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Review

Sulforaphane and Epigallocatechin Gallate Restore Estrogen Receptor Expression by Modulating Epigenetic Events in the Breast Cancer Cell Line MDA-MB-231: A Systematic Review and Meta-Analysis

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Epigenetics · Sulforaphane · Epigallocatechin gallate · Apoptosis · Breast cancer

Abstract
Background/Aims: Epigenetics refers to modifications in gene activity and expression without alteration at the DNA sequence. Environment and diet could influence gene expression. Diet modifications may be meaningful in preventing and treating chronic diseases, cancer included. Dietary bioactive compounds, such as polyphenols (e.g., curcumin, resveratrol, or epigallocatechin gallate [EGCG]) or isothiocyanate (e.g., sulforaphane [SFN]), can regulate histone acetylation. The aim of this systematic review and meta-analysis was to evaluate the effect of SFN and EGCG on breast cancer (BC) cells cultured in vitro. ***Methods:*** Due to the enormous variability observed in study protocols and the innumerable genes involved, only studies analyzing the number of apoptotic cells in the MDA-MB-231 cell line were evaluated. The effect size (ES) was computed as the ratio of means. ***Results:*** We identified 7 studies, 4 regarding the effect of 10 μM SFN on MDA-MB-231 cells (ES = 4.59, 95% confidence interval 4.05–5.20) and 3 focusing on the impact of 20 μM EGCG (ES = 2.84, 95% confidence interval 2.60–3.10). ***Conclusion:*** The findings suggest beneficial effects of dietary bioactive compounds such as SFN and EGCG and their effect on BC cells by restoring estrogen receptor gene expression, modulating epigenetic changes and events, and interfering with tumor growth rate. Publication bias limits the generalizability of the conclusions. High-quality studies are needed.

V. Gianfredi and S. Vannini contributed equally to this work.

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Introduction

Epigenetic mechanisms, such as chromatin remodeling, histone modifications, nucleosome repositioning, and direct/indirect modulation of gene expression by noncoding RNAs such as microRNAs [1], are of crucial importance in ensuring proper development and stability of tissue-specific gene expression patterns in mammals [2]. Epigenetic dysregulations can result in malignant cell transformation, providing a link between cellular adaptation to tumor microenvironment and plasticity [3].

Understanding epigenetic changes plays a central role not only in cancer etiopathogenesis, but also in cancer prevention and therapy. Modifications of specific sites of histones which are involved in the organization of the chromatin structure and in the regulation of gene transcription can induce changes in the transcription of various genes [4]. Inhibitors of histone deacetylases have been shown to reactivate the silenced tumor suppressor gene [5, 6].

Besides the biological makeup with its genetic/epigenetic dysregulations, lifestyles, such as nutrition, play a major role both in the etiopathogenesis and prevention of cancer, also influencing epigenetic events. According to the American Institute for Cancer Research (AICR) and the World Cancer Research Fund (WCRF), one-third of tumors may be attributable to nutrition [7], which means that a significant amount of tumor cases could be prevented through the adoption of a healthy diet [8]. Bioactive dietary compounds can potentially reactivate silenced genes or suppress oncogenes, restoring aberrant methylation patterns. Therefore, a new, exciting specialty termed "nutri-epigenetics," deriving from the intersection of nutritional science and epigenetics, is emerging [9, 10].

