Phytosterols and phytostanols and the hallmarks of cancer: a meta-analysis of pre-clinical animal models

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Abstract

Background and Purpose Phytosterols and phytostanols are natural products present in vegetable oils, nuts, and seeds, or added to consumer food products and intake is inversely associated with incidence and prognosis of several cancers. Randomised cancer prevention trials in humans are unfeasible due to time and cost yet the cellular processes and signalling cascades that underpin anti-cancer effects of these phytochemicals have been explored extensively in vitro and in preclinical in vivo models. Experimental Approach Here we have performed an original systematic review, meta-analysis, and qualitative interpretation of literature published up to June 2020. MEDLINE, Scopus, and hand-searching identified 408 unique records that were screen leading to 32 original articles that had investigated the effects of phytosterols or phytostanols on cancer biology in preclinical models. Data was extracted from 22 publications for meta-analysis. Key Results Phytosterols were most commonly studied and found to reduce primary and metastatic tumour burden in all cancer sites evaluated. Expression of pAKT, and markers of metastasis, angiogenesis, and proliferation were consistently reduced in breast and colorectal cancer. Very high dose treatment (not easily achievable through diet or supplementation in humans) was associated with adverse events including poor gut health and intestinal adenoma development. Conclusion and Implications Phytosterols and phytostanols are already clinically recommended for cardio-vascular disease risk reduction, and represent promising anti-cancer agents that could be delivered in clinic and to the general population at low cost, with a well understood safety profile, and now with a robust understanding of mechanism-of-action.

Introduction

Dietary intake of fruits and vegetables, and of grains and seeds, is inversely associated with cancer risk and cancer patient survival (WCRF/AICR, 2018). Research that provides mechanistic explanations for these epidemiological and clinical trial observations is incomplete, which limits translation for public health. One group of phytochemicals with purported anti-cancer activity are phytosterols and their saturated counterparts, phytostanols. Case-control studies have indicated high dietary phytosterols/stanols (PSS) intake has been associated with reduced odds of several cancers including lung (odds ratio (OR) 0.29, 95% CI 0.14-0.63) (Mendilaharsu, De Stefani, Deneo-Pellegrini, Carzoglio & Ronco, 1998), stomach (OR 0.33, 95% CI 0.17-0.65) (De Stefani et al., 2000), colorectum (OR 0.50, 95% CI 0·41-0·61) (Huang et al., 2017), and ovary (OR 0.42, 95% CI 0.20-0.87) (McCann, Freudenheim, Marshall & Graham, 2003). A recent meta-analysis indicated PSS intake imparts a non-linear reduction in pan-cancer relative risk (RR 0.63, 95% CI 0.49-0.81) with peak reduction achieved with approximately 0.5g/day or 6-7 mg kg^{-1} (Jiang et al., 2019).

Phytosterols, structurally and functionally related to cholesterol (Fig 1), are present in relatively large amounts in vegetable oils, nuts and seeds (Phillips *et al.* , 2005), with the total phytosterol content of some vegetable oils reaching values as high as 19 g kg^{-1} (Yang *et al.,* 2019). The richest sources are commercial

products supplemented with PSS (approx. 2-3 g PSS per portion) that are marketed for lowering LDL-C, and do so by 10-15 % in addition to what is achievable by statins (EFSA Panel on Dietetic Products, 2013; Han et al., 2016). Large scale manufacturing of PSS has standardised production methods which has ameliorated many problems commonly associated with studying natural products, where isolation, extraction, or synthesis methodology may vary, and final compounds applied in studies may be variable.

In 2000, Hanahan and Weinberg published 'The Hallmarks of Cancer' highlighting key characteristics of the developing tumour (Hanahan & Weinberg, 2000). Here, we use the updated categorization (Hanahan & Weinberg, 2011) to map how PSS may be interacting with cancerous and pre-cancerous cells in pre-clinical cancer models. This systematic review addresses a critical gap in the literature by collating all available information regarding the cellular and molecular response of tumours to PSS *in vivo* . Consistent evidence of a robust molecular mechanism (or lack thereof) would allow clinical research to build on the epidemiological evidence and begin evaluation of PSS as supplements that could contribute to reduced global cancer burden in the prevention setting, or as treatment adjuncts that could improve prognosis for cancer patients.

