Research Article

Reduced mammary tumor progression in a transgenic mouse model fed an isoflavone-poor soy protein concentrate

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Dietary exposure to soy has been associated with reduced breast cancer incidence. Soy isoflavones and protein components, such as protease inhibitors and the lunasin peptide, have been indicated as potential agents reducing carcinogenesis. In this study, the effect of soy-based diets was evaluated in a transgenic mouse model of breast carcinoma, overexpressing the *neu* oncogene. *Neu* female mice were fed for 20 wk a soy- and isoflavone-free diet (IFD), 4RF21 laboratory mouse diet, soy-based, thus isoflavone-rich (STD), or AIN-76-based semisynthetic diets with a soy protein isolate (SPI) or an isoflavone-poor soy protein concentrate (IPSP) as protein source. Mice were then sacrificed and tumors removed. Mammary tumor weights were not different in SPI *versus* IFD and STD fed mice. In contrast, mice fed IPSP showed reduced tumor progression *versus* IFD and STD groups ($p < 0.05$). Moreover, IPSP fed mice showed lower bromo-2′-deoxyuridine (BrdU) incorporation into breast tumor cells compared to STD and SPI fed animals ($p < 0.02$). Lung metastases were detected in 80% of IFD fed mice, in 70% of mice fed STD and SPI, and only in 50% of the IPSP fed animals. These results indicate that a diet containing an isoflavone-poor soy protein concentrate may inhibit breast tumor progression and metastasis development.

Keywords: Breast cancer / Isoflavones / Lung metastasis / Soybean proteins / Transgenic mice

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1 Introduction

Epidemiological studies showing a protective effect of fruit- and vegetable- rich diets against cancer have focused attention on the possibility that biologically active plant secondary metabolites may exert an anticarcinogenic activity [1]. Such compounds, now commonly referred to as "phytochemicals", may usefully be regarded as a new class of nutrients. From the early reports in the field, such as those by Wattenberg [2], a great deal of new evidence has become available in the past decade [3]. On the other hand, recent findings have provided inconclusive evidence, thus leading the International Agency for Research of Cancer to infer that "there is limited evidence for a cancer preventive effect of consumption of fruits and vegetables for cancer of the mouth and pharynx, esophagus, stomach, colon rectum, lung, ovary. There is inadequate evidence for a cancer-preventive effect of consumption of fruit and vegetables for all other sites" [4]. Nevertheless, the interest in the biological effects of plant constituents is constantly growing [4].

Beans from soy have been shown to carry out multiple biological activities, leading to a huge number of experimental and clinical reports. The hypothesis of an anticarcinogenic effect of soy originated from epidemiological stud-

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Abbreviations: BBI, Bowman-Birk protease inhibitors; **BrdU,** 5-bromo-2′-deoxyuridine; **DHD,** dihydrodaidzein; **IFD**, isoflavone-free diet; **IPSP**, isoflavone-poor soy protein concentrate; **MMTV,** mouse mammary tumor virus; **MRM,** multiple reaction monitoring; **SPI**, soy protein isolate; **STD**, soy-based, thus isoflavone-rich

ies showing a reduced incidence of mammary tumors in Japanese women consuming soybean-based diets [5]. This result seemed to be consistently associated with a high intake of soy-based foods in the daily diet [6]. Dietary soy is now generally believed to exert a significant preventive activity in prostate [7] and breast [8] cancer, although recent meta-analyses [9, 10] conclude that translation of these evidence into clinical recommendation is still premature. Consumption of soy food has also been associated with a reduced incidence of osteoporosis [11], but further studies are needed to confirm this preliminary evidence [12, 13]. Finally, in the case of cardiovascular prevention, there are generally positive indications [14], although a recent article from The American Heart Association cast a shadow over the cholesterol lowering potential of a soy diet [15]. These findings were recently contradicted by another meta-analysis comparing older and more recent studies on soy proteins [16].

