

Dietary Phytoestrogen Increases Tumour Size and the Frequency of Circulating Tregs in a B16-F10 Murine Melanoma Tumour Model

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Abstract

Background: Clinical studies show strong associations between hormone levels, particularly estrogens and the development of skin cancers. Cutaneous melanoma is considered a hormone-related tumour; however, their role in melanoma progression remains unclear.

Objective: To investigate the effects of a phytoestrogen-rich diet on melanoma tumour initiation and development using a syngeneic mouse model.

Patients and Methods: Mice were fed either a phytoestrogen-rich or low control diet and injected subcutaneously with 5×10^5 syngeneic melanoma cells (B16-F10). After 10–12 weeks, tumours and spleens were collected. Tumour size and weight were measured, and quantitative PCR (qPCR) was performed to analyse the expression of estrogen receptor (ER) α and β . Regulatory T cells (Tregs) from splenocytes was assessed via flow cytometry.

Results: Mice consuming the phytoestrogen-rich diet exhibited significantly larger tumours compared to those on the control diet. Phytoestrogens in the diet up regulated ER β and down regulated ER α mRNA expression in tumour tissue. A significant increase in the proportion of splenic Tregs was observed in tumour-bearing mice fed a phytoestrogen-rich diet.

Conclusion: This study highlights the influence of dietary composition on tumour growth and associated immune responsiveness, emphasising the need to account for dietary factors in experimental designs and their potential impact on tumour biology.

Keywords: Phytoestrogen, T-regulatory cells, Murine melanoma tumours, Flow cytometry.

Introduction

Genetic, inflammatory and environmental factors have a significant role in the development of cancers (1). However, the impact of dietary components on outcomes in animal experiments is often underappreciated (2). A variety of commercial rodent diet formulations are available (3–5), many of which include Soy meal as a primary protein source (48). Soy meal is rich in phytoestrogens, plant-derived compounds structurally similar to endogenous estrogens, which can exert either estrogenic or anti-estrogenic effects through their interaction with estrogen receptors α (ER α) and ER β (6-50). Estrogen receptors belong to the nuclear receptor superfamily of transcription factors (7). Their activation elicits opposing effects on cancer growth and progression. Specifically, the expression of ER β is often reduced in various cancer cells (8). According to De Giorgi and colleagues, ER β expression counteracts the proliferative effects mediated by ER α in the skin (9,10). It is well established that expression of ER α is associated with abnormal proliferation, inflammation, tumorigenesis and the development of malignancy (7,8,11). These findings indicate that the effects of estrogens on cancer growth may depend on the relative ratio of ER α /ER β expression within a given

tumour cell or tissue (12). Estrogen receptors (ERs) can translocate from the cytoplasm to the nucleus, where they bind to transcriptional control regions of DNA or interact with small RNAs, subsequently inducing the expression of specific genes. Consequently, phytoestrogens have the potential to regulate estrogen-mediated processes, including the induction of sex hormones (13). High dietary phytoestrogen exposure can interfere with measurements in studies involving estrogenic activity, potentially affecting the interpretation of animal model experiments (14). Major natural dietary sources of phytoestrogens include soybeans, wheat, potatoes, rice, alfalfa, and oats (15-49). These compounds can bind to estrogen receptors, eliciting effects in animals, humans, and cultured cells. Consequently, studies of hormone-dependent or hormone-modulated conditions, such as animal models of cancer and investigations into steroid hormones like estrogen may be significantly compromised by the presence of high levels of dietary phytoestrogen (4). Several studies have reported significant differences in experimental outcomes when comparing diets with high phytoestrogen content to those with very low phytoestrogen levels (2,3). Phytoestrogens, particularly isoflavones, are recognised as endocrine disruptors with significant pathophysiological impacts. The Environmental Protection Agency (EPA) defines endocrine disruptors as substances that alter the structure and function of the endocrine system, leading to adverse effects (16). These disruptions may be attributed to the estrogenic activity, nutrient composition, and metabolizable energy of phytoestrogen-rich diets (17-19). In animal models, flavones have been shown to disrupt lactation, alter the timing of puberty, impair the ability to produce viable and fertile offspring, influence sex specific behaviours, accelerate reproductive ageing and compromise fertility (16). The role of