Despite important achievements and improvements in clinical outcomes, cancer still imposes a heavy epidemiological and societal burden [11]. The conventional treatment shows side effects on healthy cells and, therefore, identifying alternative methodologies is crucial. For example, with few or negligible adverse effects, dietary bioactive compounds show chemopreventive effects, especially at low doses and at physiological concentrations, repressing phase 1 detoxification enzymes and inducing phase 2 enzymes, favoring cell cycle arrest, inducing apoptosis, and inhibiting cellular proliferation [9, 10, 12]. In 2004, Hu et al. [13] proved, in a murine model, that oral administration of 50 μM sulforaphane (SFN), an isothiocyanate derived from glucosinolates, induces a 20-μM plasmatic peak of the compound which, due to allosteric inhibition of histone deacetylase, epigenetically determines an increase in histone acetylation at the level of gene promoters and, as such, the activation of a number of tumor suppressor genes, including p21. Song et al. [14], utilizing a bioinformatic approach, predicted the networks and pathways affected by the administration of epigallocatechin gallate (EGCG) in tumor cells. EGCG is one of the most commonly found and active catechins, usually present in green tea. Its administration could impact on different biological processes and events such as cell cycle, cellular assembly and organization, DNA replication, recombination and repair, and cell death and survival, among others.

The aim of this systematic review and meta-analysis was to evaluate the role of two dietary bioactive compounds, SFN and EGCG, focusing on breast cancer (BC), given its epidemiological and clinical relevance. Both incidence and related mortality have increased by 18% since 2008, and the annual global burden of BC is expected to reach 3.2 million new cases by 2050 [15]. As such, the effect of SFN and EGCG in restoring estrogen receptor (ER) expression modulating epigenetic events on BC cells cultured in vitro was assessed.

