# COMPARISON OF THE PHARMACOKINETIC AND PHARMACODYNAMIC PROFILES OF RESVERATROL AT DIETARY AND PHARMACOLOGICAL DOSES

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## ABSTRACT

**Comparison of the pharmacokinetic and pharmacodynamic profiles of resveratrol at dietary and pharmacological doses.**

**by Edwina N Scott**

Epidemiological and preclinical studies suggest that dietary-derived cancer chemopreventive agents may be active at dietary and supra-dietary doses. This project therefore investigated the effect of dose on the pharmacokinetic and pharmacodynamic actions of resveratrol, a polyphenol found in grapes. An achievable dietary intake (5 mg/day in humans) and a supra-dietary dose, previously used in clinical trials, were compared in preclinical systems, in healthy volunteers and in patients. Accelerator mass spectrometry (AMS) was used in the clinical part of this project as its sensitivity allowed the safe administration of radiolabelled resveratrol to participants.

*In vitro* microarray results from human colon HCA7 cancer cells showed that resveratrol exposure for 3 months at 0.01 μM, the estimated plasma level in man after an oral 5 mg dose, altered the apoptosis, glucose transport and cell adhesion pathways. Resveratrol exposure at 1.4 μM, the plasma $C_{max}$ value in man after a 1 g dose, did not significantly alter any pathways. The equivalent doses, determined by conversion by body weight and resulting in the same tissue levels as in man, were administered to *Apc*$^{Min+/-}$ mice. Resveratrol reduced the tumour burden at a dietary dose in the presence of a high fat diet, but this was not seen when administered with a standard fat diet, nor at supra-dietary doses. The mechanisms of actions of resveratrol *in vivo* were unclear. Clinically, oral administration of [$^{14}$C]-resveratrol resulted in detectable levels in plasma, colon and prostate tissue even at the 5 mg dose. The metabolite profiles were similar between prostate tissue and plasma, with metabolites predominating. Overall, these results suggest that the dose-response relationship of resveratrol may be biphasic or U-shaped. The confirmation of detectable levels of resveratrol and its metabolite in prostate and colon tissue suggest that resveratrol could exert pharmacodynamic actions in these organs.

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## ABBREVIATIONS

| | |
|---|---|
| ACC | Acetyl-CoA Carboxylase |
| ACTL | Actin-like gene |
| ADME | Absorption, Distribution, Metabolism and Excretion |
| ALS | Acid Labile Subunit |
| AMPK | 5' Adenosine Monophosphate-activated Protein Kinase |
| AMS | Accelerator Mass Spectrometry |
| AOM | Azoxymethane |
| *APC* gene | Adenomatous Polyposis Coli |
| APC trial | Adenoma Prevention with Celecoxib trial |
| Aped | Apoptotic protease activating factor |
| APPROVe | Adenomatous Poly Prevention on Vioxx trial |
| ASB6 | Ankyrin repeat + SOCS Box-containing |
| AUC | Area Under Curve |
| AXIN | Axis Inhibitor gene |
| BAD | Bcl2-Associated Death promoter |
| B[*a*]P | Benzo[α]pyrene |
| BCRP | Breast Cancer Resistance Protein |
| BIRC3 | Baculoviral Inhibitor of apoptosis Repeat-Containing |
| CARET | Beta-Carotene and Retinol Efficacy Trial |
| CDK | Cyclin Dependent Kinase |
| CDH | Cadherin-like gene |
| CHOP | CCAAT/enhancer-binding protein-homologous protein |
| CI | Confidence Interval |
| CL/Fm | Drug clearance |
| $C_{max}$ | Maximum plasma concentration |
| COX | Cyclo-oxygenase |
| CTC | Common Toxicity Criteria |
| CYP | Cytochrome P450 enzyme family |
| DEPC | Diethyl pyrocarbonate |
| DLEC1 | Deleted in Lung and Oesophageal Cancer |
| DMBA | 7,12-Dimethylbenzoic Acid |
| DMH | 1,2-Dimethylhydrazine |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic Acid |
| DR | Death Receptors |
| ECF | Extracellular Fluid |
| EDTA | Ethylenediaminetetraacetic Acid |
| EGF | Epidermal Growth Factor |
| ELF | Embryonic Liver Fodrin |
| ELISA | Enzyme-linked immunosorbent assay |
| eNOS | Endothelial Nitric Oxide Synthase |
| EPIC | European Prospective Investigation into Cancer and Nutrition |
| ERK | Extracellular signal-Regulated Kinase |
| FAP | Familial Adenomatous Polyposis |
| FAS | Fatty Acid Synthase |
| FDA | Federal Drug Agency |
| FDR | False Discovery Rate |
| GIAPI | GPI-Anchored membrane protein |