A wide variety of dietary components present in soybeans have been indicated as potential anticancer agents, such as proteins/peptides [17, 18], isoflavones [19], phytosterols [20], saponins [21], and pytate [22]. Proteins and isoflavones have been indicated as the major soybean components possibly responsible for protection against breast cancer, since phytosterols, saponins, and phytate are poorly absorbed from the gastrointestinal tract [22 – 24].

Soy proteins bearing antitumoral activity include the Kunitz [25] and the Bowman-Birk protease inhibitors (BBI) [17, 26]; proteins are also an important source of biologically active peptides, which may be released from their parent proteins by enzymatic proteolysis, *i.e.*, during gastrointestinal digestion or food processing [27, 28]. A bioactive peptide from soybean, lunasin [18], has been shown to exhibit significant anticancer activity. A wide range of lunasin concentrations within the Glycine max cultivars and different commercial soy proteins have been described [29].

Genistein, the major soy isoflavone has been shown to reduce tumor progression in animals exposed to chemical carcinogens [30]; however, genistein can both reduce and increase cell proliferation *in vitro* based on achieved concentrations in target cells [31].

The aim of the present study was to investigate the impact on tumor progression of dietary treatments containing soy proteins with different isoflavone contents, in a transgenic mouse model of breast carcinoma.

Studies addressed to establish the potential of soy components in cancer prevention/treatment have been generally carried out on *in vitro* or *in vivo* models, with tumors being induced by chemical carcinogens [32, 33]. The potential of these compounds in cancer prevention would, however, require a more suitable model, representative of the human condition, *e.g.*, leading to metastasis development.

The model used in the present study differs from the classical type of transplantable tumors that provide a rather artificial condition, since mice overexpress an activated rat ErbB-2/neu oncogene (mouse mammary tumor virus (MMTV)-*neu*) known to play a role in human breast cancer [34]. Female mice develop multiple mammary tumors in 100% of cases in a very predictable manner [35, 36], thus making the model very useful as provider of homogenous neoplastic material. Both multifocal breast tumors and lung metastases arise in the absence of any exogenous inducing factor, thus making it a model sharing some similarities to human conditions of high breast cancer risk [37 – 39]. Moreover, MMTV-*neu* tumors bear estrogen receptors, which are present in the majority of human breast cancers [40]. This model used by us and others to test various innovative approaches [37, 41, 42] responds well to a variety of factors, from those affecting angiogenesis [39] to cytokine activation [42]. These features make this animal model an excellent tool to investigate dietary effects on tumor growth and metastases dissemination.

2 Materials and methods

2.1 Transgenic mouse lineage

Production and screening of MMTV-*neu* transgenic mice were carried out as previously described [36, 37]. MMTV-*neu* transgenic mice have been previously generated in the authors' laboratory [34]. Briefly, the construct contains a 4.6-kb cDNA encoding the activated form of the rat c-*neu* (Val to Glu change at amino acid 664), driven by regulatory regions of the MMTV-long terminal repeat (MMTV-LTR); SV40 splicing and polyadenylation signals are added downstream to the cDNA. Transgenic mice were generated by pronuclear microinjection of the construct into oocytes derived from CD1 Swiss albino mice. Tail DNA was extracted and analyzed by PCR for the presence of the transgene [36, 37].

Procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national (D.L. No. 116, G.U. Suppl. 40, February 18, 1992, Circolare No. 8, G.U. luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health-NIH Publication No. 85-23, revised 1996).