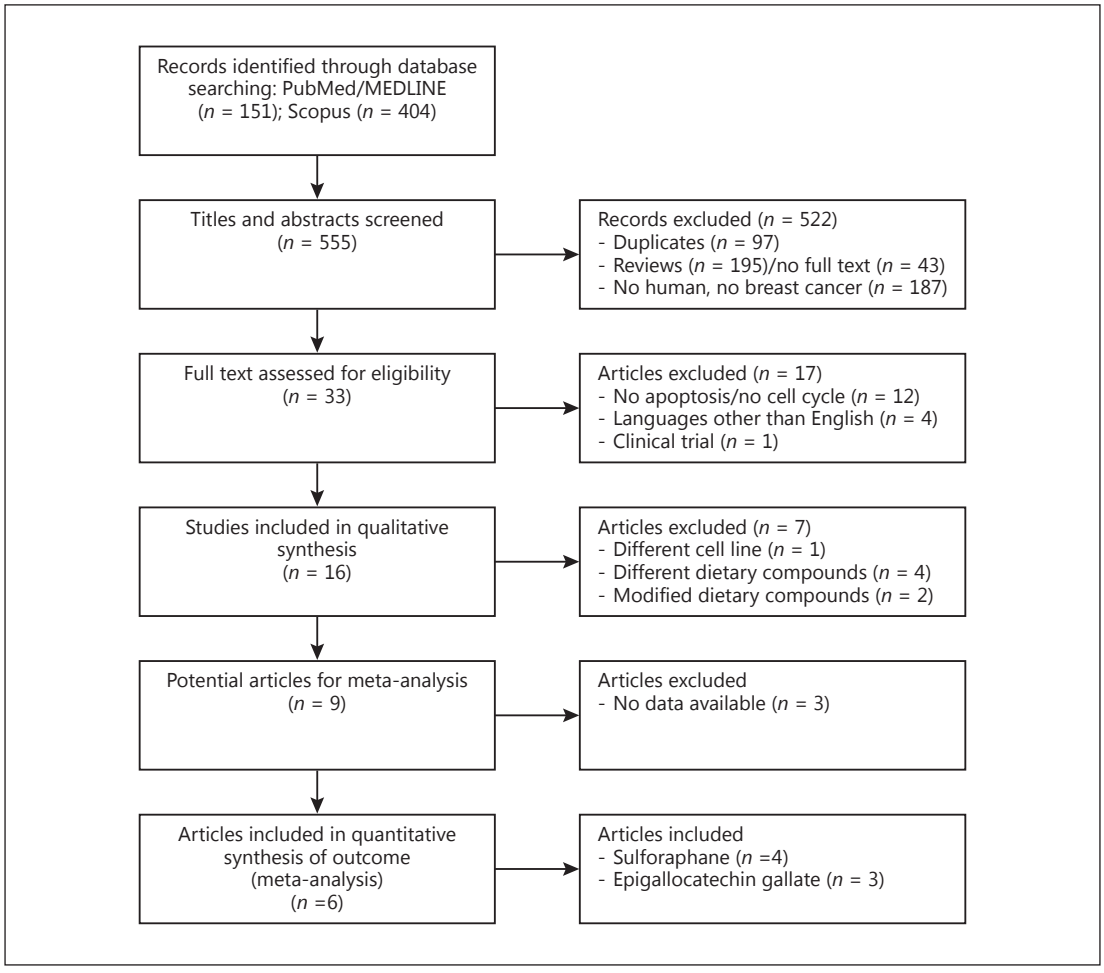


Fig. 1. Flowchart showing the study selection process.

Materials and Methods

Data Sources and Searches

This systematic review and meta-analysis was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [16].

On February 2, 2016, two researchers (V.G. and D.N.) independently searched from inception two major scholarly databases, PubMed/MEDLINE and Scopus, using ad hoc, pre-established search terms related to dietary bioactive compounds, BC, and epigenetics. Where appropriate, medical subject headings (MeSH) terms and wildcard options were used. More in details, the used string was: (dietary compound OR sulforaphane OR SFN OR epigallocatechin OR EGCG) AND (epigen* OR nutrigen* OR DNA methylation OR histone modification) AND (cancer prevention OR breast cancer OR cancer). The search was limited to articles published in English, and only full-text papers were taken into consideration for inclusion. To identify further potentially eligible studies, a manual check of the reference lists of each included study was carried out.

Study Selection

The study selection process is summarized in Figure 1. Due to the enormous variability observed in study protocols and the different roles played by the innumerable genes involved in cell cycle arrest and in the regulation of apoptosis, only studies analyzing the number of apoptotic cells in the MDA-MB-231 BC cell line were evaluated. The doses considered for inclusion of studies in this meta-analysis were selected on the

basis of physiological bioavailability evaluated after a common assumption based on 68 g of broccoli sprouts (approximately 105 mg SFN) and 3–4 cups of green tea (approximately 200 mg EGCG) [5, 17–19].

The MDA-MB-231 cell line are a mammary BC cell line derived from metastatic site with pleural effusion. The cell line is aneuploid female (modal number = 64, range = 52–68), with chromosome counts in the near-triploid range; normal chromosomes N8 and N15 are absent [20].

Initially, two researchers (V.G. and D.N.) independently screened the titles and abstracts of all papers in order to exclude studies that clearly did not meet the inclusion criteria. The full-text versions of all potentially relevant studies were screened independently by three researchers (V.G., D.N., and S.V.). Data extraction and quality assessment were carried on autonomously by three researchers (V.G., D.N., and N.L.B.). Disagreements were resolved through discussions until consensus was reached. If discrepancies still existed, the opinions of three other researchers (M.M., M.V., and A.I.) were sought for further discussion.

Articles were considered eligible if they met the following inclusion criteria: (1) evaluation of the biological effects of SFN or EGCG dietary bioactive compounds on human BC cells cultured in vitro; (2) epigenetic DNA modification induced by the considered bioactive compounds; (3) apoptosis induced by SFN or EGCG tested in vitro at least in triplicate; and (4) human BC cell line MDA-MB-231. Only papers providing a clear description of the study design and findings were considered in depth.

Exclusion criteria were: (1) full-length articles not written in English; (2) tests conducted in vivo; (3) abstracts, case reports, letters to the editor, editorials, commentaries, reviews without original data, studies with lack of control groups, or appropriate data for extraction; and (4) tests performed in cell lines different from MDA-MB-231.

Data Extraction

The data, extracted independently by two authors (V.G. and D.N.), were recorded on a standard spreadsheet. Extracted data included the main characteristic of each study (authors, year, country of publication, and study design), characteristics of the cells (cell line, gene expression), characteristics of treatment (bioactive dietary compound, dosage, duration), and the reported results (when numerical values of results were lacking, data were extrapolated from graphs). The secondary outcomes represented the effect(s) on cell cycle and/or the modification of gene expression.