| | |
|---|---|
| GORASP | Golgi Reassembly Stacking Protein |
| GST | Glutathione-S-transferase |
| GTT | Glucose Tolerance Test |
| HEEBO | Human Exonic Evidence Based Oligonucleotide |
| HEPES | 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid |
| HHV | Human Herpes Virus |
| HNPCC | Hereditary Non-Polyposis Colon Cancer |
| HPLC | High Performance Liquid Chromatography |
| HR | Hazard Ratio |
| HPV | Human Papilloma Virus |
| HSV | Herpes Simplex Virus |
| ICF | Intracellular Fluid |
| IGF | Insulin-like Growth Factor |
| IGFBP | Insulin-like Growth Factor Binding Protein |
| IGFD | Insulin-like Growth Factor Deficiency |
| IL | Interleukin |
| IFNγ | Interferon Gamma |
| IR | Insulin Receptor |
| IRS | Insulin Receptor Substrate Protein |
| k-ras | Kirsten Rat Sarcoma Viral Oncogene |
| KLF | Krűppel-Like Factor |
| LC-MS | Liquid Chromatography and Mass Spectrometry |
| LID | Hepatic specific IGF1R knockout mice |
| LoD | Limit of Detection |
| LSC | Liquid Scintillation Counting |
| MAPK | Mitogen Activated Protein Kinase |
| MeIQ | 2-Amino-3, 8-dimethylimidazo[4,5-f]quinoxaline |
| Min | Multiple Intestinal Neoplasia mouse model of intestinal cancer |
| miRNA | microRNA |
| mom | Modifier Of Min locus |
| MMR | DNA Mismatch Repair System |
| mRNA | Messenger RNA |
| MRP | Multiple Drug Resistance Protein |
| MS | Mass Spectrometry |
| MSR | Macrophage Scavenger Receptor |
| NF-κB | Nuclear Factor-kappaB |
| NMBA | *N*-nitrosomethylbenzylamine |
| NMR | Nuclear Magnetic Resonance |
| NMU | *N*-Methyl-*N*-nitrosourea |
| NO | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| NSAIDS | Non-Steroidal Anti-Inflammatory Drugs |
| OR | Odds Ratio |
| PBS | Phosphate Buffer Saline |
| PCPT | Prostate Cancer Prevention Trial |
| PCR | Polymerase Chain Reaction |
| PhIP | 2-Amino-1-methyl-6-phenylimidazo[4.5-b]pyridine |
| PI3K | Phosphoinositide 3-kinase |
| PIN | Prostate In-situ Neoplasia |
| PK | Pharmacokinetic |

| | |
|---|---|
| PKC | Protein Kinase C |
| PMA | Phorbol Myristate Acetate |
| PMT | Photomultiplier |
| PPIL | Peptidylprolyl Isomerase, Cyclophilin-Like |
| ppm | Parts Per Million |
| PRCC | Papillary Renal Cell Carcinoma |
| PS | Performance Status |
| PSA | Prostate Specific Antigen |
| PUFA | Polyunsaturated Fatty Acids |
| QC | Quality Control |
| RAB | Ras Oncogene Family |
| RASSF | Ras Association domain family |
| REDUCE | Reduction by Dutasteride in Prostate Cancer Study |
| RHOQ | Ras Homolog gene |
| RNA | Ribonucleic Acid |
| rpm | Revolutions Per Minute |
| RR | Response Rate |
| SCID | Severe Combined Immunodeficiency mice |
| SD | Standard Deviation |
| SDS | Sodium Dodecyl Sulphate |
| SEER | Surveillance Epidemiology and End Results, National Cancer Institute |
| SELECT | Selenium and Vitamin E Cancer Prevention Trial study |
| SLC2A8 | Solute Carrier, family 2 |
| SPRY | Sprouty homolog gene |
| SSC | Saline Sodium Citrate |
| SV40 Tag | Simian Virus-40 T-antigen targeted |
| TBARS | Thiobarbituric acid-reactive substances |
| TB | Tributyrin |
| TGF-β | Transforming Growth Factor Beta |
| $T_{max}$ | Time of maximum plasma concentration |
| TLN1 | Talin 1 |
| TNF | Tumour Necrosis Factor |
| TNFRSF | Tumour Necrosis Factor Receptor Super Family |
| TPA | 12-*O*-Tetradecanoylphorbol-13-acetate |
| TRAF2 | TNF Receptor Associated Protein |
| TRAIL | Tumour necrosis factor-Related Apoptosis-Inducing Ligand |
| TRAMP | Transgenic Carcinoma Mouse Prostate Model |
| TRPM | Transient Receptor Potential cation channel |
| UBXD | Ultrabiothorax Domain-containing |
| UDP | Uridine Diphosphate Glucuronosyltransferase |
| UGT | UDP-glucuronosyltransferases |
| UHL | University Hospitals of Leicester |
| UV | Ultraviolet |
| VEGF | Vascular Endothelial Growth Factor |
| V/F | Apparent volume of distribution |
| VICTOR | Vioxx In Colorectal cancer Therapy: definition of Optimal Regime trial |
| WHO | World Health Organisation |

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# 1. Introduction

# 1. Introduction

## 1.1 Carcinogenesis and chemoprevention

Cancer contributes significantly to worldwide mortality and morbidity, accounting for one in three deaths in the Western World (SEER Cancer Statistics Review, 1975-2004). Patients often present with advanced disease and despite decades of research into new therapeutic options, treatment is still usually with palliative intent in the metastatic setting. Chemoprevention, defined as the administration of natural or synthetic substances to inhibit, reverse or delay cancer, is attractive as an alternative strategy to manage the burden of cancer and was first proposed more than 30 years ago (Sporn *et al.* 76). Rather than trying to eradicate cancer, it may be easier to prevent it from occurring in the first place. Screening has been established as a similar approach and has been proven to decrease mortality from cervical, breast and colon cancer (Adami *et al.* 94, D'alo *et al.* 10, Pignone *et al.* 02). In order to devise strategies for chemoprevention, the process of carcinogenesis must first be understood.