phytoestrogens in malignancy have been examined in a range of clinical and experimental studies. For example, in a recent large prospective cohort study, increased intake of total isoflavones, daidzein, glycitein, and formononetin was found to be associated with a reduced risk of pancreatic cancer among all participants and ever smokers (20). However, in ovarian cancer, associations between intake of phytoestrogens and cancer risk showed no major aetiological role (21). In a study of genistein supplementation on genome-wide DNA methylation and gene expression in patients with localised prostate cancer there were global gene expression changes and this had effects on molecular pathways involved in prostate tumorigenesis which included developmental pathways, markers of stem cells and proliferation and transcriptional regulation. The authors identified a reduction in MYC activity and a concomitant increase in PTEN activity (22). In a breast cancer soy supplementation study, cancer-related genes and pathways were examined and high plasma genistein identified a gene-signature with overexpression of FGFR2 and cell cycle progression and proliferation genes. Therefore, for a subset of women, soy may negatively affect gene expression in breast cancer (23). Although melanoma is traditionally considered a non-hormone-related cancer, growing evidence suggests a direct association between sex hormones, particularly estrogens, and melanoma progression (24). T-regulatory cells (Tregs) are recognised as significant barriers to effective anti-tumour immune responses, contributing to the development of an immunosuppressive tumour microenvironment (TME) (25,26). Tregs have been extensively studied in the peripheral blood and immune infiltrates of various cancers, with their accumulation strongly linked to poor prognosis in melanoma, breast, and colon cancers (27-30). Intracellular metabolism plays a critical role in

determining cell activity and function. Recent studies indicate that the metabolic and functional state of Tregs is shaped by local environmental conditions and the availability of specific metabolites. These metabolites, present in both the peripheral circulation and the TME, profoundly influence Treg differentiation, and phenotype stability (25). Tregs are classically characterised as CD4⁺CD25⁺ lymphocytes expressing the forkhead/winged helix family transcription factor FOXP3. In this study, we investigated the role of dietary phytoestrogens on B16-F10 melanoma tumour growth in C57BL/6 mice, examined the expression of tumour derived ERs, and assessed the frequency of peripheral treg populations.

Patients and Methods

Cell culture: Pigmented murine melanoma cells (B16-F10; kindly provided by Professor Steven Todryk, University of Northumbria, UK) were maintained DMEM/F-12 medium supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin). To eliminate potential estrogen effects, the cells were cultured in phenol-free medium (31). Once the cells reached approximately 70% confluence, their viability (>90%) was assessed using trypan blue exclusion, and viable cells were counted with a haemocytometer.

Animals: The experiments were conducted using specific pathogen-free C57BL/6 black (6–8 weeks old) mice obtained from Jackson Laboratory 600 Main Street Bar Harbor, ME USA 04609. They were bred in the Preclinical Research Facility (PRF) at the University of Leicester and used with authority from the Home Office under the supervisor's project license P43308E3B, with approval from the institutional animal welfare and ethical review board on 11 July 2016. The mice were housed at 25°C under a 12-hour light/dark cycle and provided ad libitum access to food and water. Mice were divided randomly in two groups and fed either a

chow diet rich in phytoestrogen (5LF2; Test Diet® product, 14.3% protein, 5.8% fat, 65% carbohydrate, up to 20% Soybean meal) or a low estrogenic control diet (58R1; Test Diet® product, 14.8% protein, 4.8% fat, 73.9% carbohydrate, 0% Soybean meal) for 8 weeks, with sex and age matched between groups. Mice were then inoculated subcutaneously into the right flank with 5×10^5 B16-F10 murine melanoma cells suspended in 100 µl of PBS. Tumours were allowed to establish, and their size was measured daily using calipers until the endpoint, typically 10–14 days post-injection. Tumour volume was calculated using the formula $V = \pi/6 \times \text{length} \times \text{width}^2$. Tumour weight was measured using an analytical balance, and all weights are reported in milligrams (mg). At the conclusion of the experiment, all mice were sacrificed, and tumours and spleens were collected for analysis.