2.2 Study design

All experiments were conducted on hemizygous transgenic females, obtained by crosses between control CD1 females and transgenic males. Starting from breeding, up to the end of lactation (Day 21 postpartum), the CD1 females were fed 4RF21 laboratory mouse diet, which is soy-based and thus rich in isoflavones (Mucedola, Milano, Italy). At weaning (21 days of age), pups were screened by PCR and 40 MMTV-*neu* female mice were randomly divided into four

Table 1. Composition of IFD, STD, SPI, and IPSP diets

Ingredients (g/kg)	IFD	STD	SPI	IPSP
Corn/wheat protein	200[a)]	–	–	–
Soy protein	–	215[a)]	–	–
Soy protein isolate	–	–	200[a)]	–
Soy protein concentrate	–	–	–	200[a)]
Methionine	–	–	3	3
Unsaturated fat	85[b)]	50[b)]	52[b)]	52[b)]
Carbohydrates	630[c)]	630[c)]	650[c)]	650[c)]
Fiber	40	60	50	50
Mineral mix	35	35	35	35
Vitamin mix	10	10	10	10

IFD, isoflavone-free diet; STD, soy-based, isoflavone-rich, standard diet for mice; SPI, soy protein isolate-based diet; IPSP, isoflavone-poor soy protein concentrate-based diet.

a) The value indicates the net protein content.

b) Unsaturated fat was derived from soybean oil for IFD and STD diets and from corn oil for SPI and IPSP diets.

c) Carbohydrates were derived from wheat and corn for IFD and STD diets; SPI and IPSP are AIN-76-based semisynthetic diets where carbohydrates are constituted by sucrose and corn starch.

groups (ten animals each) and fed, *ad libitum*, the following diets:

IFD: wheat- and corn-based, soy- and isoflavone-free diet (2019 Teklad Global, Harlan Italia, MI, Italy).

STD: 4RF21 laboratory mouse diet soy-based and thus isoflavone-rich (Mucedola, Milano, Italy).

SPI: AIN-76-based semisynthetic diet containing soy protein isolate (Sigma–Aldrich, St. Louis, MO, USA) as protein source (Lab. Piccioni, Gessate, MI, Italy);

IPSP: AIN-76-based semisynthetic diet containing an isoflavone-poor soy protein concentrate (Croksoy^{R}70, Perfoods, Milano, Italy) as protein source (Lab. Piccioni), routinely used in the clinic for its hypocholesterolemic properties [43, 44].

The standard composition of IFD, STD, SPI, and IPSP is shown in Table 1. Soy protein components have been previously reported [45]. In soybean seed-derived proteins (STD diet) all the different protein fractions are clearly identified; in both soy protein isolate (SPI diet) and soy protein concentrate (IPSP diet), all the protein fractions are present as fragmented products of molecular weight $\leq$25 kDa, mostly constituted by small size peptides.

2.3 Isoflavone quantitation in diets and serum

Extraction and HPLC-UV analyses of isoflavones in the diet were performed as described [46]. Isoflavone analyses in mouse serum were instead performed by HPLC-ESI-multiple reaction monitoring (MRM) [46]. Briefly, an aliquot of 100 μL serum sample was incubated at 37°C overnight in sodium citrate buffer (25 mM, pH 5) in the presence of β-glucuronidase and sulfatase in order to hydrolyze the conjugated isoflavone metabolites. Serum samples were then extracted and the organic phase containing the metabolites submitted to HPLC-ESI-MRM analysis using an Agilent-1100 HPLC equipped with a binary pump, a thermostated column compartment, an autosampler, a diode array detector (DAD), and an Ion Trap System SL (Agilent Technologies, Palo Alto, CA, USA). The LC-MS interface was an electrospray ion source (ESI) working in negative ion mode, and the ions were analyzed in MRM.

2.4 Histopathological analysis

Animals were sacrificed at 5 months of age by cervical dislocation and all ten tumors growing in each female were removed and weighed. All tissues were examined for lesions. The heaviest and the lightest mammary tumors, lungs, and liver were fixed in 10% neutral buffered formalin. After fixation, organs were routinely processed for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin (HE). Mammary tumors were classified according to the recommendations of the Annapolis meeting [47]. For a careful examination of metastatic spread to lungs, all lobes were evaluated on a single histological section. Metastases were counted and scored as small (<10 cells), intermediate (10–100 cells), and large (>100 cells), following a classification previously established by our research group [39].