### 1.1.1 Carcinogenesis

Carcinogenesis was first described by Foulds as the process of transformation from normal tissue to malignancy, caused by multiple step-wise genetic changes (Foulds *et al.* 58). Although the three stage carcinogenesis model of initiation, promotion and progression was initially proposed for colorectal cancer (Farber 68), this model has now been accepted as applicable for malignancies in general (see Fig 1.1). Tumour initiation usually occurs when endogenous or exogenous genotoxic carcinogens cause DNA damage, which in turn may result in mutation induction leading to metaplasia and hyperplasia. This damage occurs rapidly and may be repaired before mutation

fixation. Promotion may occur due to stimulation by a non-genotoxic promoter, resulting in selective clonal expansion of the initiated cells. Histologically, promotion results in mild/moderate dysplasia and intraepithelial neoplasia. This stage is reversible and may include hyperproliferation, inflammation and tissue remodelling. Animals treated with chemical initiators or promoters alone, such as 7,12-dimethylbenz(*a*)anthracene (DMBA) and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) respectively, did not necessarily develop cancer (Goerttler *et al.* 79). The combination however, increased the risk significantly and these experiments were thus described as two-stage carcinogenesis studies. Progression is characterised by irreversible phenotypic and genotypic malignant conversion, and includes abnormalities ranging from severe dysplasia/carcinoma *in situ* to invasive metastatic cancer. The stages of promotion and progression are relatively slow compared to initiation, with the former probably occurring over years.

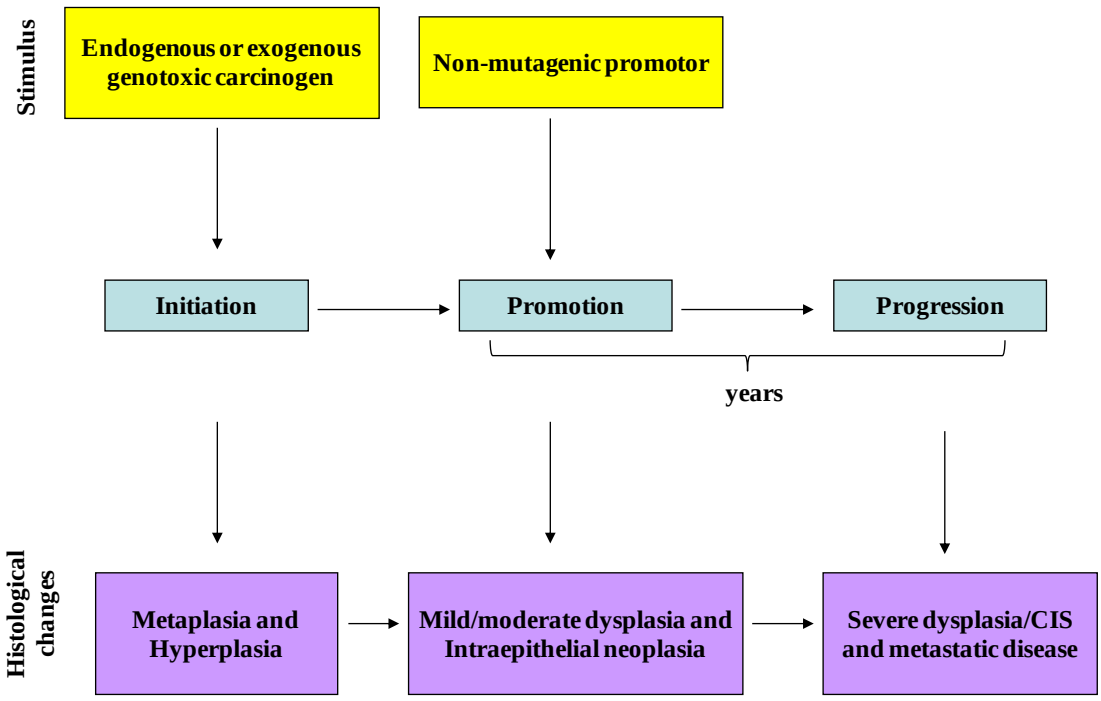



**Figure 1.1 Three stage model of carcinogenesis.** CIN = carcinoma in situ

Although carcinogenesis has been described above as a temporally organised sequential process, this does not necessarily occur clinically since humans are exposed to multiple factors simultaneously. Evidence of this comes from tumour tissue samples which contain cancer cells at different stages of carcinogenesis. This was first noted in oral squamous cell carcinomas by Slaughter, and was termed Field of Cancerization (Slaughter *et al.* 53). Oral cancer was noted to develop in mutifocal areas of precancerous change, surrounded by abnormal tissue. The different stages of carcinogenesis can also be found in the same patient but spatially distinct, as exemplified by the adenoma-carcinoma sequence for colorectal cancer, where an adenocarcinoma and an adenoma may occur at the same time in the same patient but in two different sections of the bowel (see Section 1.1.2).