RNA extraction and quantitative real-time (RT-PCR): Total RNA was extracted from melanoma tumours using TRIzol reagent (Sigma-Aldrich, UK). Genomic DNA contamination was removed using an RNase-free DNase kit (Sigma-Aldrich). A total of 3 µg of RNA was retro-transcribed into cDNA following the manufacturer's instructions (Thermo Scientific). Gene-specific amplification was carried out using the SensiMix SYBR kit (Bioline Reagents Ltd., London) and analysed on a Corbett Rotor-Gene TM6000 machine to measure the expression of Estrogen Receptor α (ER α) and Estrogen Receptor β (ER β). The $\Delta\Delta\text{CT}$ method (Livak & Schmittgen, 2001) was used for relative quantification. Samples were analysed in triplicate, and GAPDH was used as the housekeeping gene. The primer sequences used were as follows: for Estrogen Receptor β (ER β), forward 5'-CAGTAACAAGGGCATGGAAC-3' and reverse 5'-GTACATGTCCCACTTCTGACA-3'; for GAPDH, forward 5'-

CCCTTAAGAGGGATGCTGCC-3' and reverse 5'-TACGGCCAAATCCGTTTACA-3'; and for Estrogen Receptor α (ER α), forward 5'-GACCAGATGGTCAGTGCCTT-3' and reverse 5'-ACTCGAGAAGGTGGACCTGA-3'.

Flow cytometry analysis: To detect T-regulatory cells, freshly isolated splenocytes (1×10^6 cells/100 μ l FACS buffer) from tumour-bearing mice fed the respective diets were pre-blocked with an Fc receptor-specific anti-mouse CD16/32 antibody (BioLegend) for 30 minutes on ice. Following the blocking step, cells were stained with PE and APC-conjugated antibodies targeting CD4, CD25, and FOXP3 (mouse, 130-094-165) for 30 minutes on ice in the dark. After staining, the cells were washed and re-suspended in 400 μ l PBS supplemented with 3% (v/v) FCS. The stained cells were then transferred into polypropylene tubes for flow cytometry analysis. Spectral overlap of fluorochromes was compensated where necessary, and flow cytometry data were acquired using BD FACS Diva™ software version 8.0. Splenocytes from three tumour-bearing mice per dietary group were used.

Statistical analysis

The data were expressed as the mean $\pm$ SD for bar charts. Box-and-whisker plots display the distribution of the data, with the box representing the interquartile range (IQR) (25th to 75th percentile) and the line inside the box indicating the median. Whiskers extend to the minimum and maximum values, showing the full data range. Individual values are also shown. Analysis was performed using GraphPad Prism 10 (GraphPad, San Diego, California, USA). Tests for normality were conducted by inspecting QQ-plots and employing the Shapiro-Wilk test to confirm the assumption of a normal distribution. Statistical significance was determined using Unpaired t-tests (or the non-parametric equivalent) and indicated by exact p-values where alpha was set

to < 0.05 . Effect sizes were calculated using η^2 (eta squared) to quantify the proportion of variance explained by group differences. For parametric tests, η^2 was derived from the t-statistic, while for non-parametric tests, it was calculated using the Z-score (Mann-Whitney U/Wilcoxon). For qPCR analyses, the results were presented as the fold change in gene expression normalised to the housekeeping gene.

Results

A phytoestrogen rich diet enhances melanoma tumour weight and size: To investigate the impact of any difference in tumour weight of tumour bearing mice fed on chow diet rich in phytoestrogen (5LF2) or control diet low in estrogen (58R1), C57BL/6 mice were inoculated subcutaneously into the right flank with 5×10^5 B16-F10 murine melanoma cells. The results demonstrated statistically significant differences in tumour weight between the two diet groups (Figure 1).

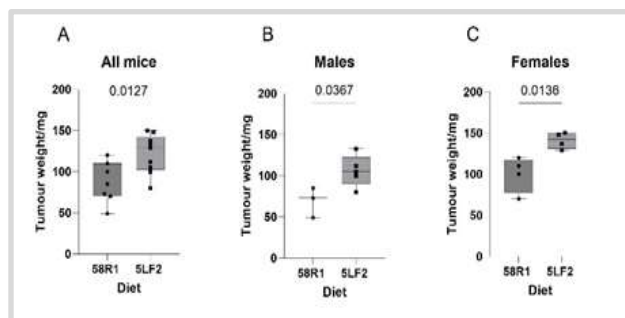


Figure 1. Melanoma tumour weight in C57BL/6 mice after administration of estrogenic or control diets. The effect of estrogenic (5LF2) and control diets (58R1) on tumour weight in melanoma-bearing mice. (A) The tumour weight in all mice following an estrogen-rich or poor diet (B) The tumour weight in males following an estrogen-rich or poor diet. (C) The tumour weight in females following an estrogen-rich or poor diet. The data are presented as box plots, including individual data points, the median, and the quartiles. Statistical significance was assessed using an unpaired t-test, with exact p values provided. * $p < 0.05$.

