoptosis in melanoma cells through an increased expression of BCL2, BCL-X(L), and survivin. *J Invest Dermatol*. 2008;128(6):1499-505.
- Hocker TL, Singh MK, Tsao H. Melanoma genetics and therapeutic approaches in the 21st century: moving from the benchside to the bedside. *J Invest Dermatol*. 2008;128(11):2575-95.
- Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose Expression of Tumor Necrosis Factor-Alpha: Direct Role in Obesity-Linked Insulin Resistance. *Science (New York, NY)*. 1993;259(5091):87-91.
- Katerinaki E, Evans GS, Lorigan PC, MacNeil S. TNF-alpha increases human melanoma cell invasion and migration in vitro: the role of proteolytic enzymes. *Brit J Cancer*. 2003;89(6):1123-9.
- Kern L, Mittenbühler MJ, Vesting AJ, Ostermann AL, Wunderlich CM, Wunderlich FT. Obesity-induced TNF $\alpha$  and IL-6 Signaling: The missing link between obesity and inflammation—Driven liver and colorectal cancers. *Cancers (Basel)*. 2018;11(1):1-21.
- Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE, et al. Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects and associated with obesity-related parameters. *Int J Obes (Lond)*. 2006;30(9):1347-55.
- Klein-Platat C, Drai J, Oujaa M, Schlienger JL, Simon C. Plasma fatty acid composition is associated with the metabolic syndrome and low-grade inflammation in overweight adolescents. *Am J Clin Nutr*. 2005;82(6):1178-84.
- Labrie F, Luu-The V, Labrie C, Pelletier G, El-Alfy M. Intracrinology and the skin. *Horm Res*. 2000;54(5-6):218-29.
- Lamarre NS, Sr MRR, Braverman AS, Gerstein MI, Mydlo JH. Effect of Obese and Lean Zucker Rat Sera on Human and Rat Prostate Cancer Cells: Implications in Obesity-Related Prostate Tumor Biology. *Urology*. 2007;69(1):191-195.
- Lappalainen T, Kolehmainen M, Schwab U, Pulkkinen L, Laaksonen DE, Rauramaa R, et al. Serum concentrations and expressions of serum amyloid A and leptin in adipose tissue are interrelated: the Genobin Study. *Eur J Endocrinol*. 2008;158(3):333-41.
- Maachi M, Piéroni L, Bruckert E, Jardel C, Fellahi S, Hainque B, et al. Systemic low-grade inflammation is related to both circulating and adipose tissue TNF $\alpha$ , leptin and IL-6 levels in obese women. *Int J Obes Relat Metab Disord*. 2004;28(8):993-7.
- Madan V, Lear JT, Szeimies RM. Non-melanoma skin cancer. *Lancet*. 2010;375(9715):673-85.
- Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. 2008;454(7203):436-44.
- Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity*. 2005;23(4):344-6.
- Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages

