Tea Polyphenols as Prostate Cancer Preventive Agents

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Abstract

Prostate cancer is an ideal candidate for chemoprevention. Tea drinking is a possible explanation for the rarity of prostate cancer among Chinese men. Tea (*Camellia sinensis*) contains flavonoid polyphenols called catechins, believed to be responsible for this anti-carcinogenesis. In black tea these catechins are oxidised into theaflavins. Catechins and theaflavins both inhibit human prostate cancer cell proliferation *in vitro*. Catechins inhibit prostate cancer in the TRAMP mouse animal model of the disease. To determine if tea polyphenols warrant investigation in large trials, evidence is required from animal models and biomarkers of cancer prevention identified in small human studies.

In the work presented here, prostate carcinogenesis was inhibited by orally administered tea polyphenols in the TRAMP mouse. Following 26 weeks of polyphenol administration median prostate masses were 0.54g, 0.28g and 1.01g for the theaflavin, catechin and control group respectively. This is the first *in vivo* evidence of prostate cancer chemoprevention by black tea theaflavins and adds to the previously published evidence for the same effect by green tea catechins. In the catechin group, this chemoprevention was associated with a significant reduction in the concentration of oxidative DNA adduct malondialdehyde-deoxyguanosine (M_1G) in prostate tissue. M_1G, a marker of oxidative DNA damage, was therefore proposed as a putative biomarker of prostate cancer chemoprevention. A human trial was then performed involving 18 men randomised to receive four weeks of catechins, theaflavins or no polyphenol prior to transurethral resection of prostate. A significant reduction in M_1G was detected in the DNA from prostate tissue of men who had received catechins.

Tea polyphenols and particularly catechins may therefore represent prostate cancer prevention agents suitable for study in a larger human intervention trial however, this finding should be first be tested in further better designed biomarker studies using this result to inform decisions on study population size.

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“Was ihn nicht umbringt, macht ihn stärker” Nietzsche

Contents

1 Introduction

1.1 Principles of carcinogenesis

Cancers are diseases of cellular function in which cells reproduce and accumulate pathologically. Escaping the normal mechanisms of homeostasis which limit cellular accumulation, cancers compromise the host by compressing surrounding structures, invading into them, spreading to distant sites and releasing aberrant chemical messengers. Although cancer represents a spectrum of diseases, certain principles are common to perhaps all cancers. Carcinogenesis begins with environmental agents, e.g., chemical, radiation or viral, inducing DNA damage within a gene involved in the control of proliferation or DNA repair. If this damage escapes repair then a stable somatic mutation is formed, this is equivalent to Friedewald and Rous's concept of "initiation" [1]. At this stage the cell is still reliant on external stimuli to allow division. This external stimulation, referred to as "promotion" by Freidewald and Rous, can represent the hormonal environment or exposure to further chemical carcinogens e.g., in food or smoke, often exerting their effect by producing inflammation. To achieve the autonomous growth that is characteristic of these diseases, a cell and its progeny must acquire additional alterations to its normal physiology through step-wise genetic mutation [2].

Mutations lead to either gain-of-function i.e., activation of oncogenes or loss-of-function i.e., inactivation of anti-oncogenes. Loss-of-function mutations of anti-oncogenes necessarily involve mutation of each of the two alleles in a diploid cell.

This two-hit hypothesis was originally proposed by Knudson to explain the pattern of occurrence of inherited retinoblastomas [3].

Each individual alteration, that endows reproductive or survival advantage upon a particular cell, leads to the clonal expansion of its progeny. In this way, carcinogenesis progresses through a process of variation generated by mutation followed by selection. This has been described as reminiscent of Darwinian natural selection [4, 5].

The accumulation of these necessary mutations, observed in carcinogenesis, implies a higher mutation rate than would normally be expected. This has led to the proposal of the “mutator phenotype hypothesis” [6, 7], in which mutations in genes responsible for DNA repair leads to genomic instability, this instability allows a higher rate of stable somatic mutation [6].

The alterations in cellular function that are necessary to achieve carcinogenesis have been categorized into six groups, i) Independence from growth signals e.g., EGF-R activation; ii) Insensitivity to antigrowth signals e.g., loss of pRb function; iii) Evasion of apoptosis e.g., loss of p53 function; iv) Limitless replicative potential e.g., up-regulated telomerase; v) Sustained angiogenesis e.g., over-expression of VEGF; and, vi) Tissue invasion and metastasis e.g., changes in expression of integrins [2].

1.2 Prostate cancer

1.2.1 Epidemiology

Prostate carcinoma is now the most common male cancer in the UK and the second leading cause of cancer related death after lung cancer [8]. The lifetime risk of prostate cancer in the UK has reached one in 14 men [8]. A UK study of 704 men diagnosed with prostate cancer found 38% presented with clinically localized potentially curable disease, 37% with locally advanced and 25% with metastatic disease [9].

A number of predisposing factors have been identified. Age is the strongest risk factor, the mean age of onset being 72-74 years [10] with >96% cases occurring in men >60 years [11]. The rate of prostate cancer diagnosis at autopsy is 20% in men 50-60 years and 50% in men 70-80 years [12].

The striking variations in prostate cancer incidence seen between different countries may reflect racial genetic differences or environmental causes. Asians have the lowest and African Americans the highest rate of prostate cancer incidence [13]. There are also racial differences in mortality rates. African-Americans have a five year survival rate of 93% compared with 98% for white Americans with prostate cancer; this may reflect the increased likelihood of African-Americans presenting with advanced stage [13].

Familial accumulation appears to be stronger in prostate cancer than in either colon or breast cancer [14]. Genetic factors have also been inferred from

twin concordance studies, linkage analysis and familial accumulation of cases. Twin studies have demonstrated that heritable factors account for 42% of the variance in prostate cancer incidence [15]. A number of putative genes are under study including the *RNASEL* gene, *HPC1* locus, the androgen receptor, the vitamin D-receptor, *CYP17* (17α-hydroxylase) and *SRD5A2* (5α-reductase) [10].

Many possible environmental risk factors for prostate cancer have been proposed. Dietary fat intake was initially implicated, however, there is contradictory evidence in the literature. Of 23 case-control studies, 13 showed a positive relationship between total fat intake and prostate cancer; and of seven cohort studies only three found such a link [16]. Consumption of the essential marine fatty acids, eicosapentaenoic acid and docosahexaenoic acid, has been linked to lower prostate cancer risk [17, 18]. A study of the counties within the USA found a significant South-North trend for prostate cancer incidence that was attributed to the protective effect of UV exposure [19]. The major source of vitamin D is from the skin where the precursor 7-dehydrocholesterol is converted to vitamin D_3 through the action of UV radiation. It has been suggested that the increased risk of prostate cancer found in northern geographical regions in African-American men may be a result of low levels of vitamin D [20].

The incidence of prostate cancer in the Chinese population in Tianjin is 1.9 per 100,000 compared with 100-139 per 100,000 in the USA [21]. The high consumption of tea and soya products amongst Chinese men has been proposed as one possible explanation for this difference in incidence. Other dietary factors

negatively associated with prostate cancer include lycopene from tomatoes, selenium and vitamin E (see section 1.8.4).

1.2.2 Pathology

Human prostate cancers are almost exclusively (98%) adenocarcinomas. These most commonly arise from the peripheral zone (68%), and less often from the transition zone (24%) or the central zone (8%) [22]. Human prostate cancer is typically multi-focal with individual foci being genetically distinct and exhibiting different grades [23]. How the disease develops is highly variable; lower grade tumours can run protracted courses over decades whereas others progress to metastasis quickly. In an attempt to predict their behaviour adenocarcinomas of the prostate are graded using the Gleason score to categorise morphology [24]. In a study of 828 cases of prostate cancer treated conservatively the ten year disease specific survival in those with a Gleason grades <=7 was 87% compared with only 34% for those with a Gleason grade >7 [25]. The cancer is staged using the TMN system revised by the American Joint Committee on Cancer in 2010 [26] which is summarised below.

Primary tumour (T)

Tx Primary tumour cannot be assessed

T0 No evidence of primary tumour

T1 Clinically inapparent tumour (not palpable or visible on imaging)

T1a Tumour incidental histologic finding in 5% or less of tissue resected (in prostatectomy)

T1b Tumor incidental histologic finding in more than 5% of tissue resected

T1c Tumor identified by needle biopsy (e.g., because of elevated PSA)

T2 Tumour confined to the prostate

T2a Tumor involves one-half of 1 lobe or less

T2b Tumor involves more than one-half of 1 lobe but not both lobes

T2c Tumor involves both lobes

T3 Tumour extends through the prostate capsule

T3a Extracapsular extension (unilateral or bilateral)

T3b Tumor invades seminal vesicle(s)

T4 Tumour is fixed or invades adjacent structures other than seminal vesicles

Regional lymph nodes (N)

NX Regional lymph nodes were not assessed

N0 No regional lymph node metastasis (lymph nodes confined to the true pelvis)

N1 Metastasis in regional lymph node(s)

Distant metastasis (M)

MX Distant metastasis cannot be assessed by any modality

M0 No distant metastasis

M1 Distant metastasis

M1a Nonregional lymph nodes

M1b Bone

M1c Other sites with or without bone disease

There is considerable difficulty in accurately assigning a TNM stage preoperatively. MRI is used to try to detect extra capsular spread however, the positive predictive value of an MRI suggesting local spread (T3) is only 70% [27]. Bone metastases are usually evaluated by Technetium-99m bone scintigraphy (bone scan) but are most sensitively detected using MRI [28]. Serum PSA concentration can be used to decide which patient to subject to further staging investigations; in one study, in the absence of bone pain only 2 of 852 patients with a PSA <20 µg/L had a metastases detectable by bone scan. Pelvic lymph node metastases are preoperatively assessed by MRI however, to most accurately stage the pelvic lymph node involvement in patients undergoing a radical prostatectomy a pelvic lymph node dissection can be performed [29]. Who exactly should be assessed by pelvic lymph node dissection is controversial and currently being evaluated.

Although independently assessed and defined, there are correlations between stage and grade, for instance, node metastases are more common in those with Gleason 8 disease than in those with Gleason 5 prostate cancer (23% v 2%) [30].

1.2.3 Treatment

The management options and clinical outcomes are determined by stage and grade of prostate cancer. The options for localised prostate cancer (T stage <3) include watchful waiting, external beam radiotherapy, interstitial radiotherapy (brachytherapy), early androgen deprivation and radical prostatectomy; due to a paucity of evidence directly comparing treatment modalities, practice and advice varies widely between practitioners.

Watchful waiting may be more suitable to “favourable risk” localised prostate cancer. In a study published in 2010 of 450 patients, favourable risk was defined as all patients with localised prostate cancer with a Gleason score <7 and PSA <10 ng/ml, as well as, patients >70 years with a Gleason score <8 and PSA<15 ng/ml. These men were treated with active surveillance. Follow up over a mean of 6.8 years showed 10 year prostate cancer actuarial survival of 97.2%, in this study radical treatment was given if men demonstrated a PSA doubling time<3 years or they progressed from T1 to T2 [31].

Radical prostatectomy is currently a recommended treatment for men with moderately and poorly differentiated prostate cancer [32]. A Scandinavian study of

695 men with T1b, T1c or T2 prostate cancer were randomised to watchful waiting or radical prostatectomy, with a mean follow up length of 6.2 years [33]. Radical prostatectomy significantly reduced disease specific mortality but not overall mortality; 62 men in the watchful-waiting group and 53 in the radical prostatectomy group died (P=0.31). The ProtecT trial is currently comparing radical prostatectomy, radical radiotherapy and watchful waiting in a 230,000 randomised controlled multi-centre trial (International Standard Randomised Controlled Trial Number 20141297).

Radical radiotherapy and radical prostatectomy for localised prostate cancer are currently equally recommended [32]. Case series have been published suggesting disease-free survival following radiation is comparable with radical prostatectomy [34, 35].

For patients with locally advanced prostate cancer improved survival can be achieved through the administration of radiotherapy and goserelin. In a trial of 415 patients with prostate cancer T1-4 with N0 or NX and M0 or MX, patients were randomised to receive radiotherapy alone or radiotherapy followed by immediate commencement of goserelin. The median follow up was 45 months, with five year survival of 79% in the dual treatment group compared with 62% in radiotherapy alone group [36].

The role of radical prostatectomy in men with T3 disease is controversial but is currently recommended by European Urology Association guidelines in men with lower risk disease i.e., Gleason grade <9, PSA <20 ng/ml. There is some evidence from patient series that the cancer specific survival rate of T3 disease treated with

radical surgery approaches that of T2 disease managed in the same way (90% vs 96% at 10 years) [37].

For those with advanced metastatic prostate cancer, the mainstay of treatment is with androgen hormone ablation therapy either by surgical castration or more commonly with the use of luteinising-hormone-releasing-hormone (LHRH) inhibitors such as goserelin, however within several years most patients' disease will progress [38]. Complete androgen blockade with an oral androgen antagonist such as bicalutamide in addition to an LHRH antagonist offer a survival advantage of less than 5% over 5 years [39]. Alternative approaches for those with disease that has escaped hormonal control are limited; some cases may respond to secondary hormonal therapy which can include anti-androgen withdrawal, oestrogens and corticosteroids [40]. The role of cytotoxic therapy is very limited, in a phase three trial, the use of docetaxel in combination with prednisolone resulted in a 2-3 month survival advantage compared with mitoxantrone [41]. For many with hormone escaped disease however the treatment is palliative [42].

1.3 Tea polyphenols

1.3.1 Classification of polyphenols

Polyphenols are a class of chemical substances naturally occurring in many edible plants, defined by the inclusion of more than one phenolic ring in their structure. Polyphenols may be subdivided into more than ten structural types which include phenolic acids, lignins, stilbenoids and flavanoids. Catechins and theaflavins are tea-derived flavanoids that contribute to its bitterness and astringency [43].

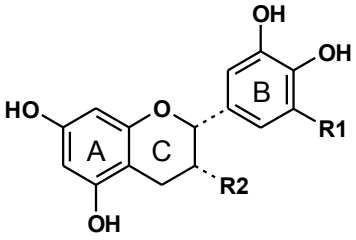
1.3.2 Tea manufacture and polyphenol content

Tea is an infusion made from the leaves of *Camellia sinensis*, after water it is the most common beverage in the world. Green tea is the non-oxidized product that is primarily consumed in China and Japan, and it represents about 20% of world tea consumption. In order to denature the oxidative enzymes found in the tea leaves they are heated. The naturally occurring polyphenols are, therefore, preserved relatively unchanged; the water extractible material from green tea contains, by dry weight, 30-40% catechins, 3% other flavanoids and 3-6% caffeine [44]. Black tea, however, is manufactured including a process known as “fermentation” that allows oxidation of a proportion of the catechins, leaving 3-10%

catechins and generating 2-6% dimeric theaflavins and >20% oligomeric thearubigins [45].

The catechins found in tea include epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG), see figure 1. The theaflavins are a mixture of theaflavin (TF), theaflavin-3-gallate, theaflavin-3'-gallate (TFG) and theaflavin-3,3'-digallate (TFdiG), see figure 2 [44]. Of these polyphenols, EGCG and TFdiG have shown the most promise as anti-carcinogenic agents.

A typical 240 ml serving of black tea brewed using 2.25 g of dry tea for four minutes has been shown to contain 18.7 mg theaflavins [46]. A capsule containing 1000 mg of theaflavins represents, therefore, approximately 53 servings. A 240 ml serving of green tea contains approximately 56 mg EGCG [47]. A capsule containing 1000 mg catechins, of which 666 mg is EGCG, represents approximately 12 servings in terms of EGCG content.



	R1	R2	MW
Epicatechin	-H	-H	290
Epigallocatechin	-OH	-H	306
Epicatechin-3-gallate	-H	gallate	442
Epgallocatechin-3-gallate	-OH	gallate	458

Gallate =

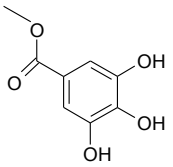
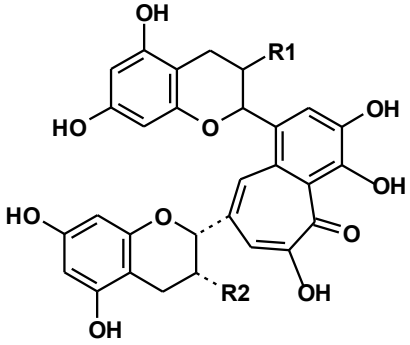


Figure 1 The generic three ring structure of green tea catechins showing A, B and C rings where R1 and R2 represent various possible side groups.



	R1	R2	MW
Theaflavin	-OH	-OH	564.5
Theaflavin-3-gallate	-gallate	-OH	716.6
Theaflavin-3'-gallate	-OH	gallate	716.6
Theaflavin-3,3'-digallate	-gallate	gallate	868.8

Figure 2 The generic six ring structure of black tea theaflavins showing where R1 and R2 represent various possible side groups.

1.3.3 Anti-oxidant properties of tea polyphenols

Many non-nutrient flavanoids are thought to exhibit anti-oxidant properties. This anti-oxidation may be demonstrated by direct scavenging and reduction of oxygen free radicals; by terminating propagatory chains of lipid peroxidation; scavenging reactive nitrogen species thereby inhibiting tyrosine nitration and DNA deamination; and, by chelating transition metal ions thus inhibiting the generation of iron-induced free radicals [48].

It is the arrangement of phenolic hydroxyl groups on the B ring of catechins that produces the low reduction potential capable of reduction reactions, this is enhanced by the addition of a gallate group to the C ring. This explains the hierarchy of antioxidant potential demonstrated by green tea catechins: EGCG = ECG > EGC > EC [48]. In reducing highly oxidizing free radicals ($R^{\bullet}$ = superoxide, peroxyl, alkoxyl or hydroxyl radicals), aroxyl radicals are formed by hydrogen donation, which then react with further radicals to produce stable quinones:

$$HO\text{-}Fl\text{-}OH + R^{\bullet} \rightarrow HO\text{-}Fl\text{-}O^{\bullet} + RH$$

$$HO\text{-}Fl\text{-}O^{\bullet} + R^{\bullet} \rightarrow O{=}Fl{=}O + RH$$

The hierarchy of catechins in lipid peroxyl radical scavenging potency is ECG = EGCG = EC > EGC which reflects the reduced ability of EGC to access lipids because of its high aqueous solubility [49]. Peroxynitrite ($ONOO^{-}$)is an oxidizing and nitrating radical generated following rapid nitric oxide production such

as in inflammation [50]. Tyrosine is particularly vulnerable to nitration by peroxynitrite forming products that are implicated in stimulating inflammatory reactions [48]. Tea catechins can inhibit this nitration with the following hierarchy of potencies ECG = EGCG > EC = EGC, this suggests that the gallate group is an important area of activity in these reactions [51].

1.4 Oral bioavailability and safety of tea polyphenols

Pharmacokinetic studies of single doses of EGCG or green tea polyphenols have demonstrated low bioavailability thought to be due to pre-systemic elimination. This elimination is saturated at repeated high doses; 800 mg as a one-off dose led to a peak plasma level of 234.9 ng/ml +/-140.9, whereas repeated daily dosing of 800 mg for four weeks led to a higher peak concentration of 390.3 ng/ml +/- 231.4 [52, 53]. This increase in peak plasma level following repeated dosing did not result from accumulation, but rather may have been due to inhibited pre-systemic elimination. There was no increase in side-effects with the EGCG dosed group compared with the placebo group other than mild nausea. Similar pharmacokinetic results and side-effect profiles were found using a tea polyphenol mixture (Polyphenon E) containing 800 mg EGCG.

Greater oral bioavailability can be achieved by administering the EGCG after an overnight fast [54]. A polyphenon E dose containing 800 mg of EGCG was well tolerated and led to a peak plasma concentration of 1.5 μg/ml after 83 minutes in fasted subjects, compared with a concentration of 294 ng/ml in the fed subjects.

Green tea polyphenols have been demonstrated to be available in human prostate tissue following oral consumption. Consumption of green tea containing 4.1 mg EGCG per kg body weight led to a mean prostate EGCG concentration of 40 pmol/g [55]. Oral doses of 600 mg EGCG orally led to a maximum plasma EGCG concentration of 321 ng/ml [56].

The bioavailability of theaflavins appears to be even lower, in healthy volunteers consuming 700 mg theaflavins, the peak plasma concentration detected was one ng/ml at two hours [57]. The role played by metabolites of catechins and theaflavins in the biological activity ascribed to them is not yet clear. Hippuric acid has been identified as the major urinary metabolite of black tea consumption [58]. Catechins are mainly excreted in urine as glucuronide and sulphate conjugates [59].

There have been 34 cases of human hepatotoxicity following green tea polyphenol ingestion between 1999 and 2008, of 32 cases that were assessable 20 were hepatocellular, 6 cholestatic and 6 mixed [60]. Evaluation in rats found increased serum transaminases indicating hepatotoxicity when high oral doses 3.5g/kg were administered, but that no such toxicity was seen at doses of 764-820 mg/kg [61]. It is thought that the mechanism of this toxicity may be a pro-oxidant activity of tea polyphenols [62]. In a recent study of 26 men given 1.3g/day orally of tea green tea polyphenols no rise in liver enzymes was observed [63]. There are no reports yet of theaflavin-related toxicity in humans.

1.5 Human prostate adenocarcinoma cell lines

1.5.1 Role of transformed cell lines in cancer chemoprevention studies

Transformed cell lines have been widely utilised in preliminary studies of the cancer preventive potential of compounds despite the fact that they represent cells from tissue that has already progressed to malignancy. Since cancer chemoprevention aims to prevent, arrest or reverse carcinogenesis, an ideal chemopreventive agent would offer a range of concentrations at which they preferentially reduced proliferation and increased the apoptosis rate of malignant and premalignant cells while leaving normal cells unaffected. Therefore transformed cell lines can rapidly and cheaply screen large numbers of agents to identify the most suitable candidates prior to progression to animal studies. The work presented here utilised two prostate transformed cell lines, LNCap and DU145.

1.5.2 LNCaP and DU145 cells

LNCaP cells were isolated in 1977 from the supraclavicular lymph node aspiration of a patient with androgen-independent metastatic prostate cancer [64, 65]. LNCaP has a mutated androgen binding domain of the androgen receptor, remain androgen sensitive and express PSA [65]. DU145 cells were originally isolated from a brain metastasis. It is not detectably androgen sensitive nor does it secrete PSA [66].

1.6 Mouse models of prostate cancer

1.6.1 The relevance of animal models to human prostate chemoprevention studies

Animal models of prostate cancer are vital to rapidly screen the potential of putative cancer preventive agents as well as to determine the molecular basis of carcinogenesis and chemoprevention. An ideal animal model in which to study prostate cancer chemoprevention should meet a number of criteria [67]. Each stage of carcinogenesis should be present and recognisable; initiation, promotion and progression. Cancer should progress from precursor lesions such as prostate intraepithelial neoplasia (PIN) and progress into less differentiated higher grade disease. Both histological and molecular features of carcinogenesis should reflect those found in human disease. Early stage disease should be androgen-responsive later escaping hormonal dependence. Carcinogenesis within the model should occur inevitably to minimise the number of animals required to analyse cancer preventive agents. Finally the model should have an intact immune system [67].

Spontaneous prostate cancer is rare in rodents. Rats do spontaneously develop the disease, however, strains have been developed with an increased rate of prostate carcinoma such as the Lobund Wistar and ACI/Seg strains [67]. The rate of carcinogenesis can be chemically increased for instance by administration of methylnitrosourea and testosterone [68]. A spontaneous prostate cancer in the inbred Copenhagen rat strain (the Dunning R-3327 tumour) was serially passaged,

by subcutaneous grafting, to develop a range of cell lines of varying differentiation and behaviours [69].

To take advantage of their shorter life cycle, a number of approaches have been used to provide mouse models of this disease. Mouse models are powerful tools for the rapid screening of potentially cancer preventive compounds. There are, however, concerns about the morphological differences between the murine and human prostates. The human prostate is a single, discrete organ that encircles the urethra at the bladder neck. Within this structure, it is arranged into zones, peripheral, transitional, central zones and anterior fibromuscular stroma [70]. The murine prostate, however, is made up of four paired, morphologically separate lobes; the anterior, dorsal, lateral and ventral lobes. It is often stated that the dorsolateral lobes are equivalent to the peripheral zone in human based on descriptive studies. Recently this has been supported by evidence, from microarray analysis, demonstrating similar gene expression profiles between these two functional compartments [71].

1.6.2 Xenograft models of prostate cancer

The development of immunodeficient mice has allowed the isolation of human prostate cancer cell lines that can be serially xenografted into these mice. The first strain to be used was the athymic BALB/c nude mouse strain, deficient in T lymphocytes, and into which was subcutaneously grafted fragments of human prostate cancer tissue [72]. Nude mice develop some extrathymic T cell function,

significant B cell function as well as increased natural killer cells and normal antigen presenting cells; the efficiency of xenografting is therefore reduced. The similar athymic NMRI strain was found to give higher rates of successful transplantation increasing the take rate from 3% in BALB/c nude mice to 38% [73].

The severe combined immunodeficiency (SCID) mouse lacks T and B lymphocyte function, with normal natural killer cells and myeloid function [74]. The crossing of this mouse with the non-obese diabetic (NOD) mouse created the NOD-SCID strain that included deficiencies in natural killer cells, complement function and antigen presenting cells, in addition to the deficits found in the SCID mouse [75]. Although the severe immunodeficiency of SCID and NOD-SCID mice renders them more susceptible to pathogens, they have the advantage that tumours develop faster and bone metastases are more likely than in athymic mice [76]. The NOD-SCID mouse has also been used as the host for orthotopic xenografts of human prostate cancer cell lines which then metastasize to bone [77].

The mouse prostate reconstitutional model takes advantage of the ability of mouse foetus urogenital sinus to develop into a prostate gland after it has been grafted under the renal capsule of an isogenic male host [78]. Recombinant retrovirus was used to introduce *ras* and *myc* oncogenes to the sinus cells prior to transplantation reliably producing poorly differentiated carcinoma in the developing prostate [78].

The development of transgenic mice that develop *de novo* prostate cancer has allowed the study of the initiation of carcinoma. It is hoped that the tumours

developed by these mice most closely replicate the complex interactions between the various cell types and tissues, which occur in human prostate carcinogenesis.

1.6.3 The TRAMP mouse

The Transgenic Adenocarcinoma of Mouse Prostate “TRAMP” mouse (strain name = C57BL/6-Tg(TRAMP)8247Ng/J) is a strain based on C57B that expresses the simian virus 40 (SV40) T/t antigen-coding region under the control of the prostate-specific rat probasin promoter [79]. The expression of the SV40 T/t antigen induces carcinogenesis due to its inhibitory interactions with the anti-oncogenes p53 [80] and Rb [81]. Such anti-oncogenes would normally act following somatic oncogenic mutation to arrest the cell cycle, allowing repair, or if this is impossible, to induce apoptosis [82]. Expression of the transgene occurs from eight weeks of age, HGPIN or prostate carcinoma can be detected at around ten weeks and metastases are reported to occur by 12 weeks with a reported 100% of animals developing metastases by 28 weeks of age most commonly in lymph nodes and lungs [83]. The TRAMP mouse has been used in trials to demonstrate prostate cancer prevention potential of green tea polyphenols, celecoxib and zinc [84-86].

1.6.4 Other transgenic murine prostate cancer models

Various strains of transgenic mice were created using a longer 12 kb fragment of the rat probasin promoter to control expression of the SV40 T antigen and including a deletion that removes t antigen expression. These strains are collectively known as the "LADY" model. Disease progression is less aggressive than in the TRAMP mouse with tumour formation ranging from 12-20 weeks [87] only one strain of LADY, 12T-10, develops metastases [88]. The reason for this less aggressive phenotype may be the loss of the t antigen expression or may be a feature of the CD1 background strain [88]. Both the TRAMP and LADY models develop a high frequency of neuroendocrine tumours; these are uncommon in humans and display a more aggressive phenotype [87].

The frequent finding of *c-myc* amplification in human prostate cancer inspired the generation of a transgenic mouse strain expressing *c-myc* under the control of rat prostate-specific C3(1) promoter [89]. However, this mouse develops a prostate epithelial cell morphology that resembles human PIN and falls short of frank carcinoma; this was thought to be due to insufficient expression of the transgene. An alternative model over expressing *c-myc* under the control of probasin based promoters shows progression to invasive disease [90]. One possible advantage that this model has over those based on targeted SV40 expression is the absence of the neuroendocrine phenotype.

1.7 Prostate cancer anti-carcinogenesis by tea polyphenols

1.7.1 Epidemiology of prostate cancer in relation to tea polyphenol intake

There has been much speculation about the cause of the low rate of prostate cancer amongst Asian men [21]. Tea, and especially the green tea popular in Asia, has attracted attention as a possible dietary factor responsible. Epidemiological studies have found inverse associations between tea intake and the incidence of a number of cancers, including pancreatic, gastric, colorectal and skin [91-96]. The epidemiological evidence linking tea drinking and a reduction in the incidence of prostate cancer has so far been inconclusive.

A prospective cohort study of 7833 men who developed 149 cases of prostate carcinoma, found black tea drinking was associated with an age adjusted relative risk of 0.6-0.8, p=0.02 [97]. In a Canadian case control study of 617 cases and 636 controls, drinking at least two cups of tea per day was associated with an odds ratio of 0.7 (95% CI 0.50–0.99) [98]. A recent case-control study of Chinese men, including 130 cases, has again linked tea drinking with lower prostate cancer incidence, the adjusted odds ratio relative to non-tea drinkers being 0.28 (95% CI = 0.17–0.47) [99]. A recent prospective cohort study of 49,920 japanese men, 40-69 years old, correlated prostate cancer incidence with green tea drinking. Green tea was not associated with localised prostate cancer incidence. However, five or more cups of green tea a day was associated with a reduced incidence (RR=0.52;

95% CI 0.28-0.96) of advanced prostate cancer compared with those drinking less than one cup a day [100].

Other studies found no risk reduction, including; an Italian case-control study of 107 cases [101]; an American case-control study of 362 cases [102] and a Canadian retrospective cohort study involving 3400 men who developed 145 cases of prostate cancer [103].

1.7.2 Tea polyphenols inhibit prostate cancer cells in vitro and induce apoptosis

After the addition of EGCG to *in vitro* prostate cancer cell culture media at 10 μM (4.58 mg/ml), proliferation inhibition of >90% has been demonstrated [104]. Apoptosis has been demonstrated in LNCaP androgen-dependent prostate cancer cells following incubation with EGCG at 92 μg/ml [105]. In DU145 androgen-independent prostate cancer cells apoptosis is reported following incubation with EGCG at concentrations of 92 μg/ml [105], 80 μg/ml [106] and 40 μg/ml [107]. Evidence from work with normal epidermal keratinocytes and normal fibroblasts suggests that this growth inhibition and apoptosis is preferentially induced in malignant cell lines compared with normal counterparts [106, 108].

Much less is known about the anti-carcinogenic effects of the theaflavins. Theaflavins produce a >90% inhibition to the proliferation of LNCaP and DU145 cells when added to cell culture media [104, 109]. Theaflavins reduce proliferation and induce apoptosis in PC-3 prostate cancer cells, the IC50 dose for growth inhibition being 40 μg/ml [110]. Theaflavin-3'-monogallate has been shown to induce apoptosis in WI38VA transformed fibroblasts at a concentration of 72 μg/ml [111]. In H661 lung tumour cells apoptosis was induced by 100 μM (approximately 72 μg/ml) theaflavins but not by 30 μM (22 μg/ml) [112].

1.7.3 The effect of tea polyphenols on animal models of prostate carcinogenesis

Evidence from animal models has demonstrated that cancer inhibition by EGCG can be reproduced *in vivo*. Athymic mice grafted with PC-3 or LNCaP human prostate cell lines demonstrated growth inhibition and even regression of the tumours when 1 mg/day EGCG was administered intraperitoneally [113]. The transgenic "TRAMP" mouse develops autochthonous carcinoma of the prostate that mimics human disease [79, 114]. This was used to demonstrate inhibition of carcinogenesis following oral administration of green tea polyphenols rich in EGCG. Not only did this intervention inhibit primary tumour growth but also it completely prevented all distant metastases [85]. Subsequently, it has been shown that the efficacy of cancer inhibition by green tea in the TRAMP mouse is greatest in the earliest stages of prostate carcinogenesis [115].

No animal models of prostate cancer have yet been used to assess the ability of theaflavins to prevent carcinogenesis. Orally administered black tea has, however, been shown to prevent, inhibit growth and induce apoptosis in ultraviolet-induced skin tumours in mice [116].

1.7.4 Human interventional trials of tea polyphenols as prostate cancer prevention agents

A recent proof-of-principle clinical trial involved 60 patients with high grade prostate intraepithelial neoplasia (HGPIN) randomised to receive 600 mg a day green tea catechins (GTC; 52% EGCG) or placebo. After one year, the 30 men receiving GTC had developed one case of prostate cancer, nine cancers were found in the placebo-treated group [117].

In another trial, 42 men with hormone-escaped prostate cancer were given 6 g of green tea per day in 6 divided doses [118]. The main finding of this trial was that there was an unacceptable rate of caffeine related side-effects. Grade 1 or 2 toxicity, occurred in 69% of patients with 6 episodes of grade 3 and 1 episode of grade 4 toxicity. This study failed to demonstrate any inhibition of carcinoma progression reflected in serum PSA concentration.

In a recent study of 26 men with prostate cancer awaiting radical prostatectomy, 1.3g polphenon E containing 800 mg EGCG, was given daily until surgery. Comparing pre and post-treatment serum, reductions were seen in the concentrations of hepatocyte growth factor, vascular endothelial growth factor, prostate specific antigen and insulin like growth factor [63].

1.8 Prostate carcinoma chemoprevention

1.8.1 Prostate cancer as a chemoprevention target

Cancer chemoprevention is the use of natural or synthetic compounds to prevent, delay or reverse the multistage process of carcinogenesis. The late onset, prolonged natural history and high incidence of prostate carcinoma in elderly men make this an ideal target for efforts to develop chemopreventive agents. With such a late average age of onset even a modest delay in carcinogenesis would lead to a significant fall in the incidence of this condition. Unfortunately this same prolonged natural history means that analysis of each agent's chemopreventive potential can take many years and involve many thousands of men.

1.8.2 5α-reductase inhibitors as prostate cancer preventive agents

The Prostate Cancer Prevention Trial (PCPT) is the only completed large phase III double-blind randomised controlled trial of prostate cancer chemoprevention. The intervention used in this trial was finasteride, a 5α-reductase inhibitor. 5α-reductase is the enzyme responsible for conversion of circulating testosterone to the more potent dihydrotestosterone (DHT), responsible for prostate epithelial proliferation [119]. Between October 1993 and March 2003 the PCPT recruited 18,882 men who were 55 years of age or older with normal digital rectal examinations (DRE) and a PSA concentration of 3.0 ng/ml or less.

Prostate cancer was detected in 18.4% of those taking finasteride compared with 24.4% of controls, this represents a 24.8% reduction of prostate cancer prevalence over a seven year period in those men taking finasteride compared with the placebo group [120]. Implementation of this finding into clinical practice has been impeded by the finding of excess cases of high grade prostate cancer (HGPC) in the finasteride treated group (6.4% compared with 5.1% in the placebo group).

It is possible that this excess of HGPC cases in the finasteride treated group is artefactual. If finasteride had genuinely induced HGPC the ratio between HGPC in the finasteride and placebo groups would have gradually increased with increasing cumulative dose over the course of the trial. In fact, the ratio was highest at 3.6 during year 2, lowest at one during year four [121]. The apparent increase in HGPC may have been due to the fact that grading of prostate tumours was more accurate in the finasteride group, if a patient had a HGPC at radical prostatectomy the likelihood that this was found at biopsy was 70.3% in the finasteride group and 50% in the placebo group [120]. Reduction of prostate volume due to finasteride may have led to this detection-bias; the excess risk of HGPC disappeared when adjusted for the number of biopsy cores and gland volume [122].

Dutasteride is an alternative 5α-reductase inhibitor that is currently under investigation in the Reduction by Dutasteride of Prostate Cancer Events (REDUCE) trial. DHT synthesis from testosterone is catalysed by 5α-reductase types I and II. Finasteride selectively inhibits the type II isoenzyme whereas

dutasteride is a dual inhibitor [123]. There is evidence that type I 5α-reductase is over-expressed in some prostate malignancies [124]. Dutasteride results in an overall 90-94% suppression of DHT compared with 67-76% by finasteride [125]. Retrospective analysis of data from three trials designed to study dutasteride efficacy and safety in patients with benign prostatic hyperplasia, suggest that prostate cancer incidence was reduced by dutasteride [126]. The REDUCE trial is a four year prospective, multi-centre, randomised, placebo-controlled phase III trial investigating dutasteride in men with an increased risk of prostate cancer. 8121 men, 50-75 years old received 0.5 mg dutasteride or placebo. Their PSA was 2.5-10 ng/ml (50-60 years) or 3.0-10 ng/ml (60-75 years), with free PSA 25% or less. A 6-12 core prostate biopsy showing no evidence of malignancy or high grade prostatic intraepithelial neoplasia must have been performed within six months prior to recruitment [127]. Men with international prostate symptom scores >25 or prostate volumes >80 ml are excluded in order to reduce the number of men requiring surgery for benign prostatic hyperplasia. The primary endpoints were the histology of prostate biopsies performed at 24 and 48 months.

The results of this trial have not yet been published however, preliminary results presented at the American Urology Association meeting in 2009 claimed a reduction in prostate cancer of 23% in the treated group. As in the PCPT, more high grade cancers were found in the dutasteride group 0.9% v 0.6% the fact that this did not reach statistical significance may reflect the trial was less than half the size of the PCPT. The most common side effects reported were erectile dysfunction and decreased libido.

1.8.3 Selenium and vitamin E as prostate cancer preventive agents

Selenium is an anti-oxidant micronutrient. Data arising from the Netherlands Cohort Study suggests an inverse relationship between selenium and prostate cancer [128]. Selenium content was measured in toenail clippings as a marker of dietary intake. 58,279 men were followed up for a mean of 6.3 years, 540 incident prostate carcinoma cases were identified. Prostate cancer incidence was reduced by 31% in the highest quintile for selenium content, although this effect was most apparent in ex-smokers and smokers rather than men who had never smoked. In a similar case-control study within the Health Professionals Follow-up Study, selenium content of toenails was negatively associated with the risk of advanced prostate cancer; comparing the highest and lowest quintiles of selenium content, the odds ratio was 0.49 [129]. The Nutritional Prevention of Cancer Study was an interventional trial that used 200 μg/day selenium supplements administered over an average of 7.5 years to 927 men; the relative risk of prostate cancer diagnosis was 0.51 [130]. However, there are a number of weaknesses in this trial: many more men in the control group underwent prostate biopsy; this could not be explained by differences in PSA levels. Prostate cancer reduction was confined to those men who were selenium deficient prior to supplementation and only those men with a baseline PSA ≤4ng/ml. Finally, the proportion of subjects reporting previous cancers was 35% lower in the selenium group than in the placebo group [130, 131].

α-Tocopherol is the most powerful anti-oxidant of the tocopherols collectively known as vitamin E. There is evidence that vitamin E protects against death from prostate cancer. A case-control study of 145 men found that prostate cancer risk was reduced in those with the highest serum α-tocopherol concentrations. The odds ratio was 0.65 for the highest versus the lowest quintiles [132]. A 17 year follow up study found that low levels of vitamin E were associated with higher rates of prostate cancer deaths in smokers (RR=8.3) [133]. A cohort study of 47,780 men (Health Professional Follow-up Study) showed that smokers were at increased risk of prostate cancer and that this increased risk was abolished by vitamin E supplementation [134]. The most powerful assessment, so far, of the effect of vitamin E on prostate cancer came in the Alpha-Tocopherol, Beta Carotene Cancer Prevention Trial [135]. This interventional trial was primarily designed to examine whether these agents could reduce lung cancer in smokers, prostate cancer incidence was a secondary endpoint. The study enrolled 29,133 male smokers 50-69 years. For those receiving 50 mg α-tocopheryl acetate supplements over a mean period of 6.1 years the prostate cancer incidence fell to 11.7 per 100,000 compared with 17.8 per 100,000 in those not receiving α-tocopherol. However, 99% of the participants were current or past smokers and it is, therefore, unknown if these results are applicable to non-smokers[135].

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) is a phase III, randomised, placebo-controlled trial of prostate cancer prevention that has recently reported [136]. The SELECT study had a 2x2 factorial design where the study population is divided into three treatment groups (200 μg selenium, 400 mg

α-tocopherol and both agents) and a placebo group to assess prostate cancer prevention [136]. 32,400 men, ≥50 years for African Americans and ≥55years for Caucasians, with normal DRE and PSA ≤4 ng/ml have already been recruited to undergo a minimum of seven years and a maximum of 12 years intervention [137]. The primary end-point of prostate cancer diagnosis was assessed by annual DRE and PSA measurement and appropriate biopsies, without an end of trial biopsy. Neither selenium nor vitamin E either alone or in combination prevented prostate cancer incidence [138].