Methods

Search strategy

A comprehensive search of online databases MEDLINE and Scopus was carried out throughout June 2020. Search terms are available in supplementary materials. The intention to review was submitted to PROSPERO on 9th June 2020 and was approved and published on 11th June 2020 with reference number CRD42020191337 (Thorne, Cioccoloni, Wallis, Websdale, Soteriou & Zulyniak, 2020) and can be accessed at crd.york.ac.uk/PROSPERO.

Study selection

Titles and abstracts were screened for inclusion criteria; *i*) original data papers (e.g. reviews were excluded at this point); *ii*) conducted in whole organism animal models; *iii*) evaluated cancer (other diseases excluded);*iv*) phytosterol, phytostanol, a derivative/metabolite, or a mixture; *v*) English language publications; vi) not published in a predatory journal, listed on the website predatoryjournals.com. Screening was performed in duplicate by independent reviewers. Discrepancies were evaluated, discussed, and agreed by all members of the research team.

Data extraction

All data were extracted in duplicate into Microsoft Excel by independent contributors. Any disagreements were resolved by discussion with full research team. Extracted data included information and measures on animal models; study design; intervention and control treatments, duration, and dose; cancer type; and outcome assessment. Effect sizes were extracted as means with measures of variance. Where effect sizes were only presented in figures, WebPlotDigitizer (v4.2) was used to extract data (Rohatgi, 2019). Where more than 1 treatment group was compared to the same control, the effect size of the highest dose was extracted, and/or the parent PSS molecule was chosen rather than derivatives.

Statistical analysis

Meta-analyses was performed in RevMan version 5.3 (The Nordic Cochrane Centre, 2014). Heterogeneity was anticipated between studies due to variation in animal models and treatments, so random-effects models were used if [?] 3 studies were available. Where fewer than 3 studies were available, a fixed effects model was applied to preserve power and minimise risk of type-1 errors (Jackson & Turner, 2017). In analysis where effect sizes were calculated using different metrics that could not be harmonised, standardized mean difference

(SMD) was used (Borenstein, Hedges, Higgins & Rothstein, 2009). Effect sizes of SMD are interpreted as mean difference in units of standard deviation (versus control) following exposure to the intervention. Degree of heterogeneity of meta-analyses were quantified using I^2. We anticipated that meta-analyses of animal studies would reflect higher levels of heterogeneity than human clinical trials (Vesterinen et al., 2014) as clinical trials aim minimise inter-population variables, whereas animal studies generally aim to minimise *intra* -study variation through the use of inbred strains, strict protocol, and controlled environments. This in turn, makes the collective assessment of animal studies susceptible to high*inter* -study variation because each study has adapted its own protocol. We applied I^2>75% as a marker of high heterogeneity for meta-analysis of animal studies (Peter, Mtewa, Nagendrappa, Kaligirwa & Sesaazi, 2020). In meta-analyses with [?] 10 comparisons per outcome and I^2>75%, sources of heterogeneity were explored and discussed (Deeks, Higgins, Altman & Group., 2019; Peter, Mtewa, Nagendrappa, Kaligirwa & Sesaazi, 2020). Assessment of publication bias was performed by visual inspection where [?] 10 studies were assessed for a single outcome.

Risk of bias

Risk of bias (ROB) was performed for experimental design and adherence to BJP and PROPSERO guidelines for animal experiments (SF1A); adherence to BJP guidelines for natural products (SF1B); adherence to BJP guidelines for immunoblotting adapted to include immunohistochemistry (SF1C).

Results

Systematic Search

336 records identified in Scopus were combined with 281 from Medline and with 22 identified through other routes (e.g. preliminary literature reviews) resulting in 408 unique records after removal of duplicates. After screening for inclusion and exclusion criteria full text of 46 records was analysed. Thirty-two were found suitable for inclusion in qualitative synthesis (summarized in Table 1) and of these 23 were appropriate for data extraction and meta-analysis. This information is summarised in the PRISMA diagram (Fig 2).