Data Synthesis and Analysis

The traditional pairwise meta-analysis method was used to directly analyze the dietary bioactive compound-induced apoptosis. A fixed-effects model was applied. The effect size (ES) in the present meta-analysis was computed as the ratio of means (RoM) with its 95% confidence intervals (CIs) [21, 22]. The RoM is defined as the mean value in the exposed group (cells treated with dietary bioactive compound) divided by the mean value in the control group (untreated cells). The statistical heterogeneity among studies was assessed by the χ^2 test and I^2 statistics. A p value <0.05 was considered statistically significant.

The meta-analysis was carried out using the open-source Review Manager (RevMan 5.3) software (The Cochrane Collaboration, The Nordic Cochrane Centre, Copenhagen). Publication bias was also assessed when appropriate by visually inspecting funnel plots [23].

Results

Study Characteristics and Quality

The initial search strategy yielded 555 records (151 from PubMed/MEDLINE and 404 from Scopus). Out of the 555 items, 97 were duplicates and were therefore excluded. After the initial screening of titles and abstracts, a further 238 items (195 reviews and meta-analyses and 43 non-full-text papers: conference papers, articles unrelated to the topic of the current systematic review) were excluded. Furthermore, another 187 articles were excluded (no human cells and/or no BC cells). Thirty-three full-text articles were retrieved and assessed. Out of these 33 articles, 24 studies were excluded for the following reason: (i) articles not written in English (4 studies, 3 in Chinese and 1 in German); (ii) articles not focusing on cell cycle and/or apoptosis (12 studies); (iii) articles investigating chemically modified dietary compounds (2 studies); (iv) quantitative data insufficient or not available (3 studies); (v) cell

Table 1. Main characteristics of the four studies included in the meta-analysis related to the role of SFN and apoptosis induction in MDA-MB-231 breast cancer cells

Reference (first author), journal, country	SFN, μM	Treated	Control	OR (95% CI)	Cell line	Treatment duration
Meeran, 2010 [25], PLoS One, USA	10	3	3	5.91 (5.07–6.89)	MDA-MB-231	6 days
Meeran, 2012 [29], PLoS One, USA	10	3	3	6.90 (4.36–10.92)	MDA-MB-231 siRNA	72 h
Meeran, 2012 [29], PLoS One, USA	10	3	3	7.91 (5.39–11.60)	MDA-MB-231 ER siRNA	72 h
Deb, 2014 [26], Tumour Biol, India	10	3	3	19.80 (4.32–90.80)	MDA-MB-231	24 h
Lubecka-Pietruszewska, 2015 [24], J Nutrigenet Nutrigenomics, Poland	10	3	3	0.87 (0.64–1.19)	MDA-MB-231	96 h

CI, confidence interval; ER, estrogen receptor; OR, odds ratio; SFN, sulforaphane; siRNA, small interfering RNA.

line other than MDA-MB-231 (1 study); (vi) clinical trial (1 study); (vii) studies assessing folic acid (1 study) or curcumin (3 studies) as dietary compounds.

Finally, 6 papers were retained for the current meta-analysis. Since one of them analyzed two different MDA-MB-231 cell lines (ER+ and ER–), we considered it as two separate studies. In conclusion, 4 studies focused on SFN and 3 on EGCG.

SFN-Induced Apoptosis in MDA-MB-231 Cells

The data extracted from the studies are shown in Table 1. All studies were carried out within the last 6 years. Two studies were conducted in the USA, 1 in Poland, and 1 in India. Referring to apoptosis, the authors indicated an increase in apoptosis rate in 4 cases, while in 1 study this effect could not be observed. Two studies analyzed the standard MDA-MB-231 cell line [24, 25], while one study [26] analyzed MDA-MB-231 cells both with and without ERα expression. The concentration of SFN, tested in the included studies, was 10 μM.