2.5 Cell proliferation

Cell proliferation in tumors was determined by immunohistochemical analysis. 5-Bromo-2′-deoxyuridine (BrdU) incorporation into cellular DNA was used as an indicator of proliferating cells [48]. Two hours before sacrifice 100 mg/kg of BrdU (Sigma–Aldrich) were given i.p. to the mice. Tumors were subsequently excised, fixed in formalin for 24 h and paraffin embedded. Five micrometer sections were cut, deparaffinized, rehydrated in increasing dilutions of ethanol, rinsed in water, incubated with 4 N HCl in PBS + 0.1% Triton X100 for 10 min and treated with proteinase K (20 μg/mL) (Roche, Basel, Switzerland) for 30 min at room temperature. Endogenous peroxidase was blocked with 10% H_2O_2 (36 vol.) in PBS for 10 min. BrdU mouse mAb (Becton Dickinson, Franklin Lakes, NJ, USA) diluted 1:25, was coupled with biotinylated mouse IgG *in vitro* (following Dako Ark-kit procedure) and incubated for 30 min at room temperature, followed by incubation with Ark-streptavidin (Dako, Glostrup, Denmark) for 30 min. The peroxidase reaction was developed using liquid DAB + chromogen (Dako) for 3 min. PBS + 0.05% Tween-20 was used for washing between steps. The sections were then washed with distilled water, counterstained with Mayer's hematoxylin, washed with tap water, dehydrated in graded ethanol and xylene and mounted with a coverslip. Nonimmune murine IgG at appropriate concentrations was used as a negative control.

Table 2. Body weight gain in mice fed IFD, STD, SPI, and IPSP diets

Age	IFD	STD	SPI	IPSP
4 wk	17.38 ± 1.76	19.03 ± 1.66	20.21 ± 3.49	20.01 ± 3.89
6 wk	23.39 ± 1.63[a)]	24.34 ± 3.34[b)]	22.42 ± 1.78	23.63 ± 2.48[c)]
8 wk	25.74 ± 2.04[a)]	26.91 ± 3.52[b)]	23.81 ± 2.48[d)]	24.58 ± 2.88[c)]
10 wk	25.48 ± 1.75[a)]	28.82 ± 3.68[b,e)]	26.42 ± 2.69[d,f)]	26.02 ± 2.07[c)]
12 wk	29.48 ± 3.33[a,g)]	29.99 ± 3.78[b,e)]	28.49 ± 3.54[d,f,h)]	28.18 ± 2.56[c,i)]

Data are expressed as mean ± SD; $n = 10$ mice/group.
a) $p < 0.0005$ *vs.* 4 wk IFD.
b) $p < 0.0005$ *vs.* 4 wk STD.
c) $p < 0.05$ *vs.* 4 wk IPSP.
d) $p < 0.05$ *vs.* 4 wk SPI.
e) $p < 0.005$ *vs.* 4 wk STD.
f) $p < 0.05$ *vs.* 6 wk SPI.
g) $p < 0.005$ *vs.* 6, 8 and 10 wk IFD.
h) $p < 0.005$ *vs.* 8 wk SPI.
i) $p < 0.05$ *vs.* 6 and 8 wk IPSP.

2.6 Statistical analyses

Data are expressed as mean ± SE. Analysis of variance (ANOVA) was used to compare tumor weights and cell proliferation among groups, followed by a Bonferroni's *post-hoc* test. A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Animal growth and energy intake

Mice gained weight without significant differences among groups (Table 2). On the basis of the metabolizable energy for each diet and the mean daily food intake of each group, daily energy intake was calculated and was 17.50, 16.90, 16.36, and 16.55 kcal/die, for IFD, STD, SPI, and IPSP diets, respectively.