1.8.4 Potential agents of prostate cancer prevention in preclinical assessment

Genistein is an isoflavone found in soybeans. Soy isoflavones display oestrogen-like activity which may explain why genistein inhibits proliferation and induces apoptosis in prostate cancer cells *in vitro* [139]. A phase II clinical trial involved 100 mg of soy isoflavone taken twice daily by 41 men with known prostate cancer, for 3-6 months. Overall, there was a decrease in the PSA rise, with PSA stabilization occurring in 83% of patients with hormone sensitive disease and in 35% of hormone refractory cases [140]. In a similar trial, 59 men with early prostate cancer were treated for 12 weeks with 60 mg of soy isoflavones, PSA was reduced or stable in 69% of treated men compared with 55% of controls [141].

Lycopene is the main red carotenoid in tomatoes and is a powerful anti-oxidant whose consumption is associated with prostate cancer risk reduction. In a case-control study of 578 cases, the highest quintile for plasma lycopene

concentration compare to the lowest, had an odds ratio of 0.75 for prostate cancer diagnosis during the 13 years follow-up [142]. The prospective Health Professionals Follow-up study has correlated lycopene consumption with prostate cancer in 47,365 men who developed 2,481 cases over seven years. The men in the highest decile for lycopene consumption, had a relative risk of 0.78 compared with the lowest decile [143].

Non-steroidal anti-inflammatory drugs (NSAIDs) exert their effect by inhibiting constitutively expressed COX-1 and especially the inducible COX-2 isoform thought to play a role in inflammation and malignant proliferation.

Epidemiological evidence has suggested that this class of drugs possess prostate cancer preventive activity. A case control study of 417 cases suggested NSAID use was associated with an odds ratio of 0.34 for developing prostate cancer [144]. In a large cohort study of 90,100 men over nine years, in the Kaiser Permanente Medical Care Program, 2,466 cases of prostate cancer were identified. Aspirin use was associated with a relative risk of prostate cancer of 0.76 [145]. A Mayo Clinic cohort study of 1,362 men over a median of 66 months identified 91 cases of prostate cancer. NSAID usage was associated with a relative risk of developing prostate cancer of 0.45 [146].

An animal model of prostate carcinogenesis, the TRAMP mouse, has demonstrated reduced tumour progression and reduced metastases following intervention with celecoxib [84].

In a pilot study, celecoxib was administered to 12 patients with biochemically relapsed prostate carcinoma after radiotherapy or surgery. Five men

had a decline in their PSA levels, three had a PSA stabilisation and three experienced an increase in PSA doubling time [147]. A trial involving 96 men after prostatectomy for cancer compared the sulindac metabolite, exisulind, with placebo. The exisulind group experienced longer PSA doubling times and delayed biochemical recurrence [148].

The side-effect profile of NSAIDs includes gastrointestinal haemorrhage, perforation and renal complications, which may limit the suitability of these agents in cancer prevention. It was thought that COX-2 specific inhibitors may represent a safer alternative to traditional NSAIDS, however, recent concern over the cardiovascular risks associated with COX-2 inhibitors have thrown this into doubt [149].

Omega-3 (n-3) fatty acids are essential unsaturated fatty acids found in fish oils. Studies have found a significant protective effect from fish or n-3 fatty acid consumption. A Japanese case-control study in 1985 of 100 cases of prostate cancer found a relative risk of 0.45 in men who consumed fish often, compared with those who never ate fish [150]. The Swedish twin registry cohort study followed 6,272 men for over 20 years identifying 466 prostate cancer cases. Men who reported fish to be a large part of their diet were at a relative risk of prostate cancer of 0.43 compared with those who never or seldom consumed fish [151]. In The Health Professionals Follow-up Study 47,882 men were followed for 12 years, although no significant protective effect was demonstrated overall, high versus low quartiles for fish consumption were at a relative risk of 0.59 of developing metastatic prostate cancer [152]. There is only one study to have demonstrated a

significant link directly between n-3 fatty acids and prostate cancer risk reduction. A case control study from New Zealand investigating 317 prostate cancer cases measured erythrocyte phosphatidylcholine levels of EPA and DHA. High EPA and DHA levels in erythrocytes were associated with a relative risk of developing prostate cancer of 0.59 and 0.62 respectively [18]. Evidence from interventional trials using fish oils has yet to be forthcoming

The major source of vitamin D is from the skin where the precursor 7-dehydrocholesterol is converted to vitamin D_3 through the action of UV radiation. A study of the counties within the USA found a significant South-North trend for prostate cancer incidence that was suggested to be due to the protective effect of UV exposure [19]. A case control study using 149 prostate cancer cases identified in the Helsinki Heart Study population compared serum levels of 25-hydroxyvitamin D (25(OH)D) in cases and matched controls. Men with 25(OH)D concentrations below the median had an adjusted relative risk of 1.7 compared with those with above median 25(OH)D concentrations [153].

In a small clinical trial of seven patients with recurrence following radical surgery or radiotherapy for prostate cancer, calcitriol ($1,25(OH)_2$ vitamin D_3) was administered for six to 15 months. Calcitriol therapy significantly increased the PSA doubling time in six of seven patients [154]. However, vitamin D supplementation was associated with dose-dependent hypercalciuria that limited the dose administered. In an effort to avoid troublesome calciuria, 22 patients with rising PSA following radical surgery or radiotherapy received a low calcium diet and weekly calcitriol for a median of ten months. PSA doubling time significantly

increased following treatment without associated hypercalcaemia [155]. Vitamin D analogues with reduced hypercalcaemia or hypercalciuria are currently being investigated.

1.9 Potential mechanisms and molecular biomarkers of prostate cancer chemoprevention by tea polyphenols

1.9.1 The role of biomarkers

To assess cancer chemopreventive agents in human trials, directly, using cancer incidence as an endpoint requires large numbers of participants in studies that last many years. In order to rapidly investigate if agents have sufficient potential to warrant such trials, biomarkers are used as surrogate endpoints in preliminary human studies. Carcinogenesis is a multistage process. Biomarkers of the efficacy of cancer chemoprevention are molecular changes whose presence correlates with an early stage of this carcinogenic process. In addition, biomarkers of carcinoma (tumour markers) are used in clinical settings to monitor disease progression. An understanding of the molecular mechanism by which tea polyphenols exert their inhibitory effect upon carcinogenesis can be used to guide a choice of putative biomarkers.

Unfortunately, numerous putative mechanisms of prostate cancer chemoprevention by tea polyphenols have been proposed and the hierarchy of importance of these molecular systems has yet to be elucidated. There have been many proposed histological, serological, tissue based and imaging biomarkers of prostate carcinogenesis [156]. In this study M_1G, IGF-1, IGFBP-3, components of the IGF signal transduction pathway (Erk and Akt), VEGF and PSA are all

investigated as possible biomarkers of prostate cancer prevention by tea polyphenols.

1.9.2 Malondialdehyde-deoxyguanosine DNA adducts (M_1G) as a prostate carcinogenesis biomarker

Tea polyphenols are powerful anti-oxidants. As oxidative DNA damage is known to be mutagenic and carcinogenic it is possible that anti-oxidation is a mechanism of cancer prevention [157]. EGCG is the most effective among the catechins in reacting with most reactive oxygen species. For example, *in vitro*, models such as human low density lipoprotein (LDL) have demonstrated that EGCG inhibits lipid oxidation [158]. Evidence from cell culture demonstrates this effect in cell lines, for example, EGCG inhibited iron induced cell membrane lipid oxidation in Jurkat T cells [159]. In human volunteers, a recent review of seven studies shows a consistent increase in plasma anti-oxidant capacity following oral consumption of green tea [160]. However the reduction in oxidative stress, following EGCG administration, measured by lipid peroxidation, was only present in rats when older animals were studied [161, 162]. The absence of antioxidant effect in young rats may suggest that EGCG only acts as an antioxidant in the presence of high oxidative stress. This reduction of excessive oxidative stress may inhibit carcinogenesis by reducing oxidative DNA damage, supplementing the diets of heavy smokers with 4 cups daily of green tea for four months reduced urinary DNA adduct (8-hydroxydeoxy-2'-deoxyguanosine) levels by 31% [163].

Similarly, theaflavins, *in vitro*, inhibit membrane lipid peroxidation, LDL oxidation and peroxide induced DNA cleavage [164, 165]. Inhibition of LDL oxidation by theaflavins has been confirmed in cell culture using macrophages [166]. However, an *ex vivo* study, using plasma from human volunteers following oral administration of black tea, failed to demonstrate an increase in anti-oxidant activity [167].

Conversely, there is evidence that the cytotoxic actions of EGCG may be related to their pro-oxidant capacity. EGCG-induced apoptosis of bronchial carcinoma cell lines was accompanied by the accumulation of H_2O_2 and apoptosis was abolished by the addition of catalase. Interestingly, catalase has no effect on theaflavin-induced apoptosis, implying the possibility of different polyphenols exerting anti-proliferative effects via different mechanisms [112, 168].

Malondialdehyde (MDA) is a product of endogenous lipid peroxidation, which forms adducts with nucleic acid bases the major example being guanosine, forming M_1G [169]. MDA can also be produced by arachidonic acid metabolism [170]. In addition, M_1G is generated by direct oxidative DNA damage [171]. MDA is mutagenic to human cells [172] and has demonstrated carcinogenic potential in mice and rats [157]. The molecular basis for this carcinogenesis is likely to be that M_1G induces DNA point substitutions (mostly G → T and G → A) if it is not corrected by excision-repair prior to DNA replication [173].

M_1G, as a measure of oxidative DNA damage, may represent a biomarker of carcinogenesis in the prostate. Oxidative DNA damage in prostate tissue, using 8-hydroxypurine lesions as the biomarker, has been demonstrated to be age-

related, and more prevalent in prostate carcinoma than in normal tissue [174, 175]. M_1G itself was shown to be more prevalent in normal breast tissue from women with breast carcinoma compared with disease-free controls [176]. There is also evidence that this biomarker may be amenable to modification using dietary intervention; orally administered selenium has been shown, in dogs, to reduce oxidative DNA damage in the prostate [177]. *In vitro* and *in vivo* using a xenograft mouse model, M_1G has been shown to be reduced in LNCaP cells following cancer inhibition by androgen manipulation with flutamide [178].

1.9.3 Insulin-like growth factor (IGF)-1 and IGF binding protein (IGFBP)-3 as prostate carcinogenesis biomarkers

The IGF axis includes two peptide growth factors (IGF-1 and 2) and six IGF binding proteins (IGFBP1-6). IGF is synthesized, under the influence of growth hormone, in the viscera, especially the liver and including the prostate. IGF acts via transmembrane receptors. It has mitogenic and anti-apoptotic effects on a number of target tissues [179]. IGF-1 stimulates growth of normal prostate epithelial cells in primary cell culture [180]. IGFBP-3 is the main circulating IGF-1 binding protein. IGFBP-3 principally acts as an inhibitor of IGF-1 function by sequestering the growth factor [179].

In DU145 prostate cancer cell culture, IGF-1 stimulates expression of PSA and the nuclear androgen receptor [181]. Histological examination of IGF-$1^{-/-}$ null mice has shown IGF-1 to be required for normal prostate development [182].

TRAMP mice that develop an SV40 T antigen transgene induced prostate cancer have raised IGF-1 (approximately 1.5 fold) compared with control mice [183]. In human studies, serum IGF-1 concentration is positively correlated with the risk of subsequently developing prostate carcinoma [184, 185], a higher IGF-1:IGFBP-3 ratio being associated with both higher prostate cancer risk and a poorer prostate cancer prognosis [186, 187]. IGFBP-3 can induce apoptosis in PC-3 prostate carcinoma cultured cells [188] and is a substrate for PSA [189], the cleaving of which by rising levels of PSA in carcinogenesis leads to increased bioavailalbility of IGF-1 [190]. One human study has suggested a negative correlation between plasma IGFBP-3 concentration and risk of prostate carcinogenesis [184].

EGCG appears to target the IGF axis. The IGF-induced, pro-proliferative expression of phosphatidylinositol-3-kinase and phosphorylation of Akt are inhibited by EGCG 10-40 μg/ml and theaflavins 10-50 μg/ml, in prostate cancer cell lines [191, 192]. In PC-3 prostate carcinoma cells p-Erk1/2, the downstream target of IGF-1, was shown to be inhibited by theaflavins at concentration of 25-50 μg/ml [110]. The impressive anti-carcinogenesis seen in the TRAMP mouse following green tea polyphenol administration is associated with the IGF-1/IGFBP-3 ratio, phosphatidylinositol 3'kinase, p-Akt, p-Erk1/2, VEGF, urokinase, plasminogen activator and matrix metalloproteinases 2 and 9 all being suppressed [193]. Subsequent immunohistochemical analysis of mouse dorsolateral prostate shows decreased IGF-1 levels [194]. In addition, EGCG appears to directly inhibit IGF receptor kinase activity [195].

Serum IGF-1 and IGF-1:IGFBP-3 ratio may therefore represent a biomarker of prostate carcinogenesis that is easily sampled clinically from a specimen of venous blood.

1.9.4 Signal transduction molecules downstream of the IGF-1 receptor (IGF-1R) as prostate carcinogenesis biomarkers

The mitogenic and anti-apoptotic effects of IGF-1 upon cancer cells depend on the transduction of this signal to elicit changes in gene expression. This transduction involves multiple intracellular phosphorylation events. Detection of the activation/suppression of this pathway can therefore be used as a biomarker of cancer prevention.

Activation of IGF-1R by IGF-1 leads to phosphorylation of insulin receptor substrate (IRS)-1 by IGF-1R. Phosphoinositide 3-kinase (PI3K) is activated by the binding of IRS-1 leading to an increase in phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 binds and consequently activates Akt by phosphorylation (p-Akt). Akt inactivates by phosphorylation several pro-apoptotic proteins such as Bad [196].

Simultaneously, autophosphorylation of IGF-1R upon activation by IGF-1 leads to recruitment of Shc which itself becomes phosphorylated. Activated Shc then binds Grb2 leading to activation of Erk1/2 by phosphorylation (p-Erk1/2) [197]. Erk1/2 is a mitogen-activated (MAP) kinase which activates, by phosphorylation, many pro-proliferative transcription factors such as c-Fos, c-Myc and Elk-1 [198].

Erk1/2 is known to be constitutively active in both androgen independent DU145 and androgen dependent LNCaP human prostate cancer cells [199]. Akt, however, is constitutively activated in LNCaP cells whereas DU145 cells show 4-5 fold less activation [200]. Reductions in the expression of both p-Akt and p-Erk1/2 have been demonstrated in the prostate of TRAMP mice who received 0.1% green tea polyphenols in their drinking water. It has been suggested that this change in expression pattern is critical for the inhibition of prostate carcinogenesis seen in the mice that received the polyphenols [193]. Immunohistochemical analysis of human prostate cancer specimen has demonstrated raised p-Akt in higher grade prostate cancer [201].

It is proposed that the mechanism by which tea polyphenols may inhibit such signal transduction agents as p-Erk and p-Akt is by the inhibition of transmembrane receptors. In human colon cancer cells, EGCG inhibits activation of receptors EGFR and HER2 which are known to act via the MAPK pathway [202].

1.9.5 Apoptosis and cleaved caspase-3 as an indicator of apoptosis induction

The induction of apoptosis within *in vitro* prostate cancer cells lines following treatment with tea polyphenols has been demonstrated [104-107, 203]. Induction of apoptosis is controlled by alterations in the balance of pro- and anti-apoptotic signalling molecules such as Bcl-2 and Bax [204]. Caspases are cysteine proteases, caspase-3 is a member of the CED-3 subfamily of caspases and is recognised as a central effector of apoptosis [205]. It is responsible for proteolytic

cleavage of a large number of substrates including PARP [206], DNA-PK [207], u1-70kDa [207], heteronuclear ribonucleoproteins C1 and C2 [208] and Rb [209].

EGCG at 10-80 μg/ml decreases Bcl-2 levels and increases Bax, caspase 8,9 and 3 in LNCaP cells [210]. Theaflavins have been shown to inhibit Bcl-2 family proteins *in vitro* [211]. Activation of caspase-3 has also been demonstrated in LNCaP and DU145 cells following exposure to genistein [212] and amygdaline [213]. In LNCaP cells theaflavins were shown to suppress Bcl-2 at the same time as upregulation of p53 and Bax resulting in activation of caspases 3 and 9 [110]. Caspase 3 deficient cancer cells do not undergo polyphenol-induced apoptosis suggesting that this apoptosis is a caspase 3 executed mechanism [214]. In a mouse xenograft model implanted with CWR22Rβ1 cells both EGCG and theaflavins, provided orally, resulted in inhibition of the tumours accompanied by reductions in PSA and Bcl-2 with a corresponding rise in Bax protein [215].

The effect of EGCG on many cell cycle regulation molecules has been characterized. EGCG has been shown to inhibit Cdk2 and Cdk4 leading to cell cycle arrest in human breast carcinoma cells [216]. Exposure of prostate cancer cell lines to EGCG at concentrations 10-80 μg/ml, increases p53 and WAF1/p21 expression, and reduces cyclin expression. These changes have been implicated in cell cycle arrest and subsequent induction of apoptosis [107, 210, 217].

1.9.6 Vascular endothelial growth factor (VEGF)

VEGF is a growth factor that plays a central role in the angiogenesis facilitating physiological and pathological tissue growth including carcinogenesis. VEGF has been found to be expressed in many cancers including colorectal, lung, breast and prostate[218]. Prostate cancers cells have been shown to over-express VEGF compared with benign prostate cells [219]. VEGF functions are mediated through the transmembrane receptor VEGFR, VEGFR-2 is the subgroup specifically implicated in angiogenesis. VEGFR-2 acts via the Akt pathway [220] to activate MAP kinases such as Erk1/2 [221].

The anti-angiogenic effect of EGCG was first suggested by Cao and Cao in each of three models of angiogenesis; bovine capillary endothelial cells stimulated with FGF2 *in vitro*; a chick chorioallantoic membrane assay, and ; VEGF induced neovascularisation of mouse cornea [222]. Inhibition of angiogenesis by EGCG has been seen in human colon cell line HT29 *in vitro* and in xenografts. EGCG 4.5-22.5 μg/ml increased endothelial apoptosis and decreased microvascular density [223].

There are a number of putative mechanisms responsible for anti-angiogenesis by EGCG. In the *in vitro* model, human microvascular endothelial cells in collagen gel matrix, anti-angiogenesis by EGCG may be partly due to downregulation of VE cadherin and Akt activation [224]. VEGF dependent phosphorylation of VEGFR-2 was inhibited by EGCG in an assay using bovine aortic endothelial cells [225]. EGCG inhibits IL-8 production and angiogenic tube

formation by human microvascular endothelial cells in a gel matrix assay [226]. Reductions in VEGF have been demonstrated *in vivo* in athymic mouse model grafted with CWR22Rβ1, following administration of EGCG and theaflavins [215]. In prostate carcinogenesis within the TRAMP mouse it has been proposed that IGF-1 dependent expression of VEGF may be responsible for the neovascularisation seen during tumour growth [190]. Downregulation of VEGF is seen in the TRAMP mouse model following green tea polyphenol administration [193].

1.9.7 Prostate specific antigen (PSA) as a biomarker of prostate cancer disease progression

PSA is a protease found in human semen and to a lesser extent in serum. Its main function is to lyse the seminal coagulum. PSA is a member of the kallikrein gene family and the PSA gene is now termed KLK3 [227]. It has widespread use in clinical practice as a tumour marker, as its expression is specific to the prostate. Serum PSA concentration correlates with the risk of current prostate cancer and its expression is reduced by successful treatment of prostate cancer [228].

PSA concentration in men 40-60 years old, without prostate carcinoma, has been correlated with their risk of developing future disease over the next 10-15years. The biological basis of this increase risk is unknown but it may reflect higher androgen exposure; inflammation and consequent oxidative stress; or it

may itself be causal [229]. Prostate inflammation due to sexually transmitted infections has been shown to increase PSA and prostate cancer risk irrespective of the pathogen involved suggesting that inflammation may be carcinogenic [230]. PSA leaking into serum due to loss of the usual unidirectional secretion by prostate epithelium cells into prostate ducts reflects inflammation; however PSA may contribute to maintaining the inflammatory process by cleaving binding proteins such as IGFBP-3 thus increasing local concentrations of growth factor IGF-1 [231]. In addition it has been demonstrated that PSA stimulates the generation of reactive oxygen species in PC-3 and DU145 cells [232].

In a chemopreventive setting it is proposed that tea polyphenols may oppose the oxidative stress associated with inflammation, and maintained by PSA, and therefore reduce the secreted PSA.

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1.9.8 Other putative mechanisms of prostate cancer chemoprevention by tea polyphenols

The expression of ornithine decarboxylase (ODC) is androgen controlled in the prostate, and is the rate-limiting step of the polyamine biosynthetic pathway. Inhibition of ODC may therefore have an anti-proliferative effect. Testosterone-induced expression of ODC is significantly inhibited by green tea polyphenols 10-80 µg/ml in cultured LNCaP cells and in rodent models [233].

Similarly, chemical inhibitors of the rate limiting synthetic enzyme, fatty acid synthase (FAS), inhibit growth and induce apoptosis. EGCG at 18-68 µg/ml inhibits FAS activity in LNCaP cells [234].

Microarray analysis of TRAMP mouse prostate has identified the expression of minichromosome maintenance protein 7 (MCM7) as being suppressed by green tea catechins, MCM7 having been previously identified as being associated with the aggressive phenotype of prostate cancer [235].

Androgens are potent stimulators of physiological and pathological prostate proliferation. Androgen receptor expression is inhibited in LNCaP cells by EGCG 4.5-9 µg/ml and theaflavin 11-22 µg/ml [109]. 5α reductase, responsible for activation of the androgen testosterone, is inhibited, *in vitro*, by TFdiG 17 µg/ml [236].

Cyclooxygenase (COX)-2 over-expression has been implicated in carcinogenesis, EGCG 4.5-45 µg/ml inhibits expression of COX-2 in LNCaP and PC-3 cells leaving COX-1 expression unaltered [237].

Matrix metalloproteinases (MMP) are known to be involved in carcinoma invasion and metastasis. MMP-2 activation is inhibited by EGCG 4.5-22.5 µg/ml in LNCaP cells [238]. Expression of prostate specific antigen (PSA), a clinical tumour marker and an activator of MMP-2 is inhibited in LNCaP cells by EGCG 4.5-9 µg/ml and theaflavins 11-22 µg/ml [109]. Urokinase is another proteolytic enzyme linked with invasive tumour behaviour that can be inhibited by EGCG *in vitro* [239].

1.10 Aims

Green tea polyphenols have been considered and tested by several groups as putative prostate cancer chemopreventive agents, whilst information of the putative prostate cancer preventive efficacy of black tea polyphenols is rare. The overall aim of the work described here was to explore the feasibility and suitability of developing green tea and/or black tea polyphenols as putative prostate cancer chemopreventive interventions. In order to achieve this aim the following experimental objectives were addressed.

Experiments with human prostate cancer cells *in vitro* were performed in which they were cultured in media to which tea polyphenols, catechins or theaflavins, had been added. Other groups have identified that tea polyphenols inhibit the proliferation and increase the apoptosis of prostate cancer cells *in vitro*. It was intended to identify molecular biomarkers of the cancer inhibitory effect, see section 1.7.2. Certain specific biomarkers chosen for analysis: the oxidative DNA adduct M_1G; signal transduction molecule p-Erk; and, the apoptosis effector molecule caspase 3, see section 1.9.

An intervention trial was performed in a population of TRAMP mice to determine whether catechins or theaflavins in the animals' drinking water inhibits prostate tumour development and metastasis *in vivo*, and whether any anti-carcinogenesis seen was associated with changes in the putative prostate chemoprevention biomarkers: prostate M_1G, prostate p-Erk and caspase-3, and serum IGF-1.

A human intervention trial was performed to investigate the extent to which any biomarker changes seen in the TRAMP mouse model, following oral tea polyphenol administration, could be found in human tissue or plasma.

Analyses were also performed determine whether catechins, theaflavins or their metabolites were detectable in the prostate tissue or serum of TRAMP mice following oral tea polyphenol administration. Similar investigations were performed on the prostate tissue, serum and urine of human volunteers who had consumed tea polyphenols.

2 Materials and Methods

2.1 Cell culture

2.1.1 Maintenance of cell lines

Human prostate carcinoma cell lines DU145 [66] and LNCaP [64] were maintained at 37˚C, in humidified atmosphere containing 5% CO_2 in modified MEM media and modified RPMI 1640 media respectively, to a confluence of approximately 75% in large Nunc filter cap flasks. Cell lines were from the American Type Culture Collection (ATCC) and once resuscitated from storage at -80˚C, cells did not exceed passage 30 throughout any series of experiments.

2.1.2 Cell passage

Cells were grown to approximately 75% confluence. Media was removed by aspiration. The flasks were rinsed twice by adding 10 ml autoclaved phosphate buffered saline (PBS) at 37˚C which was removed by aspiration. 5 ml of trypsin EDTA at 37˚C was then added. The flasks were incubated at 37˚C for five minutes. Cell detachment was achieved by agitation and confirmed by microscopy. 10 ml of appropriate media at 37˚C was added and the resulting cell suspension harvested. The cells were pelleted by centrifugation at 1000g for three minutes. The supernatant was removed by aspiration and the pellet resuspended in 5-10 ml of appropriate culture medium. This cell suspension was then used to

seed new flasks. For long term storage in liquid nitrogen, pellets of cells were resuspended in a mixture of 50% FBS, 40% culture media and 10% DMSO. The resulting suspension was stored at -80˚C for 24 hours before being transferred into liquid nitrogen.

2.1.3 Calculating cell density of suspension

DU145 and LNCaP cell suspensions were prepared and the cell density measured using a Beckman Coulter Z2™ COULTER COUNTER® Cell and Particle Counter as follows. 20 µl of cell suspension was diluted in 10 ml of ISOTON II. 0.5 ml of this diluted suspension was sampled by the cell counter and a count performed using size parameters 12.9-20 µm for DU145 cells and 10.5-20 µm for LNCaP cells. This was repeated and the mean of the two counts was calculated, the result representing the cell count of 1 µl of original suspension.

2.1.4 Incubation of DU145 and LNCaP cells with tea polyphenol solutions

For experiments measuring M_1G in prostate cancer cells in culture following incubation with tea polyphenols, 96 medium sized Nunc filter cap flasks were each seeded with one million cells, 48 with LNCaP cells, 48 with DU145 cells. For experiments generating protein lysates from prostate cancer cells in culture following incubation with tea polyphenols, 64 petri dishes were each seeded with 10^5 cells, 32 with LNCaP cells, 32 with DU145 cells. These cells were incubated in

appropriate media at 37˚C, humidified, 5% CO_2 for 24 hours prior to aspiration of the media and dosing with polyphenols.

For dosing cell culture media, stock solutions of 1 mg/ml Greenselect and 0.5 mg/ml theaflavins were prepared in distilled water, on the day of use. These solutions were sterilized using a 0.2 μm syringe filter immediately prior to use. These flasks or dishes of LNCaP or DU145 cells were then incubated in appropriate media containing 0, 3.125 μg/ml, 12.5 μg/ml or 25 μg/ml, theaflavins or catechins, for 24 or 48 hours.

2.2 Assay of M_1G adducts in DNA

2.2.1 DNA extraction from cells

Cells were harvested, pelleted and resuspended in 10 ml of appropriate culture media using cell passage protocol. This suspension was centrifuged at 1000 g for three minutes, the supernatant discarded and the pellet resuspended in ice cold PBS. This suspension was centrifuged at 1000 g for three minutes, the supernatant discarded and the pellet resuspended in 10 ml of ice cold buffer (20% PBS, 20% C1 lysis buffer in distilled water). This suspension was incubated on ice for ten minutes and then centrifuged at 1300 g for 15 minutes at 4˚C. The supernatant was discarded and the pellet resuspended in 4 ml of ice cold buffer (25% C1 lysis buffer in distilled water). The suspension was vortexed for 30 seconds and centrifuged at 1300 g for 15 minutes at 4˚C. The supernatant was discarded and the pellet stored at -20˚C overnight.

The pellet was resuspended in 5 ml G2 buffer and vortexed for 30 seconds. 95 µl proteinase K (20 mg/ml) and 16 µl RNase A (100 µg/ml) was added and the mixture incubated for two hours at 37˚C in an oscillating water bath.

A genomic-tip was equilibrated with 4 ml QBT buffer. The mixture was centrifuged at 1300 g for three minutes and the supernatant added to the tip and allowed to drip through by gravity. The tip was washed through twice with 5 ml QC buffer and then eluted with 5 ml QF buffer at 55˚C into a tube containing 3.5 ml isopropanol, using gravity flow.

The tube was inverted 20 times and centrifuged at 4000 g for 20 minutes at 4˚C. The supernatant was discarded and the pelleted washed with 1 ml of 70% ethanol. The pellet was transferred to a 2 ml tube and centrifuged at 5000 g for ten minutes at 4˚C. The supernatant was discarded and the pellet resuspended in 200 µl distilled water. DNA solutions were stored at -80˚C.

2.2.2 DNA extraction from tissue

Tissue fragments of approximately 75 mg stored at -80˚C were thawed. 9.5 ml of G2 buffer containing 200 µg/ml RNase A was added and the tissue mechanically homogenised using an electric pestle. To the homogenate 0.5 ml proteinase K was added and mixed by vortexing. This mixture was then incubated at 50˚C for two hours. The sample was then centrifuged at 4000 g for ten minutes at 4˚C and the supernatant reserved. A Qiagen genomic-tip 100/G was equilibrated with 4 ml QBT buffer and allowed to run dry by gravity flow. The sample was added to the genomic-tip and allowed to run through by gravity flow. The tip was washed twice with 7.5 ml QC buffer, again allowing the buffer to flow through by gravity. The DNA was eluted by adding 5 ml QF buffer at 50˚C and allowed to drip into 3.5 ml isopropanol at room temperature. The precipitated DNA was pelleted by centrifugation at 4000 g for 15 minutes at 4˚C, the supernatant was discarded and the pellet washed in 1 ml 70% ethanol in water. The pellet was transferred to a 2 ml tube and centrifuged at 5000 g for ten minutes at 4˚C. The

supernatant was discarded and the pellet resuspended in 200 µl distilled water. DNA solutions were stored at -80˚C.

2.2.3 Determining DNA concentration of extracts by spectrophotometry

DNA quantification was performed in a GeneQuant spectrophotometer. The coefficient for absorbance (260 nm and 280 nm) was set to zero using distilled water in a quartz cuvette. 2 µl of DNA extract was added to 98 µl of distilled water and vortexed. 100 µl of this solution was added to the cuvette. DNA concentration was calculated assuming dsDNA 50 µg/ml has an absorbance of 1.0 at 260 nm in a 10 mm pathlength cell. The ratio of absorbance at 260 nm and 280 nm was also recorded. Ratios of between 1.3 and 1.9 were deemed acceptable.

2.2.4 Immunoslot-blot for determination of M_1G adduct concentration

The method originally described by Leuratti et al. [240] and modified by Singh et al. [241] was used. A series of nine standards were prepared by diluting MDA treated calf thymus DNA (10 fmol/µg) with control calf thymus (0 fmol/µg). The standards each containing 3.5 µg DNA contained 0, 0.4, 1, 2, 3, 5, 6, 8 and 10 fmol/µg M_1G respectively. Experimental samples to be analysed were also pipetted into 3.5 µg aliquots. All standards and samples were adjusted to a final volume of 100 µl with KP buffer. 150 µl of PBS was added and DNA was sonicated for 20 minutes in an ultrasonic bath. DNA was then denatured at 100˚C

in a heating block for five minutes before being cooled on ice for ten minutes. 250 µl of 2 M ammonium acetate was added before vortexing and centrifugation at 13,000 g for 30 seconds.

Two shaped blotting papers were soaked in 1 M ammonium acetate before being placed in a Minifold II slot blotting apparatus. A shaped nitrocellulose membrane was soaked in water followed by 1 M ammonium acetate before being added to the blotting apparatus. Suction was applied to the apparatus and the DNA solutions loaded in triplicate, 143 µl (1 µg) per slot. The wells were allowed to run dry before 200 µl of 1 M ammonium acetate was added and allowed to run dry. The filter was then baked at 80˚C for 90 minutes.

The filter was bathed on a rocker in PBS-T, 5% milk powder for one hour, at room temperature. The filter was then washed twice in PBS-T for one minute. The filter was then incubated with 0.25 µg M_1G primary antibody in 40 ml PBS-T, 0.5% milk powder on a rocker for two hours at room temperature followed by overnight at 4˚C. The filter was washed with 50 ml PBS-T, once for one minute and then twice for five minutes. It was then incubated with 8.8 µg M_1G secondary antibody in 32 ml PBS-T, on a rocker at room temperature for two hours. The filter was washed in PBS-T for 15 minutes followed by twice for five minutes. Finally, the filter was incubated in 8 ml Supersignal® West Dura extended duration substrate for five minutes on a rocker at room temperature.

The chemiluminescence image was captured using a GeneGnome bioimaging system. The intensity of the signal from each slot blot was recorded

using GeneSnap and GeneTools image analysis software. The filter was washed in PBS on a rocker at room temperature for one hour.

The filter was then incubated in 50 ml PBS-PI at room temperature, on a rocker in the dark for two hours. The filter was then was washed in PBS for one hour before been transilluminated with UV light and the image captured with GeneGenius imaging system. The intensity of each slot blot was recorded using GeneSnap and GeneTools image analysis software.

2.2.5 Experimental design for analysis of the effect of tea polyphenols on M_1G concentrations in the DNA of DU145 and LNCaP cells

96 medium sized Nunc filter cap flasks were each seeded with one million cells, 48 with LNCaP cells, 48 with DU145 cells. These cells were incubated in appropriate media, see section 2.1.1, at 37˚C, in humidified atmosphere, 5% CO_2 for 24 hours prior to aspiration of the media and dosing with polyphenols.

For dosing cell culture media, stock solutions of 1 mg/ml Greenselect and 0.5 mg/ml theaflavins were prepared in distilled water, on the day of use. These solutions were sterilized using a 0.2 µm syringe filter immediately prior to use. The flasks cells were then incubated in appropriate media with polyphenols added to a final concentration of 0, 3.125 µg/ml, 12.5 µg/ml or 25 µg/ml, theaflavins or catechins. The flasks were then incubated, as above, for 24 or 48 hours. Each permutation of conditions was performed in triplicate. DNA was extracted from the

cells according to the method in section 2.2.1 and M_1G concentrations measured according to section 2.2.4.

2.2.6 Experimental design for analysis of M_1G concentrations in prostate tissue of TRAMP mice receiving theaflavins or catechins compared with controls

TRAMP mice were separated into three treatment groups and provided with drinking water containing 0.05% black tea theaflavins, 0.05% green tea catechins or no polyphenols as described in section 2.5.2. DNA was extracted from the dissected prostate tissue of 13 control mice, 14 mice that had received green tea catechins and 14 that had received black tea theaflavins.

M_1G concentrations of standards and samples were determined by three immuno-slot blots as described in section 2.2.4. A standard curve was calculated for each immuno-slot blot that related M_1G concentration to chemiluminescence. The linear equation of the standard curve best fit line was then used to calculate the M_1G concentration of the samples run in parallel.

2.2.7 Experimental design for analysis of M_1G concentrations in prostate tissue of men receiving theaflavins or catechins compared with controls

Eighteen human volunteers were recruited and separated into three intervention groups as per section 2.6.1 with each group receiving four weeks of either catechins, theaflavins or no polyphenols supplement. Following TURP operation, DNA was extracted from prostate tissue chips as per section 2.2.2. M_1G concentration was determined in this DNA as per section 2.2.4

2.3 Western blotting

2.3.1 Preparation of cell culture lysates

LNCaP and DU145 cells were seeded onto Petri dishes at a density of 1 x 10^5 cells per plate. These were incubated at 37˚C, 5% CO_2 for 24 hours in appropriate media. The media was then exchanged for media containing 0, 3.125 µg/ml, 12.5 µg/ml or 25 µg/ml catechins or theaflavins. These cells were incubated for 24 or 48 hours with each of the 32 permutations being prepared in duplicate. After incubation the media was removed by aspiration and the plates washed twice with ice cold PBS. All subsequent steps were performed on ice. The cell layer of each plate was then scraped into 200 µl of lysis buffer and incubated on ice for ten minutes, sonicated three times for five seconds with a sonication probe and incubated on ice for ten minutes again. Lysates were centrifuged at 13,000 g for ten minutes at 4˚C. The supernatants were stored at -80˚C.

2.3.2 Preparation of tissue lysates

Tissue fragments of approximately 100 mg were homogenised in 400 µl lysis buffer, on ice, using an electric pestle in a 2 ml tube. The homogenate was incubated on ice for ten minutes and then vortexed for ten seconds. The homogenate was sonicated with a sonication probe three times for five seconds

before being incubated again on ice. The lysate was centrifuged at 13,000 g for three minutes at 4˚C. The supernatant was stored at -80˚C.

2.3.3 Bradford assay of protein concentration

A series of dilutions of BSA were prepared in distilled water containing 2, 4, 5, 7, 9, 11, 15 and 20 µg/ml. Samples to be measured were diluted 1000 fold in distilled water. 800 µl of each standard and sample was mixed with 200 µl Biorad reagent. The mixtures were incubated at room temperature for five minutes before being loaded into spectroscopy cuvettes. A Cary 50Bio spectrophotometer was used to measure absorbance at 595 nm. Zero absorbance was set with a mixture containing 800 µl distilled water and 200 µl Biorad reagent prior to measurement of absorbance of standards and samples. A standard curve was drawn of optical density at 595 nm against protein concentration. The standard curve was used to calculate protein concentrations from the optical densities of samples.

2.3.4 SDS-Polyacrylamide gel electrophoresis and western blotting

Aliquots of 100 µg protein were added to 15 µl of 2x loading buffer and vortexed. The samples were then heated to 100˚C for five minutes, allowed cool and briefly centrifuged at 13,000 g. Polyacrylamide denaturing resolving gels were poured, 7 cm x 8.5 cm x 1.5 mm, containing 10% acrylamide and topped with stacking gel formed into 50 µl wells. These were used in a mini-PROTEAN 3

electrophoresis system filled with 1x running buffer. 15 µl precision plus dual colour protein markers were loaded into the first well of each gel and samples loaded into their respective wells. Proteins were resolved through the gel at 100 volts for approximately two hours. The resolved proteins were transferred to a nitrocellulose membrane by electrotransfer at 30 volts overnight. Electrotransfer took place in western transfer buffer. Membranes were then bathed in western blocking buffer on a rocker for two hours at room temperature.

2.3.5 Detection of specific proteins of interest on western blot membranes

Blots were incubated in appropriate primary antibody diluted in blocking buffer on a rocker, either for two hours at room temperature or overnight at 4˚C. The membrane was washed in 50 ml TBST for five minutes, four times, prior to being incubated with secondary antibody conjugated to horseradish peroxidise, diluted in blocking buffer, for two hours on a rocker at room temperature. The membranes were washed in 50 ml TBST for five minutes, four times. 2 ml ECL was added to each blot for one minute before the blots were used to expose photographic film for three to ten minutes to detect chemiluminescence. Films were developed in an automated developer.

Images of developed films were captured using a GeneGenius bioimaging system.

2.3.6 Stripping of antibody complexes from western blots

Membranes were incubated in stripping buffer for 40 minutes at 60˚C. The membranes were than rinsed four times in 50 ml TBST before being reblocked for two hours in blocking buffer that contained 10% milk powder.

2.3.7 Experimental design for the analysis of the effect of tea polyphenols on the expression of p-Erk1/2 and caspase-3 in DU145 and LNCaP cells

64 petri dishes were each seeded with 10^5 cells, 32 with LNCaP cells, 32 with DU145 cells. These cells were incubated in appropriate media, see section 2.1.1, at 37˚C, in humidified atmosphere, 5% CO_2 for 24 hours prior to aspiration of the media and dosing with polyphenols.

For dosing cell culture media, stock solutions of 1 mg/ml Greenselect and 0.5 mg/ml theaflavins were prepared in distilled water, on the day of use. These solutions were sterilized using a 0.2 µm syringe filter immediately prior to use. The cells were then incubated in appropriate media with polyphenols added to a final concentration of 0, 3.125 µg/ml, 12.5 µg/ml or 25 µg/ml, theaflavins or catechins. The cells were then incubated, as above, for 24 or 48 hours. Each permutation of conditions was performed in duplicate. Cell lysates were prepared as per section 2.3.1. Western blots were performed according to section 2.3.4. Each filter was incubated for 90 minutes with 1:1000 rabbit antibody anti-p-Erk1/2, followed by 90 minutes with 1:2000 anti-rabbit Ig HRP conjugated, as per section 2.3.5. The

membranes were stripped and reblocked as per section 2.3.6 and reprobed with 1:1000 anti-cleaved caspase3 Ig for 90 minutes followed by 1:2000 anti-rabbit Ig conjugated to HRP as per section 2.3.5. Following stripping and reblocking the final antibody used to probe the filters was 1:2000 mouse anti α-tubulin followed by 1:2000 horse anti-mouse conjugated to HRP. The resulting chemiluminescence was captured quantitatively as per section 2.3.5.