The studies showed a high statistical heterogeneity (p for χ^2 <0.0001, I^2 = 97%). The forest plot in Figure 2a shows a RoM of 4.59 (95% CI 4.05–5.20). The asymmetrical funnel plot (Fig. 2b) shows some potential publication bias.

EGCG-Inducted Apoptosis in MDA-MB-231 Cells

The included studies are reported in Table 2. They were conducted between 2007 and 2012 [27–29]. Two studies were conducted in USA and one in the UK. In all cases the authors indicated an increase in apoptosis rate. The concentration of EGCG, which was tested in two of the included studies [27, 29] was 20 μM, whereas Moiseeva et al. [28] used 8 μM EGCG. The studies showed a high statistical heterogeneity (p for χ^2 <0.0001, I^2 = 98%). The forest plot in Figure 3a shows a RoM of 2.84 (95% CI 2.60–3.10) for MDA-MB-231 cells treated with 20 μM EGCG. The asymmetrical funnel plot (Fig. 3b) shows some potential publication bias.

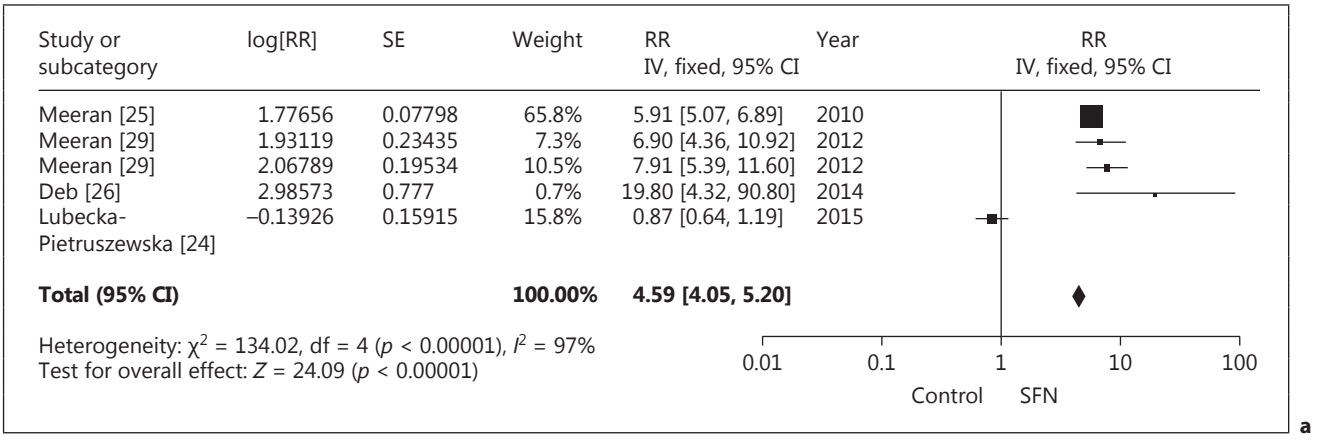
Epigenetic Changes

The epigenetic changes induced by SFN and EGCG are shown for each study in Table 3. The genes studied were phosphatase and tensin homologue (PTEN), retinoic acid receptor beta 2 (RARβ2), human telomerase reverse transcriptase (hTERT), ERα, caveolin 1 (CAV1), and interleukin 6 (IL6). After treatment, all genes but hTERT resulted upregulated. Different molecular mechanisms were involved: changes in histone marks, modulation of the transcription factors, and condensation of the chromatin structure, among others.