3.2 Isoflavone levels in diets and plasma

The isoflavone content of the diets is reported in Table 3 (expressed as aglycone concentration). As expected, isoflavones were detected only in STD, SPI, and IPSP diets, with genistein as the main component. STD diet had the highest isoflavone concentration, whereas in SPI and IPSP diets the content was very low. Table 3 also reports mean daily intake of genistein and daidzein of the experimental animals that were proportional to the concentration in the diets.

After ingestion, isoflavone glycosides are hydrolyzed by colonic bacteria to release the aglycones, *i.e.*, the only absorbable form [49]. Daidzein was only detectable as the reduced metabolite dihydrodaidzein (DHD). In contrast, no metabolite was observed in the case of genistein. Each animal was analyzed separately: mean values are reported in Table 3. In the serum of animals fed IFD nonquantifiable traces were detected. The concentrations of genistein and DHD in the sera of mice fed STD were 0.71 and 0.21 μM, respectively. Serum levels in mice fed SPI were 0.234 μM for genistein and below the limit of quantification for DHD, whereas in Croksoy^{R}70 fed animals were below the detection limit.

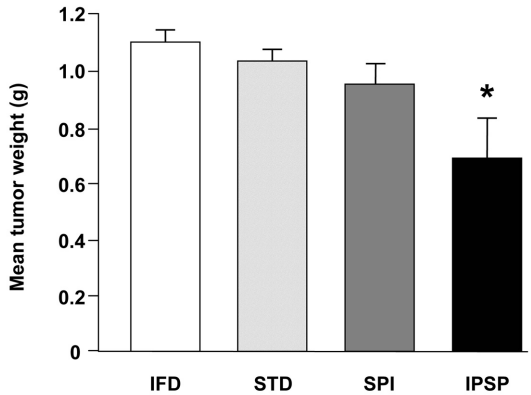

Figure 1. Mean weight of mammary tumors evaluated at the end of five months of age in MTTV-*neu* transgenic female mice fed four different diets: IFD (wheat- and corn-based, soy- and isoflavone-free), STD (soy-based, isoflavone-rich, standard diet for mice), SPI (semisynthetic diet containing soy protein isolate as protein source), IPSP (semisynthetic diet containing an isoflavone-poor soy protein concentrate as protein source). Data are expressed as mean ± SE. *$p < 0.05$ *vs.* IFD and STD groups.

3.3 Mammary tumor progression

Tumors develop in each mammary gland in 100% of mice used in this study. In line with previous studies on the same animal model fed STD diet [39], mean tumor weight of STD fed females was 1.031 ± 0.044 g (Fig. 1). Small, non-significant differences were detected between STD and IFD groups, mean tumor weights being slightly higher in the latter. The SPI diet caused a slower tumor progression com-

Table 3. Isoflavone concentration in the diets, isoflavone daily intake and concentration in the serum of treated mice

Group	Diets			Daily intake			Serum concentration
	Total isoflavones (μg/g d.w.)	Genistein (μg/g d.w.)	Daidzein (μg/g d.w.)	Genistein (mg/die)	Daidzein (mg/die)	Genistein (μM)	DHD (μM)
IFD	–	–	–	–	–	–	–
STD	502.3 ± 30.23	316.0 ± 15.22	138.5 ± 12.19	1.470 ± 0.007	0.650 ± 0.037	0.710 ± 0.063	0.210 ± 0.035
SPI	224.3 ± 17.12[a)]	157.1 ± 8.921[c)]	55.40 ± 3.620[e)]	0.740 ± 0.003[g)]	0.260 ± 0.003[i)]	0.234 ± 0.019[k)]	<LOQ
IPSP	31.20 ± 1.320[a,b)]	19.20 ± 0.856[c,d)]	9.500 ± 0.816[e,f)]	0.080 ± 0.0004[g,h)]	0.040 ± 0.0002[i,j)]	<LOD	<LOD