2.3.8 Experimental design for the analysis of protein biomarkers from prostates of TRAMP mice receiving theaflavins or catechins

TRAMP mice were separated into three treatment groups and provided with drinking water containing 0.05% black tea theaflavins, 0.05% green tea catechins or no polyphenols as described in section 2.5.2. A protein extraction was made from a fragment, approximately 75 mg of each prostate, as per the method in section 2.3.2. The protein concentration of each extract was measured as per section 2.3.3. There were 40 suitable extracts, 13 from the water fed group, 14 from the theaflavins group and 13 from the catechins group. An aliquot of 100 µg of each extract was run on an SDS-polyacrylamide gel. Five western blots containing eight protein extracts each were made as per section 2.3.4. These filters were used to detect the presence of the putative cancer prevention biomarkers p-Erk1/2, cleaved caspase 3 and VEGF, see section 1.9. The presence of the expressed TRAMP mouse transgene was also detected.

Each filter was incubated for 90 minutes with 1:1000 rabbit antibody anti-p-Erk1/2, followed by 90 minutes with 1:2000 anti-rabbit Ig HRP conjugated, as per section 2.3.5. The membranes were stripped and reblocked as per section 2.3.6 and reprobed with 1:2000 anti-SV40 T antigen for 90 minutes followed by 1:2000 anti-mouse Ig conjugated to HRP as per section 2.3.5. The membranes were stripped and reblocked as per section 2.3.6 and reprobed for 90 mins with1:1000 anti-cleaved caspase 3 followed by 90 mins with 1:2000 anti-rabbit Ig antibody conjugated to HRP. The same western blots were stripped and probed for 90 mins with anti-VEGF 1:1500 then probed for 90 mins with 1:2000 anti-rabbit Ig antibody conjugated to HRP. The presence of HRP was detected quantitatively using chemiluminescence, as per section 2.3.5.

In order to correct for unequal protein loading, the final antibody used to probe the filters was 1:2000 rabbit anti β-actin followed by 1:2000 goat anti-rabbit IgG, HRP-linked antibody conjugated to HRP. The chemiluminescence resulting from each experiment was captured on photographic film and quantified, as per section 2.3.5. The signal from each sample was divided by the corresponding signal from β-actin to correct for unequal loading of protein lysate.

2.4 Enzyme-linked immuno-sorbant assays

2.4.1 Quantikine® mouse IGF-1 solid phase ELISA

50 µl of calibrator diluent was added to each of the 96 wells in a Quantikine mouse IGF-1 microplate. Mouse plasma samples were diluted 500 fold in calibrator diluent. A serial dilution of mouse standard IGF-1 was prepared to concentrations of 1000, 500, 250, 125, 62.5, 31.3 pg/ml and a blank standard. 50 µl of each standard and sample were added to respective wells in duplicate. The plate was covered and incubated at room temperature on an orbital shaker at 500 rpm for two hours. Each well was aspirated and washed five times with 400 µl wash buffer. 100 µl of conjugate was added to each well. The plate covered and incubated at room temperature on an orbital shaker at 500 rpm. Each well was aspirated and washed five times with 400 µl wash buffer. 100 µl substrate solution was added to each well and the plate was incubated for 30 minutes at room temperature in darkness. 100 µl of stop solution was added to each well.

Optical density was measured at 450 nm and the optical density at 540 nm was subtracted from the measurements, using a microplate absorbance reader. The mean optical densities of the duplicate standards, blank and samples were calculated. The mean value for the blank wells was then subtracted from the mean values for the standards and samples. The standard values were then used to draw a standard curve. This was used to calculate the concentrations of the samples.

2.4.2 DuoSet® murine IGFBP-3 ELISA

A 100 µl aliquot of rat anti-mouse IGFBP-3 capture antibody (4 µg/ml) was added to each well of a clear plate before being covered and incubated overnight at room temperature. Each well was emptied by aspiration and washed three times with 400 µl wash buffer, each time being aspirated between washes. 200 µl of block buffer was added to each well. The plate was covered and incubated at room temperature for one hour. Each well was emptied by aspiration and washed three times with 400 µl wash buffer. The standards were prepared containing 3000, 2000, 1500, 1000, 500, 250 pg/ml and a blank. Plasma samples were then diluted 1000 fold in reagent diluent. 100 µl of standards, blank and samples were added in duplicate to respective wells. The plate was then covered and incubated for one hour at room temperature. The wells were aspirated and washed three times with wash buffer. 200 µl (200 ng/ml) biotinylated goat anti-mouse IGFBP-3 detector antibody was added to each well. The plate was then covered and incubated for two hours at room temperature. The wells were aspirated and washed three times with wash buffer. 100 µl horseradish peroxidase-streptavidin solution was added to each well, the plate covered and incubated at room temperature in darkness for 20 minutes. The wells were aspirated and washed three times with wash buffer. 100 µl substrate solution was added, the plate covered and incubated at room temperature in darkness for 20 minutes. 50 µl of 2 N sulphuric acid was added to each well.

Optical density was measured at 450 nm and the optical density at 595 nm was subtracted from the measurements, using a microplate absorbance reader. The mean optical densities of the duplicate standards, blank and samples were calculated. The mean value for the blank wells was then subtracted from the mean values for the standards and samples. The standard values were then used to draw a standard curve. This was used to calculate the concentrations of the samples.

2.4.3 Experimental design for the analysis of plasma IGF and IGFBP-3 concentrations of TRAMP mice receiving theaflavins or catechins

A group of TRAMP mice were separated into three treatment groups and provided with drinking water containing 0.05% black tea theaflavins, 0.05% green tea catechins or no polyphenols as described in section 2.5.2, 41animals were available for analysis, see section 4.1.2. The animals were exsanguinated and their plasma stored at -80ºC. IGF-1 and IGFBP-3 concentrations in plasma were estimated by the methods in sections 2.4.1 and 2.4.2.

The IGF and IGFBP-3 ELISA plates described above had capacity for 40 individuals to be analysed in duplicate therefore mouse no. 41 was excluded. The analyses therefore compared 13 control mice, 14 mice that had received theaflavins and 13 mice that had received catechins.

2.5 TRAMP mouse study

2.5.1 Maintenance of TRAMP mice

Female TRAMP mice developed on a C57B background, heterozygous for the probasin-T antigen transgene were donated by NCI animal repository. Transgenic males and the non-transgenic littermates were obtained as [TRAMP x C57B] F1. These were bred and maintained in the University of Leicester, MRC Toxicology Unit, in plastic isolators under positive pressure. The mice were kept in plastic boxes on corn bedding at 21–23°C with a 12 hr light/dark cycle. The isolation of mouse-tail DNA and PCR-based screening assay were performed by the University of Leicester technical staff [242]. Identification of individuals within a cage was achieved by ear punch configuration. Transgenic males were weaned at three weeks onto AIN93G diet [243] and recruited to study at four weeks. Each week the animals were weighed by technical staff. Monitoring for signs of illness or distress was performed daily by technical staff, such signs including piloerection, decreased activity and weight loss.

2.5.2 TRAMP mouse study design

42 male TRAMP mice heterozygotic for the probasin-T antigen transgene were recruited at four weeks of age. These mice were randomized to receive water, 0.05% Greenselect® catechins water (w/v) or 0.05% theaflavins in water

(w/v) as their sole source of fluid. At 30 weeks of age, 26 weeks on study protocol, the animals were exsanguinated by cardiac puncture under terminal halothane anaesthesia before being terminated by cervical dislocation under UK Home Office schedule one by the project licence holder, Dr T. Marczylo. The blood was placed in 1.3 ml Li-Heparin tubes and centrifuged immediately. The plasma was collected and stored at -80ºC. Dissection was performed to remove the prostate and seminal vesicles. This was achieved by reflecting the bladder, seminal vesicles and prostate mass inferiorly and severing the urethra as low as possible before it entered the pelvic floor. The bladder was then dissected off the specimen. The prostates were weighed and snap-frozen in liquid nitrogen before being stored a -80˚C. The liver, retroperitoneal lymph nodes, gut mesentery and lungs were removed and immediately placed into an excess of 10% neutral buffered formalin. Embedding of tissue in wax, cutting of tissue slices, haematoxylin and eosin staining and histological examination to detect metastases was performed by Dr. Peter Greaves.

2.6 Human investigational study of tea polyphenols in men awaiting TURP

2.6.1 Study design

An open-label randomised controlled study was performed. 18 men awaiting transurethral resection of prostate (TURP) operations were recruited. The decision to perform a TURP operation had already been taken on clinical grounds prior to inclusion in the study. At recruitment, blood was drawn and urine collected from all 18 participants.

Six participants were randomised (http://www.randomizer.org/form.htm) to receive 1 g once a day of theaflavins and six to receive 1 g once a day of Greenselect® catechins, for four weeks prior to scheduled TURP. In addition, six more participants were recruited as controls they received no trial agent.

Immediately prior to TURP operation blood was again drawn and urine collected. Following surgery all 18 patients had a sample of prostate tissue collected from the surgical specimen. The interval between the final polyphenol dose and the operation start time was approximately three to four hours.

2.6.2 Study population criteria

Inclusion criteria:

>18 years of age.

Competent to consent or withhold consent.

Having already chosen prostate biopsy and TURP prior to introduction to trial.

Given an operation date no less than four weeks from the date of biopsy.

>10g prostate tissue resected at TURP

Exclusion criteria:

Past history of allergy to any component of tea.

Already receiving tea polyphenol supplements.

Concurrent chronic disease that increases oxidative stress i.e., inflammatory bowel disease, cardiovascular disease or presence of other cancers.

Any known biochemical abnormality of liver or kidney function.

2.6.3 Recruitment and consent

Men meeting the above criteria were interviewed, introduced to the study, provided with the patient information sheet, any further questions regarding the trial were answered and their understanding of the trial verified. Following this they were invited to participate. The invited men were given a period of time no less

than 24 hours to consider their response. Those men that accepted this offer were asked to complete the consent form. They were then registered in the trial.

2.6.4 Registration

Those men that accepted the invitation to participate had their inclusion in the trial recorded in their medical notes, including the detail of any tea polyphenol administered. Participants were allocated a trial identification number. The identity of the participants was stored in the trial database. An explanatory letter was sent to their GP explaining the trial and the agent, if any, that their patient has been allocated.

2.6.5 Polyphenol administration

Those participants that were in the intervention arms of the study were requested to take 4 capsules of polyphenol totalling 1g of polyphenol (either green tea catechins or black tea theaflavins) on an empty stomach prior to breakfast each day. There were no dietary restrictions placed on the participants. On the day of surgery participants were requested to take the capsules two hours prior to arrival in hospital, in order to comply with the “nil by mouth” requirements of the anaesthetist prior to surgery.

2.6.6 Sample collection

Blood and urine was collected from participants at two time points, once prior to taking any polyphenol capsules and secondly on arrival in hospital on the day of their operation following four weeks of daily polyphenol dosing. Participants were requested to take their final polyphenol dose two hours before arriving in hospital. Blood and urine samples were collected on arrival in hospital.7.5 ml blood was collected in Li-Heparin tubes (7.5 ml). Within 30 minutes, the tubes were centrifuged at 3000 g at 4˚C for 15 minutes and the plasma collected. Samples of plasma were stored at -80˚C. Further samples of plasma had phosphate buffer added in the proportion 1:5. In addition, blood was submitted to Leicester General Hospital Department of Clinical Chemistry for analysis of PSA, Sodium, Potassium, Urea, Creatinine, Alkaline phosphatase, Alanine transaminase, Bilirubin and Albumin.

2.6.6 Trial sponsorship and agent accountability

The University Hospitals of Leicester agreed to act as sponsor for this trial. The Trust trials pharmacist maintained a record of the receipt and distribution of all trial supplies of catechins and theaflavins available for inspection by appropriate regulatory authorities.

2.6.7 Adverse Incidents, Withdrawal and Study Termination criteria:

An individual participant would be withdrawn from the study following:

- Participant request.
- Investigator determined withdrawal to be in the best interest of the participant.
- Patient was unfit for intended surgery.
- Any grade 3 and above* toxicity probably associated** with the study supplement.
- Scheduled dose delayed >12 hours.

*Grading of adverse events will be according to the NCIC Common Terminology Criteria for Adverse Events version 3.0.

**Association of an adverse event will be deemed “probably associated” with the agent if there is a timely relationship to the study agent administration, and no alternative aetiology is apparent.

The study would have been be prematurely terminated following:

- The recommendation of the ethics committee to terminate.
- Any grade 4 or 5 toxicity probably associated with study supplement.

2.6.8 Ethical approval

The trial was considered by the Leicestershire Local Research Ethics Committee One and approval granted (05/Q2501/40). Confirmation was received from the MHRA Scope meeting that clinical trial authorisation was not required for agents considered food supplements, as they are not investigational medicinal products defined by EU Directive 2001/20/EC.

2.6.9 Power Calculation

No pre-existing preliminary trials exist of biomarker characterisation following administration of tea polyphenols in human participants, on which to base a power calculation, however, this trial could form the basis of future power calculations.

Using the results obtained in this work, measuring prostate levels of DNA adduct M1G in TRAMP mice following catechin administration and comparing them with controls (see section 4.2.3), if a similar reduction in M1G was expected, 6 participants in each group would have a statistical power of only 42.2% to detect such a change using an alpha level of 0.05 [244].

2.7 Liquid chromatography and tandem mass spectroscopy (LCMSMS) for detection and quantification of tea polyphenols

2.7.1 Extraction of catechins from plasma or urine

Using Waters Oasis HLB Extraction Cartridges various solutions as detailed below were added and drawn through by negative pressure sufficient to achieve flow rates of 0.5-1 ml/minute. Each cartridge was conditioned with 1 ml methanol drawn through and equilibrated with 1 ml water drawn through. 400 µl urine or 500 – 1000 µl plasma was then added and drawn through. The cartridge was washed with 1 ml cartridge wash drawn through. The cartridge was the eluted with 2 ml cartridge eluent drawn through into a clean tube. This solution was then dried down under nitrogen gas stream at 40˚C. The residue was redissolved in 40 µl water before immediate analysis by LCMSMS.

If the urinary or plasma catechins were required to be deconjugated then 200 µl urine or plasma was mixed with an equal volume of deconjugation buffer. 4440 u β-glucuronidase and 5 u sulfatase were added and mixed. This reaction was incubated at 37˚C for 45 minutes. The mixture was then centrifuged at 16,060g for five minutes at 4˚C. The supernatant was added to a conditioned, equilibrated cartridge and drawn through. Washing, elution, drying down and redissolution were performed as detailed above.

2.7.2 Extraction of catechins from prostate tissue

The extraction of catechins from tissue was performed by a method based on that published by Chu *et al*.[245]. The tissue was covered with 800 µl of tissue extraction buffer and homogenised in a glass hand-homogeniser. The homogenate was then subjected to three bursts of five seconds with the sonication probe. The homogenate was then centrifuged at 16,060g for ten minutes at 4˚C and the supernatant collected. The organic solvents were then purged off under nitrogen gas stream at 40˚C; this was judged to be complete when the volume of the sample had halved.

If the catechins in the sample required deconjugation then 400 µl deconjugation buffer was added, in addition to 4440 u β-glucuronidase and 5 u sulfatase. Incubation and centrifugation were performed as for urine catechin deconjugation.

Whether or not used in a deconjugation reaction the sample was added to a Waters Oasis HLB Extraction Cartridge and processed as if urine.

2.7.3 Experimental design for optimisation of extraction of green tea polyphenols from biological material

In order to maximize the sensitivity of LCMSMS for tea polyphenols extracted from tissue and urine, the extraction method using Waters Oasis HLB Extraction Cartridges was altered from the method recommended by the

manufacturer. The original method included elution from the cartridge with methanol. This had been previously improved upon by the use of acetone by experiments performed by others in the group. In the work presented here, an alternative elution solvent was used, acidified acetone:methanol, to compare with 100% acetone.

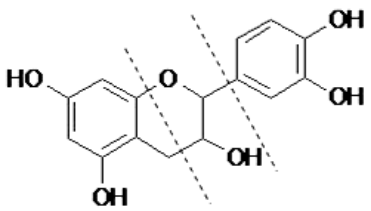
A solution was prepared containing 1.25 µg each of EGC and EC in a volume of 400 µl water. An aliquot 400 µl of this solution was added to each of two equilibrated extraction cartridges and processed as per the method for urine without a deconjugation step. Following washing of the column elution was performed using either acetone or the acidified acetone:methanol mixture. The eluents were then processed following the method for urine extraction. A third solution was prepared containing 1.25 µg of each of EGC and EC in 20µl, not subjected to an extraction method. All three solutions were analysed by LCMSMS using the method for analysis of green tea catechins and their metabolites, see section 2.7.4.

2.7.4 Liquid chromatographic and mass spectrometric conditions for analysis of green tea catechins and their metabolites

Tea catechins were separated and identified by LCMSMS using a method developed by Kaur *et.al*. [246]. 20 µl injection volumes were delivered by an automated high performance liquid chromatography (HPLC) system onto an Xterra

phenyl column. Binary gradient elution separated catechins on the column using mobile phases A and B in the following steps: 0-3.5 min 96% A; 12.5 min 83% A; 15.5% 72% A; 20 min 60% A; 25 min 50% A; 27.5-33 min 100% B followed by a reequilibration step at 96% A. The flow rate was 0.31 ml/min. Electronegative mode electrospray mass spectrometry was carried out on the eluent using the following parameters: ion spray potential -5000v; ion spray temperature 550˚C, focusing potential -350v; declustering potential -21v; electrode potential -10v; collision energy -50v and collision energy exit potential -20v. Product ion scanning was used to identify suitable daughter ions generated by fragmentation of the parental ions. Selective detection included the following mass/charge ratio transitions shown in figures 3-6.

Epicatechin (EC)



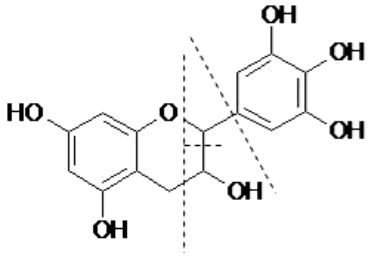
Molecular weight = 290
mz = 289

Transitions:

Epicatechin	**289→123/109**
Methyl epicatechin	**303→123/109**
Epicatechin glucuronide	**465→123/109**
Epicatechin sulfonate	**369→123/109**
Methyl epicatechin glucuronide	**479→123/109**
Methyl epicatechin sulfonate	**383→123/109**

Figure 3 The mass charge densities (mz) of EC and its proposed metabolites with transitions to fragmentation products. Diagram indicates possible sites of fragmentation explaining observed fragmentation product mz.

Epigallocatechin (EGC)



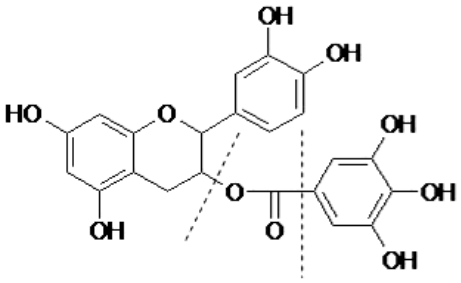
Molecular weight = 306
mz = 305

Transitions:

Epigallocatechin	**305→137/125**
Methylepigallocatechin	**319→137/125**
Epigallocatechin glucuronide	**481→137/125**
Epigallocatechin sulfonate	**385→137/125**
Methylepigallocatechin glucuronide	**495→137/125**
Methylepigallocatechin sulfonate	**399→137/125**

Figure 4 The mass charge densities (mz) of EGC and its proposed metabolites with transitions to fragmentation products. Diagram indicates possible sites of fragmentation explaining observed fragmentation product mz.

Epicatechin gallate (ECG)



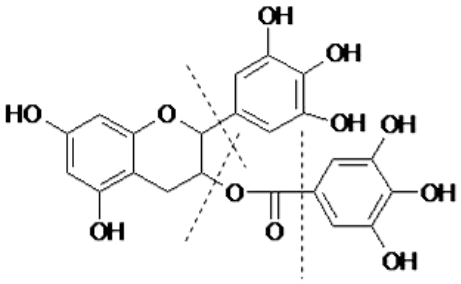
Molecular weight = 442
mz = 441

Transitions:

Epicatechin gallate	**441→169/125**
Methylepicatechin gallate	**455→169/125**
Dimethylepicatechin gallate	**469→169/125**
Epicatechin gallate glucuronide	**617→169/125**
Epicatechin gallate sulfonate	**521→169/125**
Methylepicatechin gallate glucuronide	**631→169/125**
Methylepicatechin gallate sulfonate	**535→169/125**
Dimethylepicatechin gallate glucuronide	**645→169/125**
Dimethylepicatechin gallate sulfonate	**549→169/125**

Figure 5 The mass charge densities (mz) of ECG and its proposed metabolites with transitions to fragmentation products. Diagram indicates possible sites of fragmentation explaining observed fragmentation product mz.

Epigallocatechin gallate (EGCG)



Molecular weight = 458
mz = 457

Transitions:

Epigallocatechin gallate	**457→169/125**
Methylepigallocatechin gallate	**471→169/125**
Methylepigallocatechin gallate sulfonate	**551→169/125**
Epigallocatechin gallate sulfonate	**537→169/125**
Methylepigallocatechin gallate glucuronide	**647→169/125**
Epigallocatechin gallate glucuronide	**633→169/125**
Dimethylepigallocatechin gallate	**485→169/125**
Dimethylepigallocatechin gallate sulfonate	**565→169/125**
Dimethylepigallocatechin gallate glucuronide	**661→169/125**

Figure 6 The mass charge densities (mz) of EGCG and its proposed metabolites with transitions to fragmentation products. Diagram indicates possible sites of fragmentation explaining observed fragmentation product mz.

2.7.5 Extraction of theaflavins from urine or plasma

At the time of human urine collection, antioxidation buffer was added in the proportion 1:5 buffer:urine, before storing urine samples at -80˚C. Theaflavins were extracted using a method published by Mulder et al. [57]. 5 ml of urine was added to 2.5 ml acetone and the mixture vortexed. 10 ml ethyl acetate was added and the mixture vortexed again. The upper ethyl acetate phase was then removed by pipette. A repeat extraction of the aqueous phase was performed by adding another 10 ml of ethyl acetate vortexing and removing the upper ethyl acetate phase. The two ethyl acetate fractions were pooled and dried down under a stream of nitrogen gas. The resulting residue was resuspended in 20 µl mobile phase C and analysed by LCMSMS.

2.7.6 Extraction of theaflavins from prostate tissue

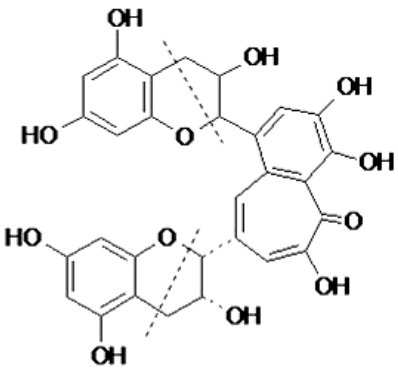
The extraction of theaflavins from tissue was performed by a method based on that published by Chu *et al*.[245]. The tissue was covered with 800 µl of tissue extraction buffer and homogenised in a glass hand-homogeniser. The homogenate was then subjected to three bursts of five seconds with the sonication probe. The homogenate was then centrifuged at 16,060g for ten minutes at 4˚C and the supernatant collected. The organic solvents were then purged off under nitrogen gas stream at 40˚C; this was judged to be complete when the volume of the sample had halved. 400 µl acetone and 1.6 ml ethyl acetate was added. The mixture was vortexed and the upper ethyl acetate phase was removed by pipette. The extraction of the aqueous phase was repeated with another 1.6 ml ethyl acetate and the two ethyl acetate fractions were pooled and dried down under a stream of nitrogen gas.

2.7.7 Liquid chromatographic and mass spectrometric conditions for analysis of black tea theaflavins and their metabolites

Black tea theaflavins were separated and identified by LCMSMS using a method modified from that used by Mulder et al. [57]. 20 µl injection volumes were delivered by an automated HPLC system onto an Xterra phenyl column. Binary gradient elution separated theaflavins on the column using mobile phases C and D in the following steps: 0% to 74% D over 50 minutes followed by 100% D for 6

minutes. The flow rate was 0.25 ml/min. Electronegative mode electrospray mass spectrometry was carried out on the eluent using the following parameters: source temperature of 120ºC, desolvation temperature 350ºC. Nitrogen was used as the nebulizer gas at 231 L/hr. The capillary voltage was 4 kV without cone voltage. Selective detection of ions was performed by multiple reaction monitoring of theaflavin parental compounds and their proposed metabolites. Selective detection included the following mass/charge ratio transitions shown in figures 7-9.

Theaflavin (TF)



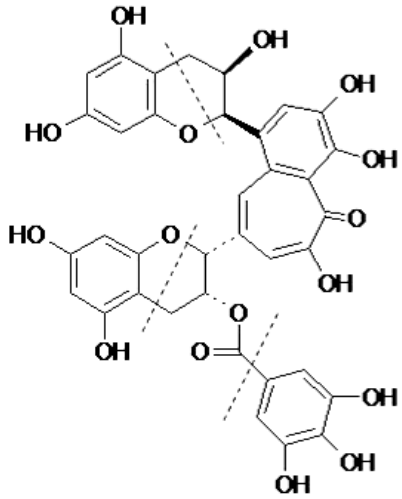
Molecular weight = 564
mz = 563

Transitions:

Theaflavin	**563→125**
Methyltheaflavin	**577→125**
Theaflavin glucuronide	**739 →125**
Theaflavin sulphonate	**643 →125**
Methyltheaflavin glucuronide	**753 →125**
Methyltheaflavin sulphonate	**657 →125**
Dimethyltheaflavin	**591 →125**
Dimethyltheaflavin glucuronide	**767 →125**
Dimethyltheaflavin sulphonate	**671 →125**
Theaflavin disulphonate	**723 →125**
Theaflavin diglucuronide	**915 →125**
Theaflavin glucuronide sulphonate	**819 →125**
Methyltheaflavin glucuronide sulphonate	**833 →125**

Figure 7 The mass charge densities (mz) of theaflavin and its proposed metabolites with transitions to fragmentation products. Diagram indicates possible sites of fragmentation explaining observed fragmentation product mz.

Theaflavin monogallate (TFG)



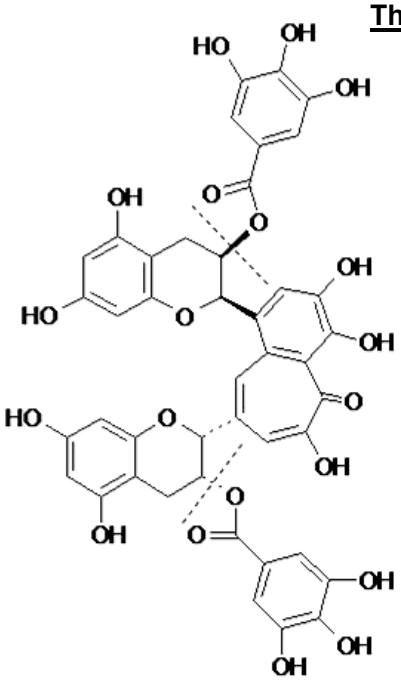
Molecular weight = 716
mz = 715

Transitions:

Theaflavin monogallate	**715 →125**
Methyltheaflavin monogallate	**729 →125**
Theaflavin monogallate glucuronide	**891 →125**
Theaflavin monogallate sulphonate	**795 →125**
Methyltheaflavin monogallate glucuronide	**905 →125**
Methyltheaflavin monogallate sulphonate	**809 →125**
Dimethyltheaflavin monogallate	**743 →125**
Dimethyltheaflavin monogallate glucuronide	**919 →125**
Dimethyltheaflavin monogallate sulphonate	**823 →125**
Theaflavin monogallate disulphonate	**875 →125**
Theaflavin monogallate diglucuronide	**1067 →125**
Theaflavin monogallate glucuronide sulphonate	**971→125**
Methyltheaflavin monogallate glucuronide sulphonate	**985 →125**

Figure 8 The mass charge densities (mz) of theaflavin monogallate and its proposed metabolites with transitions to fragmentation products. Diagram indicates possible sites of fragmentation explaining observed fragmentation product mz.

Theaflavin digallate (TFdG)



Molecular weight = 868
mz = 867

Transitions:

Theaflavin digallate	**867 →169**
Methyltheaflavin digallate	**881 →169**
Theaflavin digallate glucuronide	**1043 → 169**
Theaflavin digallate sulphonate	**947 → 169**
Methyltheaflavin digallate glucuronide	**1057 → 169**
Methyltheaflavin digallate sulphonate	**961 → 169**
Dimethyltheaflavin digallate	**895 → 169**
Dimethyltheaflavin digallate glucuronide	**1071 → 169**
Dimethyltheaflavin digallate sulphonate	**975 → 169**
Theaflavin digallate disulphonate	**1027 → 169**
Theaflavin digallate diglucuronide	**1219 → 169**
Theaflavin digallate glucuronide sulphonate	**1123 → 169**
Methyltheaflavin digallate glucuronide sulphonate	**1137 → 169**

Figure 9 The mass charge densities (mz) of theaflavin digallate and its proposed metabolites with transitions to fragmentation products. Diagram indicates possible sites of fragmentation explaining observed fragmentation product mz.

2.7.8 Experimental design for analysis of green tea catechins in TRAMP mouse plasma and prostate tissue

Following 26 weeks of dietary intervention with green tea catechins, as per the experimental design in section 2.5.2, extraction of catechins was performed on 500 µl aliquots of buffered plasma from five individual TRAMP mice who had received oral catechins; one 1000 µl aliquot of plasma pooled from such mice, and; five 500 µl aliquots of plasma from control mice, as per section 2.7.1. Deconjugation reactions followed by catechin extraction as per section 2.7.1 were performed on four 500 µl aliquots of plasma from TRAMP mice which had received oral catechins. LCMSMS analysis was performed on these samples as per section 2.7.3.

Dissected prostate tissue from five mice which had received green tea catechins was used to prepare a pair of tissue samples from each mouse, with 203-533 mg tissue in each cut sample depending on the size of the prostate tumour. Catechins were extracted from these tissue samples according to section 2.7.2. One of each pair of samples was subjected to a deconjugation step. LCMSMS analysis was performed as per section 2.7.3.

2.7.9 Experimental design for the analysis of green tea catechins in human plasma, urine and prostate tissue

Eighteen human volunteers were recruited and separated into three intervention groups as per section 2.6.1 with each group receiving four weeks of either catechins, theaflavins or no polyphenols supplement. Plasma, urine and prostate tissue chips were collected, see section 2.6.5. Using the method in section 2.7.1, extraction of catechins without deconjugation was performed on 500 µl aliquots of buffered plasma and 200 µl aliquots of buffered urine from six human subjects who had received oral catechins and six control subjects. Catechins were extracted from the prostate tissue samples which ranged from 203 mg to 533 mg as per section 2.7.2. The resulting residues were resuspended in 20 µl of water before analysis by LCMSMS, see section 2.7.4, looking for the presence of 30 different catechins.

2.7.10 Experimental design for quantification of deconjugated green tea catechins in human urine

As detailed in section 2.6, six human volunteers took 1g catechins per day for 28 days prior to donation of urine. Aliquots containing 200 µl of urine from each subject were incubated with deconjugation enzymes prior to solid phase extraction as per section 2.7.1. Following drying down of the extracted sample the residue was resuspended in 20 µl water and analysed by LCMSMS, see section 2.7.4,

looking for the presence of 30 different catechins. In parallel, samples containing 1.25 µg, 0.63 µg, 0.31 µg, 0.16 µg and 0.08 µg of each parental catechin compound (EC, ECG, EGC and EGCG) were analysed by the same LCMSMS method. The LCMSMS peak areas measured were used to derive a standard curve with which to quantify the deconjugated catechins detected in human urine. Quantification was limited to deconjugated EC, ECG, EGC and EGCG due to lack of availability of other standards e.g., methylated catechins

2.7.11 Experimental design for analysis of black tea theaflavins in TRAMP mouse plasma and prostate tissue

TRAMP mice were culled following 26 weeks of dietary intervention with black tea theaflavins, as per the study design in section 2.5.2. Theaflavins were extracted from 500 µl plasma samples of six mice which had received theaflavins in their drinking water and six mice which had not, by the method described in section 2.7.1. Theaflavins were extracted from prostate tissue of six mice which had received theaflavins and six control mice, using the method found in section 2.7.2. The masses of prostate tissue used for the extractions ranged between 194 mg and 544 mg. The resulting extracts were resuspended in 20 µl distilled water and analysed by LCMSMS, see section 2.7.7, looking for the presence of 39 theaflavin species.

2.7.12 Experimental design for analysis of black tea theaflavins in human plasma, urine and prostate tissue

As detailed in section 2.6, six human volunteers took one gram of theaflavins per day for 28 days prior to donation of plasma, urine and prostate tissue. Samples were stored at -80ºC until analysis. Theaflavins were extracted from 500 ml of plasma and 2 ml of urine from each subject and from six controls who had not received oral theaflavins, using the method in section 2.7.5. Theaflavins were extracted from prostate tissue samples from these six men using the method in section 2.7.6, in parallel with prostate tissue from six control men. The masses of tissue analysed ranged from 175 mg to 300 mg. The extracts were resuspended in 20 µl distilled water and analysed by LCMSMS as per section 2.7.7 looking for the presence of 39 theaflavin species.

2.8 Microarray gene expression analysis

2.8.1 Choice of microarray

Gene expression in human and mouse prostate following tea polyphenol administration was compared to controls using microarray analysis. Microarrays were kindly made on aldehyde slides using a Stanford type spotter and provided by Dr T. Grant, MRC Toxicology Unit, Leicester. The genome sets used to generate the microarrays were Human Exonic Evidence Based Oligonucleotide (HEEBO) and Mouse Exonic Evidence Based Oligonucleotide (MEEBO) designed in the laboratory of Alizadeh [247]. The HEEBO Set contains 48,958 and the MEEBO Set 38,467 70mer 5'amino modified oligonucleotides derived from constitutively expressed exons. Both arrays are collections of oligonucleotides based on analysis of expressed exons, allowing analysis of transcription patterns.

2.8.1 RNA extraction from tissue

Pieces of prostate tissue of approximately 100 mg were chopped with razor blades on a metal surface chilled from beneath with dry ice pellets. The chopped tise was then placed into a sterile 2 ml tube. 1 ml of TRI reagent was added and homogenised using a handheld homogeniser for 40 seconds. The tubes were then incubated at room temperature for five minutes. 200 µl 1-bromo-3-chloro-propane was then added and the tube vortexed. The tubes were then incubated at room temperature for three minutes. The sample was then loaded into a Phase Lock

Gel tube and centrifuged at 12,000 g for 15 minutes at 4ºC. The upper aqueous layer was pipetted into a fresh 1.5 ml tube avoiding the gel at the interphase. 600 µl of isopropanol was added and mixed well with the sample by inversion. The sample was incubated for ten minutes at room temperature before centrifugation at 12,000 g for ten minutes at 4ºC. The supernatant was discarded and the RNA pellet washed with 1 ml ice cold 75% ethanol in DEPC-water. After centrifugation at 12,000 g for five minutes at 4ºC the 75 % ethanol was discarded and washing step was repeated. The 75% ethanol was then discarded and the RNA was dried under negative pressure for five minutes. The RNA was then resuspended in 50 µl DEPC-water. The RNA concentration was measured by spectrophotometry. The RNA solutions were stored at -80ºC.

2.8.2 Polyadenylation of RNA

20 µg of RNA was made up to 29 µl with DEPC-water and added to 4 µl 10x A-plus reaction buffer, 10 µl 10 mM ATP and 2 µl (8 units) A-Plus Poly(A) polymerase. The total reaction was incubated at 37˚C for 20 minutes. The resulting reaction was added to 300 µl of DEPC-water and added to a microcon-10 filtration unit. The microcon unit was centrifuged at 14,000g for approximately ten minutes until approximately 10 µl of DEPC-water remained above filter. The microcon unit was then inverted and placed into a fresh tube. The fresh tube was centrifuged at 14,000 g for two minutes. The resulting eluant was dried under negative pressure for ten minutes.

2.8.3 Generation of cDNA

The purified RNA was resuspended in 13 µl DEPC-water to which was added 1 µl of 10 mM random pentadecamers and 1µl of 8 µg/µl anchored oligo $dT_{23}N_2$. This reaction was incubated at 95˚C for five minutes and 70˚C for ten minutes before cooling to 4˚C for one minute. 13 µl labelling mix, 1 µl RNAsin and 1 µl reverse transcriptase was added before incubation at 50˚C for 3 hours.

2.8.4 RNA hydrolysis and cDNA purification

10 µl of 0.5 M EDTA and 10 µl of 1 N sodium hydroxide was added to the cDNA reaction and incubated at 65˚C for 15 minutes. 25 µl of 1 M HEPES pH7.0 was then added before the reaction mix was added to 380 µl DEPC-water and transferred to a microcon-30 unit. This was centrifuged at 14,000 g for approximately five minutes. A further 380 µl DEPC-water was added and centrifugation was continued for approximately ten minutes until 10 µl remained above the filter. The microcon unit was inverted and placed into a fresh tube before centrifugation for two minutes at 14,000 g. The resulting eluent was dried under negative pressure for ten minutes.

2.8.5 Labelling cDNA with Alexa dyes

The dried cDNA pellet was resuspended in 7 µl DEPC-water. 1 µl of 1 M sodium carbonate and 2 µl of Alexa dye in DMSO (either Alexa fluor 488, 555 or 594) was added and the reaction incubated at room temperature for 1 hour in the dark. 9 µl of 4M hydroxylamine was then added and the reaction incubated at room temperature for 15 minutes in the dark.

2.8.6 cDNA hybridisation

cDNAs relating to control and intervention groups, prepared separately and labelled with particular Alexa dyes, were then combined and the mixture made up to 100 µl with DEPC-water. 10 µl 3M sodium acetate pH5.2 and 250 µl ethanol was added and the solution kept at -20˚C for 30 minutes. The solution was centrifuged at 14,000 g for 15 minutes. The supernatant was discarded and the pellet washed with 500 µl 70% ethanol at -20˚C. After centrifugation at 14,000 g for five minutes the supernatant was discarded and the wash repeated. After a final centrifugation at 14,000 g for five minutes the supernatant was discarded and the pellet dried in negative pressure for two minutes.

The pellet was resuspended in 50 µl DEPC-water, 1 µl tRNA (4 mg/ml) was added and the cDNA solution added to 380 µl DEPC-water in a Microcon-30 filtration unit. The Microcon-30 tube was centrifuged at 14,000 g until all but 15 µl had passed through the filter, approximately 8 minutes. The filter was inverted and eluted by centrifugation at 14,000 g for two minutes. The cDNA was dried down to a pellet in negative pressure for five minutes.

The pellet was resuspended in enhanced hybridization buffer. For mouse MEEBO arrays the resuspended volume was 80 µl whereas for human HEEBO array the volume was increased to 120 µl.

2.8.7 Preparation of microarray slides

Each microarray slide whether MEEBO or HEEBO was prepared by twice washing the slide in an excess of 0.2% SDS for two minutes and twice rinsing in water for two minutes. Slides were then dried by centrifugation for four minutes at 190 g. Cover slips were wased in 1% SDS for 30 minutes and rinsed in water 5 times for five minutes before being dried by centrifugation at 190 g for four minutes. A cover slip was placed on each slide covering the printed array. 35 µl of resuspended labelled cDNA was pipetted onto the slide around the cover slip and allowed to spread under the slip by capillary action. The slides were arranged into a humidified hybridization chamber and incubated at 42ºC overnight.

2.8.8 Scanning of arrays

Following hybridization the slides were washed for two minutes in excess 1x SSC, 0.03% SDS and the cover slips allowed to slide off. The slides were then transferred to 0.2x SSC for two minutes and then to 0.05x SSC for two minutes. The slides were then dried by centrifugation at 190 g for four minutes.

The slides were read within 72 hours in a microarray scanner. The intensity of fluorescence from each dye was approximately normalised by adjustment of the stimulating laser intensity. Once approximately normalised, images of the arrays were captured as .tiff files. These .tiff files were analysed using GenePix Pro 6.0 to

allow automatic algorithms to identify array spots. The fitting of features was checked manually before intensities were recorded.

2.8.9 Statistical analysis of arrays

The intensity measurements relating to each of the three wavelengths were statistically normalised using software developed by Zhang and Gant [248]. Two-sample student's t-tests were used to compare controls v each intervention group to identify candidate biomarker genes whose expression was significantly altered by intervention with dietary tea polyphenols. The rate of type I statistical error was controlled by calculation of the false-discovery rate (FDR) [249]. Having proposed a list of genes with potentially statistically significant altered gene expression, the FDR represented the proportion of that list that would be expected to have been falsely included. The arbitrary level of significance used was FDR<0.1. In addition in an attempt to highlight biological as well as statistical significance genes whose expression appeared altered less than two-fold were discarded.

Lists of genes with potentially altered expression meeting the above criteria were uploaded to the WEB-based GEne SeT AnaLysis Toolkit (Webgestalt) website. Gene ontology statistical analysis was performed to identify gene functional groups with relatively enriched gene numbers compared with the complete gene set.