Table 2. Main characteristics of the three studies included in the meta-analysis related to the role of EGCG and apoptosis induction in MDA-MB-231 breast cancer cells

Reference (first author), journal, country	EGCG, μM	Treated	Control	OR (95% CI)	Cell line	Treatment duration
Moiseeva, 2007 [28], Mol Cancer Ther, UK	8	16	16	2.00 (1.80–2.22)	MDA-MB-231	30 h
Meeran, 2011 [27], Cancer Prev Res (Phila), USA	20	3	3	6.50 (4.89–8.62)	MDA-MB-231	9 days
Meeran, 2011 [27], Cancer Prev Res (Phila), USA	20	3	3	8.62 (6.42–11.57)	MDA-MB-231	12 days
Meeran, 2012 [29], PLoS One, USA	20	3	3	6.00 (3.78–9.52)	MDA-MB-231 siRNA	72 h
Meeran, 2012 [29], PLoS One, USA	20	3	3	7.13 (4.81–10.57)	MDA-MB-231 ER siRNA	72 h

CI, confidence interval; EGCG, epigallocatechin gallate; ER, estrogen receptor; OR, odds ratio; siRNA, small interfering RNA.



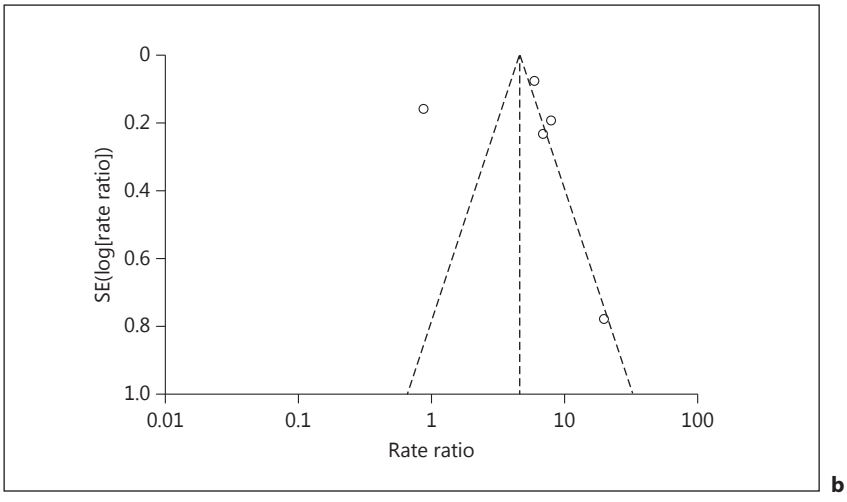
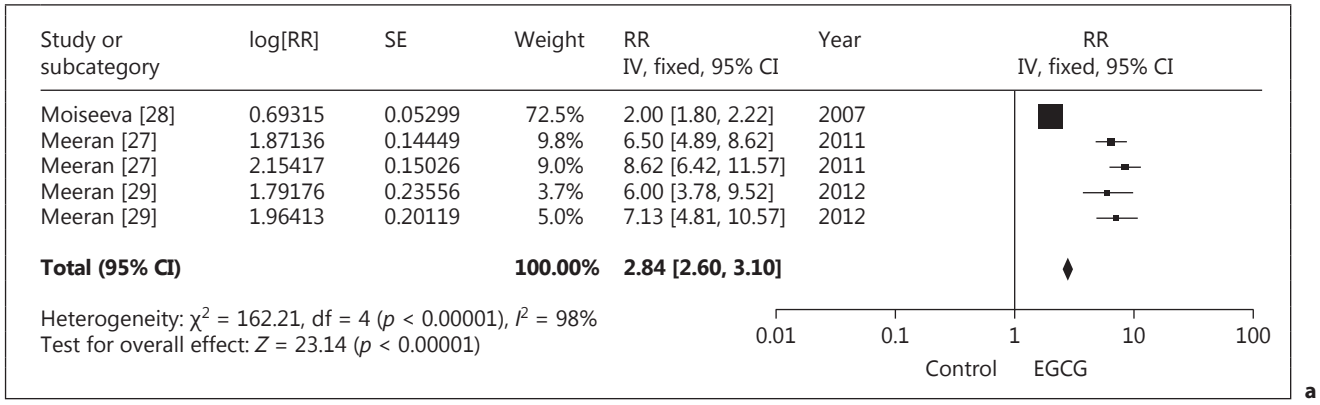


Fig. 2. Forest plot (**a**) and funnel plot (**b**) of the meta-analysis comparing the SFN-treated MDA-MB-231 cell line versus the untreated (control) MDA-MB-231 cell line in the induction of apoptosis (4 studies). CI, confidence interval; RR, rate ratio; SFN, sulforaphane.