Animal models

Mice or rats were used in all studies except for one that used zebrafish. Of the 31 studies using mammalian models, 18 employed xenograft assays, 10 induced tumours through mutagen, and three were spontaneous genetic cancer models. A total of 9 studies evaluated colorectal cancer (CRC), 9 on breast cancer (BCa), three on skin cancer/melanoma, two on lung (LCa), with gastric cancer (GCa), Ehrlich-Lettre ascites carcinoma (ECa), hepatoma (HEP), cholangiocarcinoma (CCA), ovarian cancer (OCa), pancreatic cancer (PaCa), renal cancer (RCa), and prostate cancer (PCa) each studied once. Metastasis was evaluated in three studies in the context of skin (two) and breast cancer (one).

PSS administration

The most commonly studied PSS was SITO (n=11) and its derivatives (n=7). STIG (n=4), FUCO (n=2) and PSS mixtures (n=6) were next most common, with PENI and ZGUG reported once each. CAMP, a relatively common PSS was only studied as part of PSS mixtures (n=5). Phytostanols were only assessed as mixtures. PSS were administered via three main routes; either integrated into chow per oral (PO) (n=15), oral gavage (OG) (n=5), or injection intravenously (IV) (n=1), or intraperitoneally (IP) (n=10) or as solution (n=1). The concentration of PSS to which animals were exposed varied by several orders of magnitude. Doses, normalised for a typical 65-75 kg human, ranged from the equivalent of 3mg per person per week up to 75 g per person per day.

Sustaining proliferative signalling

A range of growth factors and signalling pathways regulate the cell cycle machinery which are the effectors of proliferation. Typically, tumour proliferative index in humans can be measured by expression of cell cycle machinery proteins such as Ki67, PCNA, and CDKs. These proteins can be measured in tumour tissue by immunohistochemistry or immunoblotting, and tumour growth can be tracked non-invasively with callipers or measuring expression of light producing transgenes. Cells can also be isolated from tumours and flow sorted based on DNA content providing a measure of cell cycle kinetics in the tumour.

In our meta-analysis of 14 studies (16 comparisons; n=199) across PSS treatments we report that PSS mitigates tumour growth volume in breast (MD = -827.17 mm^3; 95% CI: -1297.26, -357.07; I2 = 100%; P<0.001), colon (MD = -1,298.56 mm^3; 95% CI: -2,156.76, -440.35; I2=96%; P=0.003), other cancers (MD = -864.21 mm^3; 95% CI: -1199.11, -215.36; I2=99%; P=0.005), and overall cancer (MD = -864.21 mm^3; 95% CI: -1137.55, -590.88; I2=99%; P<0.001), compared to controls (Fig 3A). Similarly, tumour mass was much smaller across PSS treatments in 11 studies (15 comparisons; n=222) reporting on breast (MD = -0.61 g; 95% CI: -0.93, -0.29; I2 = 93%; P<0.001), colon (MD = -3.40 g; 95% CI: -3.68, -3.12; I2=13%; P<0.001), other cancers (MD = -0.76 g; 95% CI: -1.10, -0.42; I2=97%; P<0.001), and overall cancer (MD = -1.18; 95% CI: -1.49, -0.87; I2=98%; P<0.001), compared to controls (Fig 3B). For overall total cancer mass (Fig 3A) and volume (Fig 3B), we observed very high heterogeneity (I^2>75%) which is likely attributed to differences in effect sizes between cancer models. No evidence of publication bias was observed in funnel plots for either analyses (data not shown). Tumour growth was also assessed in several studies via plasma markers CEA, CA125, and CA153. All markers were significantly reduced in PSS groups compared to controls (Fig 4A-C).

Deregulation of signalling pathways can lead to structural changes in epithelial cell organisation leading to formation of aberrant crypt foci (ACF), an early marker of CRC risk (Alrawi et al., 2006). SITO at a range of doses between 5 and 20 mg kg kg^{-1} per day (Baskar et al., 2010), and at 0.2% dw PO (Deschner et al., 1982) reduced colonic epithelial cell proliferation, ACF and crypt multiplicity, as well as tumour growth in xenograft CRC. In a DMBA mutagen model of skin cancer, STIG (0.2 – 0.4 g kg^{-1}PO) resulted in fewer and smaller skin papillomas, which were preceded by significantly longer latency period (Ali, Dixit, Ali, Alqahtani, Alkahtani & Alarifi, 2015). STIG (50 mg kg kg^{-1} IP) also slowed tumour cell doubling time in a melanoma xenograft model (Iyer & Patil, 2012). Yaccob and colleagues found a striking reduction in an NMU mutagen model breast cancer, where tumour number was reduced 10-fold suggesting that induced tumours actually regressed in the treatment group (Yaacob, Yankuzo, Devaraj, Wong & Lai, 2015).