The genetic changes that occur in carcinogenesis are different for different tumours, but they all result in the same phenotypic characteristics, which allow cancer cells to replicate in an uncontrollable manner. These characteristics were described in a seminal paper by Hanahan and Weinberg and include self sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, genome instability, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg 00). A recent paper has expanded these hallmarks to include the stress phenotypes of tumourigenesis (Luo *et al.* 09).

### 1.1.2 Chemoprevention

Chemoprevention can be divided into three stages: primary, secondary and tertiary (Gescher *et al.* 01). Primary chemoprevention refers to strategies applicable to healthy individuals which may include the general population, or subjects who are at

higher risk due to genetic or lifestyle factors. Secondary chemoprevention is applicable to patients with pre-invasive dysplasia or preneoplastic lesions, where there is a high probability that these subclinical abnormalities will develop into significant disease without intervention. Tertiary chemoprevention refers to prevention of recurrences in cancer patients who have been successfully treated.

One of the best success stories in the field of chemoprevention is that of the anti-oestrogen drugs. Hormone manipulation is now widely used for the tertiary prevention of breast cancer, with use of both anti-oestrogen receptor drugs (e.g. tamoxifen) and aromatase inhibitors (e.g. arimidex). Tamoxifen has also been investigated in multiple primary prevention studies in high risk patients with exciting results, reducing oestrogen receptor positive breast cancers by 48 % (Cuzick *et al.* 03). Although tamoxifen increased the risks of atherosclerosis, thromboembolism, endometrial carcinoma and osteoporosis, primary chemoprevention did not increase non-breast cancer mortality (Cuzick *et al.* 03). Indeed, the chemopreventive efficacy was evident at 10 years whereas most toxicities ceased after the first 5 years (Cuzick *et al.* 07). Despite these data, there is reluctance in women to accept long term administration of a pharmaceutical agent for chemoprevention purposes. In a study of 632 high risk women, only 6 % were prepared to take tamoxifen even though the participants were well educated, correctly answering questionnaires on the risks and benefits (Fagerlin *et al.* 10).

The example of tamoxifen illustrates some of the difficulties in cancer chemoprevention, that the public perception of safety may not be reassured by the scientific evidence. Tamoxifen does not seem to be acceptable to the general public

even though its efficacy has been confirmed, whereas vitamins with little demonstrable benefits in a well nourished population are commonly consumed. There is a direct relationship between the acceptability of toxicities with a chemopreventive agent and the likelihood of developing cancer – the higher the likelihood, the more acceptable the toxicities. There is also however, a psychological aspect in terms of perceived safety concerns. In this respect, consumption of dietary agents for chemoprevention may be more acceptable than that of pharmaceutical drugs.

#### 1.1.2.1 Challenges in clinical chemoprevention trials

Overall, clinical studies of chemoprevention agents have been carried out with limited success due to a variety of different reasons (Bresalier *et al.* 05). Animal models used to demonstrate efficacy do not necessarily emulate the biology of human diseases. Carcinogenesis is a multifactorial process and targeting one change may not be sufficient to prevent cancer development as parallel pathways may exist. There is also a potential delay, perhaps of more than 10 years, before appearance of chemopreventive effects in clinical trials (Collett *et al.* 99, Flossman *et al.* 07) and so the length of follow up in negative studies may have been insufficient (Cuzick *et al.* 07). Optimal duration of chemopreventive treatment, population group to recruit and time of intervention are also difficult variables to establish, with trial design sometimes governed by what is logistically possible rather than what is scientifically optimal. Compliance in human subjects is less than in animal studies especially if long term administration is necessary, which can then impact on the trial results.

The appropriate dose for administration in clinical studies is also unclear. Establishing the correct dose for maximum efficacy and minimal toxicity is of

paramount importance, as chemopreventive agents are potentially administered for very long durations to a large population, many of whom could remain healthy even without intervention. As a consequence of these considerations, there has been widespread interest in the use of dietary derived agents for the purpose of cancer chemoprevention (Hoh *et al.* 06).

The efficacy of these dietary compounds was initially based on epidemiological data, when geographical differences were noted in the distribution of different types of cancers (Sokal *et al.* 00). Migrants can develop the risks of their adopted communities, suggesting that in addition to genetics, environmental factors like diet can also be a significant contributor. The risk of colorectal cancer for example, is double in Japanese migrants to the US compared to the incidence in their native country (Flood *et al.* 00). Indeed, there has been sufficient evidence for the efficacy of fruit and vegetables for the World Health Organisation (WHO) to promote their intake as a public health measure, to decrease the risk of illnesses including cardiovascular disease and cancer (WHO Global Strategy on Diet, Physical Activity and Health 2004). In the EPIC (European Prospective Investigation into Cancer and Nutrition) study of 500,000 Europeans however, intake of fruit and vegetables did not correlate to the risk of cancer development after up to 14 years of follow up (Boffetta *et al.* 10). No intervention was involved in this trial, but participants reported their average food intake in questionnaires. Although these data were disappointing, this study reported overall cancer risk. Sub-analysis of specific tumour sites demonstrated some protection conferred by the intake of fruit and vegetables, for example in the incidence of colon cancer (Van Duijnhoven *et al.* 09).