2.8.10 Experimental design for microarray analysis of TRAMP mouse prostate gene expression following dietary intervention with tea polyphenols

In order to investigate differences in gene expression in TRAMP mouse prostate tissue following dietary intervention with tea polyphenols, they received catechins, theaflavins or no polyphenols in their drinking water for 26 weeks according to the study design in section 2.5.2. It was felt that five arrays each analysing the expression of one mouse from each group would yield sufficient statistical power to guide further investigations. Therefore, five mice from each group were selected to perform the microarray experiment. Table 1 shows the experimental design. Table 2 shows the same experiment repeated with different dye allocations to eliminate bias.

Table 1 The allocations of TRAMP mice to microarrays and the allocations of Alexa dyes (488, 555 or 594 nm) to intervention groups

Intervention group	Dye	Array 1	Array 2	Array 3	Array 4	Array 5
Controls	488	Mouse 2	Mouse 4	Mouse 5	Mouse 8	Mouse 10
Theaflavins	555	Mouse 16	Mouse 17	Mouse 19	Mouse 21	Mouse 24
Catechins	594	Mouse 28	Mouse 29	Mouse 36	Mouse 39	Mouse 41

Table 2 The allocations of TRAMP mice to microarrays and the allocations of Alexa dyes (488, 555 or 594 nm) to intervention groups

Intervention group	Dye	Array 6	Array 7	Array 8	Array 9	Array 10
Controls	594	Mouse 2	Mouse 4	Mouse 5	Mouse 8	Mouse 10
Theaflavins	488	Mouse 16	Mouse 17	Mouse 19	Mouse 21	Mouse 24
Catechins	555	Mouse 28	Mouse 29	Mouse 36	Mouse 39	Mouse 41

2.8.11 Experimental design for microarray analysis of human prostate gene expression following dietary intervention with tea polyphenols

In order to investigate differences in gene expression in human prostate tissue following dietary intervention with tea polyphenols, volunteers received catechins, theaflavins or no polyphenols four weeks according to the study design in section 2.6.1. It was felt that five arrays each analysing the expression of one individual from each group would yield sufficient statistical power to guide further investigations. Therefore, five individuals from each group were selected to perform the microarray experiment. Table 3 shows the experimental design. Table 4 shows the same experiment repeated with different dye allocations to eliminate bias.

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Table 3 The allocations of TRAMP mice to microarrays and the allocations of Alexa dyes (488, 555 or 594 nm) to intervention groups

Intervention group	Dye	Array 1	Array 2	Array 3	Array 4	Array 5
Controls	488	Subject 1	Subject 2	Subject 3	Subject 4	Subject 6
Theaflavins	555	Subject1 3	Subject 14	Subject 15	Subject 16	Subject 18
Catechins	594	Subject 7	Subject 8	Subject 10	Subject 11	Subject 12

Table 4 The allocations of TRAMP mice to microarrays and the allocations of Alexa dyes (488, 555 or 594 nm) to intervention groups

Intervention group	Dye	Array 6	Array 7	Array 8	Array 9	Array 10
Controls	594	Subject 1	Subject 2	Subject 3	Subject 4	Subject 6
Theaflavins	488	Subject1 3	Subject 14	Subject 15	Subject 16	Subject 18
Catechins	555	Subject 7	Subject 8	Subject 10	Subject 11	Subject 12

2.10 Materials

1-bromo-3-chloro-propane		*Sigma*
aadUTP	Aminoallyl deoxyuridine triphosphate	*Ambion*
Acetic acid	Analytical grade reagent glacial acetic acid	*Fisher*
Acetonitrile	HPLC grade acetonitrile	*Fisher*
Acetone	HPLC grade acetone	*Fisher*
Acidified acetone:methanol	49% acetone, 49% methanol, 2% acetic acid	
Acrylamide	Protogel ®, 30% 37.5:1 acrylamide to bisacrylamide stabilized solution	*National Diagnostics*
Ammonium acetate		*Sigma*
Ammonium persulphate		*Sigma*
Anchored oligo $dT_{23}N_2$	TTT TTT TTT TTT TTT TTT TTT TTN N 8 µg/µl	*Sigma*

Antibodies	Rabbit p-Erk1/2 antibody	*Cell Signaling* #9102
	Rabbit p-Akt (Thr308) antibody	*Cell Signaling #9275*
	Rabbit VEGF antibody	*Santa Cruz (sc-507)*
	Rabbit Cleaved caspase 3 (ASP175) antibody	*Cell Signaling* #9661
	Rabbit β-actin antibody	*Cell Signaling #4967*
	Mouse α-tubulin antibody	*Santa Cruz* (sc-5286)
	Goat anti-rabbit IgG, HRP-linked antibody	*Cell Signaling #7074*
	Horse anti-mouse IgG, HRP-linked antibody	*Cell Signaling #7076*
Antioxidation buffer	0.4 M Na_2HPO_4:0.4 M NaH_2PO_4 at pH 7.4; Ascorbate 20% w/v and EDTA 0.1% w/v	
A-Plus Poly(A) Polymerase	4 U/µl	*Epicentre Biotechnologies*
10x A-plus reaction buffer	0.5 M Tris-HCl (pH8.0), 2.5 M NaCl and 100 mM $MgCl_2$	*Epicentre Biotechnologies*
Ascorbate		*Sigma*
ATP	10 mM Adenosine triphosphate	*Epicentre Biotechnologies*
Automated film developer	Curix 60, type 9462/106	*Agfa*

β-glucuronidase	Type HP-2 from *Helix pomatia* 111,400u/ml	*Sigma*
Biorad reagent	Coomassie Brilliant Blue G-250, Phosphoric acid and methanol	*Biorad*
BSA	Bovine Serum Albumin, fraction V	*Sigma*
C1 Lysis Buffer	1.28 M sucrose, 40 mM Tris·Cl, 20 mM MgCl2, 4% Triton X-100 (v/v), pH 7.5, in distilled water	*Qiagen*
Cartridge Wash	20% methanol, 2% acetic acid in water	
Cartridge Eluent	49% methanol; 49% acetone and 2% acetic acid	
Cary 50Bio spectrophotometer		*Varian*
Cot1	mouse Cot-1 DNA 1 mg/ml	*Invitrogen*
dATP	Deoxyadenosine triphosphate	*Amersham Biosciences*
dCTP	Deoxycytidine triphosphate	*Amersham Biosciences*
Deconjugation buffer	0.4M Na_2HPO_4:0.4 M NaH_2PO_4 at pH 6.8	
DEPC	Diethyl pyrocarbonate	

DEPC-water	0.1% DEPC in distilled H2O mixed and incubated at room temperature for 1 hour. Solution was then autoclaved and allowed to cool.	
DTT	Dithiothreitol	*Invitrogen*
dGTP	Deoxyguanosine triphosphate	*Amersham Biosciences*
DMSO	Dimethyl Sulfoxide	*Sigma*
dTTP	Deoxythymidine triphosphate	*Amersham Biosciences*
DuoSet IGFBP-3 ELISA development kit Containing: Rat anti-mouse IGFBP-3 capture antibody Biotinylated goat anti-mouse IGFBP-3 detection antibody IGFBP-3 standard Streptavidin-HRP		*R&D Systems*
EC	Epicatechin	*Sigma*
ECG	Epicatechin gallate	*Unilever*
ECL reagents	ECL western blotting detection reagents	*Amersham*

EDTA	Ethylenediaminetetraacetic acid, tetrasodium salt	*Sigma*
EGC	Epigallocatechin	*Unilever*
EGCG	Epigallocatechin gallate	*Unilever*
Enhanced hybridization buffer	Vial 8 taken from FlashTag Biotin RNA labelling kit	*Genisphere*
Ethanol		*Prolabo*
Ethyl acetate	HPLC grade Ethyl acetate	*Fisher*
Foetal Bovine Serum(FBS)		*Invitrogen*
First-strand buffer	50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$	*Invitrogen*
G2 buffer	800 mM guanidine HCl; 30 mM Tris·Cl, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween-20 (v/v); 0.5% Triton X-100 (v/v), in distilled water	*Qiagen*
GeneGenius imaging system		*Syngene*
GeneGnome Bioimaging System		*Syngene*

GeneSnap and GeneTools		
	Image analysis software	*Sygene*
GeneQuant Spectrophotometer		
	Assumes dsDNA with A_{260} = 1.0 for 50 µg/ml in a 10 mm pathlength cell	*GE Healthcare 166384*
Genomic-tips	Qiagen Genomic-tip 100/G	*Qiagen* 10243
GlutaMAX™1	200mM dipeptide L-Alanyl-L-Glutamine	*Gibco 35050-038*
Glycine		*Sigma*
Greenselect®	Mixture of polyphenols extracted from green tea of which >60% are catechins and >40% total represents EGCG	*Indena*
Handheld homogeniser		
	T8 Ultra-turrax homogeniser	*Fisher*
HEEBO array	Array made from the Human Exonic Evidence Based Oligonucleotide set 48,958 probes, a collection of 70mer 5' amino modified oligonucleotide probes	

	Arrays were printed “in-house” onto three slides per array [250].	*Invitrogen*
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid	*Sigma*
HPLC system	Agilent 1100 series	*Agilent Technologies*
Isopropanol	Propan-2-ol	*Fisher*
ISOTON II	0.3% 2-phenoxyethanol, 0.03% sodium fluoride in water	*Bekman Coulter*
KP buffer	10 mM di-potassium hydrogen orthophosphate, pH 7.0, in distilled water	*Sigma*
Labelling mix	dATP 0.5 mM dGTP 0.5 mM dCTP 0.5 mM dTTP 0.2 mM aadUTP 0.3 mM DTT 10 mM in first-strand buffer	
Li-heparin tubes (7.5 ml)		
	S-Monovette®	*Sarstedt*

Li-heparin tubes (1.3 ml)		
	Micro-tube	*Sarstedt*
Lysis buffer	Sodium chloride 1.19 M, Tris 50 mM, Triton X-100 0.5% (v/v), EDTA 1 mM, EGTA 1 mM, Sodium orthovanadate 1 mM, Protease Inhibitor Cocktail 1% (v/v)	
M_1G Primary Antibody		
	Antibody specific to M_1G, raised in mouse by	Sevilla et al. [251]
M_1G Secondary Antibody		
	Horseradish peroxidise-conjugated, polyclonal goat anti-mouse Ig	*Dako*
Mass spectrometer	API2000 Triple Quadrupole mass spectrometer including air collision cell	*Applied Biosystems* / *MDS Sciex*
2-Mercaptoethanol		*Sigma*
MDA treated calf thymus DNA		
	10 fmol M_1G per mg DNA, Kindly donated by R. Singh, prepared by method described by	Seto et al.[252]

MEEBO array	Array made from the Mouse Exonic Evidence Based Oligonucleotide set 38,467 probes, a collection of 70mer 5’ amino modified oligonucleotide probes representing almost 25,000 genes. Arrays were printed “in-house” onto two slides per array[250].	*Invitrogen*
MEM Media	Modified Eagle Media. Contains GlutaMAX™1 substituted on a molar equivalent basis for L-glutamine, contains Earle's salts.	*Gibco*
Methanol	HPLC grade methanol	*Fisher*
Microarray scanner	Axon 4200 professional multi-laser scanner	*Genepix*
Microcon-10 filtration unit	Centrifuge filtration tubes designed to retain molecules >10,000 MW	*Millipore*
Microcon-30 filtration unit	Centrifuge filtration tubes designed to retain molecules >30,000 MW	*Millipore*
Microplate absorbance reader		

	FLUOstar Optima	*BMG Labtechnologies*
Milk Powder	"*Marvel*" non-fat milk powder	*Premier Brands*
Mini-PROTEAN 3		*Biorad*
Minifold II blotting apparatus		*Schleicher and Schuell*
Mobile phase A	5mM Ammonium acetate pH3; 1.75% acetonitrile 0.12% tetrahydrofuran in water	
Mobile phase B	5mM Ammonium acetate pH3.45; 58.5% acetonitrile and 12.5% tetrahydrofuran in water	
Mobile phase C	Water-acetonitrile-acetic acid, 96:2:2, v/v	
Mobile phase D	Acetonitrile-acetic acid, 98:2, v/v	
Modified MEM Media		
	Minimum Essential Medium Eagle With the following additions 10% FBS 1% 100 mM Sodium Pyruvate 0.2% Non-essential Amino Acids	*Sigma*

Modified RPMI 1640 Media

RPMI 1640 Media *Sigma*

With the follwing additions

10% FBS

1% 100 mM Sodium Pyruvate

1% 200mM GlutaMAX™1

0% Neutral Buffered Formalin

3.7% formaldehyde (w/v), 0.046 M Sodium phosphate dibasic, 0.033 M Sodium phosphate monobasic, in distilled water

Nitrocellulose Membrane

For M_1G - Whatman “PROTRAN” BA79 Pore=0.1 µm

For Western Blotting –

Whatman “PROTRAN” BA85 Pore=0.45 µm *Whatman*

Non-essential Amino Acids *HyClone*

	g/L
L-Alanine	0.89
L-Asparagine H_2O	1.50
L-Aspartic Acid	1.33
L-Glutamic Acid	1.47
Glycine	0.75
L-Proline	1.15

	L-Serine	1.05	

Nunc Filter Cap Flask

Nunclon ∆ Delta surface treatment

Large = 800 ml volume

175 cm^2 growth area

Medium= 260 ml volume

83 cm^2 growth area *Myriad*

PBS Phosphate Buffered Saline

(Dulbecco `A' Tablets)

	g/L	
Sodium chloride	8.0	
Potassium chloride	0.2	
Disodium hydrogen phosphate	1.15	
Potassium dihydrogen phosphate	0.2	
(pH 7.3)		*Oxoid*

PBS-PI 5 µg/ml propidium iodide in PBS

PBS-Tween 0.1% (v/v) Tween-20 in PBS

Pestle Cordless pellet pestle *Kontes*

Petri Dishes 100mm x 15 mm *Myriad*

Phase Lock Gel tube	2 ml tube containing “Heavy” Phase Lock Gel Tubes prepared by centrifugation at 12,000g for 30 s	*Flowgen*
Phosphate buffer	0.4 M Na2HPO4 and NaH2PO4 mixed to pH 7.4 20% ascorbate, 0.1% EDTA	
Photographic film	Hyperfilm ECL chemiluminescence film	*Amersham*
Precision plus dual colour protein marker	250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa	*Biorad*
Propidium iodide		*Sigma*
Protease Inhibitor Cocktail		*Sigma*
	AEBSF 104 mM Aprotinin 0.08 mM Leupeptin 2 mM Bestatin 4 mM Pepstatin A 1.5 mM E-64 1.4 mM	
Proteinase K	20 mg/ml stock solution	*Qiagen*
QBT buffer	750 mM NaCl, 50 mM MOPS, pH 7.0,	

	15% isopropanol (v/v), 0.15% Triton X-100, in distilled water	*Qiagen*
QC buffer	1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol (v/v), in distilled water	*Qiagen*
QF buffer	1.25 M NaCl, 50 mM Tris·Cl, pH 8.5 15% isopropanol (v/v), in distilled water	*Qiagen*
Quantikine® mouse IGF-1 solid phase ELISA		*R&D Systems*
Random pentadecamers		
	NNN NNN NNN NNN NNN	*Sigma*
Reverse transcriptase		
	Superscipt III reverse transcriptase 200U/µl	*Invitrogen*
RNase A		*Sigma*
RNAsin	RNase inhibitor 40u/µl	*Promega*
RPMI 1640	With L-glutamine and sodium bicarbonate	*Sigma*
SDS	Sodium dodecyl sulphate	*Sigma*

Shaped blotting papers		
	6.3 x 22.8 cm to fit Minifold II System	*Schleicher and Schuell*
Sodium acetate	CH_3COONa	*Sigma*
Sodium carbonate	Na_2CO_3	*Sigma*
Sodium chloride	NaCl	*Sigma*
Sodium dithionite	also known as sodium hydrosulfite $Na_2S_2O_4$	*Sigma*
Sodium hydroxide	NaOH	*Sigma*
Sodium pyruvate	100mM solution	*Sigma*
Sonication probe	UP 50H compact sonicator	*Hielscher*
Spectroscopy cuvette		
	10 x 4 x 4.5 mm REF 67.742	*Sarstedt*
Sulfatase	Type VIII from *Albalone* entrails, dissolved in water to generate solution of 0.5u/µl	*Sigma*
Supersignal® West Dura extended duration substrate		
	Prepared by mixing equal volumes of Luminol Enhancer and Stable Peroxide Buffer	*Pierce*

Syringe filter	0.2 µm pore size cellulose acetate membrane		*Schleicher and Schuell*
TBST	50mM tris (pH 7.5), 150 mM sodium chloride and 0.1% tween-20 in distilled water		
TEMED	N,N,N',N'-Tetramethylethylenediamine		*Sigma*
Tetrahydrofuran	Oxacyclopentane, C_4H_8O		*Fisher*
Theaflavins	11% theaflavin	MW= 564.5	
	28% theaflavin-3-monogallate	MW= 716.6	
	16% theaflavin-3'-mongallate	MW=716.6	
	45% theaflavin-3,3'-digallate	MW= 868.8	
	7% moisture		*Unilever*
Tissue extraction buffer	Equal volumes of Methanol:ethylacetate (2:1) and 0.3M Sodium dithionite; 0.1% EDTA		
TRI reagent	TRI Reagent is a registered trademark of Molecular Research Center, Inc.		*Sigma*
Triton X-100	Octyl phenol ethoxylate		*Sigma*
Trypsin-EDTA (10x)	0.5% Trypsin (w/v), 5.3 mM EDTA Mycoplasma and Porcine parvovirus tested.		

	1x Trypsin-EDTA prepared with PBS	*Gibco*
Tris	Tris Hydroxymethylaminoethane	*Sigma*
Tris HCl	Tris hydroxymethylaminoethane hydrochloride	*Sigma*
Tween®-20	Polyoxyethylene sorbitan monolaurate	*Sigma*
Ultrasonic bath		*Ultrawave*
Water	Dispensed from Barnstead NANOpure Diamond water purifier	*Triple Red*
Waters Oasis HLB Extraction Cartridges		
	1cc capacity containing 30 mg Oasis HLB 30µm sorbent	*Waters*
WebGestalt website	WEB-based GEne SeT AnaLysis Toolkit Online software available at : http://bioinfo.vanderbilt.edu/webgestalt/ Pathways identified by using the Kyoto Encyclopedia of Genes and Genomes database	*Zhang et al.[253]*
Western Blocking Buffer		

5% milk powder (w/v) in TBST

Western Loading Buffer

187.5 mM Tris-HCl (pH 6.8), 6% SDS (w/v),
30% glycerol (v/v), 0.03% bromophenol blue (w/v),
0.3 M dithiothreitol in distilled water

Western Polyacrylamide denaturing resolving gel

10% Acrylamide (w/v), 1x running buffer,
1% SDS (w/v), 0.076% ammonium persulphate
(w/v), 0.05% TEMED (v/v) in distilled water

Western Polyacrylamide stacking gel

3.75% Acrylamide (w/v), 1x stacking buffer,
1% SDS (w/v), 0.08% ammonium persulphate (w/v),
0.08% TEMED (v/v) in distilled water

Western Reservoir Buffer

0.19 M Glycine, 25 mM Tris, 0.1% SDS (w/v),
in distilled water

Western Running Buffer

0.4 M Tris Buffer, pH 8.8, in distilled water
(70 mM Tris-HCl + 330 mM Tris)

Western Stacking Buffer

0.1M Tris Buffer, pH 6.8, in distilled water

(95.2 mM Tris-HCl + 4.8 mM Tris)

Western Stripping Buffer

62 mM Tris and 2% SDS (w/v),

0.8 % (v/v) 2-mercaptoethanol added immediately

prior to use, in distilled water

Western Transfer Buffer

48 mM Tris, 39 mM glycine, 20% methanol (v/v),

3.7% SDS (w/v), in distilled water

Xterra phenyl column (2.1 mmx150 mm; 3.5 µm pore) *Waters*

3 Identification of biomarkers of cancer chemoprevention by tea polyphenols in human prostate cancer cells in culture

3.1 Introduction

To attempt to identify a putative biomarker of cancer prevention by tea polyphenols, their effect upon oxidative DNA adduct, M_1G, was investigated in human prostate cancer cells *in vitro*. As detailed in the introduction, section 1.9.2, oxidative DNA adducts in prostate tissue have been shown to be more prevalent in prostate carcinoma than in normal tissue [174, 175]. M_1G concentration in DNA from breast tissue taken from a breast containing a breast carcinoma was higher than the concentration from normal controls [176]. Also, orally administered selenium has been shown, in dogs, to reduce oxidative DNA damage in the prostate [177].

M_1G is, therefore, a putative biomarker shown to be related to carcinogenesis and amenable to change by dietary agents. The null hypothesis was that M_1G concentrations in DNA from cells incubated with tea polyphenols would be unchanged compared with control cells, however, it was expected that antioxidant tea polyphenols would reduce oxidative damage and therefore oxidative adducts such as M_1G.

3.2 The effect of tea polyphenols on M_1G concentrations in DU145 and LNCaP cells

LNCaP or DU145 cells, incubated in appropriate media containing catechins or theaflavins at concentrations of 0, 3.125 µg/ml, 12.5 µg/ml or 25 µg/ml for 24 or 48 hours, were prepared in triplicate and their M_1G concentrations measured according the experimental design in section 2.2.5.

Figure 10 shows M_1G standards in an immuno-slot blot with its corresponding propidium iodide stain. Figure 11 shows a blot prepared in parallel with the standards that contains DNA from DU145 cells grown in media for 24 hours, containing 0, 3.125 µg/ml, 12.5 µg/ml or 25 µg/ml.

The mean fluorescence of the propidium iodide stained slots under UV light was calculated, as was the ratio between the fluorescence of each slot and the mean. The chemiluminescence from each slot, controls and standards was then divided by this ratio in order to correct for unequal DNA loading.

The adjusted chemiluminescence from M_1G controls was then used to draw a standard curve, an example of which is shown in figure 12. However, it was clear from this standard curve that the control calf thymus DNA, thought to have minimal oxidative damage, did not give a zero signal for M_1G.

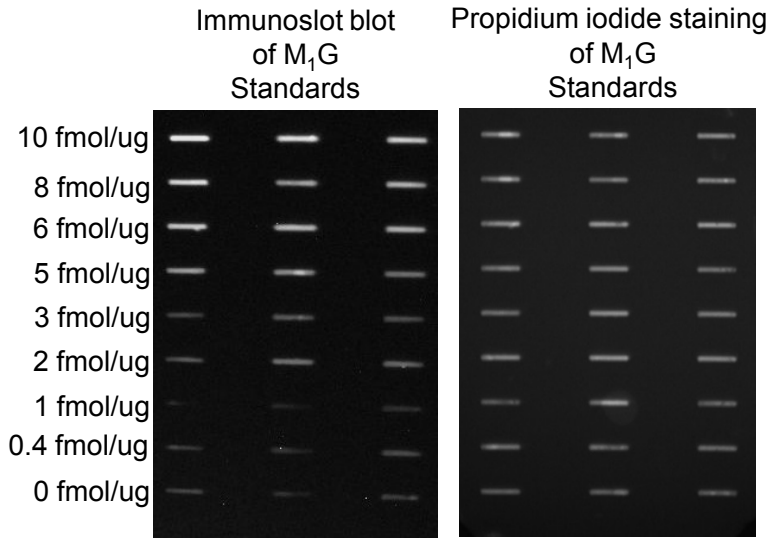

Figure 10 Immunoslot blot to detect M_1G concentration and propidium iodide staining of the same slot blot of M_1G standards prepared using calf thymus DNA.

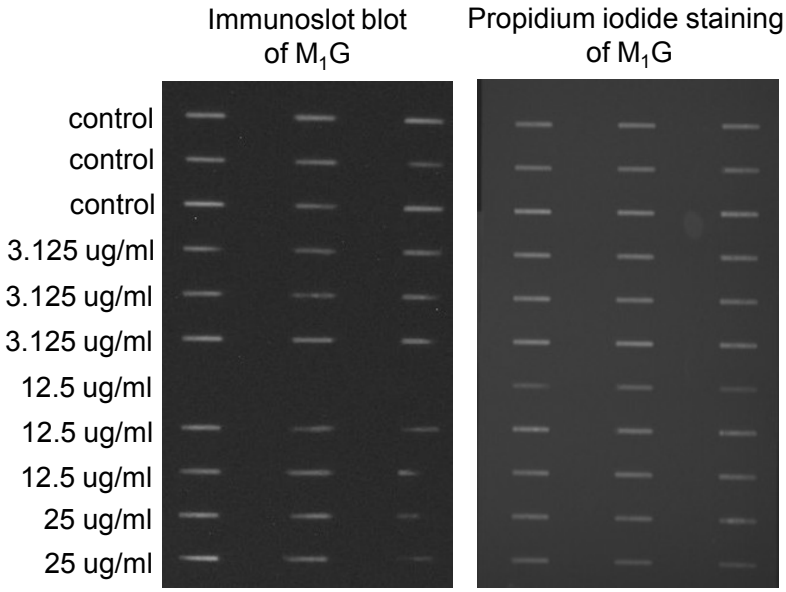

Figure 11 Immunoslot blot to detect M_1G concentration and propidium iodide staining of DNA from DU145 cells incubated for 24 hours with varying concentrations of catechins

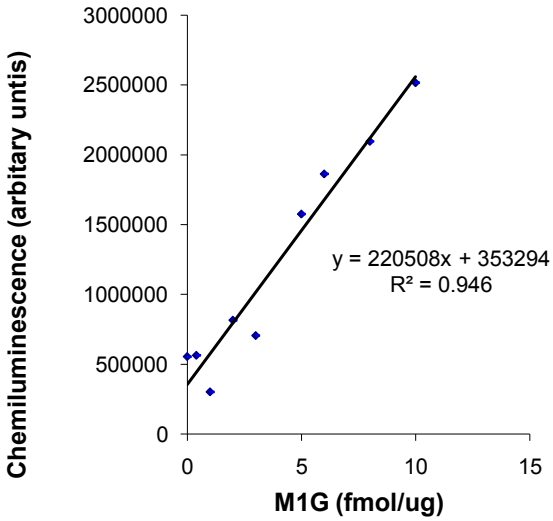

Figure 12 Standard curve of chemiluminescence from M_1G standards against M_1G concentration

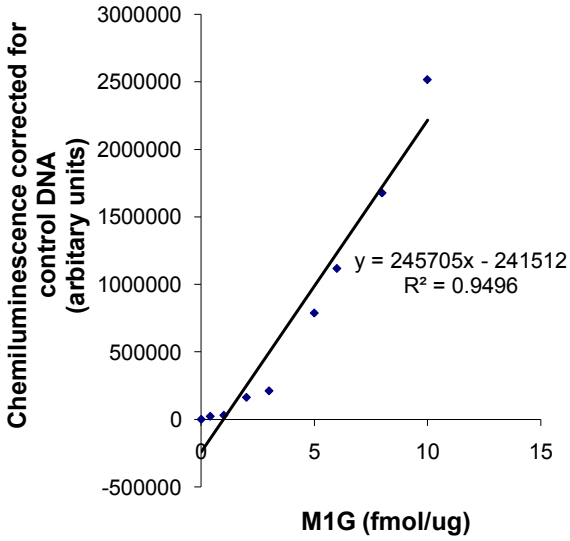

Figure 13 Standard curve of M_1G standards chemiluminescence corrected for signal from control calf thymus DNA

The signal measured from the 0 fmol/μg M_1G standard was used to calculate what proportion of each of the other standards' signal was due to the presence of the control calf thymus DNA. This variable proportion was subtracted from the signal of each of the other M_1G standards according to their composition of control calf thymus DNA and M_1G standard DNA. The derived standard curve is shown in figure 13.

This derived standard curve was then used to calculate the M_1G concentrations of the experimental samples. Figures 14-21 show the means of M_1G concentrations in DNA from experimental samples, using each permutation of DU145 and LNCaP cells, catechins and theaflavins, and, 24 hours and 48 hours incubation.

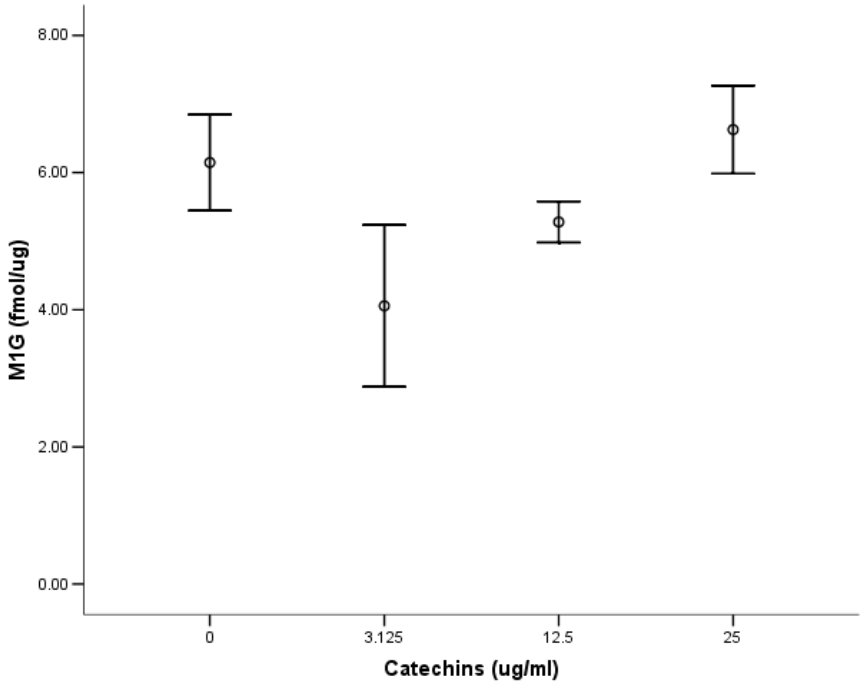

Figure 14 M_1G concentration in DNA from DU145 cells cultured for 24 hours in media containing various concentrations of catechins (mean +/- SEM), differences between groups did not reach statistical significance.

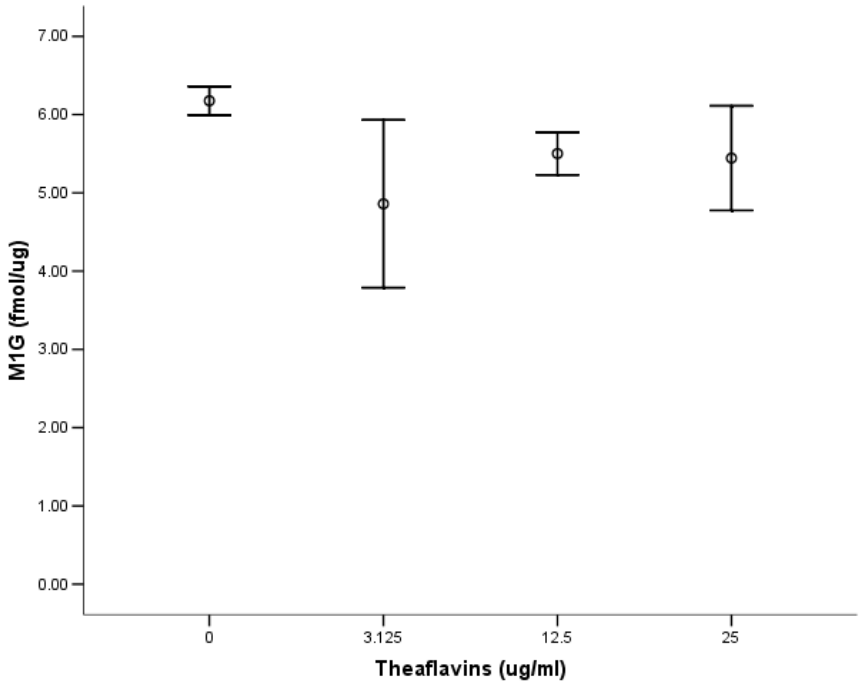

Figure 15 M_1G concentration in DNA from DU145 cells cultured for 24 hours in media containing various concentrations of theaflavins (mean +/- SEM), differences between groups did not reach statistical significance.

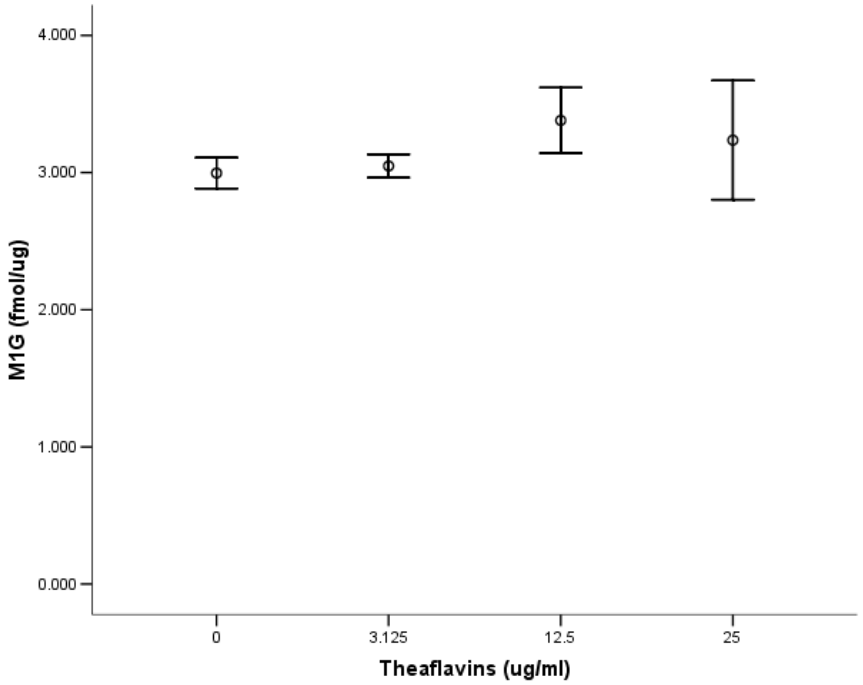

Figure 16 M_1G concentration in DNA from LNCaP cells cultured for 24 hours in media containing various concentrations of theaflavins (mean +/- SEM), differences between groups did not reach statistical significance.

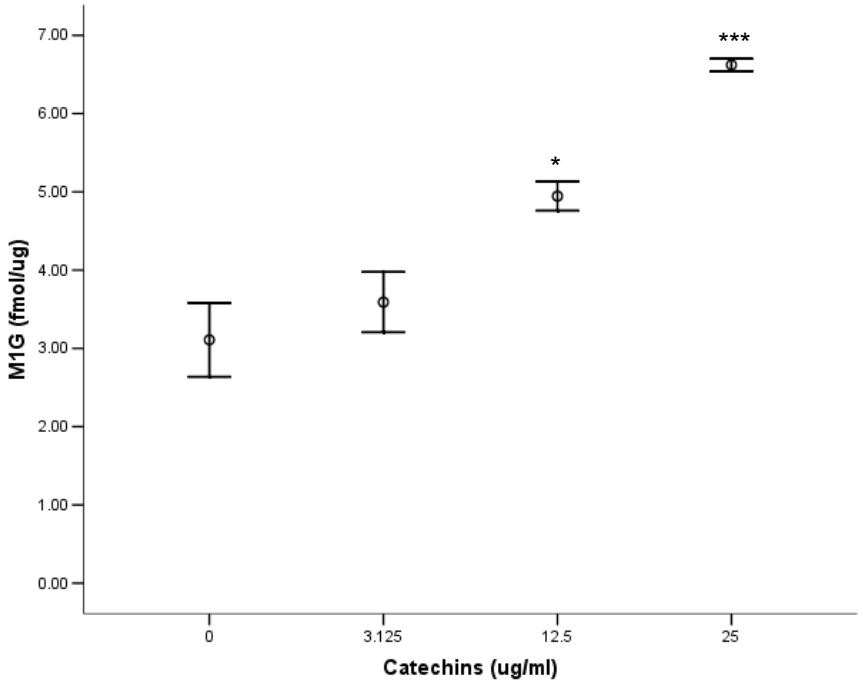

Figure 17 M_1G concentration in DNA from LNCaP cells cultured for 24 hours in media containing various concentrations of catechins (mean +/- SEM). Analysis by one-way ANOVA demonstrated that significant difference existed between the groups $p<0.001$. Post hoc analysis by Tukey's HSD test revealed that this difference was located between control cells and those incubated with 12.5 µg/ml catechins and, between control cells and those incubated with 25 µg/ml catechins (* $p=0.016$, *** $p<0.001$).

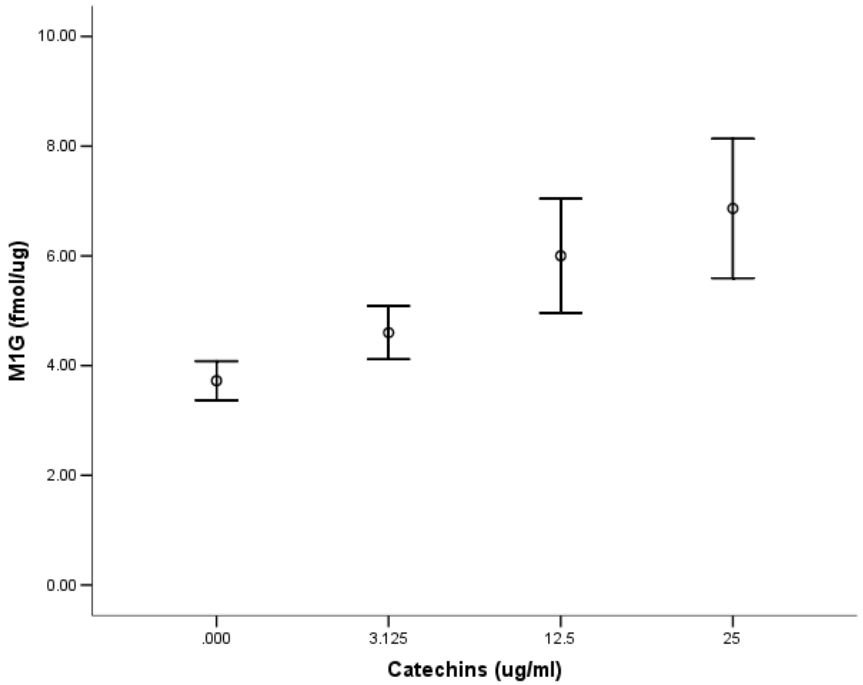

Figure 18 M_1G concentration in DNA from LNCaP cells cultured for 48 hours in media containing various concentrations of catechins (mean +/- SEM), differences between groups did not reach statistical significance.

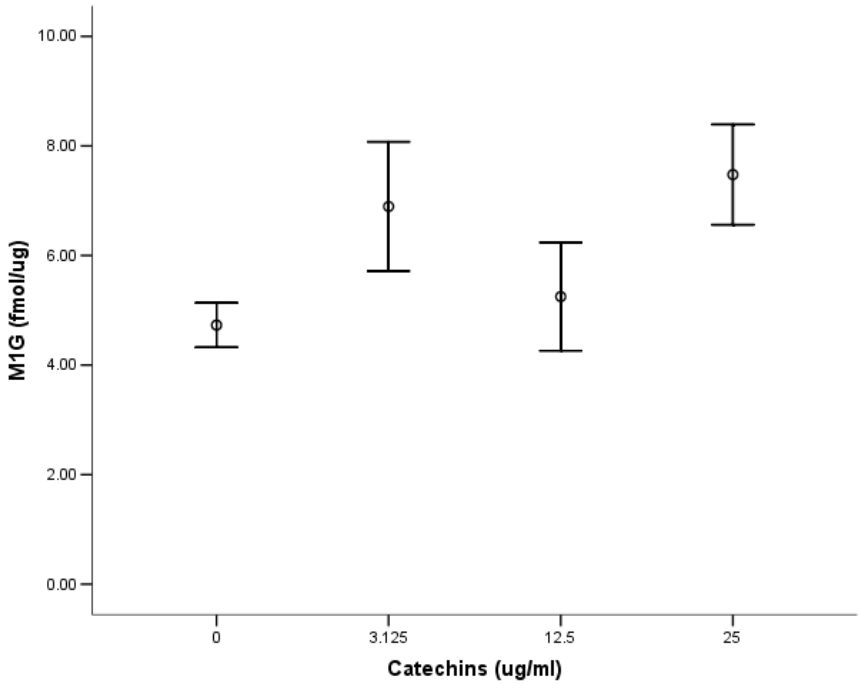

Figure 19 M_1G concentration in DNA from DU145 cells cultured for 48 hours in media containing various concentrations of catechins (mean +/- SEM), differences between groups did not reach statistical significance.