Expression of cellular proliferation markers such as Ki67, PCNA, components of the cell cycle machinery, and proliferation promoting oncogenes were evaluated in several studies. When amalgamated we found a significant reduction in Ki67 (SMD = -11.74; 95% CI: —22.29, -1.20; p=0.03; Fig 3C) and PCNA (SMD = -4.02; 95% CI: -5.51, -2.53; p<0.0001; Fig 3D). Ki67 was evaluated by WB or IHC in 4 studies, investigating 20, 40, 60, 80, 100 mg kg^{-1}treatments. Ki67 and PCNA were significantly reduced across all studies. Interestingly, Ki67 was reduced by FUCO in LCa in a convincing dose-dependent manner (Mao et al., 2019) as was PCNA by PENI dose (15 mg or 30 mg kg^{-1} weekly) and frequency (15 mg kg^{-1} once/week or three/week) in CRC xenograft HCT116 (Couder-Garcia, Jacobo-Herrera, Zentella-Dehesa, Rocha-Zavaleta, Tavarez-Santamaria & Martinez-Vazquez, 2019). Aside from Ki67 and PCNA, Cyclin D1, a proliferation control protein, was reduced by 50% in the tumours of SITO treated KCa models (Sharmila & Sindhu, 2017b), but was unchanged in another study where very high PSS containing chow was provided to Apc^{min} mice (Marttinen et al., 2014) (Table 1).

A range of studies have considered tumour cell proliferation after exposing models to PSS mixtures, which are arguably more representative of typical human exposure. In physiological doses PSS mixtures exerted their inhibitory effects on tumour growth and cancer risk in different cancer types like cholangiocarcinoma and breast cancer (Kangsamaksin, Chaithongyot, Wootthichairangsan, Hanchaina, Tangshewinsirikul & Svasti, 2017) (Kazłowska, Lin, Chang & Tsai, 2013). In an MNU carcinogen induced model of CRC, mice were fed with different doses of PSS in feed (0.3%, 1% and 2% dw) of a PSS mixture (60% SITO, 30% CAMP, 5% STIG) preneoplastic lesion formation was reduced (Janezic & Rao, 1992). Ju et al evaluated exposure to

very high doses of a PSS mixture (9.8 g/kg SITO+ 0.2g/kg STIG) in chow and observed reduced tumour area in BCa xenograft mouse (Ju, Clausen, Allred, Almada & Helferich, 2004), and Yaacob who applied somewhat lower doses (40 mg kg^{-1}: 53% SITO, 16% CAMP, 26% STIG) also found reduced tumour volume and number in an MNU mutagen model of BCa (Yaacob, Yankuzo, Devaraj, Wong & Lai, 2015).

At cancer sites where exposure to dietary compounds is considered highest, such as the GI tract, some studies found PSS mixtures to not be so effective. Rats fed with 24mg/rat/day of a PSS mixture (55% SITO, 41% CAMP, 4% STIG) developed a similar tumour burden to their controls and were likely to suffer complications from poor gut bacterial health (Quilliot, Boman, Creton, Pelletier, Floquet & Debry, 2001). Notably, CAMP made up a large proportion of this PSS mixture. A phytosterol mixture again containing high CAMP, provided at exceptionally high levels via chow (20mg/mouse/day, which when calculated by weight by weight, is equivalent to 70g/person/day), was found to promote tumour formation in the Apc^{min} mouse model (Marttinen et al., 2014). In an experimentally matched study by the same group, the same dose of phytostanols was also found to promote tumour formation in the same model (Marttinen et al., 2013).