Dietary compounds, such as curcumin from turmeric or lycopene from tomatoes, are an attractive option as chemopreventive agents since they are considered to have a favourable safety profile with no evidence that they are harmful. In contrast, significant toxicities were noted for some pharmaceutical drugs when used in the chemopreventive setting. Rofecoxib (Vioxx) for example, was licensed as a non-steroidal anti-inflammatory drug by the US Federal Drugs Agency (FDA) in 1999 and used by 84 million people (http://www.merck.com). Despite its marketing for daily use in chronic conditions such as osteoarthritis, clinical trial data submitted for its licensing noted toxicity for only up to 6 months (Laine *et al.* 99). When administered at the same dose but for far longer duration in a chemoprevention trial of patients with previous colonic adenomas in the APPROVe (Adenomatous Polyp PRevention On Vioxx) study (Bresalier *et al.* 05), unexpected side effects occurred. Rofecoxib increased the risk of cardiovascular disease, but this was only evident if used for more than 18 months. This example illustrates how a licensed drug, with a proven safety record in the therapeutic setting, can still exert unexpected serious side effects in the chemoprevention setting due to the longer treatment durations.

Clinical trials of dietary chemoprevention agents to date have administered these compounds both in their natural food matrices and in a purified form. Resveratrol for example, is a polyphenol found in grapes and has been administered as a single agent in capsules (Boocock *et al.* 07a), or as a natural constituent of lyophilised grape powder (Zern *et al.* 05). It may be difficult to extrapolate data from studies where agents were administered in their natural food matrices since if any efficacy was noted, it was not possible to definitively attribute this to any particular component. Furthermore, it would be difficult to accurately quantify the doses administered over a

chronic duration as identical, as natural variations will occur in foods between crops. In a 4 month study in premenopausal women for example, flaxseed and wheat bran supplementation were investigated for their effects on oestrogen metabolism, as a surrogate marker for the risk of breast cancer (Haggans *et al.* 00). Nutritional analysis showed that the carbohydrate, protein and fat contents could vary by up to two fold in the supplements taken by participants within the same treatment group.

Even if the active agent was identified and its concentration standardised, concentrations of other constituents may differ which could influence the pharmacokinetic properties or the efficacy of the active agent. The prevalence of obesity in the Western world also suggests that the advice to increase intake, or avoid consumption, of certain food types has not been adhered to. In one study in the US of 154,649 adults for example, 27 % of the interviewees were obese and 62 % were overweight, leaving only 11 % of the participants being of healthy weight (Singh *et al.* 10). Long term adherence to a daily intake of a certain food for chemoprevention purposes, albeit with proven efficacy, may therefore be poor. The widespread use of vitamins and supplements in the US (Loya *et al.* 09) however, suggests that dietary agents administered in a purified form may be preferable for many people and could result in better compliance.

#### 1.1.2.2 Selection of dose for use in clinical chemoprevention trials

Dietary chemoprevention agents have been administered in clinical efficacy trials at either dietary or supra-dietary doses (Zern *et al.* 05, Boocock *et al.* 07a). The dose escalation seen in the classical development of cancer therapeutic drugs would stop at the maximum tolerated dose, but this would not be appropriate for chemoprevention

purposes, especially for primary chemoprevention as subjects would not be able to tolerate prolonged treatment at such high doses. Selection of the appropriate dose is important as supra-dietary doses may exert effects very different to those elicited by dietary doses. In endothelial progenitor cells for example, resveratrol exerted biphasic effects on the nitric oxide (NO) pathway *in vitro* with stimulation of nitric oxide synthase (NOS) expression at 1 μM but inhibition at 60 nM. Similarly, resveratrol administered at 10 mg/kg in rats increased endothelial NOS expression in injured arteries and increased the number of endothelial progenitor cells in the circulation (Gu *et al.* 06), but a higher dose of 50 mg/kg failed to elicit these responses.

The relevance of results from the use of supra-dietary doses is also unclear as there are no safety data for their long term administration, which would be necessary for their use as chemoprevention agents. In addition, there is no epidemiological evidence to support their purported efficacy and it cannot be presumed that more is better. Beta-carotene for example, has been shown in preclinical studies to be a lung cancer chemoprevention agent, inhibiting the development of lung cancer in mice induced by tobacco nitrosamine (Conaway *et al.* 98). As a result of these encouraging preclinical data, beta-carotene entered clinical evaluation in four lung cancer chemoprevention trials which recruited over 100,000 subjects in total. The doses used were 20-50 mg per day and although there is no recommended daily allowance for beta-carotene, these doses were far in excess of the normal human dietary intake of 1.2 mg (Kirsh *et al.* 06). It was unclear how the doses for these chemoprevention trials were selected. Whilst no difference was noted in two studies (Hennekens *et al.* 96, Lee *et al.* 99), supplementation significantly increased the risk of lung cancer incidence in smokers in the other two trials (Omenn *et al.* 96, The Alpha-Tocopherol,

Beta Carotene Cancer Prevention Study Group 94). Some authors have postulated that the increased risk of lung cancer noted in the smoker cohort recruited into the beta-Carotene and Retinol Efficacy Trial (CARET) was due to an inappropriately high dose (Omenn *et al.* 96, Goodman *et al.* 03).