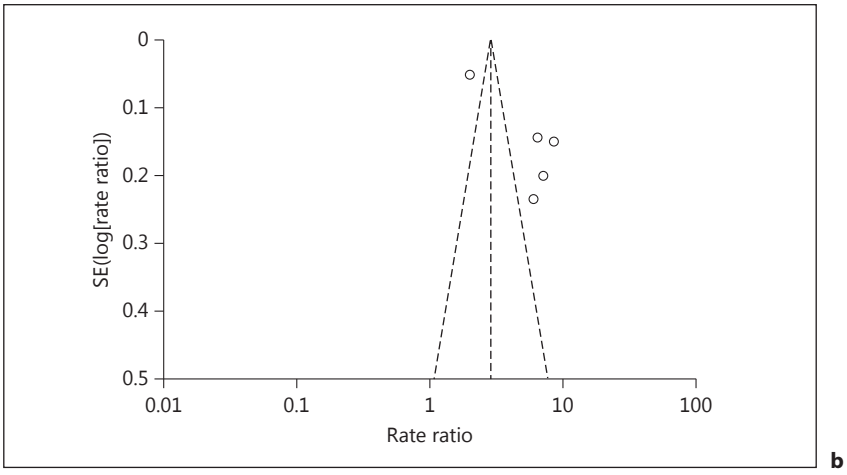


Fig. 3. Forest plot (**a**) and funnel plot (**b**) of the meta-analysis comparing the EGCG-treated MDA-MB-231 cell line versus the untreated (control) MDA-MB-231 cell line in the induction of apoptosis (3 studies). CI, confidence interval; EGCG, epigallocatechin gallate; RR, rate ratio.

Epigenetic Changes Induced by SFN

SFN has an impact on PTEN and RARβ2 expression, which finely modulate the methylation machinery and its core components (p21, p53, DNA methyltransferase 1 [DNMT1]), acting in a dose-dependent and in a dose-independent way, respectively. Moreover, methylation at the level of PTEN and RARβ2 promoters can confer an invasive potential to all cells. Even though PTEN and RARβ2 encode proteins potentially able to indirectly reduce the level of DNMT1 by downregulating the intracellular oncogenic mitogen-activated protein kinase/activator protein 1 signaling cascade, this hypothesized effect could not be detected in the MDA-MB-231 cell line. No DNMT1 suppression was noticed, while p21 was found to be upregulated through the p53-independent pathway [24].

Furthermore, SFN leads to reactivation of ERα, whose expression was found to be consistently correlated with ERα promoter hypomethylation and hyperacetylation [29]. Moreover, SFN repressed hTERT expression in a dose- and time-dependent way, with demethylation occurring mainly at the level of the first exon, facilitating the binding of the repressor CTCF and leading to a decrease in DNMT1 and DNMT3a [25]. Finally, SFN led to an upregulated expression of CAV1 [26].

Epigenetic Changes Induced by EGCG

EGCG did not prove to be a powerful epigenetic modulator, limited to increase IL6 expression [28]. Concerning hTERT, EGCG led to an increased binding of repressors such as MAD1 and E2F-1 and to a decreased binding of activators such as c-MYC [27].