Combined, the broad consensus in the published data indicate that PSS are anti-proliferative in vivo, and reduced tumour growth is associated with lower expression of proliferative markers such as Ki67 and PCNA. However, in some studies performed at very high doses, especially in mixtures containing high CAMP or CAMS concentrations, PSS appeared to be either ineffective, led to gut health complications, or in two cases promoted tumour growth and activation of oncogene expression and activity. The route of administration for high doses appeared to be an important determinant. The GI tract is exposed to highest doses of orally administered compounds, which may explain why doses equivalent to 70g/person/day of plant stanols, or plant sterols, was associated with intestinal tumour formation. We are unaware of studies that have directly compared different routes of PSS administration to explore this hypothesis further. Globally, these data indicate that PSS dose, frequency and route of administration are likely to be important variables to consider in human studies, especially if pharmacological approaches where maximum tolerable doses are considered.

Resisting cell death

Programmed cell death is important for managing malignant and pre-malignant cells. Cancer cells become resistant to death signals by increasing the expression of anti-apoptotic proteins, and reducing expression of pro-apoptotic proteins. Bcl2 and Bclxl for example maintain mitochondrial membrane stability (thus preventing the release of cytochrome c into the cytoplasm, a very early event in apoptosis) and Bad and Bax oppose this mechanism by destabilising mitochondrial membrane integrity. Caspases are then activated and enact orchestrated cellular destruction. *In vivo* , cell death can be assessed longitudinally by tumour growth assays, on flow sorted tumour cells labelled for various stages of apoptosis, or by measuring expression of protein regulating apoptosis.

Across all studies we identified, tumours of PSS treated animals had significantly greater expression of pro-apoptotic proteins and reduced expression of anti-apoptotic signals (Fig 5). There was a significant decrease in expression of Bcl2 (SMD = -3.14; 95% CI: -5.40, —0.89; p=0.006; Fig 5A) and in BclxL (MD = -0.43 DU; 95% CI: —0.51, -0.35; p<0.0001; Fig 5B) in the treated animals relative to controls. Both of these proteins function to maintain mitochondrial membrane integrity and resist apoptosis. For the pro-apoptotic proteins, Bax was strongly induced (SMD = 7.86; 95% CI: 2.69, 13.03; p=0.003; Fig 5C), whilst Bad was also induced (MD = 0.14 DU; 95% CI: 0.07, 0.21; p<0.0001 Fig 5D).

Downstream of mitochondrial membrane integrity regulation, caspase expression also regulates apoptosis. In PSS treated Casp3 (SMD = 6.09; 95% CI: 2.04, 10.14; p=0.003; Fig 5E) and Casp9 (SMD = 5.30; 95% CI: 1.74, 8.86; p=0.004; Fig 5F) were both significantly higher than in controls. PSS derivatives appeared important in the magnitude of pro-apoptotic effect observed. Dolai *et al.,* was the only study included using a PSS derivative, DAUC, and evaluated its effect on apoptosis proteins at 50 mg and 100 mg kg^{-1} per day IP. A clear dose dependant effect was seen with greater increases in Casp3, Casp9, and Bax expression and

decreases in anti-apoptotic Bcl2 in the highest treatment group (Table 1). Ma *et al.,* established that either DAUC or DAUL at 60 mg kg^{-1} IP resulted in a greater Casp3 and Casp9 activation than SITO, which was accompanied by significant decreases in PI3K/Akt signalling that were not present in the SITO group (Ma et al., 2019).