d.w., dry weight; DHD, dihydrodaidzein; LOQ, (signal/noise = 10): 0.04 μM for genistein and 0.08 μM for DHD; LOD, (signal/noise = 3): 0.018 μM for genistein and 0.035 μM for DHD.
a) $p < 0.0005$ *vs.* STD total isoflavone concentration.
b) $p < 0.0005$ *vs.* SPI total isoflavone concentration.
c) $p < 0.0005$ *vs.* STD genistein concentration.
d) $p < 0.0005$ *vs.* SPI genistein concentration.
e) $p < 0.0005$ *vs.* STD daidzein concentration.
f) $p < 0.0005$ *vs.* SPI daidzein concentration.
g) $p < 0.0005$ STD genistein daily intake.
h) $p < 0.0005$ *vs.* SPI genistein daily intake.
i) $p < 0.0005$ *vs.* STD daidzein daily intake.
j) $p < 0.0005$ SPI daidzein daily intake.
k) $p < 0.0005$ *vs.* STD genistein serum concentration.

Table 4. Frequency, number and size of lung metastases in MMTV-*neu* treated mice

Group	Mice with metastases	Large (>100 cells)	Medium (10–100 cells)	Small (<10 cells)	Total metastases
IFD	8/10	29	15	1	45
STD	7/10	33	10	0	43
SPI	7/10	35	27	1	63
IPSP	5/10	10	10	2	22

pared to both IFD and STD fed mice, but this difference did not reach statistical significance. In contrast, in IPSP fed mice, tumor progression was markedly reduced compared to all the other groups (mean tumor weight: 0.69 ± 0.14 g) and significantly different from both the IFD and STD (Fig. 1), while no significance was reached *versus* SPI ($p = 0.154$). Histologically, tumors were solid adenocarcinomas without differences among the four groups.

3.4 Pulmonary metastasis development

Pulmonary metastases, a characteristic feature of this breast cancer model [36, 39], were detected in mice from all groups, but the incidence was different (Table 4). The lowest number was found in IPSP group where only five mice developed pulmonary metastases. Metastases were quantified and their size was evaluated. Number and size of metastases were similar in IFD and STD fed mice. SPI fed animals had the highest number, whereas females fed IPSP developed the lowest number of metastases (35% of those detected in SPI and 50% of those found in IFD and STD groups).

3.5 Cell proliferation

Results of cell proliferation studies are shown in Fig. 2. No differences were observed among STD, SPI, and IFD groups. In contrast, mice fed IPSP showed a significantly lower BrdU incorporation into cell DNA of breast tumors compared to STD and SPI groups ($p < 0.02$).

4 Discussion

Although recent epidemiological data seem to show a trend toward a reduction [50], breast cancer still affects over 200 000 women in the US every year [51]. In addition to aging of the population and putative transforming factors acquired from the environment or genetically determined, other factors related to diet and body weight [52] may affect breast cancer incidence. There is, therefore, considerable interest in dietary approaches affecting, to a significant extent, breast cancer risk, particularly for predisposed females [8]. Approaches to chemoprevention/treatment range from antagonism to proteins with stimulatory properties on metastases development, *e.g.*, endothelin [53], to

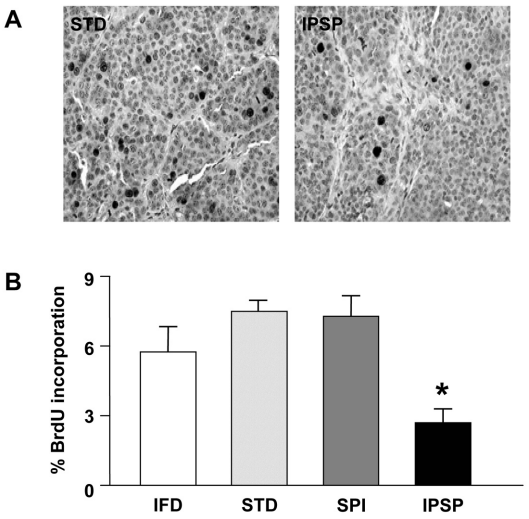

Figure 2. Immunohistochemical analysis of cell proliferation. BrdU incorporation into cellular DNA was used as indicator of proliferating cells. Panel A: examples of BrdU immunostaining in tumors removed by STD or IPSP fed mice (200 × magnification). Panel B: mean values of percent BrdU positive cells in MTTV-*neu* mice fed IFD, STD, SPI, and IPSP diets. Data are expressed as mean ± SE. *$p < 0.02$ *vs.* STD and SPI group.