- as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 2002;23(11):549-55.
- Moore RJ, Owens DM, Stamp G, Arnott C, Burke F, East N, et al. Mice deficient in tumor necrosis factor- $\alpha$  are resistant to skin carcinogenesis. *Nat Med.* 1999;5(7):828-31.
- Mueller MM. Inflammation in epithelial skin tumours: old stories and new ideas. *Eur J Cancer.* 2006;42(6):735-44.
- Nissan MH, Pratilas CA, Jones AM, Ramirez R, Won H, Liu C, et al. Loss of NF1 in cutaneous melanoma is associated with RAS activation and MEK dependence. *Cancer Res.* 2014;74(8):2340-50.
- Okumura M, Yamamoto M, Sakuma H, Kojima T, Maruyama T, Jamali M, et al. Leptin and High Glucose Stimulate Cell Proliferation in MCF-7 Human Breast Cancer Cells: Reciprocal Involvement of PKC- $\alpha$  and PPAR Expression. *Biochim Biophys Acta.* 2002;1592(2):107-16.
- Palmieri G, Ombra M, Colombino M, Casula M, Sini M, Manca A, et al. Multiple Molecular Pathways in Melanomagenesis: Characterization of Therapeutic Targets. *Front Oncol.* 2015;5:1-16.
- Price RS, Cavazos DA, Angel RED, Hursting SD, deGraffenried LA. Obesity-related systemic factors promote an invasive phenotype in prostate cancer cells. *Prostate Cancer and Prostatic Diseases.* 2012;15(2):135-43.
- Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* 2010;141(1):39-51.
- Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nat Med.* 2013;19(11):1423-37.
- Rehman AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. *Lancet.* 2008;371(9612):569-78.
- Ribas A, Flaherty KT. BRAF targeted therapy changes the treatment paradigm in melanoma. *Nat Rev Clin Oncol.* 2011;8(7):426-33.
- Roberts DL, Dive C, Rehman AG. Biological mechanisms linking obesity and cancer risk: new perspectives. *Annu Rev Med.* 2010;61:301-16.
- Rossi JM, Galon J, Turcan S, Danan C, Locke FL, Neelapu SS, et al. Abstract LB-016: Characteristics of the pretreatment tumor microenvironment may influence clinical response in patients with refractory large B cell lymphoma treated with axicabtagene ciloleucel (axi-cel) in the pivotal ZUMA-1. *Cancer Research.* 2018;78(13 Supplement): p. LB-016-LB-016.
- Rueden CT, Schindelin J, Hiner MC, DeZonia BE, Walter AE, Arena ET, et al. ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinf.* 2017;18(1):1-26.
- Rundhaug JE, Fischer SM. Molecular mechanisms of mouse skin tumor promotion. *Cancers (Basel).* 2010;2(2):436-82.
- Ryan MB, Finn AJ, Pedone KH, Thomas NE, Der CJ, Cox AD. ERK/MAPK Signaling Drives Overexpression of the Rac-GEF, PREX1, in BRAF- And NRAS-Mutant Melanoma. *Mol Cancer Res.* 2016;14(10):1009-1018.
- Sergentanis TN, Antoniadis AG, Gogas HJ, Antonopoulos CN, Adami HO, Ekbom A, et al. Obesity and risk of malignant melanoma: a meta-analysis of cohort and case-control studies. *Eur J Cancer.* 2013;49(3):642-57.
- Sharma SD, Katiyar SK. Leptin deficiency-induced obesity exacerbates ultraviolet B radiation-induced cyclooxygenase-2 expression and cell survival signals in ultraviolet B-irradiated mouse skin. *Toxicol Appl Pharmacol.* 2010;244(3):328-35.
- Sheen YS, Liao YH, Liao JY, Lin MH, Hsieh YC, Jee SH, et al. Prevalence of BRAF and NRAS mutations in cutaneous melanoma patients in Taiwan. *J Formos Med Assoc.* 2016;115(2):121-7.
- Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer.* 2006;42(6):717-27.
- Uguen A, Talagas M, Costa S, Samaison L, Paule L, Alavi Z, et al. NRAS (Q61R), BRAF (V600E) immunohistochemistry: a concomitant tool for mutation screening in melanomas. *Diagn Pathol.* 2015;10:121-131.
- Volkova E, Robinson BA, Willis J, Currie MJ, Dachs GU. Marginal effects of glucose, insulin and insulin-like growth factor on chemotherapy response in endothelial and colorectal cancer cells. *Oncol Lett.* 2014;7(2):311-20.
- Vona-Davis L, Rose DP. Angiogenesis, adipokines and breast cancer. *Cytokine Growth Factor Rev.* 2009;20(3):193-201.
- Wellbrock C, Karasarides M, Marais R. The RAF Proteins Take Centre Stage. *Nat Rev Mol Cell Biol.* 2004;5(11):875-85.
- Whitwam T, Vanbrocklin MW, Russo ME, Haak PT, Bilgili D, Resau JH, et al. Differential Oncogenic Potential of Activated RAS Isoforms in Melanocytes. *Oncogene.* 2007;26(31):4563-70.
- Wolin KY, Colditz GA. Can weight loss prevent cancer? *Br J Cancer.* 2008;99(7):995-9.
- Wu Y, Potempa LA, Kebir DE, Filep JG. C-reactive protein and inflammation: conformational changes affect function. *Biol Chem.* 2015;396(11):1181-1197.



Serum from morbidly obese patients affects melanoma cell behavior *in vitro*

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Yamada T. [Serum amyloid A (SAA)--pathogenicity and implication of appearance in plasma]. *Rinsho Byori*. 2006;54(5):509-12.

Yamada T. Serum amyloid A (SAA): a concise review of biology, assay methods and clinical usefulness. *Clin Chem Lab Med*. 1999;37(4):381-8.

Zhang Y, Daquinag A, Traktuev DO, Amaya-Manzanares F, Simmons PJ, March KL, et al. White adipose tissue cells are recruited by experimental tumors and promote cancer progression in mouse models. *Cancer Res*. 2009;69(12):5259-66.

Zhu N, Lalla R, Eves P, Brown TLH, King A, Kemp EH, et al. Melanoma cell migration is upregulated by tumour necrosis factor-alpha and suppressed by alpha-melanocyte-stimulating hormone. *Br J Cancer*. 2004;90(7):1457-63.

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