In the all-mice group, the 5LF2 diet resulted in a mean tumour weight 34.84 grams higher than the 58R1 diet ($P = 0.0127$), with a moderate effect size (Figure 1A, $R^2 = 0.3680$). In males, the mean

difference was 37.00 grams ($P = 0.0367$), with a larger effect size (Figure 1B, $R^2 = 0.5442$), while in females, the mean difference was 41.00 grams ($P = 0.0136$), showing the largest effect size (Figure 1C, $R^2 = 0.6655$). In all groups, the unpaired t-tests yielded significant P values ($P < 0.05$), indicating that the 5LF2 diet had a notable impact on tumour weight. These findings suggest that the 5LF2 diet significantly influenced tumour weight, with the most pronounced effect observed in female mice. The analysis of tumour size across all mice, males, and females fed either a phytoestrogen-rich 5LF2 diet or a control 58R1 diet revealed significant differences in tumour size between the two diet groups Figure 2. For all mice, the 5LF2 diet resulted in a mean tumour size 48.56 mm³ larger than the 58R1 diet ($P = 0.0258$), with a moderate effect size (Figure 2C, $R^2 = 0.3074$). In males, the 5LF2 diet caused a mean tumour size increase of 61.60 mm³ ($P = 0.0159$), showing a large effect size (Figure 2B, $R^2 = 0.6485$). In females, the 5LF2 diet led to a 50.75 mm³ larger tumour size ($P = 0.0238$), with a strong effect size (Figure 2C, $R^2 = 0.6010$). The unpaired t-tests for all groups showed significant differences ($P < 0.05$), indicating that the 5LF2 diet significantly influenced tumour size. These results suggest that the phytoestrogen-rich 5LF2 diet significantly impacted tumour size across all groups, with the most pronounced effect in males, followed closely by females.

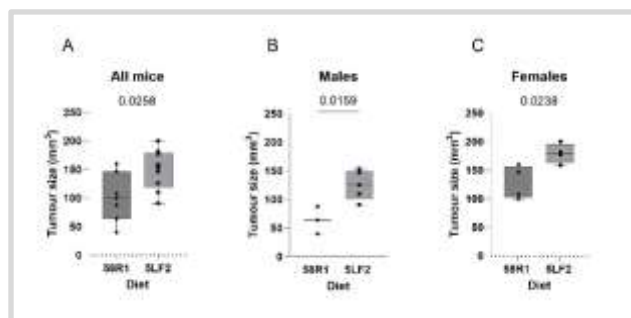


Figure 2. Effect of consuming a phytoestrogenic diet on tumour size in C57BL/6 mice. The effect of estrogenic (5LF2) and control diets (58R1) on tumour size (mm³) in melanoma-bearing mice. (A) The tumour size (mm³) in all mice following an estrogen-rich or poor diet (B) The tumour size (mm³) in males following an estrogen-rich or

poor diet. (C) The tumour size (mm³) in females following an estrogen-rich or poor diet. The data are presented as box plots, including individual data points, the median, and the quartiles. Statistical significance was assessed using an unpaired t-test, with exact p values provided. * $p < 0.05$.

A phytoestrogen diet is associated with upregulated estrogen receptor β in B16-F10 tumours:

As a transcription factor, ER β regulates the transcription of various genes, which binds to estrogen response elements (ERE) upstream of the target genes (Hayashi et al., 2003). Therefore, we investigated the effect of a diet rich in phytoestrogen on the expression of ER β in mice bearing melanoma tumours. To investigate the effect of a high phytoestrogen diet (5LF2) and control low phytoestrogen diet (58R1) on ER β mRNA levels, melanoma tumours were isolated, and fold change quantified using qPCR. The comparison of fold change in mRNA expression of ER β between the 5LF2 and 58R1 diets showed significant differences in both male (Figure 3B) and female mice (Figure 3C), but no significant difference in the overall group (Figure 3A). In male mice, the 5LF2 diet significantly increased ER β expression compared to the 58R1 diet ($P = 0.0155$), with a large effect size ($R^2 = 0.9692$). A similar significant increase was observed in female mice ($P = 0.0188$, $R^2 = 0.9627$). However, in the combined data for all mice, no significant difference was found ($P = 0.1828$), likely due to higher variability in this group. Overall, the 5LF2 diet had significantly upregulated tumour ER β mRNA expression, in males and females individually with ER β expression higher in females than males for both diets.