hormonal components [54], and to general advice on food consumption and life habits [55].

In view of the epidemiological link between soy consumption and reduced breast cancer incidence, a number of investigators have attempted to evaluate major soy components, in effort to affect breast cancer development. Among these, isoflavones have been probably the most widely studied [32]. Isoflavones, particularly genistein and daidzein, directly affect the mitotic process in growing cells, and in view of their similarity to estrogens, may bind to estrogen receptors, particularly the estrogen receptor β (ERβ) [56]. At low concentrations isoflavones appear to act solely as estrogen agonists, while at higher concentrations they have nonhormonal activities independent of ER, the end result often being suppression of cellular growth [57]. While a number of *in vivo* findings have indicated a potential activity of soy isoflavones on established breast cancer [58], other studies have provided inconsistent or possibly negative data [57, 59]; further, direct mutagenic activity of isoflavones was shown in cultured cells [60]. Animal findings compound the lack of reliable data on intervention studies in women at high risk with this type of dietary approach [32]. A further consideration is that soy is the only leguminous seed with a significant content of isoflavones [61], while the other major grain legumes, among these lupin, peas, and others, provide only negligible amount of isoflavones [62]. Since these other dietary components are a major protein source in populations with a low breast cancer risk, it remains to be established whether isoflavones are really the major protective component.

Tumor-prone transgenic animals have recently been shown to be superior in regard to validation of therapeutic strategies *versus* other traditional approaches which bear little, if any, resemblance to the human condition [63].

The MTTV-*neu* transgenic mice spontaneously develop both multifocal breast tumors and lung metastases [36, 39] closely resembling the HER-2/*neu* overexpressing breast cancer, a subset of human breast cancer that is highly aggressive and associated with a shorter survival [64]. Tumors arising in MMTV-*neu* mice are estrogen receptor positive and respond to antiestrogenic treatments [41]. For these reasons, this seemed an appropriate animal model to test the impact of soy-based diets with different isoflavone content on breast tumor development. In this model, tumors arise very early in life at each mammary gland [39], therefore the evaluation of the mean tumor weight after 4 months of dietary treatment was chosen as adequate endpoint. This approach was the same used by our group for many other studies with this same animal model [36 – 39, 41].

In the present study, the effect on tumor progression of three soy-based diets characterized by different isoflavone contents was tested, and compared to that of a control diet without soy and isoflavones (IFD). The diets were not isocaloric; particularly, the IFD was higher in fat compared to the other diets and this is known to be associated with increased experimental tumorigenesis [65]. However, animal growth was similar among groups and the daily caloric intake was only slightly increased in IFD mice compared to the other groups. Therefore, results should not have been affected by this slight unhomogeneity among groups.

In every group, tumors developed at each mammary gland in 100% of mice, without histological differences, all being solid adenocarcinomas. This result was expected, based on previous studies on MMTV-*neu* mice [39, 41].