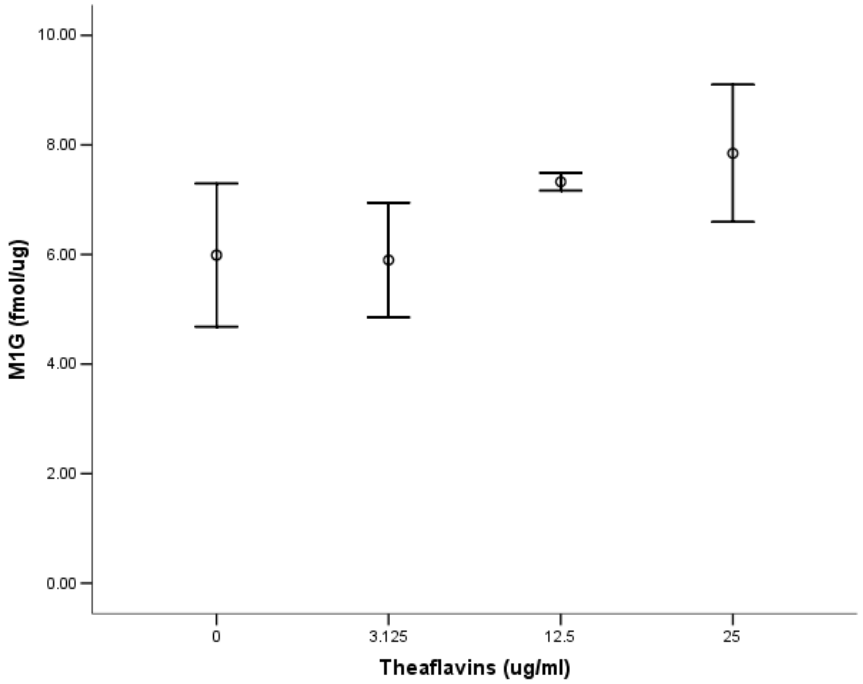

Figure 20 M_1G concentration in DNA from DU145 cells cultured for 48 hours in media containing various concentrations of theaflavins (mean +/- SEM), differences between groups did not reach statistical significance.

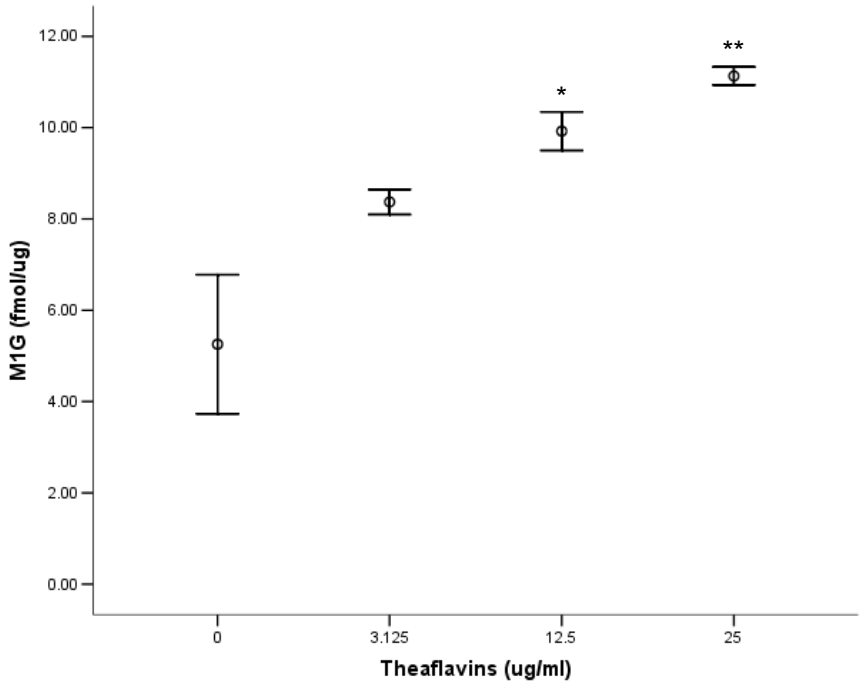

Figure 21 M_1G concentration in DNA from LNCaP cells cultured for 48 hours in media containing various concentrations of theaflavins (mean +/- SEM). Analysis by one-way ANOVA demonstrated that significant difference existed between the groups p=0.005. Post hoc analysis by Tukey's HSD test revealed that this difference was located between control cells and those incubated with 12.5 µg/ml catechins and, between control cells and those incubated with 25 µg/ml catechins (* p=0.015, ** p=0.004).

Analysis of the concentration of M_1G DNA adducts in the DNA from cultured human prostate cancer cells failed to demonstrate any consistent reduction following incubation with either catechins from green tea or theaflavins from black tea. There was, however, a trend observed in the adduct concentrations from LNCaP cells, higher concentrations being seen in cells incubated with higher concentrations of tea polyphenols, both catechins and theaflavins. Analyses of the mean M_1G adduct concentrations from cells in all eight experiments using one-way ANOVA found significant differences in two experiments both involving LNCaP cells. LNCaP cells incubated in higher concentrations of catechins for 24 hours, see figure 17, showed significant increases in M_1G concentrations. LNCaP cells incubated with higher concentrations of theaflavins for 48 hours, see figure 21, also showed significant increases in M_1G concentrations.

3.3 The effect of tea polyphenols on the expression of p-Erk1/2 and caspase-3 in DU145 and LNCaP cells

To attempt to identify further putative biomarkers of tea polyphenol mediated cancer prevention, the effects of catechins and theaflavins upon various proteins involved in carcinogenesis were studied in *in vitro* human prostate cancer cells.

p-Erk1/2 is a signal transduction molecule in the IGF-1 receptor pathway. IGF-1 is known to be associated with prostate cancer cell proliferation in culture and prostate carcinogenesis in murine models and man, see section 1.9.4. Activation of Erk1/2 by phosphorylation is under the influence of IGF-1. Reduction in the expression of p-Erk1/2 has been demonstrated in the prostate of TRAMP mice who received 0.1% green tea polyphenols in their drinking water [193].

Caspase-3 is known to be a central effector of apoptosis. Activation of caspase-3 has been demonstrated in LNCaP and DU145 cells following exposure to genistein [212] and amygdaline [213], see section 1.9.5.

It was expected that antioxidant tea polyphenols would reduce p-Erk1/2 and induce Caspase 3 expression in LNCaP and DU145 cells incubated with tea polyphenols. The null hypothesis, however, was that expression of these putative biomarkers would be unchanged compared with control cells.

Western blots of cell lysates were prepared in duplicate, of each of the 32 permutations of LNCaP or DU145 cells, catechins or theaflavins in the media, polyphenol concentrations of 0, 3.125, 12.5 or 25 µg/ml with an incubation period of 24 or 48 hrs according to section 2.3.7. Figures 22-25 show the

chemiluminescence captured on photographic film following incubation of each of the eight blots with anti-p-Erk1/2.

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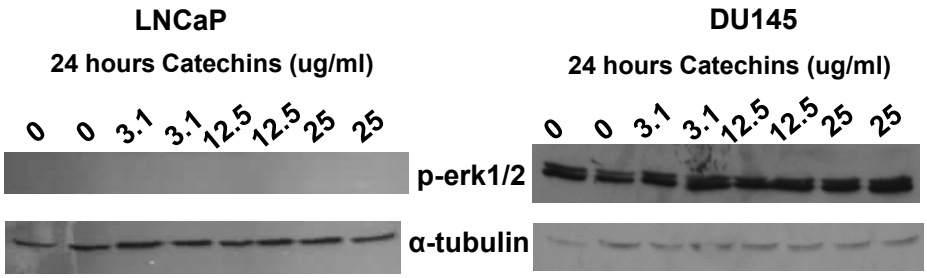


Figure 22 Western blot of cell lysates from LNCaP and DU145 cells treated with catechins for 24 hours, probed with anti-p-Erk and anti-α-tubulin, differences between groups did not reach statistical significance.

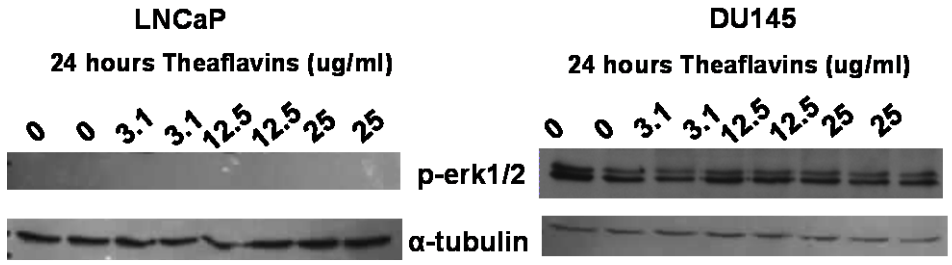


Figure 23 Western blot of cell lysates from LNCaP and DU145 cells treated with theaflavins for 24 hours, probed with anti-p-Erk and anti-α-tubulin, differences between groups did not reach statistical significance.

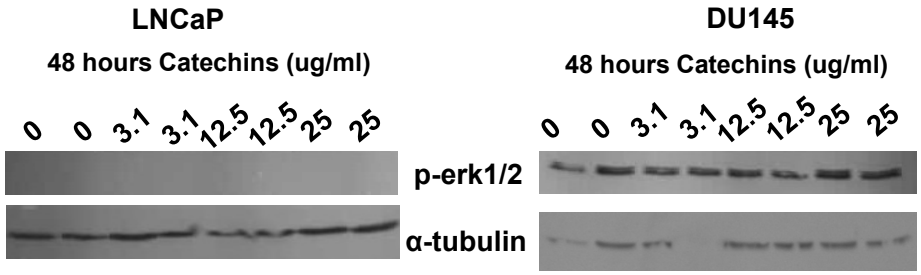


Figure 24 Western blot of cell lysates from LNCaP and DU145 cells treated with catechins for 48 hours, probed with anti-p-Erk and anti-α-tubulin, differences between groups did not reach statistical significance.

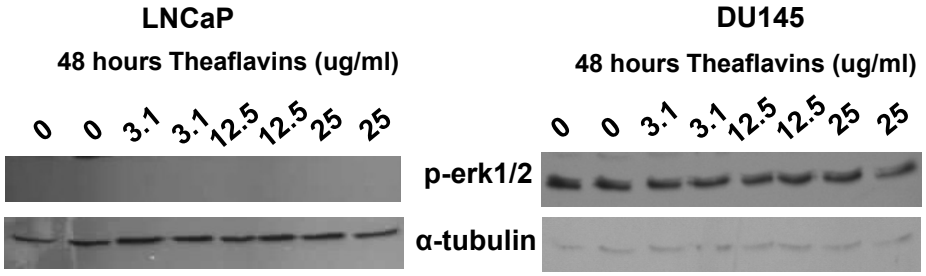


Figure 25 Western blot of cell lysates from LNCaP and DU145 cells treated with theaflavins for 48 hours, probed with anti-p-Erk and anti-α-tubulin, differences between groups did not reach statistical significance.

pErk1/2 expression was not demonstrated in LNCaP cells despite successful electrotransfer of protein being verified by the presence of strong reaction with anti-α-tubulin antibody . In DU145 cells there were easily detectable levels of intracellular pErk1/2. Equal loading of lysate was confirmed by the presence of equal reaction to anti-α-tubulin. There was no evidence that the accumulation of this intracellular signal transduction molecule is reduced by tea polyphenols.

The same blots having been stripped and reblocked were probed again with anti-cleaved caspase 3. Figure 26-29 shows the results of this experiment.

There was no expression of cleaved caspase 3 detectable in LNCaP or DU145 cells, incubated with catechins or theaflavins for 24 or 48 hours. As a positive control, mouse prostate lysates were also analysed by western blot and probed with anti-cleaved caspase-3, see section 4.2.6.

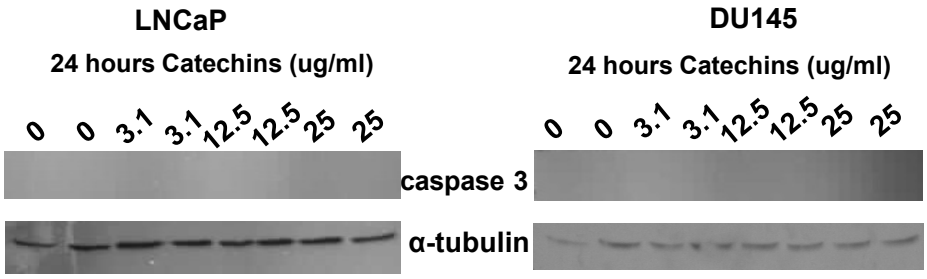


Figure 26 Western blot of cell lysates from LNCaP and DU145 cells treated with catechins for 24 hours, probed with anti-caspase 3 and anti-α-tubulin, differences between groups did not reach statistical significance.

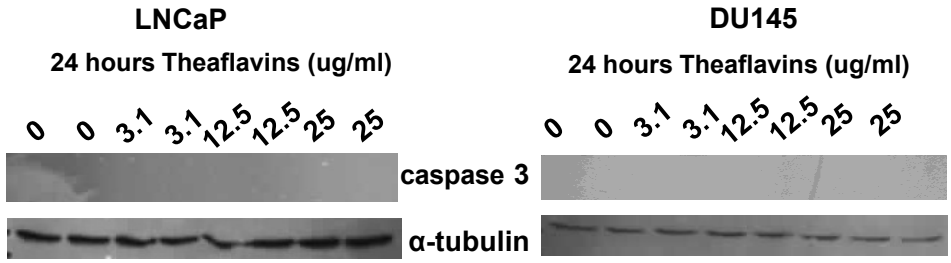


Figure 27 Western blot of cell lysates from LNCaP and DU145 cells treated with theaflavins for 24 hours, probed with anti-caspase 3 and anti-α-tubulin, differences between groups did not reach statistical significance.

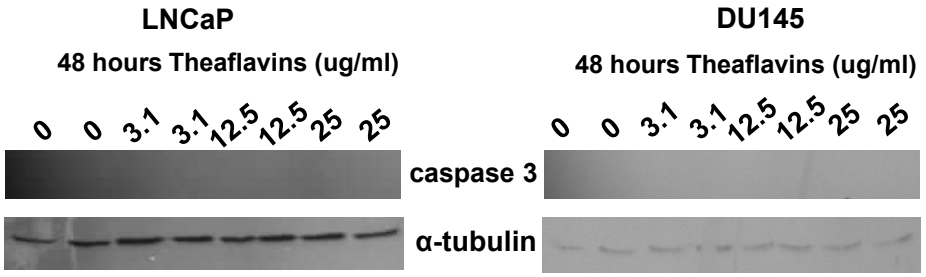


Figure 29 Western blot of cell lysates from LNCaP and DU145 cells treated with theaflavins for 48 hours, probed with anti-caspase 3 and anti-α-tubulin, differences between groups did not reach statistical significance.

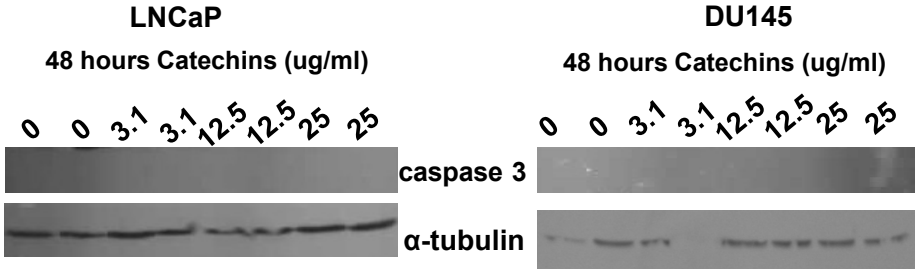


Figure 28 Western blot of cell lysates from LNCaP and DU145 cells treated with catechins for 48 hours, probed with anti-caspase 3 and anti-α-tubulin, differences between groups did not reach statistical significance.

3.4 Discussion

The identification of biomarkers of cancer chemoprevention is important for the efficient preliminary testing of potential prostate cancer preventive agents, see section 1.9.1. The long natural history of prostate cancer would impede the study of many potential chemopreventive agents in human populations due to the time and money involved in conducting clinical trials. In the work presented here experiments using prostate cancer cells in culture were performed to try to identify biomarkers of prostate cancer chemoprevention *in vitro* that could be proposed for *in vivo* animal and human studies.

The tea polyphenols, catechins and theaflavins, are thought to possess antioxidant properties, see section 1.3.3. It was therefore, expected that concentrations of the oxidative DNA adduct M_1G would fall in the DNA of cells incubated with these polyphenols, see section 1.9.2. This change in M_1G could then be proposed as a marker demonstrating the effect of tea polyphenols.

Analysis of DNA from DU145 human prostate cancer cells after incubation with 3.125, 12.5 or 25 μg/ml theaflavins or catechins failed to identify any significant change in M_1G. This may be due to tea polyphenols not acting as active antioxidant molecules in the intracellular environment or that the concentration of polyphenols was insufficient for this activity to significantly alter the oxidative status of the DNA. It was decided that increasing the concentration of the polyphenols above 25 μg/ml would not provide biologically relevant data because these concentrations would be unlikely to be achieved *in vivo*.

The incubation of LNCaP human prostate cancer cells with catechins for 24 hours or theaflavins for 48 hours produced a dose dependent increase in the M_1G concentration that was statistically significant. There was also a similar trend in the results from LNCaP cells incubated with catechins for 48 hours and with theaflavins for 24 hours; these trends did not themselves achieve statistical significance. The results taken together seem to suggest that catechins and theaflavins, at concentrations in excess of 3.125 µg/ml, increase the M_1G concentration in the DNA of cultured LNCaP cells.

Unless this effect was in some way specific to M_1G, this result may be due to tea polyphenols possessing pro-oxidant properties at the concentrations used in these experiments. The antioxidant activity of flavonoids like tea polyphenols is described in section 1.3.3. In the presence of superoxide anions, aroxyl radicals of flavonoids may be generated, which then mutually react removing radicals from the environment and terminating radical chain reactions. However, an alternative reaction may take place in the presence of copper ions which are found in the calf serum added to culture media, see section 2.1.1. A flavonoid aroxyl radical is generated by copper ion reduction. This radical then interacts with oxygen producing the stable flavonoid quinone and a superoxide anion.

$$\text{HO-Fl-OH} + \text{Cu(II)} \rightarrow \text{HO-Fl-O}^{\bullet} + \text{Cu(I)}$$

$$\text{HO-Fl-O}^{\bullet} + \text{O}_2 \rightarrow \text{O=Fl=O} + \text{O}_2^{\bullet -}$$

$$\text{O}_2^{\bullet -} + \text{Cu(I)} \rightarrow \text{H}_2\text{O}_2 + \text{Cu(II)}$$

$$\text{H}_2\text{O}_2 + \text{Cu(I)} \rightarrow \text{OH}^{\bullet} + \text{OH}^{-} + \text{Cu(II)}$$

This redox cycling of Cu(I)/Cu(II) has previously been implicated in the flavonoid-mediated generation of highly reactive radicals and the subsequent generation of the DNA adduct 8-hydroxydeoxyguanosine [254].

There was an opportunity missed that future studies could address to analyse this apparent pro-oxidant effect and the extent to which it reflects *in vivo* metabolism. For instance, investigating the effect of adding oxidative inhibitors such as catalase to culture media may provide evidence one way or the other for the validity of the above proposed pro-oxidant mechanism. In addition, it has been suggested that the generation of oxidants such as hydrogen peroxide is a not only due to the availability of transitional metals in commonly used cell culture medium but also due to their relative deficiency in antioxidants and therefore is not representative of *in vivo* metabolism [255]; it would be interesting to investigate the effect of correcting deficiencies in selenium, vitamin C and vitamin E.

The technique used for quantification of the M_1G in DNA samples relied on standards of known M_1G concentration being analysed. The technique employed used various masses of DNA of know M_1G concentration with calf thymus DNA being added to each sample in order to make each sample contain 3.5 μg DNA. It was assumed that the calf thymus DNA would contain minimal oxidative damage and therefore would not test positive for significant amounts of M_1G. However, in practice the calf thymus DNA produced a significant background signal. This signal was therefore subtracted from the signal of the standards in order to correct for this. If the signal from the calf thymus DNA was spurious then this procedure would have rendered the quantification more accurate. If, however, the calf

thymus DNA had become oxidized such that the signal was genuine, then this casts doubt on the quantification procedure. Although this doubt exists concerning the absolute values, there can be confidence in the findings that relatively increased concentrations of M_1G are found in LNCaP cells incubated with catechins for 24 hours or theaflavins for 48 hours.

It was thought that exposure to tea polyphenol may inhibit activation of Erk1/2 to p-Erk by proposed mechanisms detailed in 1.9.4. However, the experiments investigating p-Erk1/2 expression *in vitro* and the effect of tea polyphenol exposure were invalidated by the poor choice of positive control, this case tubulin. The correct approach would be to have used total Erk1/2 expression as the control as in other publish work [256]. In any case, no expression of p-Erk1/2 was demonstrated in LNCaP cells, so this work was unable to address whether tea polyphenols can inhibit Erk1/2 activation. Despite evidence that green tea polyphenols inhibit carcinogenesis *in vivo* [193] by suppression of p-Erk1/2 there was also no evidence of reduced expression in DU145 cells following exposure to green or black tea polyphenols in the work presented here. However, even if a reduction in p-Erk expression had been demonstrated the use of a time point such as 24 hours would not have been sensitive to specific reductions in p-Erk as these would have been expected to have occurred within minutes of polyphenol exposure [257]; indeed if a reduction in p-Erk had been demonstrated then this may well have been due to non-specific polyphenol toxicity.

Another significant technical deficiency in the western blot data presented here was the omission of the protein size markers used, the lack of which makes it impossible to verify the identity of the bands shown.

4 The effect of tea polyphenols on prostate carcinogenesis and biomarkers of carcinogenesis in the TRAMP mouse model

4.1 The influence of catechins and theaflavins on prostate carcinogenesis in the TRAMP mouse

4.1.1 Introduction

To examine the extent to which tea polyphenols prevent prostate cancer in humans using prostate cancer incidence as an endpoint would take years and require large numbers of subjects. Animal models can be used to more rapidly examine if particular agents have potential chemopreventive properties in order to exclude certain agents from further study. The transgenic TRAMP mouse was selected as such a model of prostate cancer, see section 1.7.3. It has previously been reported that 0.1% green tea polyphenols in the drinking water of TRAMP mice was sufficient to abolish metastatic spread of prostate carcinomas [85] see section 1.7.3.

In order to examine the influence of tea polyphenols upon prostate carcinogenesis in the TRAMP mouse, 42 such mice were randomised into three groups of 14 for 26 weeks according to the study design in section 2.5.2. One group received their drinking water without additives, one received water containing 0.05% black tea theaflavins and one received water containing 0.05%

green tea catechins as their sole fluid source. These low concentrations were chosen due to the limited solubility of theaflavins in water and the fact that 0.05% is within an order of magnitude of the concentration of green tea polyphenols already shown to have cancer preventive effects [85]. The mice were dissected to investigate prostate cancer chemoprevention by both green tea catechins and black tea theaflavins, in terms of inhibition of both the primary tumour and the rate of metastases.

4.1.2 Exclusions of TRAMP mice from the study protocol

All but six mice completed the study protocol, two from each intervention group. Table 5 shows the indications for removing these mice from the protocol for culling. Mouse number 42 was subsequently excluded from analysis because it died of unknown causes without preservation of tissues being possible immediately following death.

Table 5 Fate of TRAMP mice that failed to complete study protocol

Mouse Number	Intervention Group	Days on Study Protocol	Indication for premature culling
7	Water	138	Weight loss
42	Water	46	Found dead, deteriorated
14	Theaflavins	169	Weight loss

27	Theaflavins	95	Piloerection
32	Catechins	141	Weight loss, urinary retention
35	Catechins	162	Piloerection

4.1.3 The effect of oral tea polyphenols on TRAMP mice body weight

Mice were weighed each week during the 26 weeks of dietary intervention. Figure 30 shows the mean body weights of the mice in the three groups each week of the study. There were no significant differences between the final mean body weights of each group analysed by one way ANOVA or by the non-parametric Kruskal-Wallis test.

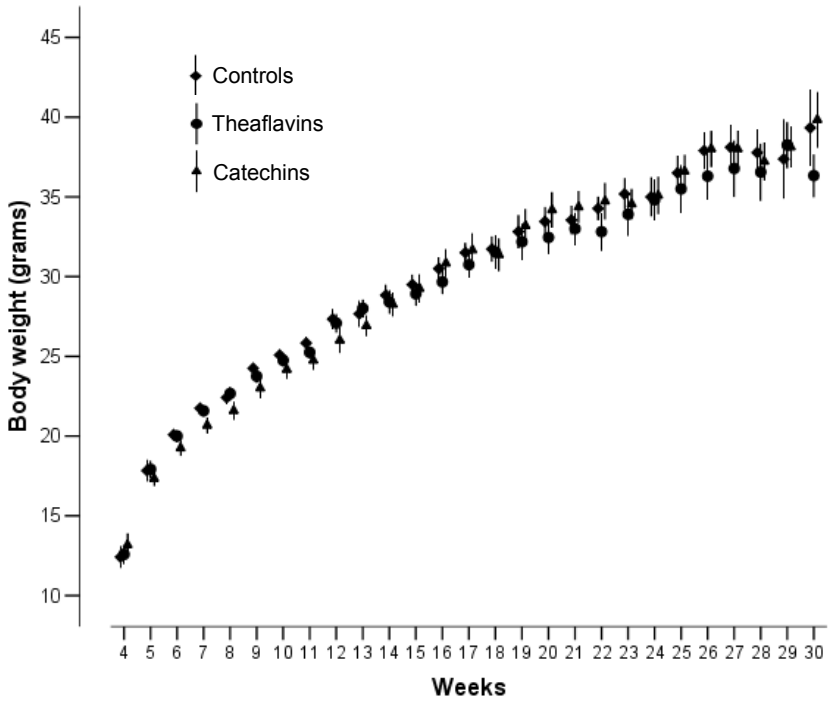


Figure 30 TRAMP mouse mean weekly body weights during 26 weeks of dietary dietary intervention (mean +/- SEM), differences between groups did not reach statistical significance.

4.1.4 Prostate weights of TRAMP mice receiving theaflavins or catechins

Following exsanguination, all 41 mice included in the analysis were dissected to remove their prostates and seminal vesicles. Figure 31 demonstrates the typical appearance of prostate tumour invading into seminal vesicles in these animals. Further dissection was then performed along the line of demarcation between the prostate tumour and the seminal vesicles. The prostate tumour mass was then weighed.

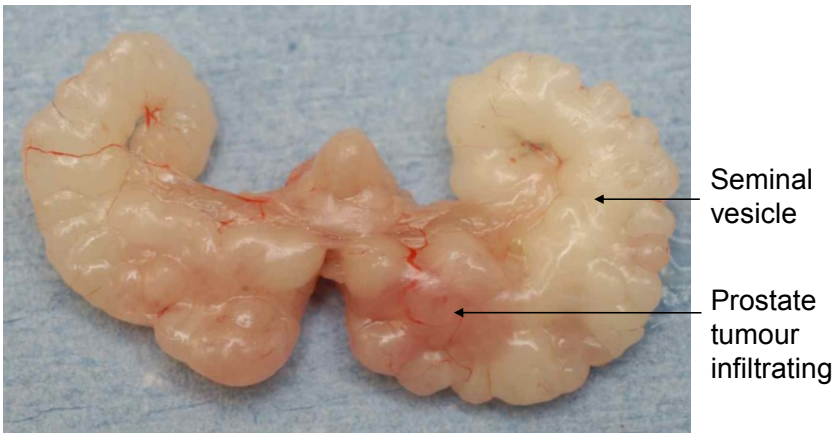


Figure 31 Prostate and seminal vesicles dissected from a TRAMP mouse after exsanguination at 30 weeks of age, following 26 weeks of dietary intervention. Demarcation is seen between prostate tumour and seminal vesicles.

The data from prostate tumour mass weights was not normally distributed, it was therefore analysed using non-parametric statistical tests. The median prostate tumour mass weights were 1.01g for control mice, 0.54 g for mice in the theaflavins

group and 0.28 g for mice in the catechin group. The reduction in tumour weights of the two intervention groups compared with the control group was statistically significant, see figure 32. There was, however, no significance difference between the median tumour mass weights of the two intervention groups.

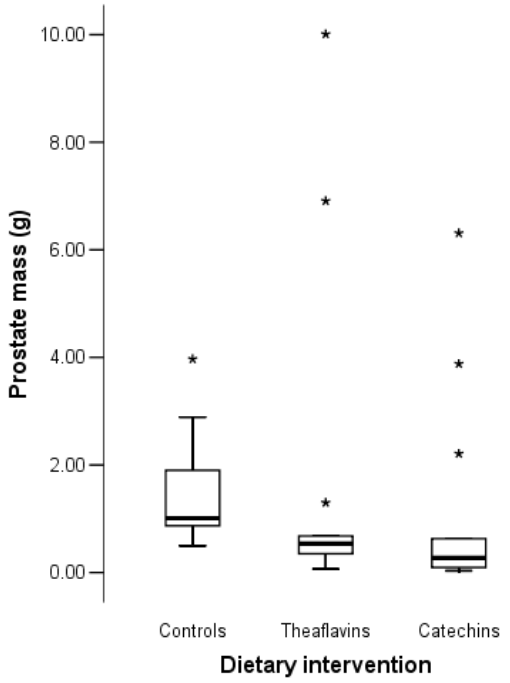


Figure 32 Prostate tumour mass from TRAMP mice after 26 weeks of dietary intervention with theaflavins, catechins or no polyphenol. In each group the bold line represents the median, the box represents the range between the 1st and 3rd quartiles, the bars indicate the data range excluding outliers which are represented by asterisks. Analysed overall using the non-parametric Kruskal-Wallis test significant differences were shown to exist between the groups, p=0.006. Post hoc analysis by Mann-Whitney U test showed this difference to be due to significantly smaller tumour masses in the two intervention groups compared with controls, p=0.008 for both.

4.1.5 Prostate carcinoma metastases in liver, retroperitoneum, mesentery and lung in TRAMP mice receiving theaflavins or catechins

Only seven TRAMP mice from the 41 animals included in the analysis were found to have histologically detectable metastases. The locations of the metastases detected are found in table 6. The number of animals affected with metastases was markedly fewer than expected on the basis of publish histological analysis of TRAMP [114] [85]. The low rate of observed metastases severely reduced the power of this experiment to detect differences between the metastases rates in the three intervention groups. No difference in metastases rates was therefore observed.

Table 6 Presence of metastases in TRAMP mice following 26 weeks of dietary intervention

Dietary Intervention	Mouse Number	Organs Containing Metastases
Water	4	Liver, Lung and Lymph Nodes
Water	7	Lung and Lymph Nodes
Water	9	Lung
Theaflavins	16	Lymph Nodes
Theaflavins	24	Lymph Nodes
Catechins	31	Liver
Catechins	38	Liver and Lymph Nodes

4.2 Identification of biomarkers of prostate cancer prevention by tea polyphenols in TRAMP mice

4.2.1 Introduction

Evidence presented in section 4.1 suggested that tea polyphenols can inhibit TRAMP mouse carcinogenesis. Performing a similar experiment in humans would take years to perform. In order to work towards establishing a similar effect in humans, the aim of this work was to identify biomarkers of chemoprevention of carcinogenesis in TRAMP that had completed the protocol in 2.5.2.

No murine homologue of human PSA has so far been identified. As an alternative prostate tumour marker, IGF-1 and its antagonising binding protein IGFBP-3 has been used in mice. It is known that this growth factor plays a role in prostate carcinogenesis and that it is raised in TRAMP mice compared with non-transgenic controls. It was also previously demonstrated that IGF-1 was reduced and IGFBP-3 was raised in TRAMP mice receiving green tea polyphenols [85].

Oxidative DNA adduct, M_1G, is a putative biomarker associated with carcinogenesis and amenable to change by dietary agents, see section 1.9.2. Experiments with human prostate cancer cells in culture failed to demonstrate a consistent change in M_1G following incubation with tea polyphenols, see section 3.1. However, cell culture experiments may not represent very closely cancer chemoprevention *in vivo*.

It has been previously reported that providing 0.1% green tea polyphenols in the drinking water of TRAMP mice can reduce the progression of primary prostate

carcinoma and abolish metastases [85]. This chemoprevention was found to be associated with reduced expression of certain signal transduction molecules including p-Erk1/2 and p-Akt [193].

The aim of this work, therefore, was to detect any change in serum IGF-1/IGFBP-3, prostate DNA M_1G concentrations, prostate expression of p-Erk1/2, VEGF and caspase-3

4.2.2 Plasma IGF and IGFBP-3 concentrations of TRAMP mice receiving theaflavins or catechins

The concentration of IGF-1 and IGFBP-3 in the plasma of TRAMP mice receiving 0.05% theaflavins, 0.05% catechins or plain drinking water was compared, see section 2.5.2 for TRAMP study protocol and section 2.4.3 for the experimental design.

The IGF standard curve is shown in figure 33. The linear equation derived from the standard curve was then used to determine the IGF-1 concentrations of 13 control mice, 14 mice that received 0.05% theaflavins and 13 mice that received 0.05% catechins.

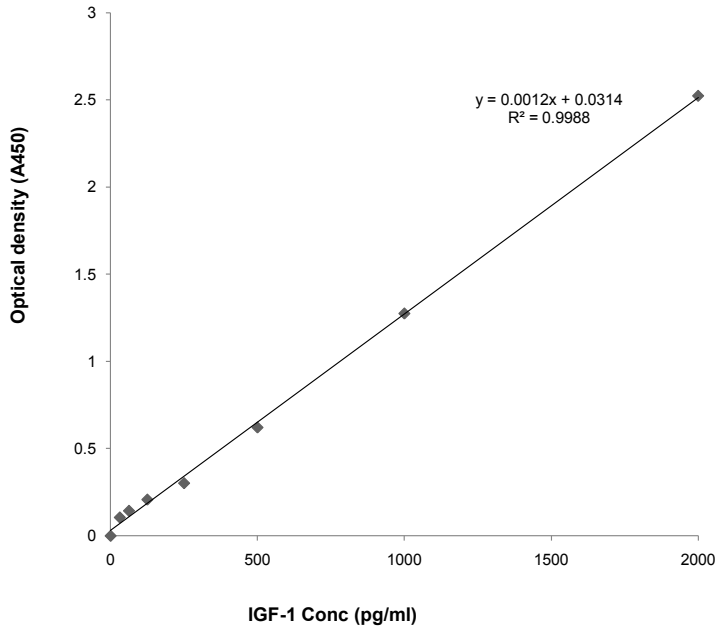


Figure 33 Standard curve of murine IGF-1 standards against optical density of ELISA dye reaction at 450 nm.

Figure 34 shows the mean concentrations of IGF-1 of the mice in each dietary intervention group. There was no significant difference between the mean IGF-1 concentrations of the three intervention groups by parametric or non-parametric analysis.

Plasma concentrations of IGFBP-3 were determined for the same samples as analysed for IGF-1 concentration. A solid phase ELISA (DuoSet) development kit was used according to the method in section 2.4.2. The standard curve is shown in figure 35. The linear equation derived from the standard curve was used to determine the IGF-1 concentrations of the same 40 of the 41 TRAMP mouse plasmas as were analysed for IGF-1 concentration. Figure 36 shows the mean concentrations of IGFBP-3 of the mice in each dietary intervention group. There

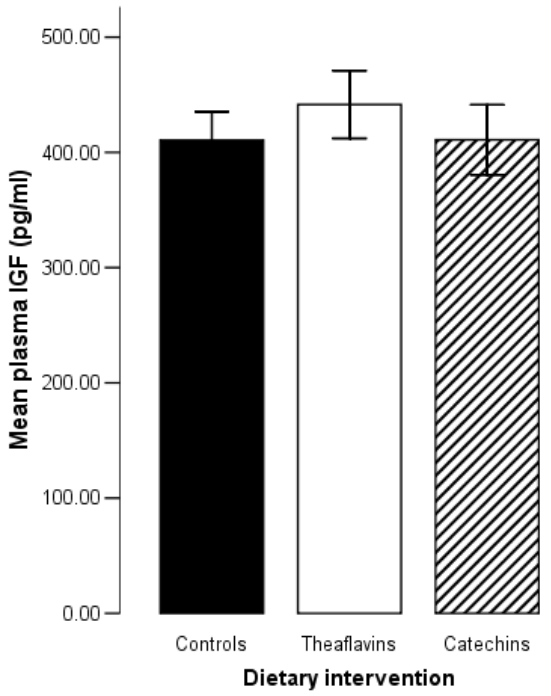


Figure 34 Mean TRAMP mouse IGF-1 concentrations and error bars following dietary intervention for 26 weeks (mean +/- SEM), differences between groups did not reach statistical significance.

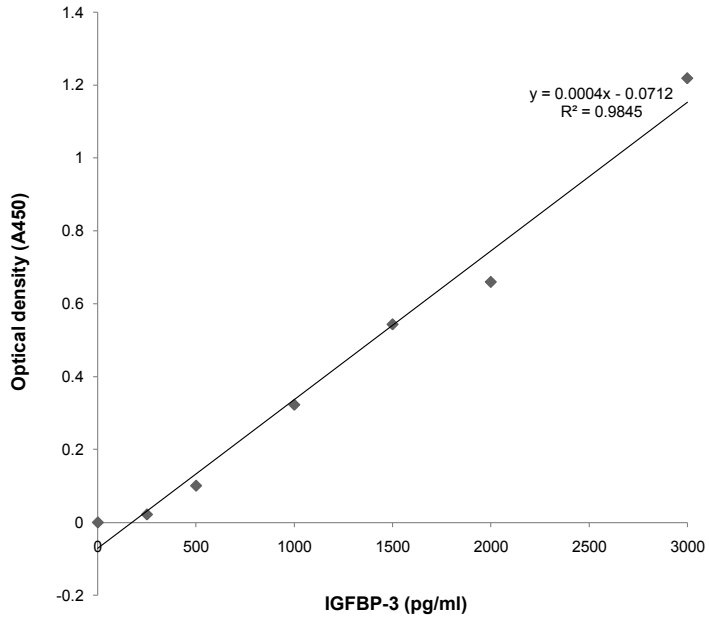


Figure 35 Standard curve of IGFBP-3 detected by ELISA relating fluorescence at 450 nm with IGFBP-3 concentration.

were no significant differences between the mean IGFBP-3 concentrations of the two intervention groups and the control group.

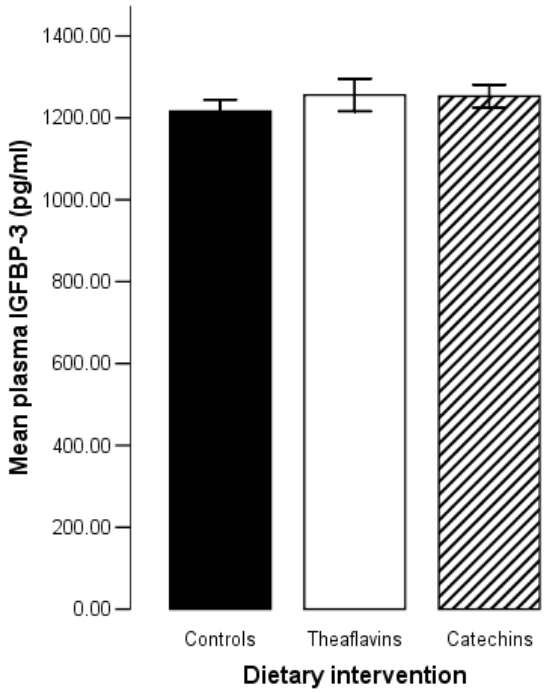


Figure 36 Mean TRAMP mouse IGFBP-3 concentrations and standard error bars following dietary intervention for 26 weeks (mean +/- SEM), differences between groups did not reach statistical significance.

As IGFBP-3 may function by reducing the bioavailability of IGF-1 the ratio of the two factors may represent a biomarker of cancer prevention. Figure 37 shows the mean ratios of IGF-1:IGFBP-3 in the two intervention groups and the control group. There was also no significant difference in the ratio of IGF-1 to IGFBP-3 by parametric or non-parametric analysis.

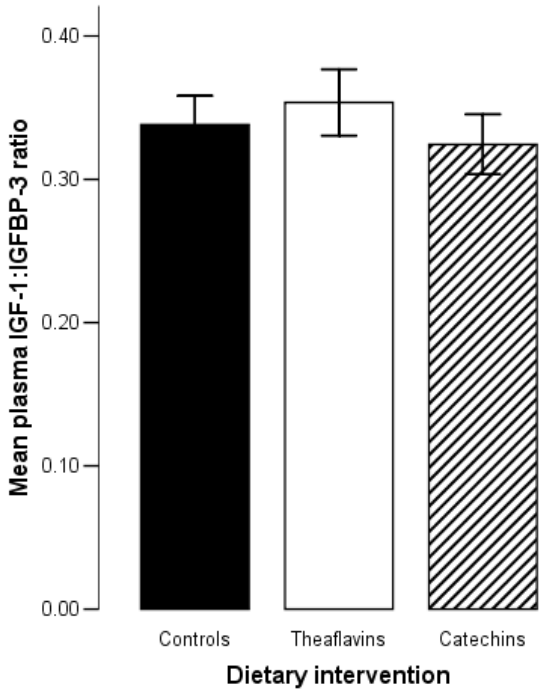
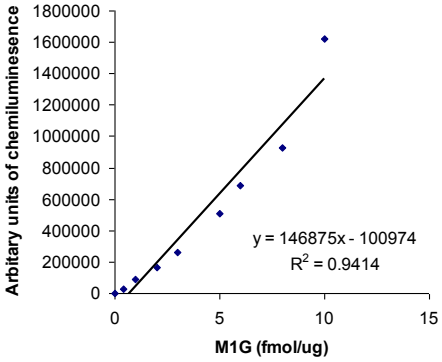
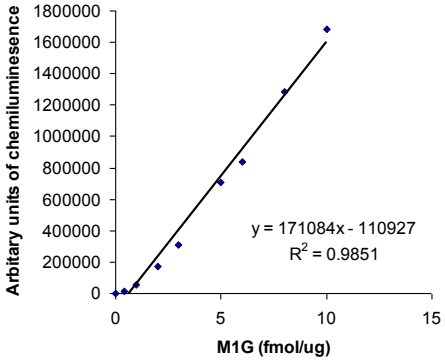


Figure 37 Mean TRAMP mouse IGF-1:IGFBP-3 concentration ratios following dietary intervention for 26 weeks (mean +/- SEM), differences between groups did not reach statistical significance.