Other hallmarks

Proliferation and cell death were the most heavily studied hallmarks identified during the systematic search. Other hallmarks were less extensively studied, yet important discoveries have been made. Four studies measured markers of metastasis or direct metastatic colonisation, and three evaluated markers of angiogenesis. PSS treated animals had significantly reduced metastatic colonisation (SMD = -1.34; 95% CI: -1.91, -0.77; $p<0.0001$; Fig 6A) from models of PCa (Awad, Fink, Williams & Kim, 2001), BCa (Han et al., 2018), and melanoma (Sundstrom et al., 2019). At the molecular level MMP2 and MMP9 were found significantly reduced in BCa xenograft models ($p<0.00001$ for both; Fig 6B-C). Expression of Snail, a transcription factor that drives EMT, was significantly reduced by SITO in PaCa BXPC3 xenografts, as were markers of EMT such as vimentin (Cao et al., 2018) (Table 1). ERK activation is associated with tumour cell angiogenesis and the metastatic epithelial-mesenchymal transformation (EMT) and Sharmila and Sindhu (2017) found SITO reduced pERK. Sundstrøm*et al.* (2019) also observed pERK reduction in brain metastases by SITO which was accompanied by fewer brain metastases in the PSS treated group (Sundstrom et al., 2019). Cao and colleagues evaluated GSK3β signalling in the context of metastasis formation and EMT in a PaCa xenograft model (Cao *et al.* , 2019). SITO given daily by IP at 80mg/kg significantly reduced E-cadherin and increased vimentin. The angiogenesis factor VEGF, was reduced in the tumours of PSS treated mice relative to controls was observed in two separate BCa models (4T1 and MCF7) treated PO with 50mg/kg and 100 mg/kg of DAUL (Han et al., 2018), and in a RCa model with 20 mg/kg PO of SITO (Sharmila & Sindhu, 2017b) ($p<0.0001$; Fig 6D). Furthermore, STIG was shown to reduce CCA CD31+ vessels, suggesting a disruption in tumour blood vessel formation (Kangsamaksin *et al.* 2017) but not by a SITO:STIG:CAMP mixture in MMTV-PyMT Tg mice (Llaverias et al., 2013) (Table 1).

Broad impact oncogenes

We found a number of oncogenes and tumour suppressor genes were evaluated as secondary endpoints in numerous studies. In vitro, PSS have been found to supress activity of AKT and NFκB pathways, and to act as PARP inhibitors, and these roles are evaluated here.

Excessive pAKT leads to enhanced tumour growth, resistance to death signals, metastasis, and angiogenesis (Revathidevi & Munirajan, 2019). Our meta-analysis of 3 pAKT studies (n=42 animals) indicated that pAKT levels were 45% lower in PSS treated groups (95% CI: -67%, -23%; $p<0.0001$; Fig 7A). In our qualitive assessment, we noted that PI3K (an oncogene on the same pathway as AKT) was also downregulated (Han et al., 2018) and hypophosphorylated in PSS treated animals (Ma et al., 2019) (Table 1). Phosphorylation of NFκB's regulatory and transcription factor subunits was reduced by PSS in BCa and PaCa models. In BCa xenograft DAUL (100 mg kg^{-1} day; 7.5g equ. human dose) reduced phosphorylation of IKKα/β, IkBa, and p65 (Han et al., 2018). In mammary gland extracts of the genetic BCa PyMT Tg model PSS treatment significantly impaired NFκB activity (Llaverias et al., 2013) under high fat diet conditions (Table 1). In the PaCa model, signalling by Nrf2-ARE was not altered by SITO, but NFκB activity was reduced. However, as reported earlier for proliferation, the CAMP rich high dose PSS mixture indicated that phytosterols given at high doses (20mg per mouse per day) did not lead to suppression of oncogene expression of function (EGFR, b-catenin, cycline D1 or pERK) (Marttinen et al., 2014). Furthermore, the sister paper published the year before indicated that high phyto*stanol* (8g kg^{-1} dw; 20mg/day/mouse; 92% CAMS, 8% STAN) led to significant increases in pro-proliferative proteins including EGFR and Cyclin-D1 (Marttinen et al., 2013).

PARP is over expressed in many cancers and is a therapeutic target in the treatment of several cancer types with the use of PARP inhibitors. Our meta-analysis of 2 studies found nuclear PARP to be significantly

reduced by PSS (SMD = -17.14; 95% CI: -24.03, -10.26; p<0.0001 Fig 7B) and cytoplasmic/cleaved PARP significantly increased (SMD = 6.22; 95% CI: 1.60, 10.83; p=0.008 Fig 7C). HCT116 cells are ATM-deficient indicating a sensitivity to DNA repair inhibiting drugs. Couder-Garcia and colleagues provided the only study that considered both dose and frequency of administration (Table 1). Interestingly cleaved PARP induction was strongly induced, and to a similar extent in both the frequent administration group (15 mg kg^{-1} 3 per week: 10.9-fold) and the high dose group (30 mg kg^{-1} once per week: 10.6-fold). As this study varied the frequency and dose of PENI administration it provided valuable information on how administration regimen should be considered in translational first-in-human studies.