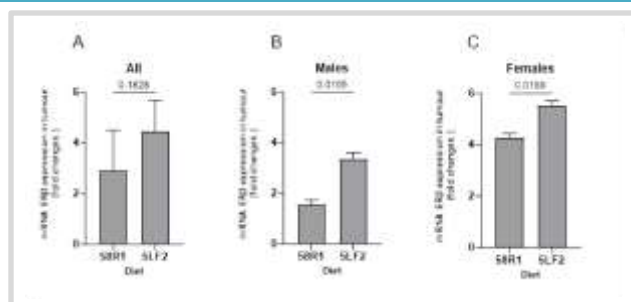


Figure 3. Effect of estrogenic (5LF2) and control (58R1) diets on ER β mRNA expression in B16-F10 tumours from melanoma-bearing mice (All n=4, Females n=2, Males n=2). The study examined the impact of estrogenic (5LF2) and control (58R1) diets on ER β mRNA expression in B16-F10 tumours from melanoma-bearing mice. Fold changes in ER β mRNA expression were assessed using the $\Delta\Delta CT$ method, with normalisation to GAPDH. Results showed differences in tumour ER β expression across (A) all mice, as well as in (B) male and (C) female subgroups, following either an estrogen-rich or estrogen-poor diet. Data are presented as mean $\pm$ SD (n = 3). Statistical significance was determined using an unpaired t-test, with exact p-values reported (*p < 0.05).

Consumption of a phytoestrogenic diet significantly increases splenic Tregs in tumour bearing mice: Splenic Tregs (CD4+CD25+ cells as a percentage of CD4 T cells) were next examined using flow cytometry across all mice, male, and female animals fed either the phytoestrogen-rich 5LF2 diet or the control 58R1 diet and showed significant differences between the two diet groups (Figure 4, Supplementary Figure 1). For all mice, the 5LF2 diet resulted in an increase of 1.743% in the Treg percentage compared to the 58R1 diet (P = 0.0397), with a moderate effect size ($R^2 = 0.2687$). In males, the 5LF2 diet caused a 2.080% increase in the Treg percentage (P = 0.0441), reflecting a moderate effect size ($R^2 = 0.5180$). In females, the 5LF2 diet led to a 1.950% increase in Treg percentage (P = 0.0453), with a moderate effect size ($R^2 = 0.5140$). These significant differences indicate that the 5LF2 diet significantly influenced the percentage of splenic Tregs. These results suggest that the 5LF2 diet has a significant effect on the percentage of Tregs across all groups, with

moderate effect sizes observed in both male and female mice.

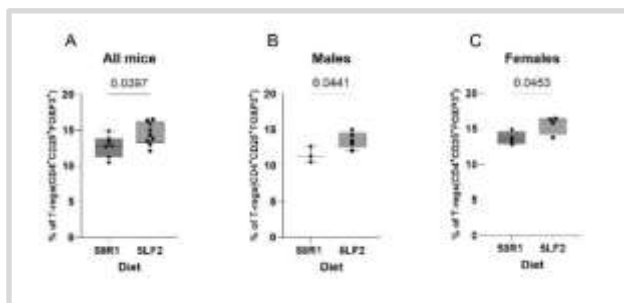


Figure 4. Flow cytometry analysis of Treg cell populations in splenocytes of tumour bearing mice fed on estrogen rich (5LF2) and poor (58R1) diets. The effect of estrogenic (5LF2) and control diets (58R1) on Treg frequencies in melanoma-bearing mice. (A) Splenic CD4+CD25+Foxp3+ cells in all mice following an estrogen-rich or poor diet (B) Splenic CD4+CD25+Foxp3+ cells in males following an estrogen-rich or poor diet. (C) Splenic CD4+CD25+Foxp3+ cells in females following an estrogen-rich or poor diet. The data are presented as box plots, including individual data points, the median, and the quartiles. Statistical significance was assessed using an unpaired t-test, with exact p values provided. *p < 0.05.