Table 3. List of epigenetic modification induced by dietary bioactive compounds

Reference (first author)	Gene	Epigenetic modification post treatment	Gene expression	Test
Lubecka-Pietruszewska, 2015 [24]	PTEN	dose-dependent decrease in methylation by 22% at level of PTEN promoter	dose-dependent increase by 72% of mRNA PTEN	methylation-sensitive restriction analysis and quantitative real-time PCR
	RARβ2	dose-independent decrease in methylation by 25% at level of RARβ2 promoter	dose-independent increase by 95% of mRNA RARβ2	
Meeran, 2010 [25]	hTERT	dose- and time-dependent decrease in methylation by 50% at level of hTERT promoter in CTCF region	dose- and time-dependent downregulation of hTERT expression	real-time PCR and bisulfite sequencing analysis
Meeran, 2012 [29]	CpG islands of ERα	increase in acetylation of ac-H3, ac-H3K9, and ac-H4; increase in methylation by 54.02 ± 2.36% at level of promoter sites	reactivation in ERα-negative human breast cancer cells	real-time PCR, 5-methyl cytosine immunostaining and South-Western dot blot analysis for 5-methyl cytosine
Deb, 2014 [26]	CAV1	demethylation at level of CAV1 promoter	23.3-fold upregulation of CAV1 expression	real-time PCR and methylation-specific PCR
Moiseeva, 2007 [28]	IL6	–	three-fold IL6 expression increase	real-time PCR
Meeran, 2011 [27]	CpG islands of hTERT	dose-dependent decrease in methylation; time-dependent decrease in acetylation of ac-H3, ac-H3K9, and ac-H4	increased binding of repressor MAD1; decreased binding of c-MYC activator; increased binding of repressor E2F-1	real-time PCR, bisulfite sequencing analysis, and chromatin immunoprecipitation analysis

ac-H3, acetyl H3; ac-H3K9, acetyl H3 at lysine 9; ac-H4, acetyl H4; CAV1, caveolin 1; ER, estrogen receptor.

Discussion

The results of the present systematic review and meta-analysis show that SFN and EGCG cause induction of ER expression restoration in the MDA-MB-231 cell line, modulating epigenetic events and changes. These bioactive dietary compounds act as modifying specific histone sites and alter the expression of specific genes. Furthermore, they have tissue-specific effects. This further confirms that the induction of these effects may rely on mechanisms and epigenetic pathways specific for breast tissue.

SFN indeed epigenetically modulates PTEN and RARβ2, with a RoM of 4.59 (95% CI 4.05–5.20), whereas EGCG is a less powerful epigenetic modulator with a RoM of 2.84 (95% CI 2.60–3.10) in MDA-MB-231 cells. A potential research prospect could be the investigation of the consumption of these compounds.

Dietary bioactive compounds in co-exposure to anticancer drugs may have a synergistic effect [29]. This could demonstrate the importance of a targeted diet during chemotherapy as BC treatment. The combined effect of dietary bioactive compounds, and some selected anticancer drugs during a course of chemotherapy, may allow lower doses of antiblastics, potentially reducing their adverse events. Furthermore, some dietary bioactive compounds have also been considered as a starting point for the synthesis of new and more powerful anticancer molecules with smaller side effects.

However, even though our study was characterized by some strengths (such as a systematic search and a meta-synthesis), it is plagued by some shortcomings, which include, above all, the high statistical heterogeneity. This was found probably because of the different recruited sample size, the different apoptotic assays (e.g., caspase induction/activity, mitochondrial potential, DNA fragmentation, etc.), different study designs, and different ESs. Another weakness is given by the finding of publication bias, which hinders generalization of the results and calls for caution in their interpretation.

Conclusion

The results of the present systematic review and meta-analysis seem to suggest beneficial effects of dietary bioactive compounds such as SFN and EGCG and their role in BC cells by restoring ER gene expression, modulating epigenetic changes and events, and interfering with tumor growth rate. However, it is also clear that in this type of molecular biology studies, the use of a robust study design is essential to achieve more consistent and reproducible results between the various research groups. On the basis of the above-mentioned limitations, including the evidence of publication bias, further high-quality studies are needed to reproduce and confirm these results.

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Disclosure Statement

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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