4.2.3 M_1G concentrations in prostate tissue of TRAMP mice receiving theaflavins or catechins compared with controls

The effect of oral tea polyphenols on the concentration of M_1G in TRAMP mouse prostate was investigated using the experimental design in section 2.2.6. DNA samples were successfully prepared from 38 mouse prostate samples, 11 from control TRAMP mice that had received plain drinking water, 14 from mice that had received theaflavins and 13 from mice that had received catechins. The mean M_1G concentrations of DNA from TRAMP mouse prostate in each of the three intervention groups is shown in figure 39. It can be seen that M_1G concentrations in the DNA of prostate tumour masses from TRAMP mice that had received catechins were significantly lower than the concentrations detected in control mice. Whilst the M_1G from the theaflavin intervention group was also lower than the control group this difference did not reach significance. A possible relationship between M_1G concentrations from TRAMP mouse prostates, across all groups, and the weight of prostate tumour from which they came was investigated by Spearman's Rank correlation test. A significantly positive correlation was demonstrated and is shown in figure 40 graph A. Analysing the groups separately demonstrates that although there is a suggestion of a relationship between M_1G and prostate weight seen in all three intervention groups this correlation only reaches significance in the theaflavin intervention group.





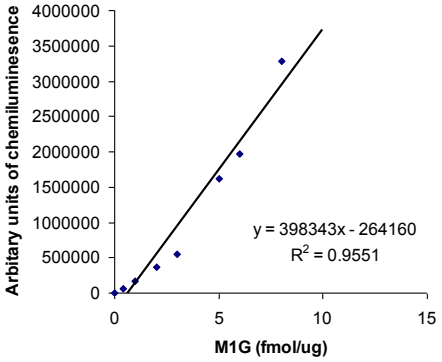


Figure 38 Standard curves of known M_1G concentrations against units of chemiluminescence following immuno-slot blot

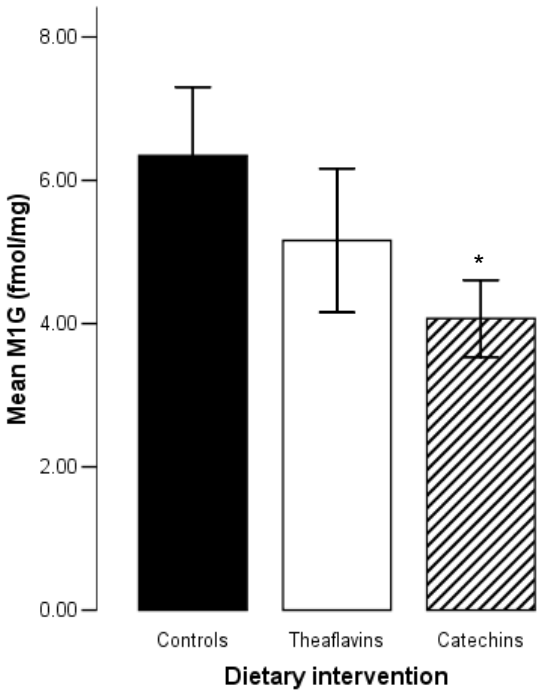


Figure 39 Mean M_1G concentrations in DNA from prostate tumour masses in TRAMP mice after 26 weeks dietary intervention (mean +/- SEM). Using the Kruskal-Wallis non-parametric test or the parametric ANOVA test to analyse the three data sets without *a priori* expectation demonstrates no statistically significant difference between the groups. However, when *post-hoc* analyses were performed using Student's t test to detect reductions in M_1G in the intervention groups compared with controls, the catechins group is shown to differ significantly from the control group the difference observed between the theaflavins group and the control group did not reach this level of significance (* p=0.045).

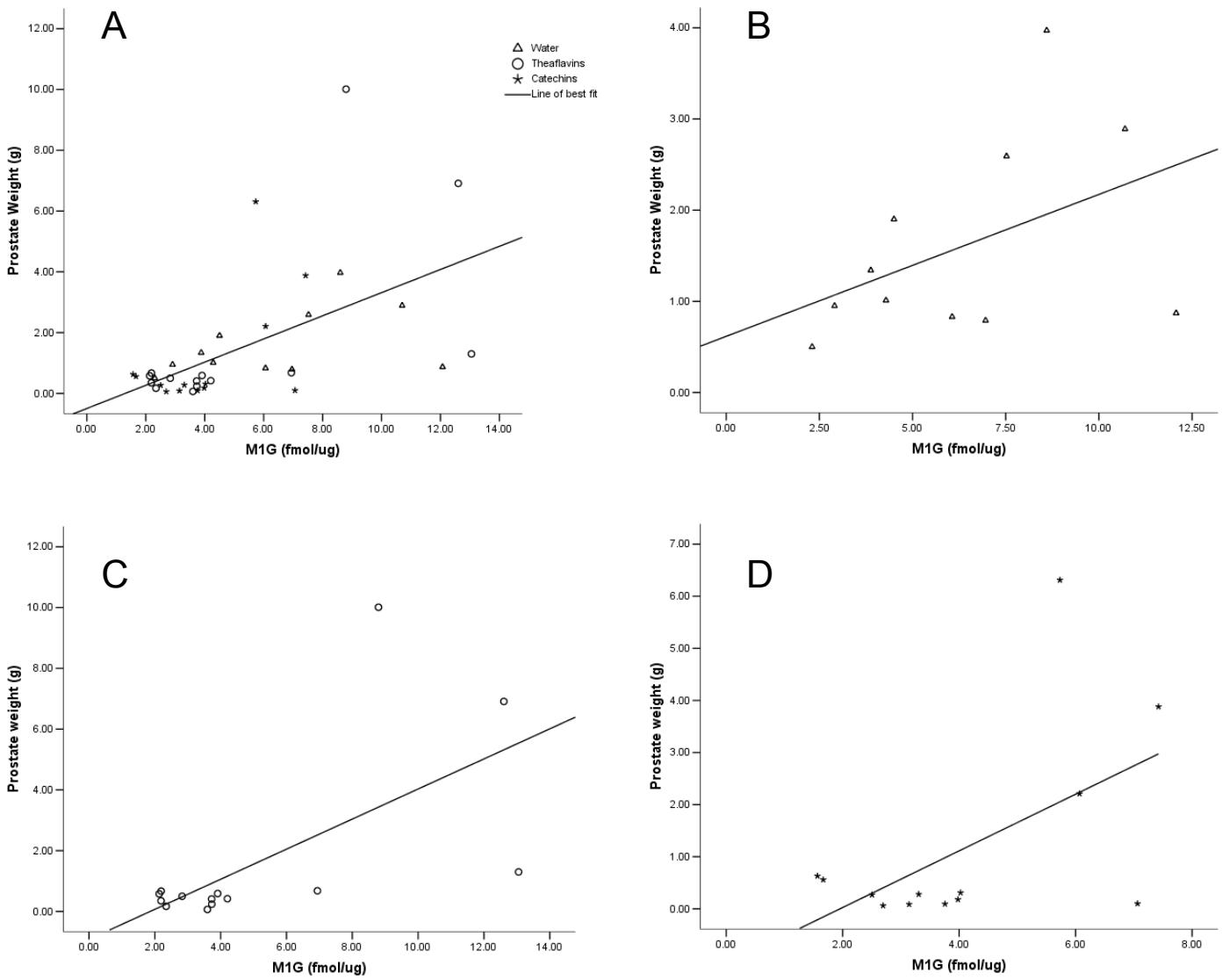


Figure 40 Correlation between M_1G in TRAMP mouse prostate DNA and the weight of the prostate tumour mass. Graph A: When data from groups are combined this correlation is statistically significant using Spearman's rank correlation test (correlation coefficient = 0.605, n=38, $p<0.001$). Spearman's test was chosen as it is a non-parametric statistical method that makes no assumptions about the distribution of the data. Graphs B,C and D: However, when data from the three intervention groups are analysed separately the trend only reaches significance in the theaflavin intervention group (Water group-graph B, correlation coefficient = 0.427, n=11, p=0.19; Theaflavin group-graph C, correlation coefficient = 0.587, n=14, p=0.027; Catechin group-graph D, correlation coefficient = 0.291, n=13, p=0.334)

4.2.4 Protein biomarkers from prostates of TRAMP mice receiving theaflavins or catechins

To investigate the effects of green tea catechins and black tea theaflavins in the drinking water of TRAMP mice upon the expression of p-Erk1/2, caspase-3 and VEGF in prostate tissue. Western blots were prepared with protein lysates from this prostate tissue and probed with antibodies against these various protein biomarkers. See section 2.3.8 for the experimental design and methods. In addition the expression of the TRAMP transgene SV40 T antigen was quantified to exclude alternative mechanisms by which tea polyphenols might have reduce prostate carcinogenesis.

Figure 41, 43, 45 and 47 show the results of probing the blots with anti-p-Erk1/2, anti-T antigen, anti-cleaved caspase 3 and anti-VEGF respectively. The quantified signal intensities were divided by the values corresponding to β actin expression to correct for unequal gel loading. Figures 42, 44, 46 and 48 show the mean corrected p-Erk 1/2, T-antigen, cleaved caspase 3 and VEGF signals respectively. Analysis using both parametric ANOVA and non-parametric Kruskal-Wallis tests show that there are no significant differences in the p-Erk 1/2, T antigen, cleaved caspase 3 or VEGF expression between the groups.

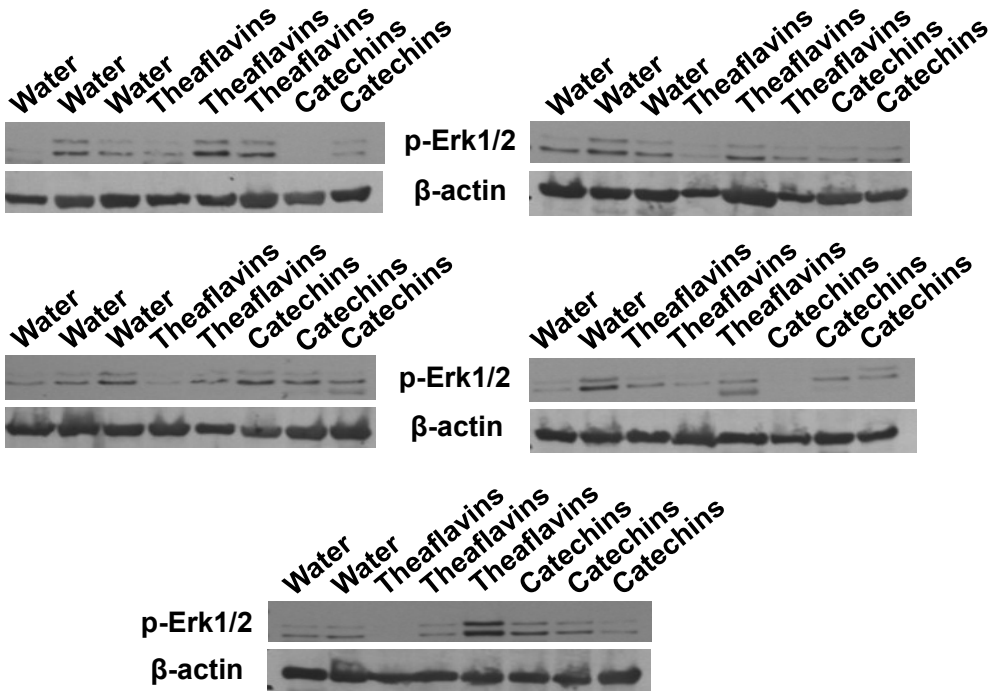


Figure 41 Western blots of protein from TRAMP prostate tumour masses following 26 weeks of dietary intervention. Each band is from an individual mouse. Blots were probed with antibodies against p-Erk1/2 and β-actin.

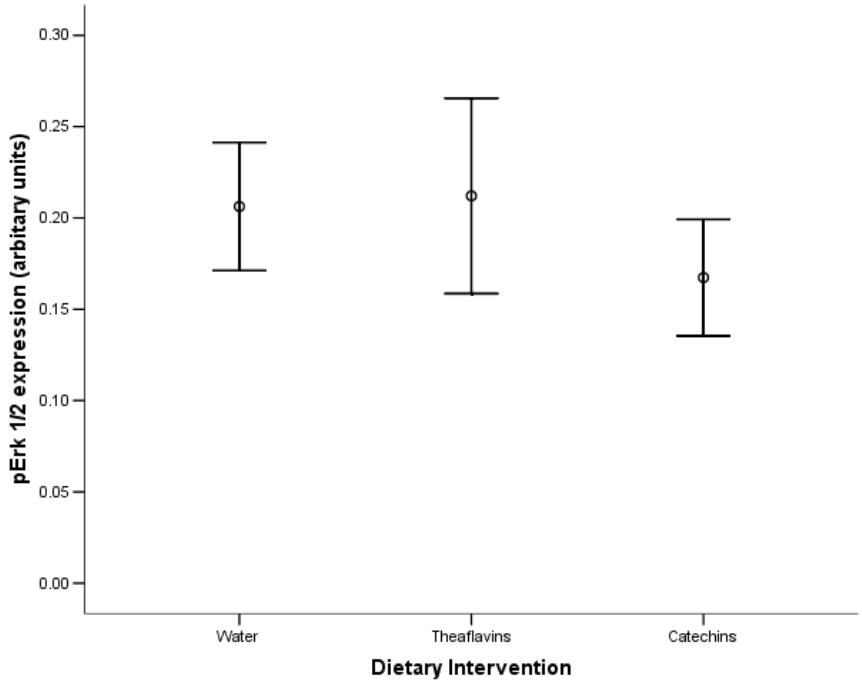


Figure 42 p-Erk 1/2 expression in TRAMP mouse prostate tumour masses following 26 weeks of dietary intervention, values were divided by β-actin expression to correct for any unequal loading, (mean +/- SEM). Differences between groups did not reach statistical significance.

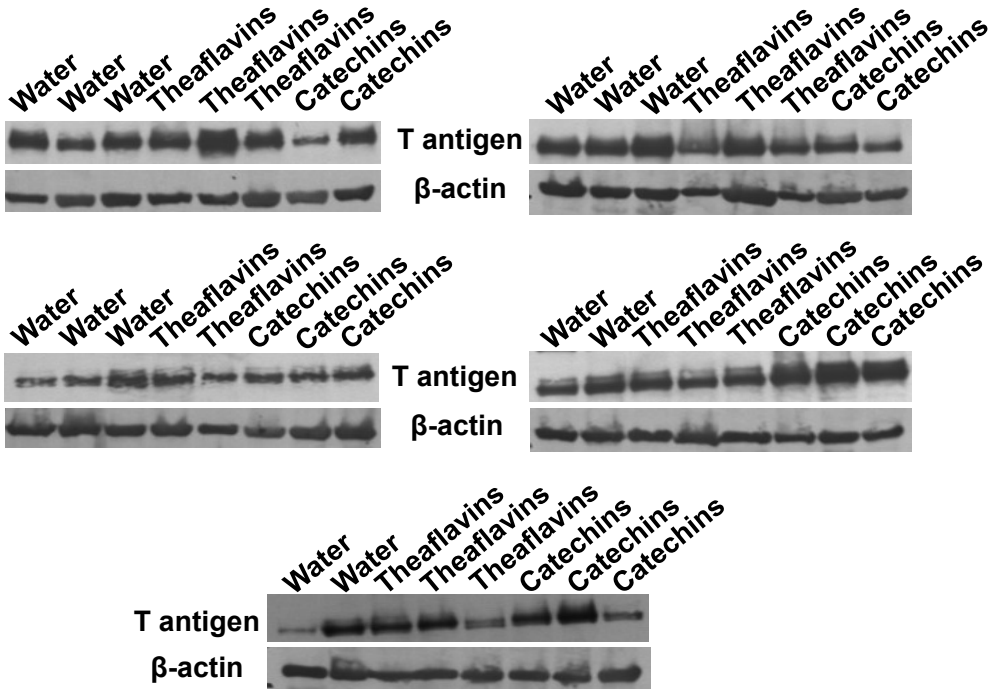


Figure 43 Western blots of protein from TRAMP prostate tumour masses following 26 weeks of dietary intervention. Each band is from an individual mouse. Blots were probed with antibodies against T antigen and β-actin.

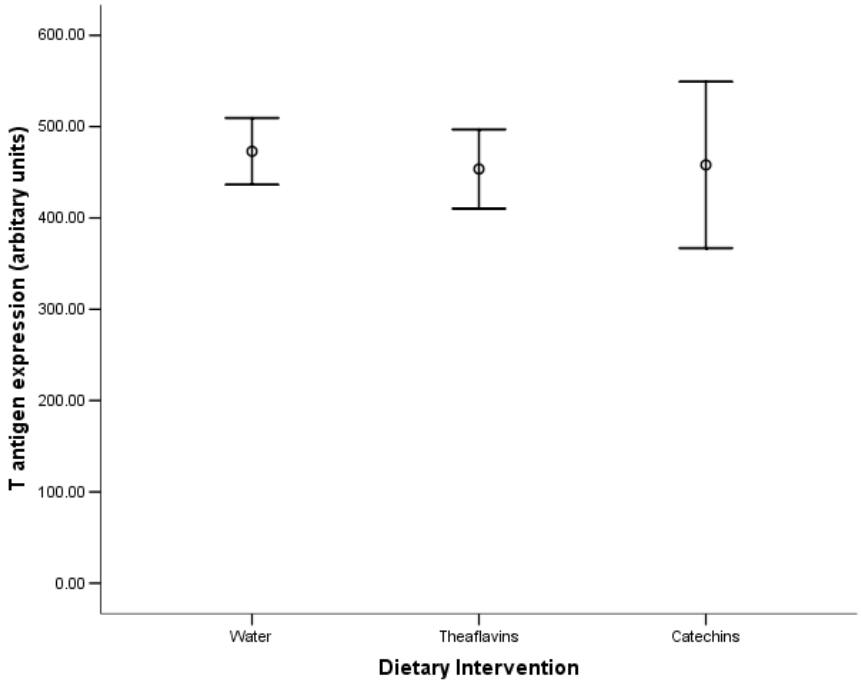


Figure 44 T antigen expression in TRAMP mouse prostate tumour masses following 26 weeks of dietary intervention, values were divided by β-actin expression to correct for any unequal loading, (mean +/- SEM). Differences between groups did not reach statistical significance.

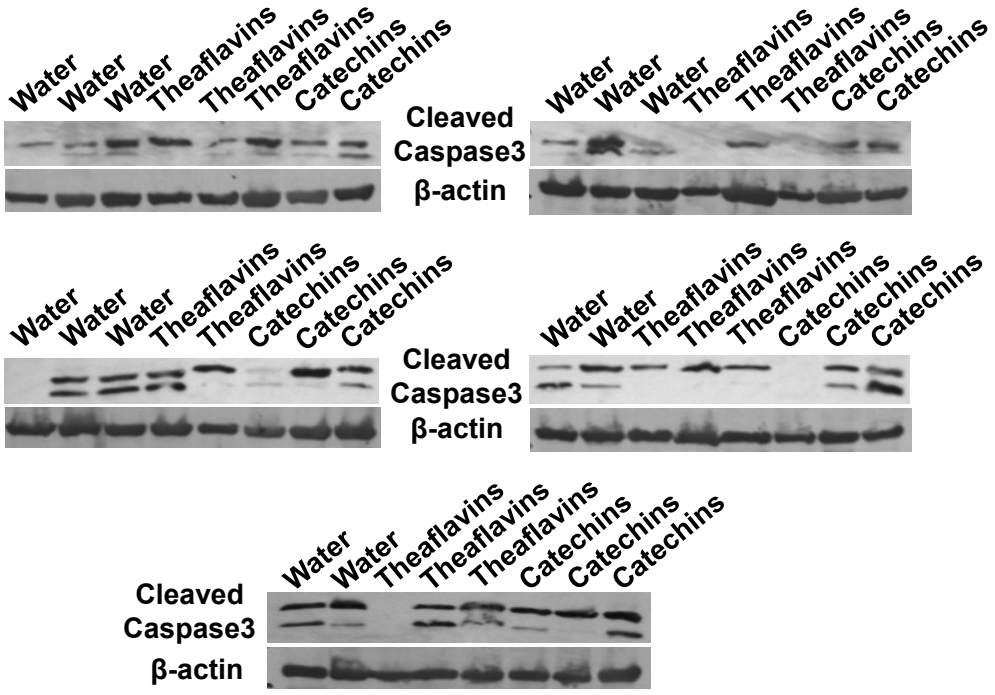


Figure 45 Western blots of protein from TRAMP prostate tumour masses following 26 weeks of dietary intervention. Each band is from an individual mouse. Blots were probed with antibodies against cleaved caspase 3 and β-actin.

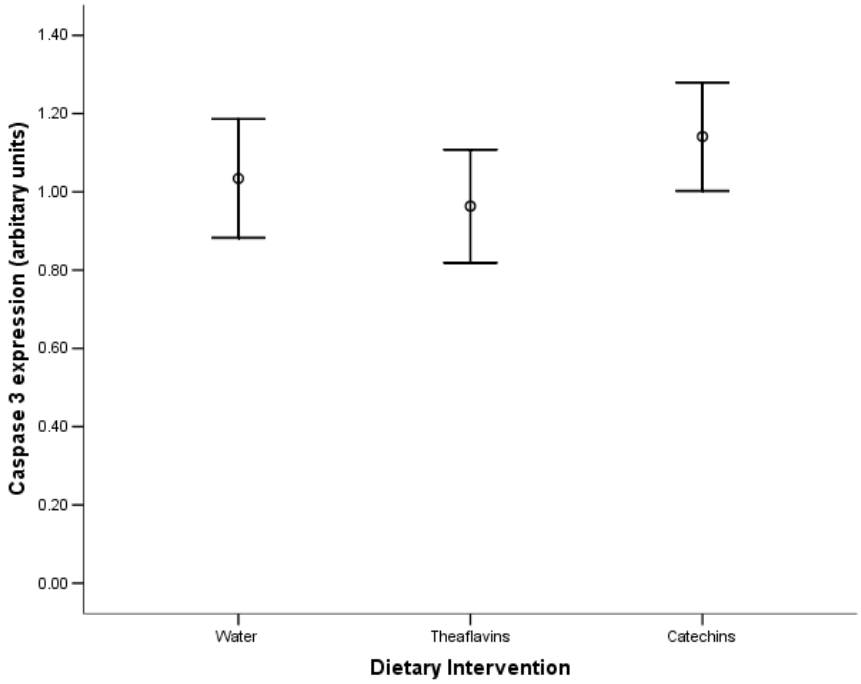


Figure 46 Activated caspase 3 expression in TRAMP mouse prostate tumour masses following 26 weeks of dietary intervention, values were divided by β-actin expression to correct for any unequal loading, (mean +/- SEM). Differences between groups did not reach statistical significance.

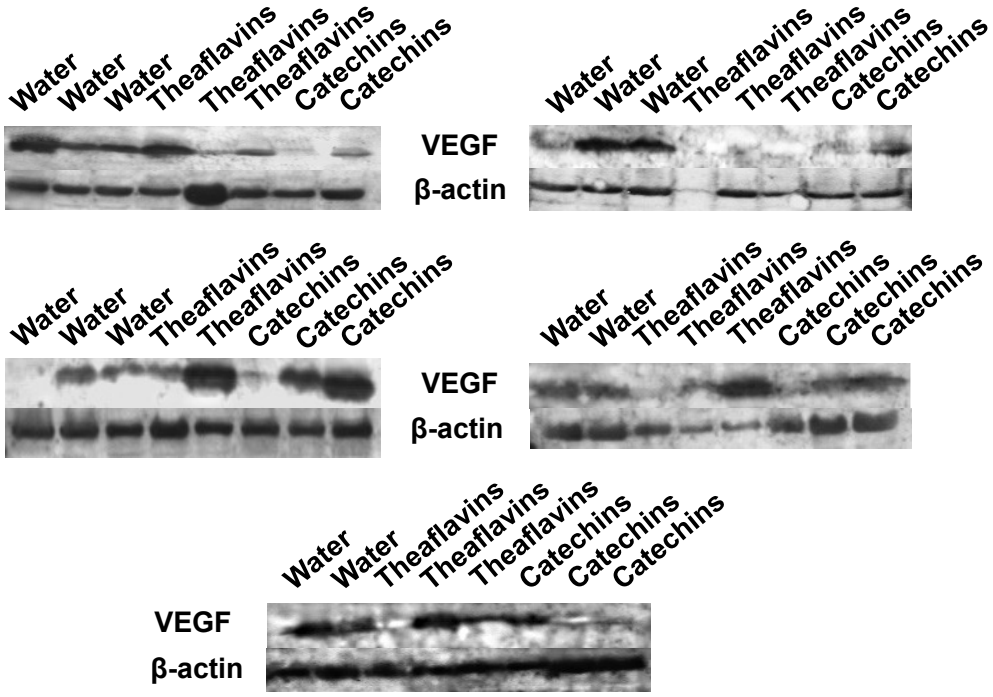


Figure 47 Western blots of protein from TRAMP prostate tumour masses following 26 weeks of dietary intervention. Each band is from an individual mouse. Blots were probed with antibodies against VEGF and β-actin.

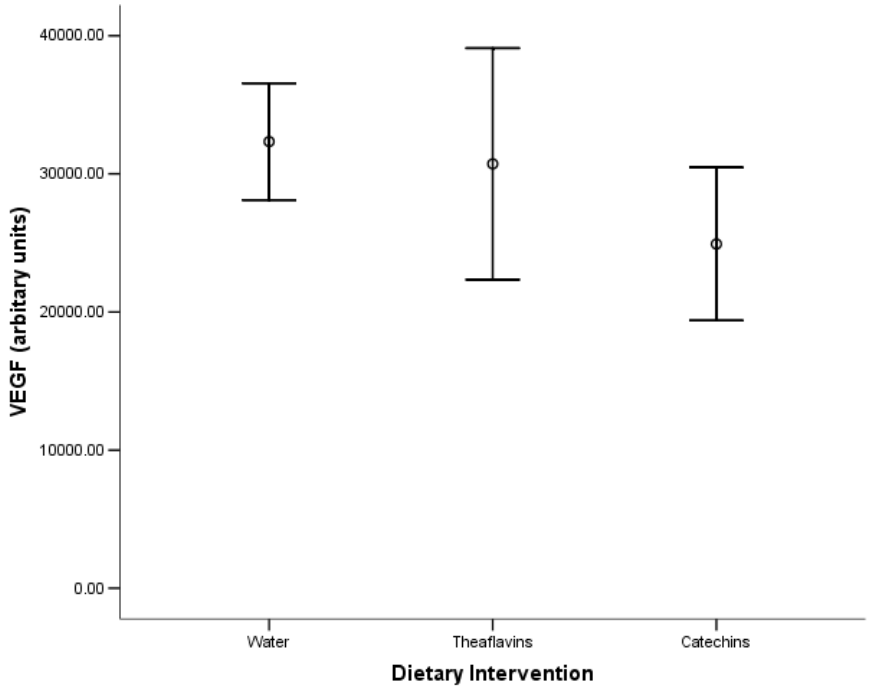


Figure 48 VEGF expression in TRAMP mouse prostate tumour masses following 26 weeks of dietary intervention, values were divided by β-actin expression to correct for any unequal loading, (mean +/- SEM). Differences between groups did not reach statistical significance.

4.2.5 Microarray analysis of TRAMP mouse prostate gene expression following dietary intervention with tea polyphenols

TRAMP mice were subject to 26 weeks of dietary intervention with 0.05% theaflavins, 0.05% catechins or no polyphenols in drinking water according to the protocol in section 2.5.2. Microarrays were used to rapidly screen the entire expressed population of mRNAs from the TRAMP prostates to identify genes whose expression was altered by the interventions, see section 2.8.10 for the study design and methods. These genes could then be proposed as potential biomarkers of prostate cancer prevention by tea polyphenols, worthy of further characterisation.

The arrays were scanned, generating lists of dye intensities thought to quantitatively reflect expression intensities. The data was analysed by two sample t-tests with calculation of FDR. Significance was set at FDR<0.1, excluding genes whose expression appeared altered less than two-fold.

Arrays 1-5 suggested the genes listed in table 7 were significantly altered in expression following dietary intervention with green tea catechins.

Table 7 Gene expression altered in prostate tissue of TRAMP mice following dietary intervention using green tea catechins, as detected by arrays 1-5

Gene Symbol	Fold change	Gene Symbol	Fold change
Ppwd1	0.26	9830107B12	0.39
A430106F12Rik	0.26	6430604K15Rik	0.39
Ube2v2	0.27	Olfr1365	0.39
Gas2l3	0.28	scl0001065.1_54	0.39
Sec10l1	0.29	Pfkfb1	0.40
Tanc1	0.30	Dtl	0.40
Olfr1515	0.31	BC031575	0.41
BC051076	0.31	Heatr3	0.42
Zfp278	0.32	Arl6ip2	0.42
Lin28b	0.32	Exoc8	0.43
Traf7	0.32	A430055H19Rik	0.43
Zc3hav1l	0.32	9530079D04Rik	0.43
Pcdh10	0.32	Olfr1065	0.43
Cspg6	0.33	ESCO1	0.43
1810019D21Rik	0.33	Olfr955	0.45
V1rc8	0.33	Zfp273	0.45
4430401P03Rik	0.34	AI467503	0.45
Pcdhga7	0.34	AA410130	0.46
Lamp3	0.34	4930505D03Rik	0.47
Lrrc52	0.34	Siat5	0.48

V1rh17	0.35	Ugt1a13	0.48
4930528G23Rik	0.35	Slc9a3r1	0.49
Mnat1	0.35	A930014D08Rik	0.49
Tas2r106	0.36	Crot	0.49
Asb5	0.36	AW210570	1.95
Olfr845	0.36	Tln	2.07
Gpbp1	0.37	BC037393	2.75
C230062I16Rik	0.37	Etv6	2.91
Rasgef1a	0.37	Olfr694	3.27
Dpysl3	0.37	Galns	4.42
Krtap21-1	0.37	Zfp236	5.86
Heatr1	0.38		

Gene ontology statistical analysis was performed to identify certain functional groupings that were over-represented in the above list compared with the expected frequency derived from the composition of the entire MEEBO gene set. Figure 49 highlights the functional groupings in which the gene list in table 7 were found to be statistically significantly enriched. The constituent genes in the functional groups highlighted in figure 49 are shown in table 8.

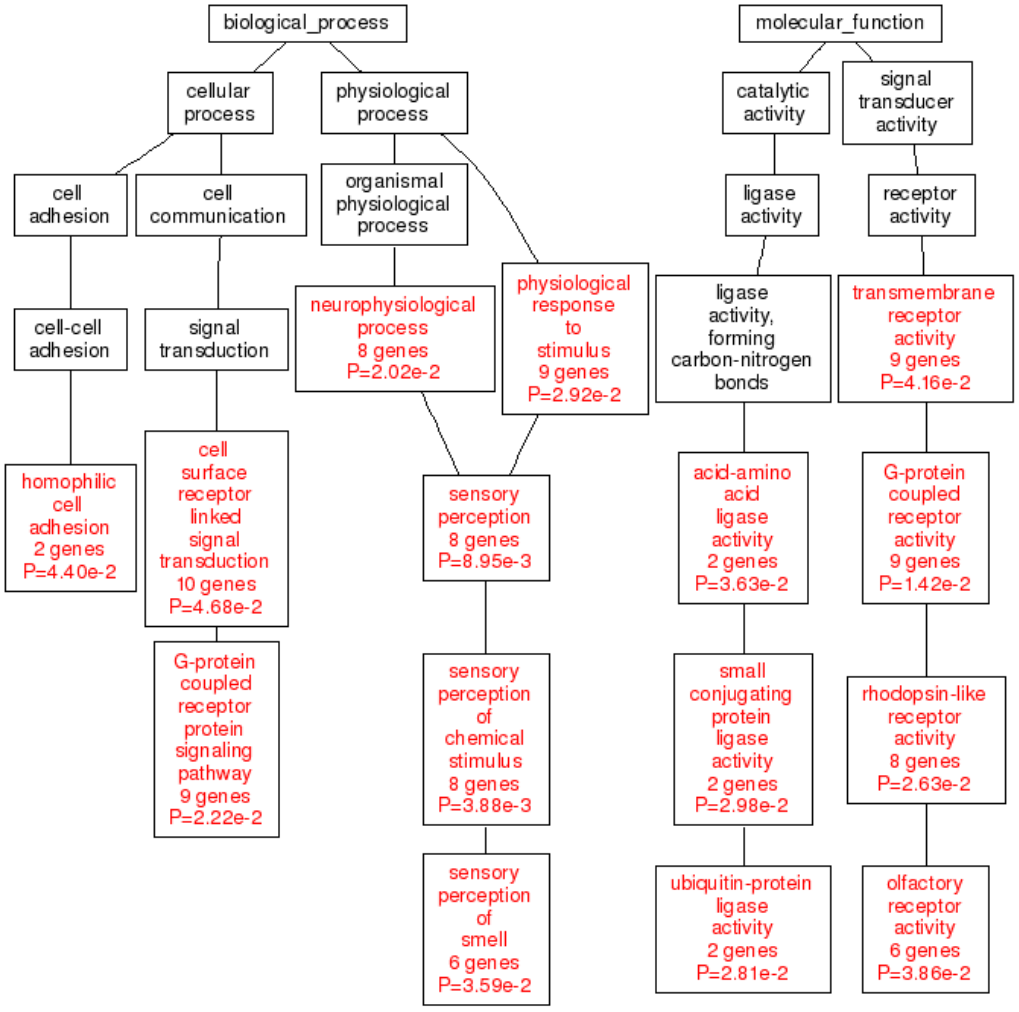


Figure 49 Functional groups of genes whose members were over-represented in the list of genes (see table 5) with altered expression in TRAMP mouse prostate following dietary intervention with green tea catechins, including p values (Red boxes indicate $p<0.05$)

Table 8 The constituent genes within the functional groupings identified in figure 49

Functional Grouping	Gene Symbol	Gene Name	Change in Regulation
Homophilic cell adhesion	Pcdh10	protocadherin 10	down
	Pcdhga7	protocadherin gamma subfamily A, 7	down
G protein coupled receptor protein signalling pathway	V1rc8	vomeronasal 1 receptor, C8	down
	Olfr845	olfactory receptor 845	down
	V1rh17	vomeronasal 1 receptor, H17	down
	Olfr1515	olfactory receptor 1515	down
	Olfr955	olfactory receptor 955	down
	Olfr1065	olfactory receptor 1065	down
	Tas2r106	taste receptor type 2 member 106	down
	Olfr694	olfactory receptor 694	up
	Olfr1365	olfactory receptor 1365	down
Sensory perception of smell	Olfr845	olfactory receptor 845	down
	Olfr694	olfactory receptor 694	up
	Olfr1515	olfactory receptor 1515	down
	Olfr1065	olfactory receptor 1065	down
	Olfr1365	olfactory receptor 1365	down
	Olfr955	olfactory receptor 955	down
Ubiquitin-protein ligase activity	Ube2v2	Tnf receptor-associated factor 7	down
	Traf7	ubiquitin-conjugating enzyme E2 variant 2	down
Olfactory receptor activity	Olfr955	olfactory receptor 955	down
	Olfr1365	olfactory receptor 1365	down
	Olfr1515	olfactory receptor 1515	down
	Olfr694	olfactory receptor 694	up
	Olfr845	olfactory receptor 845	down
	Olfr1065	olfactory receptor 1065	down

Arrays 1-5 failed to identify any genes, whose expression in prostates from mice which had received theaflavins, significantly differed from controls. Arrays 6-10 identified no genes whose expression significantly differed in the catechin treated group compared with controls. Arrays 6-10 suggested that only one expressed sequence was significantly altered in expression following dietary intervention with black tea theaflavins, Riken cDNA 9330159M07. This sequence, whose function is not known, showed a 1.5 fold increase in expression.

4.3 Identification of orally administered tea polyphenols in the TRAMP mouse

4.3.1 Introduction

Data from this work and elsewhere suggest that in order to inhibit processes associated with carcinogenesis, tea polyphenols are required to be present at concentrations in excess of 25 µg/ml, see section 1.7.2 and chapter 3. Evidence presented in section 4.1.2 suggested that orally administered polyphenols can reduce prostate carcinogenesis in TRAMP mice. LCMSMS analysis of material from these TRAMP mice aimed to demonstrate that tea polyphenols can be absorbed orally and penetrate tissue. It also aimed to investigate which metabolites are measurable *in vivo,* and in the case of green tea polyphenols, to make some estimations of the concentrations achieved following oral adminstration.

4.3.2 Optimisation of extraction of green tea polyphenols from biological material

Two alternative extraction techniques were compared in order to optimize the extraction yield of subsequent experiments. See section 2.7.3 for the experimental design. Figure 50 shows the LCMSMS traces recorded. The recovery yields, estimated by measurement of peak areas, are presented as extraction efficiencies in table 9.

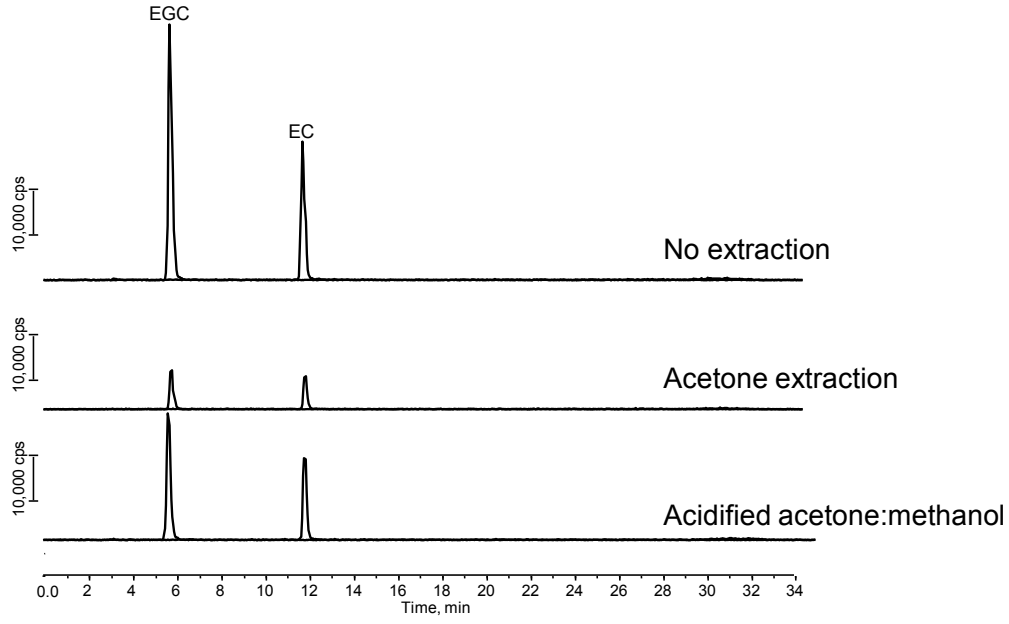


Figure 50 Aliquots of 1.25 µg of each of EGC and EC were subjected to extraction using acetone elution and acidified acetone:methanol elution. LCMSMS analyses compared these two catechin extraction methods with identical unextracted standard. The above LCMSMS signal traces are each composites of two traces (EGC trace m/z 305/125 and EC trace m/z 289/109).

Table 9 Efficiencies of two catechin extraction methods

	Standard	Acetone elution		Acetone:methanol elution	
	peak area	peak area	efficiency (%)	peak area	efficiency (%)
EGC	74,680	11,501	15.4	38,432	51.5
EC	39,229	10,093	25.7	24,561	62.6

The acidified acetone:methanol elution method demonstrated superior recovery of the green tea catechins EGC and EC. This method was therefore adopted for the subsequent extraction of all green tea catechins from both tissue and urine samples.

The efficiencies of the extraction techniques were calculated by spiking 400 µl urine and 200 mg homogenised tissue with 1.25 µg each of EC, EGC, ECG and EGCG. The catechin solutions were extracted using the urine extraction method, see section 2.8.1, and the tissue extraction method, see section 2.8.2. The residues, each resuspended in 20 µl water, were analysed by LCMSMS, see section 2.8.3. This procedure was performed in triplicate. Figure 51 shows the LCMSMS trace of one of these repeats. Table 10 shows the peak areas relating to each of the four compounds in triplicate.