The data described here suggests that PSS, particularly the glucoside derivatives of SITO, may be natural PARP inhibitors useful in the treatment of cancers characterised by mutations in DNA repair genes such as BRCA1/2. SITO, DAUC and DAUL all influenced PARP activity by promoting significant decreases in nuclear (active) PARP, and increases in its cleaved (inactive) form.

Mechanistic insights

Several mechanistic insights into how PSS may alter proliferation of tumour cells were provided during the systematic review and details are reported in Table 1. Proliferation of oestrogen receptor positive breast cancer MCF7 xenografts was inhibited by SITO provided in chow at 9.8g kg^{-1} dw. Interestingly, SITO treatment led to 35% lower circulating levels of exogenously introduced oestradiol, suggesting the antiproliferative actions of PSS could have been indirect via promoting oestradiol clearance (Ju, Clausen, Allred, Almada & Helferich, 2004). Mitochondrial function was also found significantly impaired by SITO in a model of melanoma brain metastasis. In this study the authors discovered that mitochondrial membrane integrity was impaired by SITO and this led to oxidative stress mediated apoptosis (Sundstrom et al., 2019). The high PSS dose studies performed in the Apc^{min} mouse found increases in activity (phosphorylation) and expression of pro-proliferative oncogenes including EGFR, and ERK1/2. Activation of the same proteins, and others (c-jun, c-fos, JNK, and p38) in a KCa model was significantly reduced by prolonged exposure (44-weks) to SITO (20 mg kg^{-1} PO 3 per week) (Sharmila & Sindhu, 2017a). These dose dependent differences in oncogene activation indicate a potential non-linear relationship, and activity and expression of such tumour markers should be assessed in clinical trials.

Evaluation of methodology

Heterogeneity

As expected, high levels of heterogeneity (I^2>75%) were observed in the majority of our meta-analyses — all of which contained < 10 studies for each outcome. However, we also demonstrate consistent directionality of effects between studies within each meta-analysis. This suggests that despite *inter* -study differences in experimental design that underlie the high levels of heterogeneity, the administration of PSS consistently confers protective effects against the hallmarks of cancer. Although the present study was not powered to investigate sub-groups across most analyses, future studies may be adequately powered to identify key experimental features that drive heterogeneity.

Risk of bias and adherence to guidelines for reporting on natural products, animal research, and immunoblotting

According to BJP and PROSPERO guidelines for declaration of transparency and scientific rigour (BJP, 2018b), animal research (BJP, 2018a), use of natural products (BJP, 2020), and use of immunoblotting and immunohistochemistry (BJP, 2018c) we developed a 57 point survey (Figure 8) that was completed in duplicate by two independent researchers. Four papers did not report ethical approval for their research and corresponding authors did not respond via contact details provided in the manuscripts. Our selection criteria we found there is a low risk of bias associated with our study in terms of reporting study design (Fig 8A).

Low ROB was also found for PSS origin, purity and measurement methods as these criteria were used to exclude manuscripts that did not report these characteristics, and/or evaluated effects plant/food extracts rather than pure PSS. Few records evaluated PSS toxicity, pharmacokinetics, dosage rationale, or compared to clinically effective drug (Fig 8B). However, given the long-term use of PSS in the cardiovascular disease setting, these characteristics have been reported extensively elsewhere. Moreover, antibody validation was not reported in any study, immunoblots were always cropped (Fig 8C). We do not see this as a particular limitation here as antibodies against common oncogenes such as pAKT and VEGF have been extensively published previously.