**1.1.2.3 Selection of efficacy biomarkers for use in clinical chemoprevention trials**

In an effort to advance the development of dietary agents in chemoprevention, biomarkers have been investigated as surrogate endpoints of efficacy. Ideally, such efficacy biomarkers should be intrinsically related to the mechanisms of action responsible for the chemopreventive effects, but identification and selection of appropriate markers has proven to be difficult. Phytochemicals have been shown to interfere with numerous carcinogenic pathways in different cell types, but in most cases the mechanistic hierarchy is not understood. It has therefore been difficult to mine data arising from such experiments for the identification of relevant biomarkers. Instead of investigating specific mechanisms, an alternative strategy is for global analyses of genomic or proteomic changes induced by these phytochemicals, which may be more helpful in pinpointing the most important pathways and may highlight new targets that may otherwise be overlooked.

A second issue which complicates the selection of biomarkers for clinical evaluation is that concentrations and treatment durations employed in *in vitro* studies are often inconsistent with those used in animal models. Pharmacokinetic data have shown that phytochemicals can mediate chemopreventive efficacy in rodent models at sub micromolar ($<10^{-7}$ M) plasma or target tissue concentrations after chronic exposure, yet *in vitro* studies typically make use of higher concentrations (in the $10^{-5}$ M range)

for shorter periods (Gescher *et al.* 03). This discrepancy raises concerns as to the *in vivo* relevance of any mechanisms identified *in vitro*. Folic acid for example, inhibited DNA synthesis and induced cell cycle arrest in Caco-2 colon cancer cells *in vitro* when administered at 0.6-10 µg/mL for 48 h (Akoglu *et al.* 01). In rats, dietary supplementation with folic acid (8 mg/kg/day) from 4 weeks of age significantly increased the burden of azoxymethane (AOM) induced intestinal tumours compared to the folate deficient group (Le Leu *et al.* 00). Folate analysis of whole blood in the folate supplemented or folate deficient rats yielded levels of 0.7 and 0.1 µg/mL respectively. These examples highlight the difficulty in applying *in vitro* data to *in vivo* situations: despite the administration of similar concentrations, folic acid exerted antiproliferative actions after short term treatment *in vitro*, but accelerated tumour growth after long term treatment *in vivo*.

### 1.1.3 Colorectal carcinogenesis and its chemoprevention

Colorectal cancer is the third most common cancer in the UK with 36,109 new cases diagnosed in 2004 (CancerStats Monograph 2004, Office for National Statistics). The adenoma-carcinoma sequence of malignant transformation in colorectal cancer was first proposed by Hill *et al.* in 1978 based on histopathological and epidemiological evidence (Hill *et al.* 78). Since then, a vast array of specific genetic and environmental factors have been identified as being involved in this sequence and chemopreventive strategies have been devised to target these (see Figure 1.2).

Knowledge of this sequence is by no means complete. Familial Adenomatous Polyposis (FAP) for example, is an autosomal dominantly inherited condition where due to a mutation in the adenomatous polyposis coli (APC) gene, patients develop

hundreds of adenomas, some of which then transform into invasive tumours (Kinzler and Volgelstein 96). Patients with identical truncating mutations however, may exhibit different phenotypes, suggesting that unknown factors are modulating the genotypic expression (Nishisho *et al.* 91). There is also interaction between genetic and environmental factors, with for example a two-fold increase in colorectal cancer incidence in Japanese migrants to the US, compared to the incidence in their native country (Flood *et al.* 00).

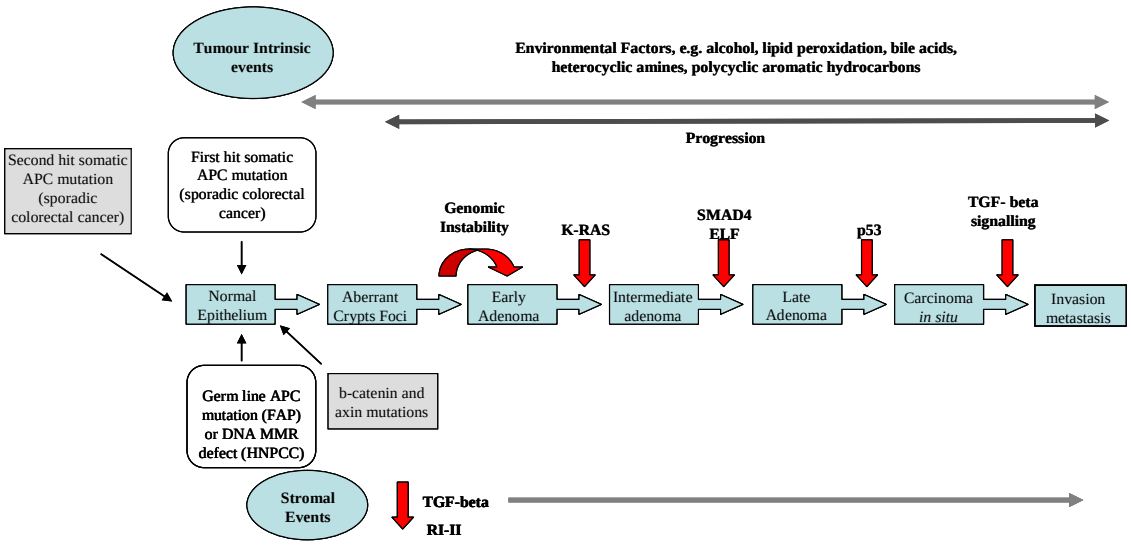



**Figure 1.2 Adenoma-carcinoma sequence of colorectal carcinogenesis (adapted from Hill *et al.*)** APC, adenomatous polyposis coli; ELF, embryonic liver fodrin; FAP, familial adeomatous polyposis; HNPCC, hereditary non-polyposis coli; DNA MMR, deoxyribose nucleic acid mismatch repair system; K-ras, Kirsten rat sarcoma viral oncogene; TGF-beta, transforming growth factor beta. Somatic mutations accounting for sporadic colorectal cancer are shown in the top half of the figure on the left, whilst familial colorectal cancers are shown in the bottom half. See Sections 1.1.2.1 and 1.1.2.2 for further details.