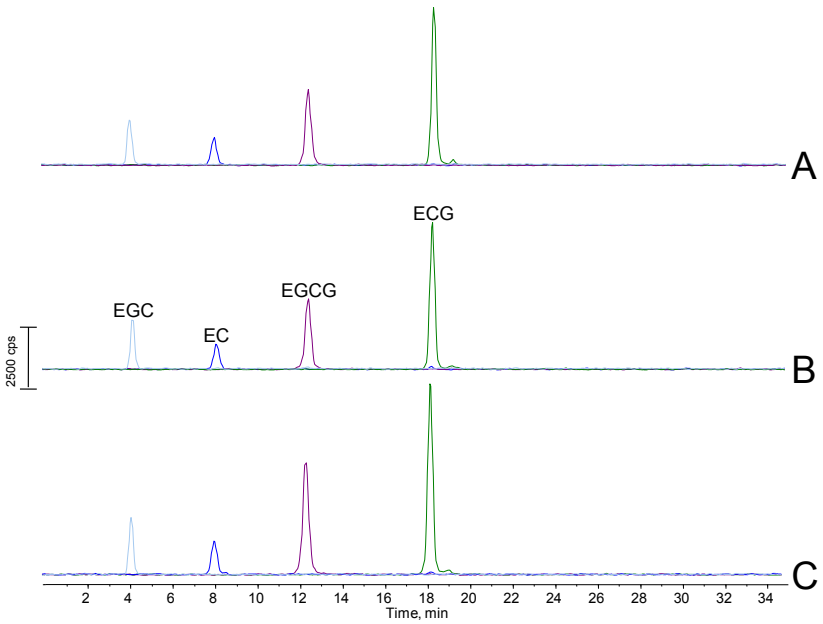


Figure 51 LCMSMS chromatograms where each of the three traces A,B and C is a composite of four traces corresponding to four catechins (EC trace m/z 289/109, EGC trace m/z 305/125, ECG trace m/z 441/125 and EGCG m/z 457/125) **A** 1.25 µg of each of EC, EGC, ECG and EGCG was spiked into 200 mg homogenised prostate tissue and extracted using the method in section 2.8.2; **B** 1.25 µg of each of EC, EGC, ECG and EGCG was spiked into 400 µl urine and extracted using the method in section 2.8.2 and ; **C** 1.25 µg of each of EC, EGC, ECG and EGCG as standards.

Table 10 Extraction efficiencies of catechin extraction methods

	Mean peak areas of standards	Mean peak areas of standards extracted from urine	Mean peak areas of standards extracted from prostate tissue
EC	27023	21345 (79%)	21848 (81%)
EGC	34650	30111 (87%)	29403 (85%)
ECG	143566	105686 (74%)	107582 (75%)
EGCG	102182	64072 (63%)	64559 (63%)

4.3.3 Green tea catechins in TRAMP mouse plasma and prostate tissue

TRAMP mice were subjected to dietary intervention with green tea catechins for 26 weeks, as per the study design in section 2.5.2. LCMSMS analysis of plasma and prostate tissue as per the experimental design in section 2.7.8 failed to identify any of 30 green tea catechins or their metabolites.

4.3.4 Black tea theaflavins in TRAMP mouse plasma and prostate tissue

TRAMP mice received black tea theaflavins in drinking water for 26 weeks, as per the study design in section 2.5.2. LCMSMS analysis of plasma and prostate tissue was performed as per the experimental design in section 2.7.11.

Figures 52 and 53 show examples of the traces seen in these experiments. Judging by the similarity of the retention times of the peaks detected by mass spectroscopy, the liquid chromatography failed to separate theaflavin compounds. Three high signal-to-noise-ratio peaks from this LCMSMS analysis of mouse number 25. Despite the shortcomings of the experiment this is tentative, preliminary evidence of the presence of three theaflavins (methyltheaflavin monogallate glucuronide, theaflavin monogallate glucuronide sulphonate and theaflavin sulphonate). Similar peaks were found in the samples from all six mice that received theaflavins. No corresponding peaks were detected in the samples extracted from plasmas of the six control mice.

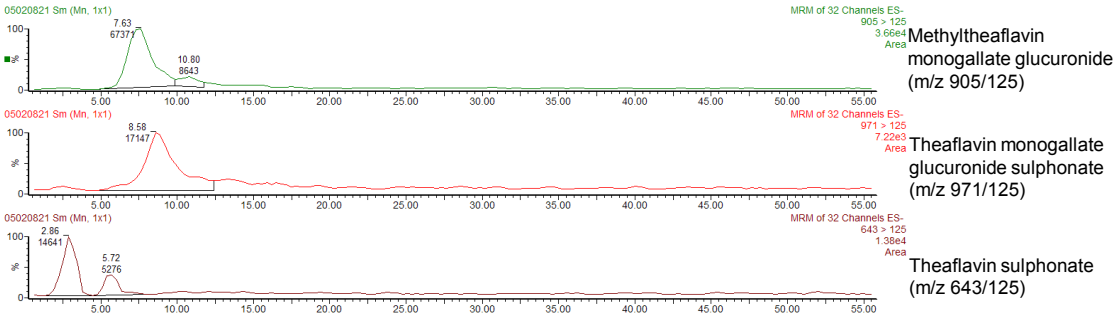


Figure 52 LCMSMS analysis of theaflavins extracted from the plasma of TRAMP mouse no.25, following 26 weeks of dietary intervention with 0.05% theaflavins dissolved in the sole drinking water source. Each trace represents a different m/z transition corresponding to an individual theaflavin. The LCMSMS method attempted to detect 39 separate theaflavin species simultaneously. Three example traces are shown demonstrating peaks of high signal-to-noise ratio however the technique failed to separate the peaks on the basis of retention times.

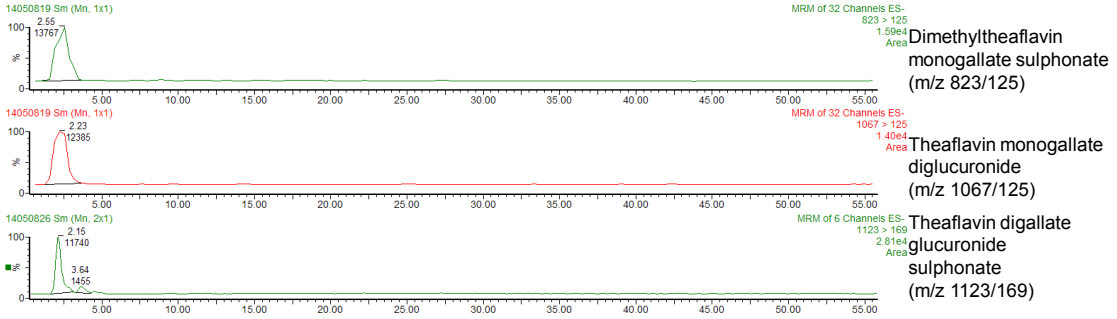


Figure 53 LCMSMS analysis of theaflavins extracted from the prostate tissue of TRAMP mouse no.25, following 26 weeks of dietary intervention with 0.05% theaflavins dissolved in the sole drinking water source. Each trace represents a different m/z transition corresponding to an individual theaflavin. The LCMSMS method attempted to detect 39 separate theaflavin species simultaneously. Three example traces are shown demonstrating peaks of high signal-to-noise ratio however the technique failed to separate the peaks on the basis of retention times.

4.4 Discussion

The natural history of prostate cancer in the human male stretches over decades as would definitive trials of cancer chemoprevention. The use of the transgenic TRAMP mouse as a model of prostate in this work has allowed the rapid investigation of tea polyphenols with putative cancer preventive activity.

Following 26 weeks dietary intervention with either theaflavins, catechins or no polyphenols all the TRAMP mouse prostates analysed in this work demonstrated tumours that were replacing normal tissue; unfortunately photographs of whole tumours, representative of each intervention group, were not recorded. The masses of these tumours varied widely and with a non-normal distribution. This presented challenges to the statistical analysis as the expected parametric comparison of means was precluded. Non-parametric comparison confirmed that following intervention with either theaflavins or catechins TRAMP prostate tumours were smaller than those in the control group. This inhibition of carcinogenesis was of a significant scale; the median prostate weights of the theaflavin and catechin groups were 0.54g and 0.28g respectively compared with 1.01g for the control group. This confirms previous evidence [85] that oral green tea polyphenols can inhibit TRAMP mouse prostate carcinogenesis and represents the first time that theaflavins have been shown to inhibit prostate carcinogenesis *in vivo*.

There was no attempt made to establish if this reduction in tumour size was due to reduced proliferation or increased apoptosis. This could have been investigated by immunohistochemical analysis of the prostate tumour tissue

harvested, using antibodies against Ki-67, a marker of proliferation [258] and TdT-mediated dUTP-biotin nick end -labelling (TUNEL) assay of DNA fragmentation to establish the rate of apoptosis [259].

A weakness of the experimental design was that with only one opportunity to establish tumour size i.e., post mortem, there was no longitudinal analysis of changes in the tumours over time. *In vivo* volumetric assessment of TRAMP mice tumours, following green tea polyphenol administration, with magnetic resonance has previously been performed by Gupta *et al*. using a 1.5 Tesla imager with a custom-built small animal receiver coil [85]. Unfortunately, such equipment was not available to the experiments presented here. Beyond simple assessment of tumour volumes, the use of *in vivo* positron emission tomography could allow longitudinal analysis of tumour metabolism under the influence of tea polyphenols [260]. Alternatively, in order to analyse the effect over time of polyphenol exposure, biomarkers could have been serially sampled at various time points throughout the study. This however would have required several animals per time point which would have greatly increased the total number of animals required for the study. It was not felt that this approach would have been compatible with the ethical framework of “reduction, refinement and replacement” commonly referred to as the 3R’s, that govern animal experimentation, first proposed by Russell and Birch [261].

The tumour weights measured in this work were much larger and the effect of green tea polyphenols more modest than those reported by Gupta *et al*. [85] who found a mean tumour weight of 76 mg in control mice and 27.5 mg in the

TRAMP mice that received the polyphenols. It is not known why this difference in tumour weights was found between the two experiments, however, the tumour weights recorded in this work were in keeping with other published work like Chiang et al. whose control mice developed tumours with a mean weight of 2.09 grams [262].

It was expected that this study would be able to investigate for reductions in TRAMP prostate cancer metastases following oral tea polyphenol administration, however the rate of metastases in the animals studied only reached seven out of 41 animals at 30 weeks of age. Previous work had found 100% of TRAMP mice harbour metastases in lungs or lymph nodes at 28 weeks [83] and 65% have lung metastases at 32 weeks [85].

It is not known why the unexpectedly low rate of metastases was observed in this work. One possible explanation may be genetic drift of the colony of TRAMP mice that were used. Genetic drift causing the loss of expression of the deleterious transgene is a constant threat to transgenic mouse colonies. By following advice from the Greenberg Lab, the risk of this is minimised in the maintenance of TRAMP colonies. Female heterozygotes for the transgene (TRAMP+/-) are backcrossed with non-transgenic C57BL/6 breeder males a proportion of the offspring will be male heterozygotes for experimentation. Indeed all the TRAMP mice used in these experiments were shown to express the T-antigen transgene. This procedure, however, does not prevent the drift of the background strain to produce a phenotype which interacts with the transgene in an unpredictable way.

The work presented here found no difference in IGF-1 or IGFBP-3 concentrations in the serum of TRAMP mice who had received either tea polyphenol compared with controls. This was in contrast to Gupta et al. [85] who reported that following 24 weeks of oral green tea polyphenols IGF-1 was decreased by approximately 1/3 and IGFBP-3 was increased approximately 1/3. The attempt to reproduce these results was made more difficult by being unable to trace the commercial ELISA kit for murine IGFBP-3 that was reported to have been used.

Data from a recent study investigating the cancer preventive effects of genistein and resveratrol in a transgenic rat model of prostate cancer also failed to find a difference in plasma IGF-1 following administration of polyphenols. However, they simultaneously found a reduction in prostate tissue IGF-1 concentration. They suggest that IGF-1regulation occurs at the prostate tissue level and tissue IGF-1 may be a better cancer biomarker [263].

The oxidative DNA adduct M_1G was measured as a putative biomarker of prostate chemoprevention, section 1.9.2. This adduct was found to be significantly decreased in prostate tumour DNA in TRAMP mice that had received green tea catechins compared with controls. The mice that had received black tea theaflavins demonstrated a trend for lower M_1G compared with controls but this difference did not reach significance. The size of the study may not have been large enough to detect this more modest difference. The smaller reduction in M_1G by theaflavins than by catechins is in keeping with the reduced prostate tumour inhibition demonstrated by the comparison of prostate tumour weights. The idea

that M_1G concentration may reflect carcinogenesis was supported by the finding that prostate tumour weight was positively correlated with M_1G when all three groups were analysed together. When the groups were analysed separately, this correlation was only significant in the theaflavin group. It is not possible to say if the absence of significance was simply a reflection of the small numbers of mice in each group and lack of statistical power.

The possible correlation between inhibition of carcinogenesis and prostate M_1G concentrations supports previous work showing a reduction in the adduct in xenografted LNCaP cells on a mouse host, following flutamide administration [178]. This represents evidence that M_1G may indeed be used as a biomarker of prostate carcinogenesis prevention.

As discussed in section 1.9.2, the mechanism by which M_1G DNA adducts are thought to contribute to carcinogenesis is by inducing DNA point substitutions [173]. An individual cell may be subject to 10^5 oxidative events per day from oxidative radicals [264] if these events cause oxidative modification of a DNA base then adducts such as M_1G can be formed [171]. Failure of accurate excision repair prior to DNA replication leads to a point mutations mostly G → T and G → A). This hypothesis could be tested by determining DNA adduct levels from prostate tumour specimens and correlating these levels with mutation analysis from the same DNA by analysing restriction fragment length polymorphism, single-strand conformation polymorphism and gene sequencing of PCR amplified genes of interest.

Other possible prostate cancer chemoprevention biomarkers were sought by analysing a number of proteins p-Erk1/2, cleaved caspase 3 and VEGF in murine prostate tissue. The expression of none of these proteins was found to be altered by tea polyphenols. This is in contrast to published *in vitro* evidence that suggests that EGCG at concentrations of 22.9 μg/ml can induce the expression of p-Erk1/2 in PC-3 cells [265] and that theaflavins concentrations of 25-50 μg/ml can induce apoptosis in LNCaP cells by downregulation of pErk1/2 [110]. It may be that the concentrations of tea polyphenols achieved *in vivo* following were not sufficient to replicate these results, however, this result also failed to duplicate the findings of Adhami et al. [193] who found pErk1/2 to be inhibited in the TRAMP mouse prostate tumour following oral administration of green tea polyphenols. The reason for this disparity is not clear. The blots shown in figure 47 analysing the expression VEGF are poor quality, possibly due to the same western blots being stripped of previous antibodies and re-probed with anti-VEGF. These blots should have been repeated prior to VEGF analysis, in order to establish with any confidence that a change in expression had been excluded.

Initial microarray analysis suggested that there were a group of genes related to olfactory receptors (Olfr955, Olfr1365, Olfr1515, Olfr694, Olfr845 and Olfr1065) whose expression was significantly altered in the prostates of mice that had received catechins compared with controls. Unfortunately this result could not be replicated in the repeat experiment in which the dye markers were allocated to the intervention groups differently; therefore it is possible that this observation was due to heterogeneous uptake of dye by certain expressed sequences rather than

genuine differences in expression patterns between the intervention groups. It is interesting to note however, that a prostate specific G-protein coupled receptor (PSGR) with homology to olfactory receptors has been found to be over-expressed in human prostate cancer, and has previously been proposed a biomarker of prostate carcinogenesis [266]. The functional role of PSGR in prostate cancer is not known. Many G protein coupled receptors, however, are known to transduce extracellular growth signals involved in growth and differentiation which are principle elements of carcinogenesis; and, over-expression of certain G protein coupled receptors (such as protease-activated receptor, PAR1) has been correlated with carcinogenesis [267]. The G protein coupled olfactory receptors identified by the initial microarray analysis therefore seem worthy of further investigation by northern blotting or quantitative RT-PCR in order to fully characterise their expression in prostate tissue following intervention with green tea catechins. It should be re-emphasized, however, that the repeat arrays performed in the work presented here did not confirm any change in olfactory receptor expression in the prostates of TRAMP mice following tea polyphenol administration; any suggestions for further studies are therefore purely speculative.

LCMSMS analysis of green tea catechins failed to identify any polyphenols in the plasma and prostate tissue of TRAMP mice that had received tea polyphenols in their drinking water. Work presented in chapter 5 demonstrates the ability of the technique to detect catechins in plasma at concentrations less than 1 µg/ml. With the polyphenol solutions being available to the animal *ad libitum*; the time interval from dosing to peak plasma/tissue concentrations being unknown;

and the half life of the agents during excretion being unknown; it is impossible to draw conclusions from the absence of detectable catechins in the samples taken. In particular, it cannot be asserted that the prostate cancer chemopreventive effect seen in TRAMP mice requires tissue concentrations of catechins considerably less than the concentrations needed to inhibit *in vitro* prostate cancer cells discussed in section 1.7.2.

LCMSMS analysis of black tea theaflavins extracted from both plasma and prostate tissue was disappointing in that the peaks demonstrated were all close to the solvent front suggesting a failure of the liquid chromatography. It is therefore impossible to claim to have established the identity of the peaks seen. It is however, interesting that no such peaks were identified in the samples from control mice providing the most tentative of evidence suggesting the presence of theaflavins in the material tested. It would seem to be worthy of further work to properly optimise of the method of theaflavin extraction and detection in order to pursue this initial indication.

5 The effect of tea polyphenols on biomarkers of carcinogenesis in human subjects

5.1 Monitoring for toxicity of oral tea polyphenols in humans

5.1.1 Introduction

Following the oral administration of tea polyphenols to human subjects biochemical monitoring was performed to allow certain adverse reactions to be detected. This was undertaken as a patient safety precaution so that, if necessary, the study could be terminated prematurely. The study was not powered to exclude tea polyphenols toxicity.

5.1.2 Biochemical markers of toxicity in human subjects receiving oral tea polyphenols

18 volunteers were randomised to receive one gram theaflavins per day, one gram catechins per day or no polyphenols supplement, as described in section 2.6.1. A series of markers of toxicity were measured before and after the dietary interventions. Plasma sodium, potassium, urea, creatinine, albumin, alkaline phosphatase, alanine transaminase and bilirubin were all measured by Leicester University Hospitals Clinical Chemistry laboratories. The post-intervention results

are expressed as a proportion of the pre-intervention measurements and figures 54 to 63 show the mean changes in each marker

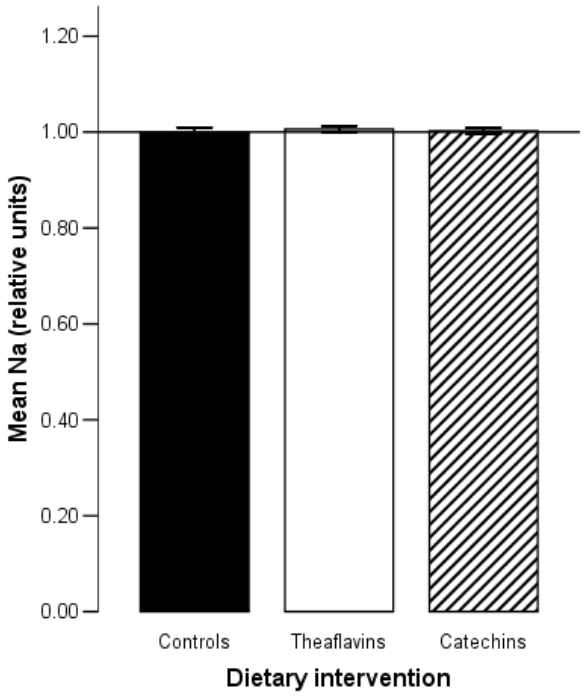


Figure 55 Mean plasma sodium change after 28 days of dietary intervention with tea polyphenols. Post-intervention Na expressed as proportion of pre-intervention Na (mean change +/- SEM). Differences between groups did not reach statistical significance.

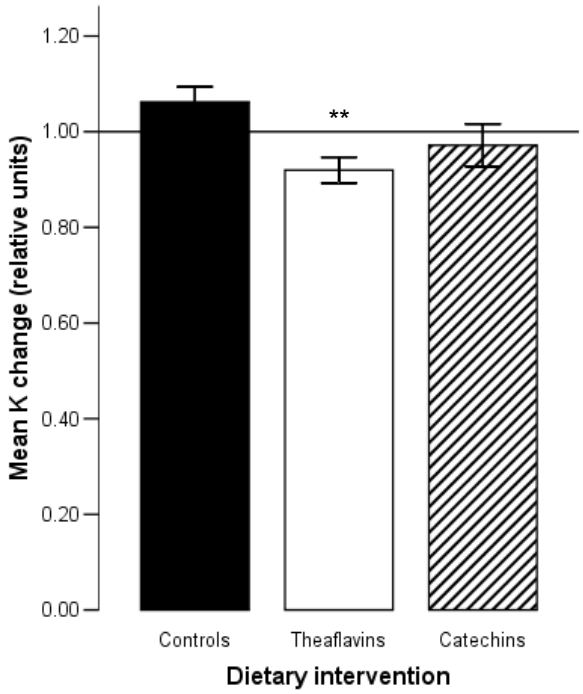


Figure 56 Mean plasma potassium change after 28 days of dietary intervention with tea polyphenols. Post-intervention potassium expressed as proportion of pre-intervention potassium, (mean change +/- SEM). Analysed overall using the Kruskal-Wallis test suggested that significant differences existed between the groups, (p=0.025). *Post-hoc* analysis using the Mann-Whitney U suggested that this significance was due to reduced potassium concentration in the theaflavin groups compared to the controls (** p=0.002). No significant differences were demonstrated between the catechin groups and control, and the two intervention groups.

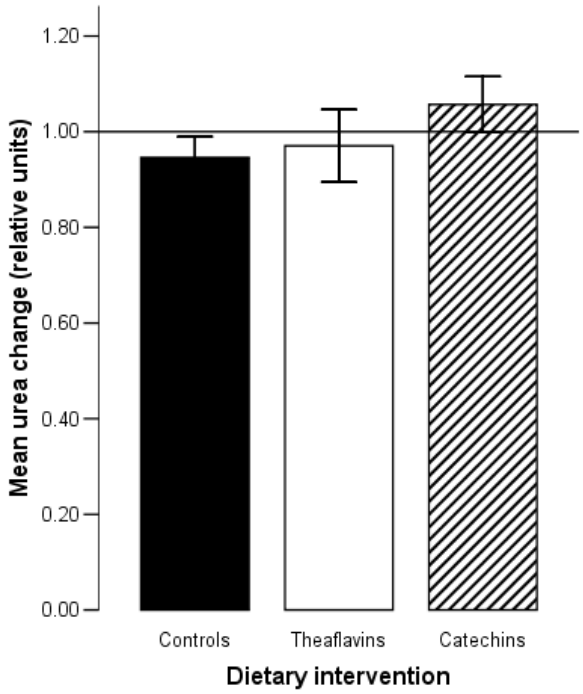


Figure 57 Mean plasma urea change after 28 days of dietary intervention with tea polyphenols. Post-intervention urea expressed as proportion of pre-intervention urea (mean change +/- SEM). Differences between groups did not reach statistical significance.

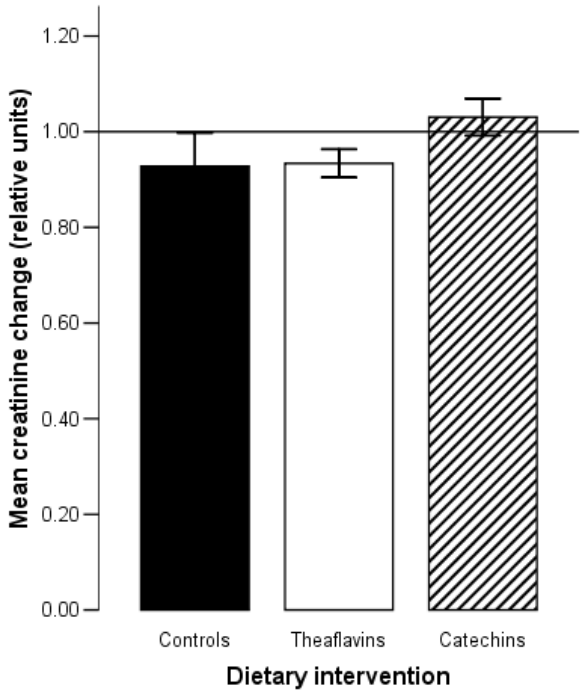


Figure 58 Mean plasma creatinine change after 28 days of dietary intervention with tea polyphenols. Post-intervention creatinine expressed as proportion of pre-intervention creatinine, (mean change +/- SEM). Differences between groups did not reach statistical significance.

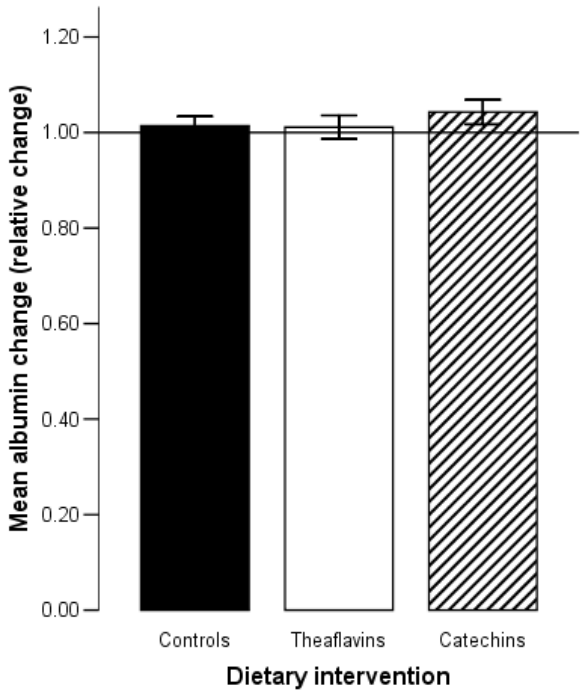


Figure 59 Mean plasma albumin change after 28 days of dietary intervention with tea polyphenols. Post-intervention albumin expressed as proportion of pre-intervention albumin, (mean change +/- SEM). Differences between groups did not reach statistical significance.

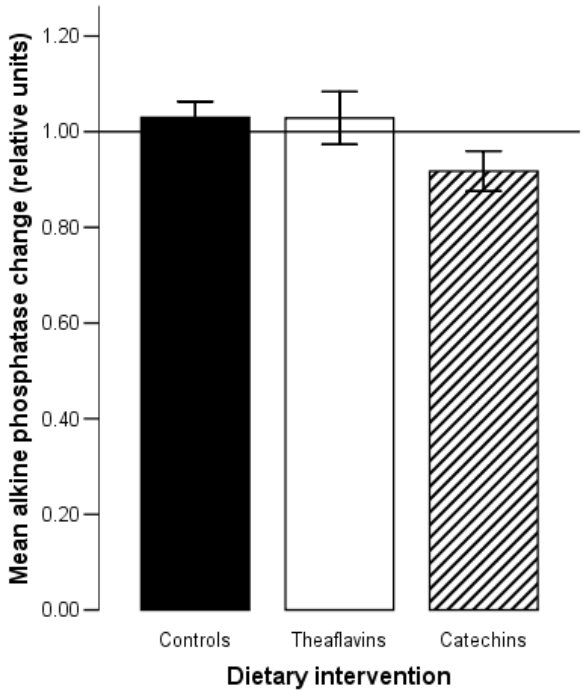


Figure 60 Mean plasma alkaline phosphatase change after 28 days of dietary intervention with tea polyphenols. Post-intervention alkaline phosphatase expressed as proportion of pre-intervention alkaline phosphatase, (mean change +/- SEM). Differences between groups did not reach statistical significance.

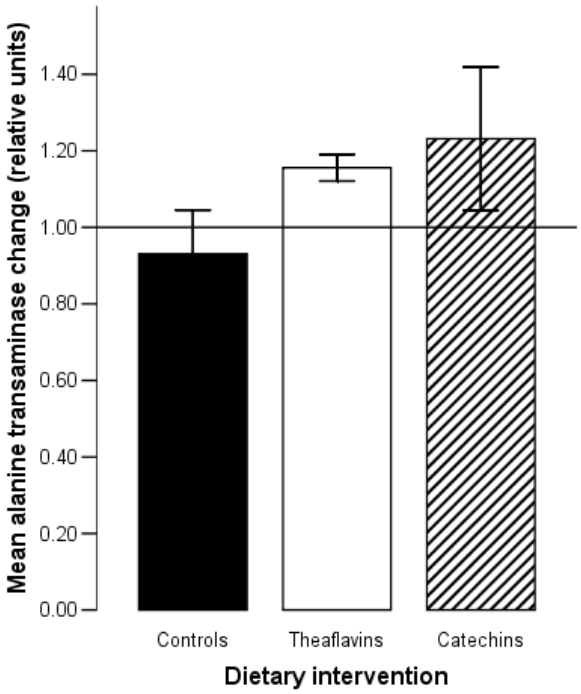


Figure 61 Mean plasma alanine transaminase change after 28 days of dietary intervention with tea polyphenols. Post-intervention alanine transaminase expressed as proportion of pre-intervention alanine transaminase, (mean change +/- SEM). Differences between groups did not reach statistical significance.

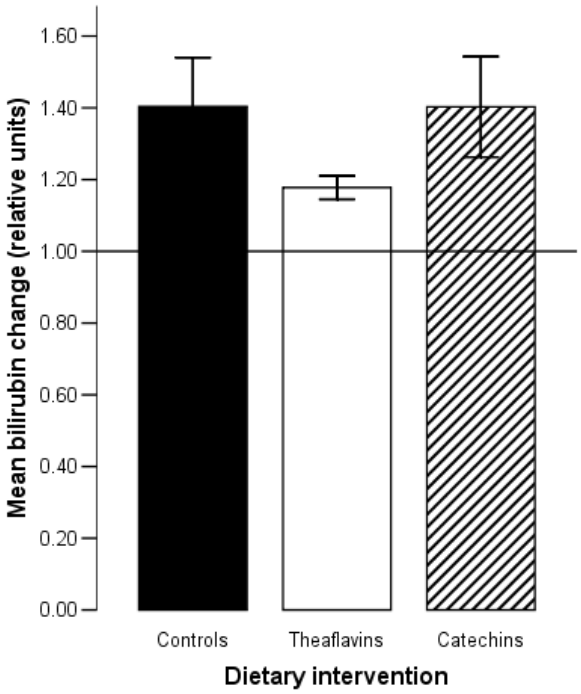


Figure 62 Mean plasma bilirubin change after 28 days of dietary intervention with tea polyphenols. Post-intervention bilirubin expressed as proportion of pre-intervention bilirubin, (mean change +/- SEM). Differences between groups did not reach statistical significance.

Given that there were only six patients in each intervention group, the data were compared using the non-parametric Kruskal-Wallis test. The only parameter that demonstrated statistically significant differences between groups following the 28 days of dietary intervention was potassium, which was significantly lower in the theaflavin group compared with the controls. Given that this represents a post-hoc analysis, after the consideration of nine variables, the Mann-Whitney U test can be expected to underestimate the p value. The small size of the change can be appreciated from the means and medians seen in table 11, the mean reduction in plasma potassium concentration in the post-intervention theaflavin group compared with the pre-invention group was 8%.

Table 11 The average changes in the plasma potassium concentration expressed as post-intervention potassium concentration as a proportion of pre-intervention concentration.

	Control	Theaflavins	Catechins
Mean plasma K change	1.06	0.92	0.97
Median plasma K change	1.06	0.95	0.98

5.2 Identification of biomarkers of prostate cancer prevention by tea polyphenols in humans

5.2.1 Introduction

In order to demonstrate human prostate cancer prevention by tea polyphenols a study would be required lasting many years and involving many thousands of patients. Before such work could be justified more preliminary evidence of human prostate cancer prevention must be demonstrated by looking for differences in cancer prevention biomarkers following oral administration of tea polyphenols.

Evidence presented in section 4.1 suggested that tea polyphenols can inhibit TRAMP mouse carcinogenesis. The concentration of oxidative DNA adduct, M_1G, was found to be significantly reduced in the DNA of TRAMP mouse prostates following dietary intervention with green tea catechins and non-significantly reduced by theaflavins, see section 4.2. Furthermore the concentration of M_1G in the DNA from these tumours was significantly correlated with the masses of the tumours, see section 4.2. M_1G was therefore identified as a potential biomarker which could be applied to early human studies of prostate cancer prevention.

The work presented here aimed to demonstrate differences in putative biomarkers of prostate carcinogenesis human prostate following the administration of tea polyphenols. The biomarkers chosen were M1G, section 1.9.2, and the human prostate tumour marker PSA, see section 1.9.7. In addition, novel

biomarkers were sought using microarray analysis of the expressed prostate genome.

5.2.2 The effect of oral tea polyphenols on the M_1G concentrations in human prostate tissue

The effect of oral green tea catechins and black tea theaflavins on prostate M_1G concentrations was investigated in eighteen men, randomised to receive theaflavins, catechins or no polyphenol see section 2.6.1 for the study design. M_1G concentrations of standards and samples were determined by using the method in section 2.2.

A standard curve was calculated that related M_1G concentration to chemiluminescence, see figure 62. The linear equation of the standard curve best fit line was then used to calculate the M_1G concentration of the samples run in parallel. Figure 63 shows the mean concentrations and standard errors of the three intervention groups. Post-hoc analyses demonstrated that the catechin group had significantly lower concentrations of M_1G compared with controls.

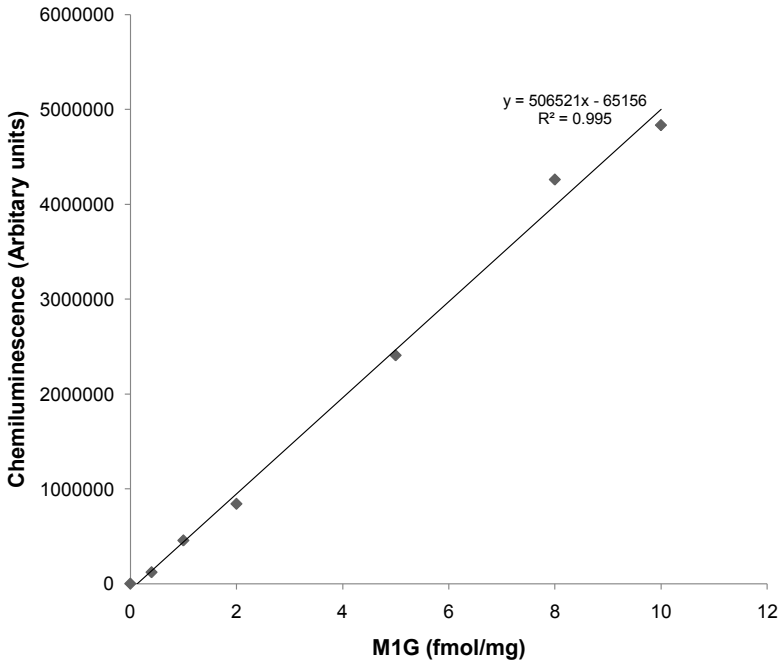


Figure 62 Standard curve of chemiluminescence from M_1G standards against M_1G concentration.

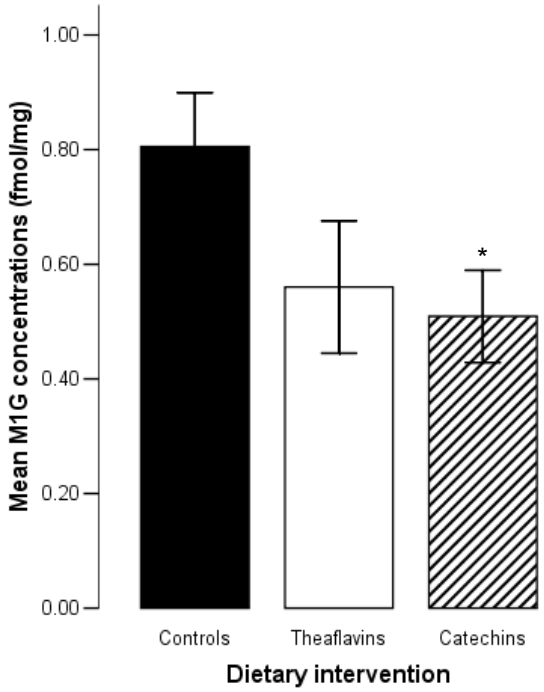


Figure 63 M_1G concentrations in prostate DNA from men who had received 28 days dietary intervention with tea polyphenols, (mean +/- SEM). Analysed overall without *a priori* expectation, using the ANOVA or Kruskal-Wallis tests, no significant differences were detected between the groups. However, *post-hoc* analyses by Student's t test, to detect reductions in M_1G in the intervention groups compared with controls, shows that the M_1G concentration of catechins group is significantly lower than the control group (* p=0.04). The difference observed between the theaflavins group and the control group did not reach the level of significance.

5.2.3 Microarray analysis of human prostate gene expression following dietary intervention with tea polyphenols

Following four weeks of dietary intervention with catechins, theaflavins or no polyphenols, human prostate tissue was collected at TURP operations see section 2.6.1 for the study design. Microarrays of gene expression were used to rapidly screen the entire expressed population of mRNAs from the human prostates to identify genes whose expression was altered by the interventions. These genes could then be proposed as potential biomarkers of prostate cancer prevention by tea polyphenols, worthy of further characterisation. See section 2.8.11 for the array allocation and experimental design. The experiment was then repeated using the same RNA extracts but using different labelling Alexa dyes for each intervention group to reduce the possibility of labelling bias simulating the appearance of altered gene expression.

None of the expressed sequences screened on the microarrays were significantly altered in expression when men who received polyphenols were compared with those who acted as controls.

5.2.3 The effect of tea polyphenols on the tumour marker PSA

Following four weeks of dietary intervention with catechins, theaflavins or no polyphenols, human plasma was collected for measurement of PSA. Figure 64 shows the result of the intervention, the post-intervention values being expressed as a proportion of the pre-intervention values. The data were compared using the non-parametric Kruskal-Wallis test. There were no significant differences between the groups.

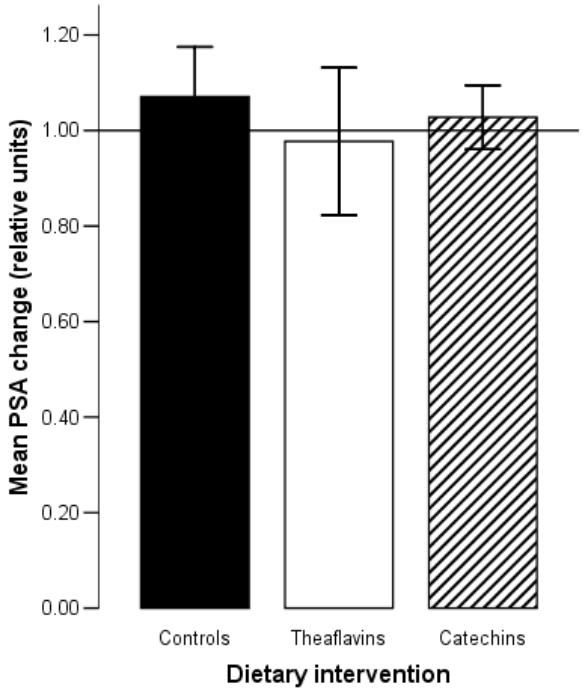


Figure 54 Mean PSA change after 28 days of dietary intervention with tea polyphenols. Post-intervention PSA expressed as proportion of pre-intervention PSA (mean change +/- SEM). Differences between groups did not reach statistical significance.

5.3 Identification of orally administered tea polyphenols in human subjects

5.3.1 Introduction

Data from this work and elsewhere suggest that in order to inhibit processes associated with carcinogenesis, tea polyphenols are required to be present at concentrations in excess of 25 µg/ml, see section 1.7.2 and chapter 3. Evidence presented in section 4.1.2 suggests that orally administered polyphenols can reduce prostate carcinogenesis in TRAMP mice while simultaneously reducing the concentration of the putative biomarker M_1G in prostate DNA, see section 4.2.2. Evidence presented in section 5.2.2 suggests that orally administered tea polyphenols can similarly reduce levels of M_1G in human prostate. LCMSMS analysis of material from these human subjects aimed to demonstrate that tea polyphenols can be absorbed orally and penetrate tissue. It also aimed to investigate which metabolites are measurable *in vivo,* and in the case of green tea polyphenols, to make some estimations of the concentrations achieved following oral adminstration.

5.3.2 Identification of green tea catechin metabolites in human urine

Six human volunteers took 1g catechins per day for 28 days prior to donation of urine and six volunteers acted as controls. LCMSMS was used to look for the presence of 30 green tea catechins in this urine, see section 2.7.9.

Figure 65 shows the catechin compounds detected in the urine from one of these human volunteers (patient 7). None of the identified catechins were detected in the urine from control volunteers; however, not all the metabolites were present in all six of the patients' urines who had received catechins. Table 12 indicates the number of patients' urines in which each metabolite was present. In total three native catechins and 16 catechin metabolites were identified.