A phytoestrogen diet is associated with downregulated estrogen receptor α in B16-F10 tumours: We next investigated the effect of a diet rich in phytoestrogen on the expression of ER α in mice bearing melanoma tumours (Figure 5). The comparison of fold change in mRNA expression of ER α between the 5LF2 and 58R1 diets showed significant differences in male and female mice, but no significant difference in the overall group. In male mice (Figure 5B), the 5LF2 diet significantly decreased ER α expression compared to the 58R1 diet (P = 0.0203), with a large effect size ($R^2 = 0.9599$). Similarly, in female mice (Figure 5C), a significant decrease was observed (P = 0.0105, $R^2 = 0.9792$). However, in the combined data for all mice, no significant difference was found (P = 0.0788), likely due to higher variability in this group due to sex specific ER α levels. Overall, the high phytoestrogen 5LF2 diet resulted in significant downregulation of ER α mRNA expression in both male and female groups.

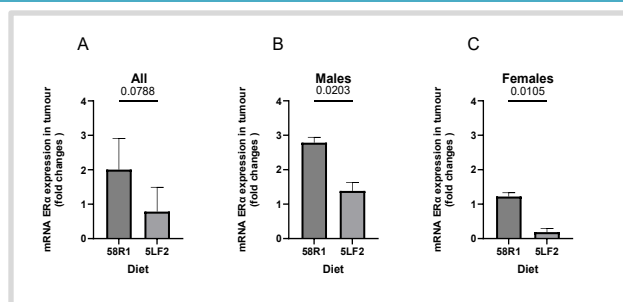


Figure 5. Effect of estrogenic (5LF2) and control (58R1) diets on ER α mRNA expression in B16-F10 tumours from melanoma-bearing mice (All n=4, Females n=2, Males n=2). The study examined the impact of estrogenic (5LF2) and control (58R1) diets on ER α mRNA expression in B16-F10 tumours from melanoma-bearing mice. Fold changes in ER α mRNA expression were assessed using the $\Delta\Delta\text{CT}$ method, with normalisation to GAPDH. Results showed differences in tumour ER α expression across (A) all mice, as well as in (B) male and (C) female subgroups, following either an estrogen-rich or estrogen-poor diet. Data are presented as mean $\pm$ SD (n = 3). Statistical significance was determined using an unpaired t-test, with exact p-values reported (*p < 0.05).

Discussion

The aim of this study was to investigate the impact of phytoestrogen-rich diets on melanoma tumour growth. We show that B16-F10 tumours were larger in size and unregulated ER β mRNA and down regulated ER α . In addition, splenic Tregs isolated from tumour-bearing mice showed an increased frequency in the CD4 $^{+}$ T cell population. This agrees with previous studies that suggest animal diets containing phytoestrogens can significantly influence the outcomes of tumour studies and hormonal cellular endpoints. Therefore, diet selection is critically important, and can directly affect experimental results (3). Despite studies which indicate the anti-angiogenic and anti-cancer effects of consuming a diet rich in phytoestrogen, there is ongoing concern about the potential risks associated with consuming high levels of these compounds (32,33). Commercial rodent diets formulated with soy as a protein source are typically provided to animals daily, resulting in the

consumption of large doses of phytoestrogens, particularly isoflavones (34). These results in a sustained high serum concentration of isoflavones compared to animals fed on free or low soy diet (4). Thigpen and colleagues (2004) found that dietary isoflavones can affect the reproductive, skeletal, and cardiovascular systems (3). As a result, this may influence and alter the outcomes of experiments focused on comparative estrogenicity, endocrine disruption and carcinogenicity. Sex-related factors are intriguing aspects of melanoma tumour growth. Premenopausal women developed melanoma tumours more slowly than men and experience better survival rates, potentially due to the influence of sex hormone levels and the expression of estrogen receptors (35). These observations support the role of sex hormones in melanoma development and progression (11). However, our measurements of tumour weight revealed that mice fed a high estrogenic diet (5LF2) had larger tumours compared to those fed on the 58R1 diet. This aligns with previous work showing that B16 tumours grow more rapidly in female C57BL/6 mice than in males. They also demonstrate that sex and estrogen receptors signalling mechanisms may impact tumour development and immune cell infiltration (36). Our results align with a study reporting an increase in the incidence of vulvar carcinomas in female 129/J mice fed soy protein containing daidzein and genistein for three months, compared to other groups fed phytoestrogen-free diets (3). Female athymic nude mice fed dietary phytoestrogens across a wide concentration range (125–1,000 μg) exhibited increased tumour size, comparable to the estradiol control group. Long-term exposure to dietary soy isoflavones significantly enhances proliferation of estrogen-dependent tumours and increased total plasma genistein concentrations (37). Similarly, soy-derived isoflavones, with genistein as a key component, stimulated tumour