### 1.1.3.1 Environmental factors

Dietary carcinogens exist or are formed in a variety of foods with red meat, smoked/burnt food, fat, refined carbohydrate and alcohol all being implicated as risk factors for colorectal cancer (Goldin-Lang *et al.* 96). One of the largest epidemiological studies carried out involving 478,040 adults from 10 European countries and followed up for a mean of 4.8 years (Norat *et al.* 05) confirmed a

significant positive association between red meat intake and colorectal cancer (>160 g/day versus <20 g/day, HR 1.35). Manipulating the lifestyle of a population however, may be difficult even if convincing data of harm are presented to the public. Smoking for example, has been known to cause lung cancer since 1950 (Doll and Hill 50). Sixty years later and despite multiple public health campaigns, 45 % of cancer survivors, surveyed in a study of 2188 individuals with a previous history of solid tumours from multiple primary sites, continued to smoke (Tseng *et al.* 10).

The mechanism through which red meat consumption increases the risk of colorectal cancer development has not been elucidated, but a number of possibilities have been suggested. Fats contained in red meat may undergo oxidative degradation, resulting in the production of fatty acid radicals which can then damage DNA (de Assis *et al.* 09). Oxidative stress can also be generated by inflammation and in patients with ulcerative colitis for example, the risk of colorectal cancer is increased by up to 10-fold compared to that of the general population (Seril *et al.* 03). Antioxidants were therefore postulated to be chemopreventive agents by counteracting the carcinogenic actions of oxidative damage. Selenium, an antioxidant and an essential mineral, was investigated in a trial of 1312 patients with no history of colorectal cancer (Reid *et al.* 06). Selenium decreased the risk of colorectal adenoma formation after an average follow up of 7.9 years, but only in participants with low baseline selenium levels or who were smokers.

Dietary red meat or fat consumption may also increase the risk of colorectal cancer by stimulation of bile acid production, which is required for food digestion. Deconjugation and dehydroxylation of primary bile acids in the colon by anaerobic

bacteria produces secondary bile acids (Nagengast *et al.* 95). The exact carcinogenic mechanisms of secondary bile acids are unclear, but they have been shown to cause DNA damage in humans (Bernstein *et al.* 05). In animals, bile acids induced colorectal tumours whilst ursodeoxycholic acid, an agent which binds bile acids, is an effective chemopreventive agent (Loddenkemper *et al.* 06). Although a phase 1 study of ursodeoxycholic acid for 3 weeks in healthy volunteers demonstrated no changes in faecal secondary bile acid levels (Hess *et al.* 04), a phase 3 study involving 1285 patients with a previous history of colorectal adenoma demonstrated that ursodeoxycholic acid administered for 3 years significantly reduced recurrence of high grade dysplastic adenomas (OR 0.61) (Alberts *et al.* 05).

Calcium and fibre have been proposed as chemopreventive agents by binding colonic bile acids and other carcinogens (Burkitt *et al.* 69). Fibre in addition is thought to reduce faecal transit time, thereby decreasing the duration of carcinogen exposure. The role of fibre as a chemopreventive agent in colorectal cancer assessed in animal models and from epidemiological data is unclear, with some studies showing efficacy whilst others showed no effect, or even a pro-carcinogenic effect (Jacobs *et al.* 83, Galloway *et al.* 86, Fuchs *et al.* 99). A randomised study of calcium and fibre supplementation for 3 years was carried out in 665 patients with a history of colorectal adenomas, with adenoma recurrence as the primary end point (Bonithon-Kopp *et al.* 00). There was a non-significant trend for calcium to reduce the risk of recurrence, but fibre supplementation surprisingly increased the risk (OR 1.67, p=0.042). The authors proposed that this unexpected pro-carcinogenic effect could be due to increased levels of short-chain fatty acids by bacterial fermentation of the fibre supplement, leading to decreased luminal pH and therefore intracellular pH of colonic

epithelial cells. This acidification could then stimulate colonic cell proliferation by altering DNA synthesis (Jacobs *et al.* 86).

Heterocyclic amines (e.g. 2-amino-3, 8-dimethylimidazo[4,5-f]quinoxaline, MeIQx and 2-amino-1-methyl-6-phenylimidazo[4.5-b]pyridine, PhIP) and polycyclic aromatic hydrocarbons (e.g. benzo[*a*]pyrene, B[*a*]P) are formed within and on the surface of meat cooked at high temperatures. These have been shown in animals to be mutagenic and carcinogenic, inducing formation of aberrant crypts as well as malignancies in the colon in a dose-dependent manner (Hasegawa *et al.* 94). Data from humans however, are contradictory from case-control studies where the intake of potential carcinogens from meat could only be estimated retrospectively from dietary questionnaires. One study showed no association (Augustsson *et al.* 99), whilst other studies (Sinha *et al.* 05, Gunter *et al.* 05, Martinez *et al.* 07) showed positive dose-dependent associations with adenoma formation, with odds ratios of up to 2.82.