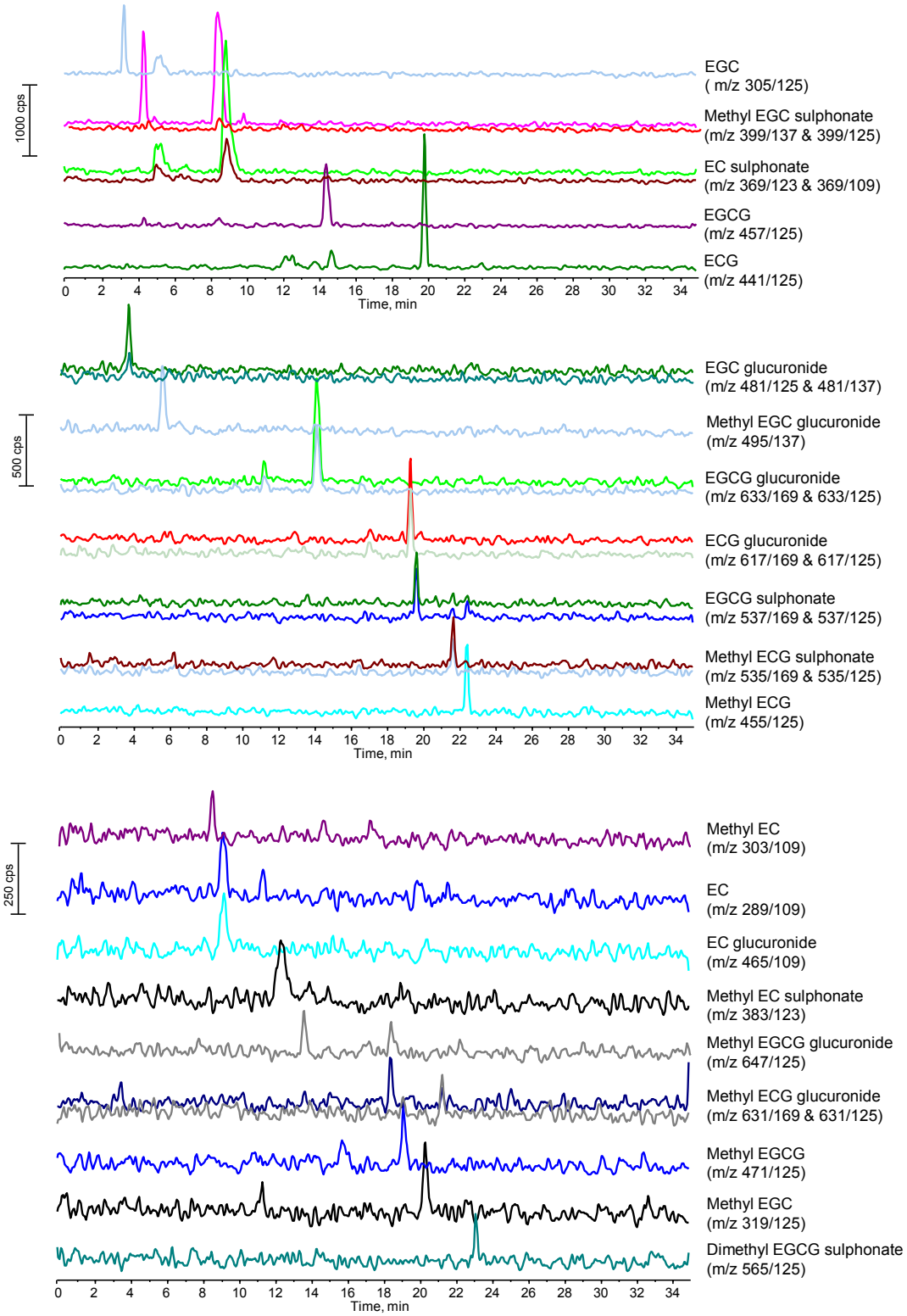


Figure 65 LCMSMS analysis of catechins extracted from the urine of human subject no. 7, following 4 weeks of dietary intervention with 1g catechins orally daily. Each trace represents a different m/z transition corresponding to an individual catechin. The LCMSMS method attempted to detect 30 separate catechin species simultaneously. Those traces showing peaks with high signal-to-noise ratio are shown above. The peak heights are determined by the count per second recorded for each m/z transition. The traces are shown in three groups, each group is presented using a different y-axis scale (counts per unit height).

Table 12 Frequency of detection of catechin metabolites in urine samples from six human volunteers following oral catechin administration

Catechin	Number of patients' urine in which catechin present
ECG	5
EC sulphonate	5
Methyl EGC	5
EGC glucuronide	5
Methyl EGC	5
EGC	4
EGCG	4
EGCG glucuronide	4
ECG glucuronide	4
Methyl EGCG glucuronide	4
EC	3
EC glucuronide	3
Methyl EC sulphonate	3
Methyl ECG glucuronide	3
Methyl EC	2
Methyl EGCG	2
EGCG sulphonate	2
Methyl ECG sulphonate	2
Methyl ECG	2

5.3.3 Quantification of deconjugated green tea catechins in human urine

In order to quantify the catechins detected in the urine of human subjects that had received oral green tea catechins a deconjugation reaction was used prior to LCMSMS, see section 2.7.10 for the experimental design. Figure 66 shows the deconjugated catechin compounds detected in the urine from patient 7. Figure 67 shows the standard curves for each of the four parental molecules (EC, EGC, ECG and EGCG). The LCMSMS peak areas corresponding to deconjugated catechins in subjects' urine were compared with the standard curves allowing quantification of the parental molecules found in urine. The peak areas and calculated catechins concentrations for each subject's urine are shown in table 13.

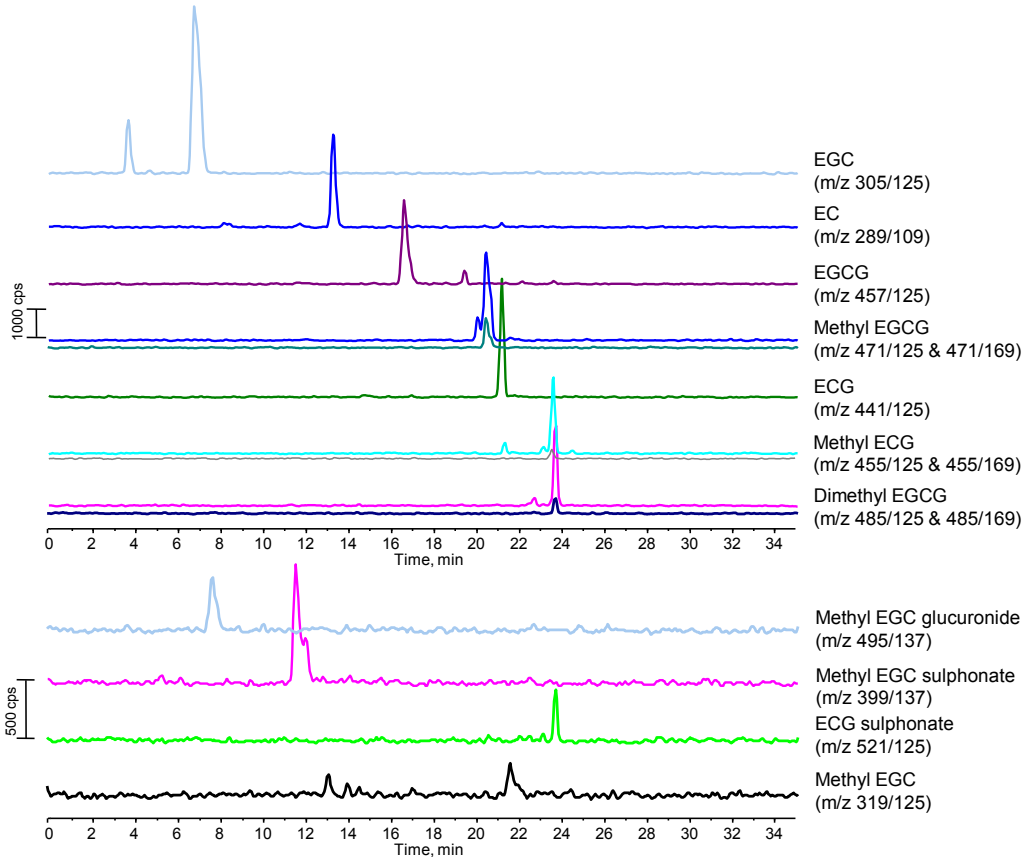


Figure 66 LCMSMS analysis of catechins extracted from the urine of human subject no. 7, following 4 weeks of dietary intervention with 1g catechins orally daily and subsequently subjected to a deconjugation reaction. Each trace represents a different m/z transition corresponding to an individual catechin. The LCMSMS method attempted to detect 30 separate catechin species simultaneously. Those traces showing peaks with high signal-to-noise ratio are shown above. The peak heights are determined by the count per second recorded for each m/z transition. The traces are shown in three groups, each group is presented using a different y-axis scale (counts per unit height).

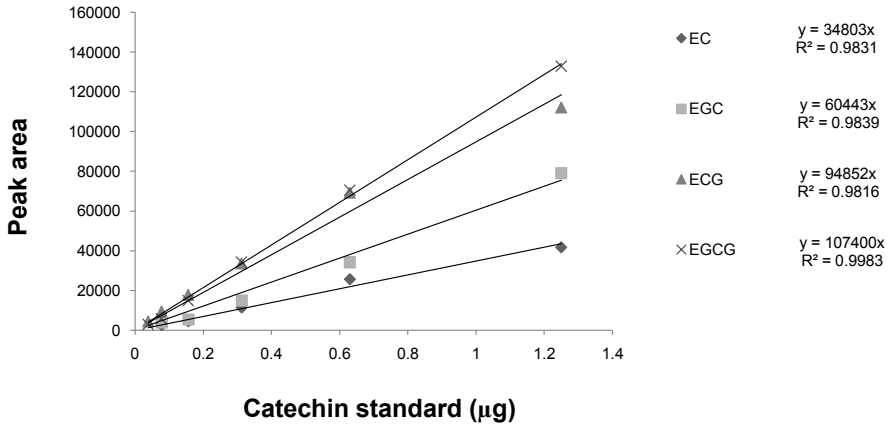


Figure 67 Standard curves of four parental catechin compounds analysed by LCMSMS.

Table 13 The peak areas and derived concentrations of each deconjugated catechin compound in each subjects urine.

Subject	Catechins							
	EC		EGC		ECG		EGCG	
	(peak area)	(μg/ml)	(peak area)	(μg/ml)	(peak area)	(μg/ml)	(peak area)	(μg/ml)
7	52575	7.55	16035	1.33	55077	2.90	6417	0.30
8	0	0	0	0	0	0	0	0
9	6759	0.97	25093	2.08	3649	0.19	8558	0.40
10	24465	3.51	85695	7.09	15329	0.81	15196	0.71
11	9072	1.30	16718	1.38	1531	0.08	3826	0.18
12	4900	0.70	10302	0.85	0	0	0	0

5.3.4 Identification of green tea catechins in human plasma

Six human volunteers took 1g catechins per day for 28 days prior to donation of plasma and six volunteers acted as controls. LCMSMS was used to look for the presence of 30 green tea catechins in this plasma, see section 2.7.9 for the experimental design.

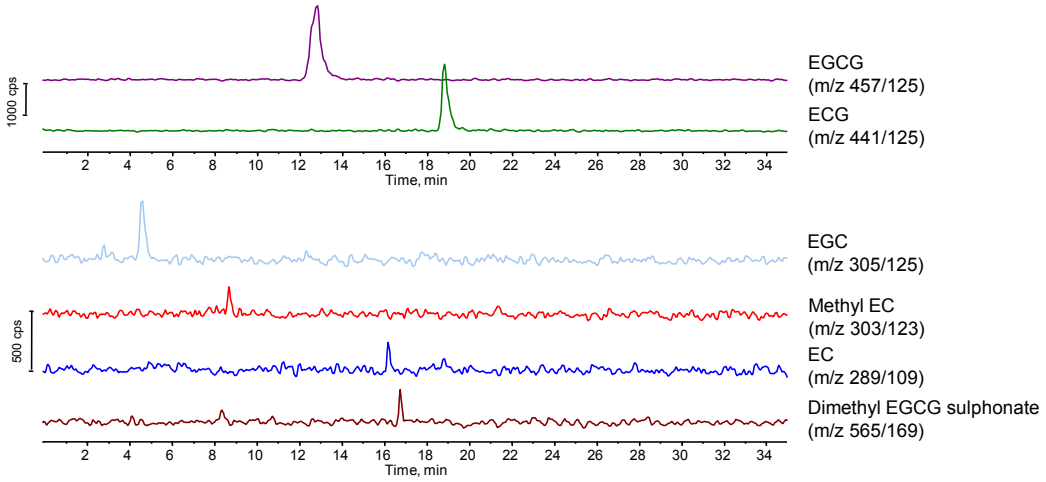


Figure 68 LCMSMS analysis of catechins extracted from the plasma of human subject no. 9, following 4 weeks of dietary intervention with 1g catechins orally daily. Each trace represents a different m/z transition corresponding to an individual catechin. The LCMSMS method attempted to detect 30 separate catechin species simultaneously. Those traces showing peaks with high signal-to-noise ratio are shown above. The peak heights are determined by the count per second recorded for each m/z transition. The traces are shown in two groups, each group is presented using a different y-axis scale (counts per unit height).

Figure 68 shows the catechin compounds detected in the plasma from one of these human volunteers (patient 9). No catechins were detected in the plasma from control volunteers. Table 14 shows the frequency with which each catechin compound was found in the plasma samples from the 6 human subjects given

1g/day catechins. In total, from the 30 compounds sought, all four native catechins and three catechin metabolites were identified.

Table 14 Frequency of detection of catechin metabolites in plasma samples from six human volunteers following oral catechin administration

Catechin	Number of patients' plasma in which catechin present
ECG	3
EGCG	2
EGC	1
EC	1
Methyl EC	1
Methyl EGC	1
Dimethyl EGCG sulphonate	1

Quantification was performed by comparison of the peak areas from the catechin traces detected in human plasma with the simultaneously run standard curve see figure 67. Since sulphonated and glucuronidated metabolites were not present in significant amounts in human plasma, no deconjugation reaction was performed. Table 15 shows the concentrations of parental catechin molecules found in plasma.

Table 15 The peak areas and derived concentrations of each parental catechin compound in each subjects plasma.

Subject	Catechins							
	EC		EGC		ECG		EGCG	
	(peak area)	(μg/ml)	(peak area)	(μg/ml)	(peak area)	(μg/ml)	(peak area)	(μg/ml)
7	0	0	0	0	6409	0.53	4955	0.71
8	0	0	0	0	0	0	0	0
9	3224	0.15	9061	0.48	49700	4.11	89900	12.92
10	0	0	0	0	0	0	0	0
11	0	0	0	0	3251	0.27	0	0
12	0	0	0	0	0	0	0	0

5.3.5 Analysis of green tea catechins in human prostate

Six human volunteers took 1g catechins per day for 28 days prior to donation of prostate tissue and six volunteers acted as controls. LCMSMS was used to look for the presence of 30 green tea catechins in this plasma, see section 2.7.9. None of the 30 catechins sought were detectable in these prostate tissue samples.

5.3.6 Analysis of black tea theaflavins in human urine, plasma and prostate tissue

As detailed in section 2.6, six human volunteers took 1g theaflavins per day for 28 days prior to donation of urine, plasma and prostate tissue. LCMSMS was used to look for the presence of 39 theaflavin species in these samples and in the samples of six control volunteers, see section 2.7.12 for the experimental design and methods.

Figure 69 shows examples of the traces obtained from analysis of the plasma of subject 13, although similar traces were obtained from plasma samples of all six subjects. Figure 70 shows two of the traces obtained from analysis of the prostate tissue of subject 13. No corresponding peaks were present in the analyses of plasma and prostate tissue from the six control subjects. There were no theaflavin compounds detected in any of the urine samples.

All the peaks demonstrated here, appeared soon after the solvent front indicating the lack of method optimisation. Whilst the traces are consistent with the presence of theaflavin compounds in plasma and prostate, confirmation would require the development of a robust LCMSMS method.

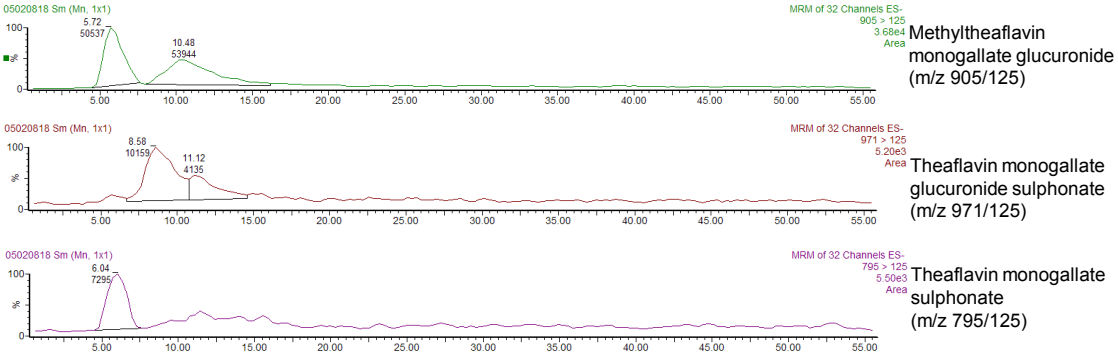


Figure 69 LCMSMS analysis of theaflavins extracted from the plasma of human subject no. 13, following 4 weeks of dietary intervention with 1g theaflavins orally daily Compounds were identified by LCMSMS following 4 weeks of dietary intervention with 1g theaflavins orally daily. Each trace represents a different m/z transition corresponding to an individual theaflavin. The LCMSMS method attempted to detect 39 separate theaflavin species simultaneously. Three example traces are shown demonstrating peaks of high signal-to-noise ratio however the technique failed to separate the peaks on the basis of retention times.

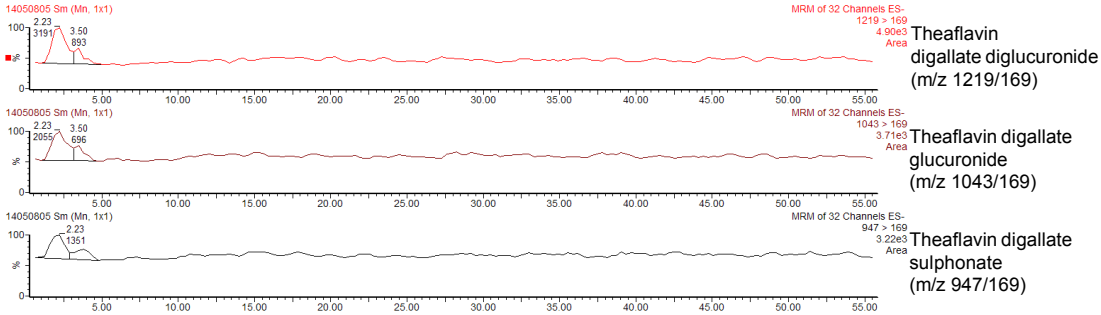


Figure 70 LCMSMS analysis of theaflavins extracted from the prostate tissue of human subject no. 13, following 4 weeks of dietary intervention with 1g theaflavins orally daily Compounds were identified by LCMSMS following 4 weeks of dietary intervention with 1g theaflavins orally daily. Each trace represents a different m/z transition corresponding to an individual theaflavin. The LCMSMS method attempted to detect 39 separate theaflavin species simultaneously. Three example traces are shown demonstrating peaks of high signal-to-noise ratio however the technique failed to separate the peaks on the basis of retention times.

5.4 Discussion

This study of male volunteers separated into three intervention groups, one that received green tea catechin, one that received black tea theaflavins and a control group contained only six individuals in each groups. The low power of this study makes statistical analysis dubious and it is impossible to draw clear conclusions.

This study was not designed or powered to establish to presence or absence of toxicity to humans following ingestion of tea polyphenols, however, toxicity data was collected at the beginning and end of each participant's involvement in the study in accordance with ethical requirements. Had the study involved a medicinal product, as defined by the MHRA applying EU Directive 2001/20/EC, then the level of toxicity monitoring performed in this study would have been inadequate. A study of a medicinal product would have required clinical monitoring and biochemical toxicity data to have been collected at regular intervals throughout the study. Despite not being required by the licensing authorities, the lack of continuous toxicity monitoring is a significant weakness of this study.

The one biochemical parameter measured that showed a statistically significant change was plasma potassium concentrations, which was lower in the human volunteers who had received theaflavins compared with controls. It is important to note, however, that since eight biochemical markers of toxicity were monitored and compared it is possible that the result could represent a type I statistical error, there is no other published evidence of a significant decrease in plasma potassium following tea polyphenol ingestion. Also, in considering this

result we must distinguish between statistically significant and clinically significant changes. The normal range quoted by Leicester Royal Infirmary clinical laboratories for plasma potassium is 3.5-5.4 mM. The bottom of the normal range is 35% lower than the top of the normal range, this puts into context the 8% lower plasma potassium concentrations in those who had received theaflavins. Overall, therefore, there was no evidence of toxicity following ingestion of tea polyphenols by the volunteers in this study.

Evidence from chapter 4 supported the role of M_1G as a biomarker of prostate cancer prevention in the TRAMP mouse by correlating prostate tumour weights with intervention with tea polyphenols. Comparison of M_1G concentrations in the DNA of prostate tissue of men who had received tea polyphenols with those that had not, revealed lower values in both intervention groups compared with controls, however, this only reached possible significance in the group of men that had received green tea catechins.

It should be noted that the comparisons between the means of three groups involve comparisons between three pairs of means. The more comparisons performed, the greater the risk of a type I statistical error. The use of ANOVA/Kruskal Wallis tests to analyse all the groups simultaneously avoids this possibility, when this analysis was performed on the M_1G data no significant differences were identified. However, it may be argued that it was reasonable to limit the analysis to comparison of two pairs of means i.e., catechin group v controls and theaflavin group v controls. When this type of analysis was applied using serial t-tests then significance was identified between the catechin group and

the controls; given only two comparisons were made the risk of a type I error was deemed small.

If this result was confirmed by a larger study, this would represents evidence that M_1G can be utilised as a biomarker of prostate cancer prevention by catechins not only in mice but also in humans. The reduction in M_1G seen in the men that received theaflavins did not reach significance; this may reflect a reduced chemopreventive potential compared with catechins, demonstrated in TRAMP mice as detailed in chapter 4. It is possible that a larger study would demonstrate the significance of this more modest reduction in adduct concentration. The preliminary evidence presented here that tea polyphenols reduce M_1G in human prostate DNA could form the basis for a power calculation when planning the optimum size of further studies.

This evidence represents only indirect evidence for green tea catechins as human prostate cancer preventive agents and it may be argued that more compelling evidence was provided by Bettuzzi et al. [117] who found that, in patients with HG-PIN, catechins significantly reduced the number of men developing invasive prostate cancer (1 of 30 v 9 of 30). However, it is noticeable that there has been no published repetition of this experiment in the intervening years since the publication of the original paper. Further evidence is necessary to justify any investment in a confirmatory larger study.

The absence of a change in plasma PSA concentration in men who received tea polyphenols compared with controls is predictable given the brief intervention interval. Bettuzzi et al. [117] also failed to find any change in PSA

even after one year's administration of green tea catechins, given that a strong cancer preventive effect was observed over this time period it suggests that PSA may not be an appropriate biomarker of cancer prevention despite its widespread use as a clinical biomarker.

Analysis of human urine following administration of oral green tea catechins using LCMSMS provided clear evidence of urinary excreted catechins. Catechins were found in both native as well as metabolised forms. Catechins showed evidence of *in vivo* sulphonation, methylation and conjugation with glucuronide as was predicted by the availability of hydroxyl groups on the native catechins. It is not known to what extent the metabolites rather than the native catechins are responsible for the biological processes associated with catechin administration. Enzymatic deconjugation to remove glucuronides and sulphonates allowed quantification of total catechin concentrations in urine. This procedure found the following ranges, EC 0-7.6 µg/ml, EGC 0-7.1 µg/ml, ECG 0-2.9 µg/ml and EGCG 0-0.7 µg/ml. Many fewer catechin species were detected in plasma, however each native species was detect and quantification suggested the following ranges EC 0-0.2 µg/ml, EGC 0-0.5 µg/ml, ECG 0-4.1 µg/ml and EGCG 0-12.9 µg/ml. These findings provided evidence of the bioavailability of the catechins administered. They also provided evidence of a range of endogenously generated catechin metabolites that may or may not share biological activity with their parental compounds. The metabolites were represented in greater numbers in urine probably due to an increase in polarity and therefore aqueous solubility allowing renal excretion.

This trial was designed to measure polyphenol concentrations at only one time point; therefore it is impossible to know whether the low concentrations of polyphenols detected represented low bioavailability of polyphenols or whether the sample collection time point missed the peak urine/plasma/tissue levels. A robust study design would have involved dosing human volunteers followed by regular plasma and urine collection in order to establish the pharmacokinetics of catechin and theaflavin preparations used in this study. In particular, identification of the time interval to peak plasma concentration after oral dosing and knowledge of the half-life of polyphenols during excretion, would have allowed the collection of prostate tissue to have been rationally timed relative to the final polyphenol dose. The weakness of the study design used in this work makes it impossible to correlate the low levels of polyphenols demonstrated, with the effects demonstrated upon prostate cancer prevention biomarkers.

Analysis of theaflavins in human urine, plasma and prostate tissue was inadequate due to lack of optimisation of the LCMSMS technique. The preliminary inconclusive analysis performed provides the suggestion that theaflavins are present in plasma and prostate tissue but there was no such evidence from urine. This result requires replication with robust LCMSMS techniques, but if it were to be believed then this may reflect that the size of the theaflavin molecules does not favour renal clearance.

6 General Discussion

Prostate cancer is the ideal target for chemoprevention; it is common, with a late onset and a prolonged natural history. Any delay in the onset of the disease due to chemoprevention would effectively reduce the incidence. Nutrichemicals have been investigated for this role, in the hope that they will inhibit carcinogenesis while being zero or low toxicity agents, acceptable to the healthy population. Promising evidence from epidemiological studies and *in vitro* studies suggesting catechins from green tea and theaflavins from black tea have anti-carcinogenic properties, see section 1.7, have raised hopes that these agents may prove to be useful prostate cancer preventive agents.

Before a decision can be taken to progress these agents to human clinical trials, there is certain pre-requisite evidence that must be sought. Promising anti-carcinogenic effects established *in vitro* must be tested in an appropriate animal model of the disease; secondly biomarkers of prostate carcinogenesis amenable to alteration by oral tea polyphenols must be identified. The results presented here have attempted to address these two unresolved areas.

Work presented here using the TRAMP mouse model demonstrated that orally administered catechins and theaflavins can both individually inhibit prostate carcinogenesis *in vivo,* see discussion 4.4. For theaflavins, this represents the first time that they have been shown to inhibit prostate carcinogenesis in a model animal system. For catechins, this confirms the results of other published work showing that catechins reduce prostate cancer size in the TRAMP model [85].

The evidence that tea polyphenols inhibit prostate carcinogenesis in an animal model begs the question of whether this effect could be duplicated in humans. Unless a human trial of many years duration can be performed, this question cannot be definitively answered. However, in order to pursue this possibility, biomarkers of the observed murine prostate cancer inhibition by tea polyphenols where sought, in order that they could be deployed in a human trial. Evidence was demonstrated that the oxidative DNA adduct, M_1G could play this role. In TRAMP mice, concentrations of this adduct in prostate tissue were significantly reduced following administration of oral catechins from 4 weeks to 30 weeks of age, see discussion section 4.4.

This finding was in contrast to other work presented here that failed to show any consistent change in the M_1G from human prostate cancer cells cultured *in vitro* with tea polyphenols compared with controls. It is possible that significant results, from M_1G or protein biomarkers, in cell culture could have been observed had higher concentrations of polyphenols been utilised. However, there is evidence that these concentrations are not relevant to possible future human dosing regimens, see discussion 3.4. Also, a potential confounding factor was demonstrated in work presented here showing that tea polyphenols when in high concentrations such as 25 µg/ml can display pro-oxidant properties *in vitro*, see discussion 3.4, rather than acting as anti-oxidants as they have in other assay systems, see section 1.3.3.

This same molecular marker was studied in human prostate tissue following one month of oral administration of either catechin or theaflavin. A reduction of

M_1G concentration was found following oral catechin administration, see discussion 5.4. It is tempting to speculate that this predicts a similar prevention of prostate carcinogenesis in humans as previously demonstrated in mice. However, it should be reemphasized that the human trial presented here had only six members in each group, making any conclusions extremely tentative. Of course, even the most robust biomarker evidence can never definitively address the issue of chemopreventive efficacy but it can demonstrate that agents such as catechins are worthy of further study in larger trials.

If further biomarkers of prostate carcinogenesis could be identified and shown to be altered by tea polyphenols the argument in favour of larger clinical trials would be strengthened. The work presented here unsuccessfully attempted to identify a number of other biomarkers. There was a preliminary suggestion from TRAMP mouse microarray analysis that there may be genes with homology to olfactory receptors whose expression is altered by tea polyphenols, see discussion 4.4, but this result was not validated in repeat experiments.

There are a number of candidate agents for human trials of prostate cancer prevention. Currently the 5α reductase inhibitors finasteride and dutasteride both have shown to reduce prostate cancer incidence in large clinical trials of prostate cancer incidence, see section 1.8. However, with both agents there are concerns about increased high grade cancers although it has been proposed that this is due to sampling error due to prostate volume reduction; also, both these agents produce sexual side effects such as erectile dysfunction. It is likely that both these issues will greatly reduce the acceptability of these agents to men targeted for

prevention programmes. The public may well prefer the option of a minimal toxicity nutrichemical preventive agent even if it was less potent. In order to produce a clinically significant reduction in prostate cancer incidence a future prevention strategy may choose to use a cocktail of low toxicity nutrichemicals. However, clinical trials should establish the safety and efficacy of each agent individually prior to the proposition of combinations.

The animal model and human biomarker evidence presented here suggests the hypothesis that tea polyphenols, and in particular green tea catechins, act as antioxidants *in vivo* following oral administration and that they have anti-carcinogenic properties that are worthy of further study. Future studies of tea polyphenols could aim to address some of the shortcomings of the work presented here to affirm the preliminary positive findings. Further experiments could try to identify the most important molecular mechanism responsible for the well established anti-carcinogenesis effect by tea polyphenols seen in prostate cancer cells *in vitro*. Such experiments should concentrate on molecular changes within minutes of tea polyphenol exposure in order to avoid non-specific toxic effects. The suggestion of *in vivo* anti-carcinogenesis by tea polyphenols presented here should lead to larger animal studies to investigate the longitudinal cancer preventive effect of tea polyphenols over time by serial assessment of cancer growth using MRI. Similarly, larger animal studies would allow potential biomarkers such as prostate DNA M_1G to be investigated at several time points over the study duration. The preliminary data presented here suggesting a reduction in human prostate M_1G following oral tea polyphenol administration could

be used in power calculations to provide a rational basis on which to decide on an appropriate study population size in further human studies.

7 Abbreviations

BSA	Bovine serum albumin, fraction V
CPS	Counts per second
DRE	Digital rectal examination
DNA	Deoxyribonucleic Acid
EC	Epicatechin
ECG	Epicatechin-3-gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin-3-gallate
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
Gd-chelate	Gadolinium chelate
GTC	Green tea catechins
HGPC	High grade prostate cancer
HGPIN	High grade prostate intraepithelial neoplasia
LCMSMS	Liquid chromatography and tandem mass spectrometry
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IGFBP-3	Insulin-like growth factor binding protein -3
LCMSMS	Liquid chromatography and tandem mass spectroscopy
LDL	Low density lipoprotein
M_1G	Malondialdehyde-deoxyguanosine DNA adducts

MDA	Malondialdehyde
mz	Mass charge ratio
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween-20
PCPT	Prostate Cancer Prevention Trial
PSA	Prostate specific antigen
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
TF	Theaflavin
TFG	Theaflavin-3-gallate
TFdiG	Theaflavin-3,3’-digallate
TRAMP	Transgenic Adenocarcinoma of Mouse Prostate
TURP	Transurethral resection of prostate
w/v	weight /volume
v/v	volume /volume

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Appendix 1

Example of patient information sheet and consent form

Patient Information Sheet and Consent Form (Catechins)

Can Dietary Supplements from Tea Prevent Prostate Cancer?

Introduction:

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Purpose of the study:

The prostate gland sits below the bladder in men. In the UK 1 in 14 men will develop prostate cancer in their lifetime. This study aims to find out if chemicals from tea called catechins have the potential to prevent prostate cancer in healthy men. To do this, the study will need to answer two questions. Firstly, can catechins be absorbed from capsules into the blood and prostate tissue? Secondly, do these catechins alter chemical markers in the blood, urine and prostate? Changes in these markers are thought to show that there is a cancer-preventive effect.

Why have I been chosen?

You have been chosen because you meet the following criteria:

- You and your consultant have decided your condition should be treated by having a prostate biopsy followed by a prostate operation called a transurethral resection of prostate (TURP)
- It will be at least four weeks from the date of biopsy until the date of the operation.
- You have no allergy to tea
- You are otherwise fit and well

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

In the work proposed here patients will be recruited who are awaiting both prostate biopsies and subsequent prostate operations. Catechins supplements will be administered, by mouth, for four weeks up to and including the date of their operation. The ability of the catechins to be absorbed by the patients will be determined by measuring their concentrations in blood and prostate tissue. Changes in chemical markers from the blood and prostate tissue will show if catechins may be having a cancer preventive effect.

Men who agree to take part will have their details registered in the trial database held by Mr. J.F. Thorpe at The Department of Cancer Studies and Molecular Medicine, 5th Floor RKCSB, Leicester Royal Infirmary, Leicester. All your information is confidential and all samples will only be labelled with your anonymous trial identification number.

You will be given at least 24 hours to consider whether you would like to participate.

You will be asked to make one extra visit to the hospital to be enrolled in to the trial if you agree to participate. Your travel expenses will be paid for this extra visit to a maximum of £15. You will then have blood taken and asked to provide a urine sample for measurement of markers of prostate cancer prevention. This amount of blood will be about 3 teaspoons worth.

Whilst you are having your prostate biopsy 10 to 12 pieces of tissue are commonly sampled. The trial will involve taking two extra pieces at the same procedure.

You will be issued with 250mg capsules of catechins. You should take four capsules every morning, every day up to and including the day of surgery. This should last approximately four weeks.

When you are admitted for surgery you will be asked to provide another similar sample of blood and urine. After the operation a sample of prostate tissue will be collected from the surgically removed tissue.

Nothing else will be asked of you.

The tissue and blood collected will be temporarily stored during the study. Unless you give separate permission, the tissue, blood and urine will be destroyed at the end of the trial, approximately autumn 2006.

What do I have to do?

You may drive, drink alcohol as you do normally and continue in any activity in which you normally participate. You should continue to take any regular medication.

You should take four capsules, every morning, until your operation.

Will I receive payment for the tissue that I donate for this research study?

You will not receive any payment for the tissue. The tissue is a gift - neither yourself nor your relatives will benefit from any inventions that result from the use of the tissue.

What is the dietary supplement that is being tested?

Catechins are naturally found in green tea but they are also in ordinary black tea available in the UK. There are no recorded side effects from taking caffeine-free tea extracts.

There is evidence from the laboratory that these supplements can prevent prostate cancer.

You will be issued with capsules containing 250mg of catechins. The dose is FOUR CAPSULES PER DAY, taken together.

What are the alternatives for treatment?

You have been invited to participate in this trial because you and your consultant have already chosen the biopsy and TURP operation for the treatment of your condition. You will not be denied any treatment that you would have otherwise received because of participation in this trial.

What are the side effects of any treatment received when taking part?

There are no known side effects of caffeine-free tea extracts. If you become concerned that you may be experiencing an unexpected side effect then you should contact the principal investigator. In the event of an emergency contact your GP.

What are the possible disadvantages and risks of taking part?

If you have private medical insurance you should check with the company before agreeing to take part in this trial. You will need to do this to ensure that your participation will not affect your medical insurance.

What are the possible benefits of taking part?

It is important to realise that it is not anticipated that the men participating in this trial will derive any benefit themselves from this treatment. If this study shows that these supplements from tea have potential as prostate cancer prevention agents further trials will be required.

What if new information becomes available?

Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, the principal investigator will contact you and discuss with you whether you want to continue in the study. If you decide to withdraw your doctor will make arrangements for your care to continue. If you decide to continue in the study you will be asked to sign an updated consent form.

Also, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He will explain the reasons and arrange for your care to continue.

What happens when the research study stops?

After your operation you will not be provided with further capsules. Your consultant will continue your care and advise if any further treatment is recommended.

What if something goes wrong?

If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you. If taking part in this research project harms you, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

Your own GP and any other hospital consultants who are treating you will be notified of your participation in the trial. All information, which is collected, about you during the course of the research will be kept strictly confidential. Any information about you, which leaves the hospital, will have your name and address removed so that you cannot be recognised from it.

The researchers involved in this study, and possibly responsible regulatory authorities will require limited access to your medical notes only where it is relevant to your participation in this study.

What will happen to the results of the research study?

The results of the study are likely to be published in a scientific journal. A copy of the published results can be obtained by contacting the principal investigator. You will not be identified in any report/publication.

Who is organising and funding the research?

The research is organised by the Cancer Biomarkers and Prevention Group, Department of Cancer Studies and Molecular Medicine, University of Leicester. Funding has been arranged from The Hope Foundation for Cancer Research in Leicestershire and Rutland, The British Urological Foundation and The Prostate Research Campaign UK.

Who has reviewed the study?

All research that involves NHS patients or staff, information from NHS medical records or uses NHS premises or facilities must be approved by an NHS Research Ethics Committee before it goes ahead. Approval does not guarantee that you will not come to any harm if you take part. However, approval means that the committee is satisfied that your rights will be respected, that any risks have been reduced to a minimum and balanced against possible benefits and that you have been given sufficient information on which to make an informed decision.

If you need more information, please contact:

Dr. J.F. Thorpe (Principal Investigator)

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Urology Section
Department of Cancer Studies & Molecular Medicine
University of Leicester
Clinical Sciences Unit
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW

0116 258 4617 jkm7@le.ac.uk

Thank you for reading this!

Should you wish to participate in this study, you will be given a copy of this information sheet and a signed consent form to keep.

Patient name, address, DOB (or ID label)

Centre Number: _____
Study Number: _____
Patient Identification Number for this trial: _____

CONSENT FORM

Title of Project:
(to be clear and consistent with other documentation relating to the trial)

Name of Researcher / Principal Investigator:

Please initial box

1. I confirm that I have read and understand the information sheet dated ..... version ..... for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that I may withdraw my consent to my tissue being used at any time without justifying my decision and without affecting my normal care and medical management. ☐

4. I agree to donate the tissue samples as detailed below and allow their use in medical research as described in the Patient Information Leaflet. ☐

5. I understand that the tissue is a gift and that I will not benefit from any intellectual property that results from the use of the tissue. ☐

6. I understand that tissue samples will not be used to undertake any genetic tests whose results may have adverse consequences on my or my families insurance or employment. ☐

7. I understand that if research using my tissues produces information, which has immediate clinical relevance to me, I will be informed by my hospital consultant or GP and be given an opportunity to discuss the results. ☐

8. I understand that sections of any of my medical notes may be looked at by responsible individuals from the research team, or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

9. The samples which I hereby consent to donate are: ☐

……

……SAMPLES OF PROSTATE TISSUE……

10. I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Researcher	Date	Signature

Original for researcher/site file/CRF
copy for patient, copy for hospital notes

Patient Information Sheet and Consent Form (Theaflavins)

Can Dietary Supplements from Tea Prevent Prostate Cancer?

Introduction:

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Purpose of the study:

The prostate gland sits below the bladder in men. In the UK 1 in 14 men will develop prostate cancer in their lifetime. This study aims to find out if chemicals from tea called theaflavins have the potential to prevent prostate cancer in healthy men. To do this, the study will need to answer two questions. Firstly, can theaflavins be absorbed from capsules into the blood and prostate tissue? Secondly, do these theaflavins alter chemical markers in the blood, urine and prostate? Changes in these markers are thought to show that there is a cancer-preventive effect.

Why have I been chosen?

You have been chosen because you meet the following criteria:

- You and your consultant have decided your condition should be treated by having a prostate biopsy followed by a prostate operation called a transurethral resection of prostate (TURP)
- It will be at least four weeks from the date of biopsy until the date of the operation.
- You have no allergy to tea
- You are otherwise fit and well

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

In the work proposed here patients will be recruited who are awaiting both prostate biopsies and subsequent prostate operations. Theaflavin supplements will be administered, by mouth, for four weeks up to and including the date of their operation. The ability of the theaflavins to be absorbed by the patients will be determined by measuring their concentrations in blood and prostate tissue. Changes in chemical markers from the blood and prostate tissue will show if theaflavins may be having a cancer preventive effect.

Men who agree to take part will have their details registered in the trial database held by Mr. J.F. Thorpe at The Department of Cancer Studies and Molecular Medicine, 5th Floor RKCSB, Leicester Royal Infirmary, Leicester. All your information is confidential and all samples will only be labelled with your anonymous trial identification number.

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You will be issued with 250mg capsules of theaflavins. You should take four capsules every morning, every day up to and including the day of surgery. This should last approximately four weeks.

When you are admitted for surgery you will be asked to provide another similar sample of blood and urine. After the operation a sample of prostate tissue will be collected from the surgically removed tissue.

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What is the dietary supplement that is being tested?

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Thank you for reading this!

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Patient name, address, DOB (or ID label)

Centre Number: _____
Study Number: _____
Patient Identification Number for this trial: _____

CONSENT FORM

Title of Project:
(to be clear and consistent with other documentation relating to the trial)

Name of Researcher / Principal Investigator:

Please initial box

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_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Researcher	Date	Signature

Original for researcher/site file/CRF
copy for patient, copy for hospital notes