Isoflavones did not seem to exert a protective effect against tumor progression, since even the diet with the highest isoflavone content (STD) did not inhibit tumor development. These data are in line with previous results [57, 59, 66], but are in contrast with other animal studies indicating a chemopreventive effect by isoflavones [67 – 70]. In those investigations, timing of exposure seemed to be crucial, neonatal and prepubertal isoflavone consumption being necessary to observe a protective effect [70]. All the animals used in our study had been exposed from conception to weaning, through the mothers fed STD diet, to a concentration of isoflavones potentially adequate to provide a chemopreventive effect [71]; nevertheless, no differences were observed between IFD and STD groups. No variations in tumor development *versus* IFD were also detected in SPI group, exposed after weaning to an intermediate concentration of isoflavones. A significant reduction in size of the primary tumors after treatment was instead observed with a soy-based, isoflavone-poor diet (IPSP). These data are supported by a significantly reduced cell proliferation, as assessed by BrdU incorporation. Moreover, IPSP fed

mice displayed a lower incidence of lung metastases compared to all the other groups, suggesting a potential effect of this dietary treatment on metastasization. Altogether, based on our results, isoflavones do not appear to be involved to a significant extent in the reduced tumor progression induced by soy diets. This conclusion is supported by pharmacokinetic considerations. Mice on the isoflavone-poor diet consumed approximately 0.12 mg/day of isoflavones. Based on body weight, this amount is apparently not different from the 50 mg/day typically consumed by populations with a high isoflavone intake [72]. It should be noted however that pharmacokinetic is determined not only by body weight, but also by other variables (including blood volume and plasma kinetics) and is identified by the so-called Mordenti's function ($Y = aW^b$) [73]. Upon this correction, isoflavone blood levels in IPSP fed mice are no more than 10% of those measured in men exposed to similar doses. These considerations are supported by previous mouse studies [74] and by our results indicating that a daily intake of 0.12 mg/die of isoflavones in IPSP fed mice is associated with isoflavone blood levels below 0.05 μM (Table 3) *versus* 0.5 μM in humans exposed to 50 mg/day of isoflavones [72]. The isoflavone plasma levels typically found in populations consuming soy foods are therefore much higher than those found in IPSP fed mice and can be approximately estimated in between those measured in ISP and STD fed mice.

Among factors having an impact on tumor growth, the activated status of HER-2 and possibly of downstream factors such as mitogen-activator protein kinases p38 [75] and p27 [76], may be possibly affected by IPSP. In addition, a previous study by our group indicated the unusual sensitivity of this tumor to angiogenic factors as well explained by the vascular targeted, angiostatin that besides delaying primary tumor growth, more significantly inhibited the appearance of lung metastases [39]. Protein components in IPSP may therefore directly influence cell proliferation in a spontaneous, highly proliferative mammary tumor, by interfering with some downstream events caused by the activated rat *neu* oncogene.

A limitation of the present study is that the protein/peptide components possibly responsible for the observed effect have not been yet identified. Investigations on this regard are currently in progress. Candidate proteins/peptide bearing antitumoral activity include the Kunitz [25] and the BBI [17, 26]. Other anticarcinogen soy protein components may be a tridecapeptide (MITLAIPVNKPGR) that stimulates phagocytosis of human neutrophils isolated from a trypsin digest of soybean proteins [28] and lunasin, a unique 43-amino acid peptide that can arrest mitosis after transfection into human mammalian cells. Lunasin is directly active on mouse skin cancer after local application [18]. There is, however, no evidence for a direct activity after oral administration, since lunasin needs internalization through an RGD cell adhesion motif, followed by co-localization with hypo-acetylated chromatin.

In conclusion, the reported findings provide, to the best of our knowledge, the first experimental indication that the protein component of a legume-based diet may be directly responsible for a significant reduction of breast cancer development in a model with spontaneous, not chemically induced, disease. More specifically, the almost complete absence of isoflavones, still an ambiguous dietary factor in breast cancer chemoprevention, clearly indicates that epidemiological and prospective studies linking soy intake and breast cancer risk should give more intense consideration to the protein component of the diet under evaluation. This appears particularly desirable after the recent indication that even women with a high risk of hereditary breast and ovarian cancer, *i. e.*, BRCA1/2 positive, appear to adhere to a minimal extent to any use of dietary supplements that may be possibly indicated as suitable for breast cancer prevention [77].

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