progression and prevented tumour regression in a mammary cancer model, resulting in significantly larger tumours compared to controls after three months of feeding (38). In contrast, our findings do not agree with work that showed dietary supplementation with isoflavones resulted in the development of smaller tumours in a dose-dependent manner (2.5-20% soybean protein) in an experimental metastasis model (39). This discrepancy may be attributed to the lower number of B16-F10 cells for tumour implantation (0.5×10^5), which was nearly 10 times fewer than the numbers used in our study (39). Additionally, their study employed a mouse melanoma B16 cell line which is different from the B16-F10 line and used lower doses of soybean protein in their diets compared to those used in this study (up to 20%). Importantly their study used an intravenous injection model which contrasts with the subcutaneous injection model used in this study. Another study supporting a protective role of phytoestrogens showed that the administration of 15 mg/kg of a soybean-based diet for five days reduced tumour-induced angiogenesis in syngeneic 6-8-week-old female C57BL/6 mice intraperitoneally injected with 1×10^5 B16-F10 cells (40). However, the study employed the less aggressive parental B16-F0 cell line, (31). Furthermore, the previous study injected 1×10^5 B16-F10 cells intraperitoneally, five times fewer cells than used in our study, and employed a different injection site. Collectively, these differences suggest that the effect of dietary phytoestrogens may depend on factors such as the route of injection, duration of exposure, soybean diet dosage, number of cells used, and the specific animal model. Our investigation revealed that mice fed a high-estrogenic diet (5LF2) exhibited a significantly higher proportion of splenic Tregs compared to mice on a low-estrogenic diet (58R1). This result concurs with a previous study demonstrating that estrogen (17- β -estradiol, E2) administered at

physiological doses enhances Treg expansion and upregulates Foxp3 and IL-10 expression in multiple tissues of immunocompetent ovariectomized female mouse models (41). Additionally, others have reported that increased estrogen (17- β -estradiol, E2) levels stimulate Foxp3 expression in both naïve and syngeneic pregnant female C57BL/6 mice. This finding is particularly significant given the accumulation of FoxP3+ Tregs in tumours is a well-established predictor of poor prognosis in various cancers (26,42,43). Our results revealed significantly higher ER β mRNA expression in tumours from mice fed a high-estrogenic diet (5LF2) compared to a low-estrogenic diet (58R1), with females exhibiting higher expression than males fed on the same diets. Similar findings were obtained by de Giorgi et al. (2009) that reported higher ER β mRNA levels in primary compared with metastatic melanomas (9,10). Additionally, immunohistochemistry has confirmed the presence of ER β protein, but not ER α , in human malignant melanoma cells (45). ER β is thought to play a protective role in tumour suppression by reducing uncontrolled proliferation and enhancing apoptotic activity, with its activation shown to inhibit cutaneous melanoma cell growth (8,46). Conversely, ER α mRNA expression was lower in tumours of mice fed a phytoestrogen-rich diet compared to controls, and expression was also lower in males compared to females. This finding contrasts with studies suggesting that ER α promotes the proliferation of various cancerous cells (47). The findings from our study are consistent with existing literature, which suggests that mouse melanoma tumours express both estrogen receptors (ER α and ER β). Specifically, the interaction of phytoestrogens, which are chemically similar to estrogen, in the diet appears to stimulate a decrease in ER α expression, potentially promoting cell proliferation and enhancing tumour progression.

This is accompanied by an increase in the splenic Treg population. In contrast, the expression of ER β may counteract tumour growth mechanisms. This could occur through the activation of ER β or by disrupting the activity of ER α , potentially through the formation of ER α /ER β heterodimers. Such interactions may exert an anti-proliferative effect, leading to reduced tumour growth and a lower percentage of splenic T-regs. This study adopts a well-established murine melanoma model, with dietary manipulation to simulate phytoestrogen exposure and the quantification of immune cell subsets. We incorporate efforts to adhere to the 3Rs (Replacement, Reduction, and Refinement) by using the minimum number of animals necessary to achieve meaningful results, and statistical tests appropriate for small sample sizes were applied. However, there are several limitations and although the all-mouse group included larger numbers (>6), stratified sex-based analysis involved a smaller sample size per group. Therefore, the stratified analysis should be interpreted more cautiously. Additionally, the analysis of ER expression examined only mRNA expression and not protein expression and therefore expression may not fully reflect functional ER activity. We also note the limited scope of immune cell profiling and the lack of long-term tumour monitoring.