Conclusions

SITO is the most common phytosterol found in foods and we found SITO was strongly associated with the inhibition of several cancer hallmarks including: resisting cell death, sustaining proliferative signalling, inducing angiogenesis, and activating invasion and metastasis (graphical abstract). Given the wide range of PSS available in nature, estimated at over 200 (Moreau et al., 2018), to progress these compounds as anti-tumour agents into clinical practice, some decisions need to be made. Should new and relatively understudied PSS be characterised in an attempt to identify PSS with the most potent anti-tumour PSS? Or is the evidence now sufficient that SITO can be evaluated in clinical trials as an adjunct to existing treatment? If translatable to humans, the evidence presented here suggests that relatively modest daily PSS intake will suppress oncogenic signalling and suppression of multiple cancer hallmarks, leading to reduced cancer risk and burden. There is also convincing evidence that PSS would synergise with several existing therapies such as PARP inhibitors (Couder-García, Jacobo-Herrera, Zentella-Dehesa, Rocha-Zavaleta, Tavarez-Santamaría & Martínez-Vázquez, 2019), gemcitabine (Cao et al., 2018), and vemurafenib (Sundstrom et al., 2019). However, the cellular receptors for PSS have not been clearly identified in the context of cancer cell biology. Previously, PSS were shown to dampen the effect of oxysterols in breast cancer (Hutchinson, Lianto, Moore, Hughes & Thorne, 2019), and oxysterols are now considered strong mediators of the pathophysiology of several cancers (Baek et al., 2017; He et al., 2019; Segala et al., 2017). Further mechanistic evidence could be provided by applying emerging technologies such as phage display high throughput screens (Dilly et al., 2017) to panels of PSS to identify cellular protein receptors to which PSS directly bind. An alternative mechanism of action is integration into cellular membranes. Disruption of the plasma membrane would impair signalling by oncogenic signalling pathways including AKT (Fakih, Sanver, Kane & Thorne, 2018), and disruption of mitochondrial membrane integrity promotes oxidative stress and tumour cell specific apoptosis (Sundstrom et al., 2019). Given the well understood toxicity profile of PSS, combined with their now >20-year use in clinic to reduce cardio-vascular disease risk, and the plethora of preclinical in vivo evidence we have summarised here, it is timely to consider PSS as adjuncts to cancer therapies.

Figure Legends

Figure 1. Molecular structure of cholesterol and common dietary plant sterols and stanols. Reproduced from (Hutchinson, Lianto, Moore, Hughes & Thorne, 2019) with authors' permission.

Figure 2. PRISMA flow diagram showing searching, screening, eligibility, and inclusion process.

Figure 3. Forest plot of tumour size and proliferation markers after plant phytosterols and stanols administration. (A) Mean difference in change between PSS treatment and control of tumour volume (mm^3) according to cancer type. (B) Mean difference in change between PSS treatment and control of tumour weight (g) according to cancer type. (C) Standard mean difference in change between PSS treatment and control of PCNA proliferation marker. (D) Standard mean difference in change between PSS treatment and control of Ki67 proliferation marker.

Figure 4. Forest plot of cancer serum biomarkers after plant phytosterols and stanols administration. (A) Standard mean difference in change between PSS treatment and control of CEA. (B) Standard mean difference in change between PSS treatment and control of CA125. (C) Mean difference in change between PSS treatment and control of CA153.

Figure 5. Forest plot of apoptosis markers after plant phytosterols and stanols administration. (A) Standard mean difference in change between PSS treatment and control of Bcl-2. (B) Mean difference in change between PSS treatment and control of Bclxl. (C) Standard mean difference in change between PSS treatment and control of Bax. (D) Mean difference in change between PSS treatment and control of Bad. (E) Standard mean difference in change between PSS treatment and control of Caspase-3. (F) Standard mean difference in change between PSS treatment and control of Caspase-9.

Figure 6. Forest plot of metastasis and metastasis markers after plant phytosterols and stanols administration. (A) Mean difference in change between PSS treatment and control of metastasis number. (B) Mean difference in change between PSS treatment and control of MMP2. (C) Mean difference in change between PSS treatment and control of MMP9. (D) Standard mean difference in change between PSS treatment and control of VEGF.

Figure 7. Forest plot of pAKT and PARP expression after plant phytosterols and stanols administration. (A) Standard mean difference in change between PSS treatment and control of pAKT. (B) Standard mean difference in change between PSS treatment and control of nuclear PARP. (C) Mean difference in change between PSS treatment and control cleaved PARP.

Figure 8. Risk of bias analysis and adherence scores for British Journal of Pharmacology criteria for animal research, immunoblotting, and research on natural products.

Figure 9. Graphical abstract. Reproduced from (Hanahan & Weinberg, 2011) with copyright permission.

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