Conclusions

Despite significant contradictions in findings regarding the effects of dietary phytoestrogens on tumour growth, there is growing clinical and preclinical evidence suggesting that phytoestrogen-rich diets in animal models may influence cancer research outcomes. Variations in dietary isoflavone levels, particularly in soy-based laboratory diets, have been identified as a key factor contributing to inconsistent results across studies.

Recommendations

This study highlights the need to consider animal

diets as an essential experimental variable that should be carefully controlled to ensure reproducible and reliable animal models. For certain experimental designs, an isoflavone-free or low diet may be required to prevent dietary interference with experimental outcomes. Therefore, selecting an appropriate diet is crucial for the validity and reliability of carcinogenicity studies.

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Ethical clearance: Approval of the programme of work was granted by the institutional Animal Welfare and Ethics Subcommittee (item AWERB/15/24) and by the Secretary of State of the UK Home Office (license P43308E3B) on 11 July 2016.

Conflict of interest: None.

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الغذاء لغري لهذا يتوسعون في د من حجم لورام في ادفيم عدال تخاللي ا تيل تنظيمي قدوارة في نموذج ورم هي النوم
الفاري B16F10

¹ فاه عدیجسین، ²لهوی تشاردماشادو

لہجہ خاص

التهبيّة تُظهِرُ الدُّرُوسُ السَّيْرِيَّةَ اِتِّبَاطًا وَثِيقًا بَيْنَ مَتَبَيِّاتِ الْمَرَمَنَاتِ، وَخُصَّةً الْعِتْرُوجِيْنَ، وَتَطَوُّرِ سَرَطَنَاتِ الْجُدِي. يُعْبَرُ الْوَرَمُ الْهَيْفِي (الْجُدِي) **Cutaneous melanoma** (مِنْ أَلْوَرَامِ الْمَتَبِّطِ قَبْلَ مَرَمَنَاتٍ؛ وَمِثْلُكَ الْفَزَالُ أَدْوَارُ هَلْ يَتَطَوَّرُ سَرَطَانُ الْجُدِي زَالِغٌ وَفِي وَضَحِ لِحْدِ الْبِنِ).

الأهداف: موال تحقيق من تأثير النظام الغذائي الغني بالدهن على مستويات ومنتجات في بدء وتطور الورم لدى الفئتين يجب ان تكون متماثلة في حيوان (syngeneic mouse model).

لوحى ولطرق: يتم غنيء افران املنظام غاى غنى يلفو بسواستروچجات اونظام غاى غنى فافض(لمرافة) وتم قن ملك حتالاج لبـ 5 × 105 خاليا مالانوما تهمكة (حجيا) B16-F10 (بعد 10-12 ليوغاتم جمع الورام ولاحظ الاتم في اس حجم ووزن الورام، كما اجرى بفاعل الطولوي مرزنتلى لسسل الكمي) qPCR (تحليل لتعير مسقبالت البتروجين) α ER و بت متقيد مال الى التلطيبة النخية Tregs (من الى الطرح الهاسه خدم فى اس التفقق الخلوى) flow cytometry).

النتائج: أظهرت لقعز ان التي ملت لعلقت للنظام الغظلي لا غيب لعلقت ولتروجينات أوراماً أكبر حجماً قارن قبلك لتتغذت غنى للنظام الغظلي المراقب. أدت الفئوس استروجين انتقي للنظام الغضائى لا عذى انتعير مسبق لالاستروجين ER β وضخنت عير ER α في أنسجة لورم كالم لوحظت زيادة لغير عفين سبة اللى الى المتطبة العظمية (Tregs في الطحال لدى افعزان ال حامله لالور الملتي استملكت النظام الغضائى الغنى بالفئوس استروجينات.

الاستنتاج يحصل ط مذهب الدراسات الضوء في اهي تغيير لم ين ات النظام الغنيمة في من موال ورام ولت جة العالم اعني قال من طب طب ووتأكد في لاجة الى مراعاة اقر ليعب النظام الغنيمة عي تضمن ليعت جارب لم ل هلمن ثلثي مضمحل في في لول لوي ال ورم.

للتلخيصات هتت اسي: السترو حن زلميني، ال لفي التي لتي في ال تبلي، أورا م ال لني و لم لذي في نله، ق ي اس التفق ال لفي.

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