#### 1.1.3.2 Genetic factors

Most cases of colorectal cancers are sporadic, but genetic changes are nonetheless noted in all cases. Two types of genetic mutations occur in colorectal tumours, namely chromosomal instability and microsatellite lesions. Chromosomal instability, which occurs in 85 % of colorectal cancers, includes chromosomal translocation, chromosomal amplification and allelic loss, which then leads to loss of tumour suppressor genes. Chromosomal instability is usually sporadic but can be inherited, as in the case of FAP patients. In these patients, mutation of the APC tumour suppressor gene on chromosome 5q 21 results in C-terminally truncated proteins. APC is involved in the Wnt pathway (Fodde *et al.* 02) and can affect the *c-myc*

oncogene, $\beta$-catenin, AXIN2 gene and cyclin D1 proteins downstream. FAP patients can express different phenotypes due to different mutations of the same gene (Kinzler *et al.* 96), but treatment for all is colectomy as penetrance is nearly 100 %.

Microsatellite lesions refer to mutations within a gene and these may be repaired by mismatch repair enzymes (MMR), which correct any mismatched nucleotides inserted during DNA replication. High frequency of microsatellite lesions occur in patients with MMR defects which may be sporadic, or familial as in Hereditary Non-Polyposis Colorectal Cancer (HNPCC) patients. A study of 607 patients showed that individuals with tumours with high frequency of microsatellite lesions were more likely to have right-sided poorly differentiated cancers, which were less likely to metastasise compared to tumours with normal or low levels of microsatellite lesions (Gryfe *et al.* 00). The authors proposed that high frequency microsatellite instability was associated with better stage-adjusted prognosis because MMR defects resulted in specific genetic mutations, e.g. β-catenin and TGFβ, that are different compared to those found in tumours with chromosomal instability, e.g. APC and p53. These differences in genetic mutations then lead to different histological features, different responsiveness to chemotherapy and ultimately, different tumour behaviour and patient prognosis.

Genetic polymorphisms may also alter the risk of colorectal cancer development. Cyclo-oxygenase 2 enzyme (COX-2) for example, is known to be a central factor in colorectal carcinogenesis by mediating inflammation and oxidative damage. Different polymorphisms in the gene encoding COX-2 have been associated with different risks of colorecal cancer development in a study of Caucasian Americans

(Goodman *et al.* 04). The role of this enzyme in carcinogenesis was further confirmed by the impressive *in vivo* results of its inhibitors as chemopreventive agents in the $Apc^{Min+/-}$ (Multiple Intestinal Neoplasia) mice (see Chapter 1.1.3.3, Jacoby *et al.* 00). These data were confirmed clinically in FAP patients who were administered celecoxib, a COX-2 inhibitor, for 6 months which resulted in a reduction in the mean number of colorectal polyps by 28 %, compared to 4.5 % in the placebo group (Steinbach *et al.* 00).

The role of COX-2 inhibitors in patients with previous history of sporadic colorectal adenomas however, is more controversial (see Section 1.1.2.1). In the APPROVe study and the APC (Adenoma Prevention with Celecoxib) trials, both were stopped prematurely due to unexpected cardiovascular toxicities (Bresalier *et al.* 05, Bertagnolli *et al.* 06). As a consequence a tertiary chemoprevention trial of rofecoxib in patients with previous colorectal cancer, the phase 3 VICTOR (Vioxx In Colorectal cancer Therapy: definition of Optimal Regime) study, was also stopped earlier than planned (Kerr *et al.* 07).

Other major genes which are involved in chromosomal instability of colorectal cancer include DCC/MADH2/MADH4 on chromosome 18q, tumour protein (p53) on chromosome 17q and k-*ras* oncogene. Understanding of the genetic basis of colorectal cancer has not yet resulted in any targeted gene therapy, but knowledge of the genetic basis of FAP has allowed development of a widely used rodent model of colorectal cancer, the $Apc^{Min+/-}$ mouse.

### 1.1.3.3 *Apc*$^{Min+/-}$ mice as an animal model of colorectal cancer

The *Apc*$^{Min+/-}$ mouse is a genetic model of colorectal carcinogenesis, where the autosomal dominant Multiple Intestinal Neoplasia (*Min*) mutation in the Adenomatous Polyposis Coli (*Apc*) gene was originally induced by the chemical carcinogen ethylnitrosourea which alkylates DNA (Moser *et al.* 90, Su *et al.* 92). The survival of these mice is shortened to approximately 4 months by the development of multiple intestinal adenomas, the numbers of which are influenced by the Modifier of Min (*Mom*) loci (Dietrich *et al.* 93). The *Apc*$^{Min+/-}$ mouse has been widely used in chemoprevention studies and may be more clinically relevant than carcinogen-induced murine models, as human tumours can result from mutation of the APC gene but rarely from exposure to the chemicals administered to these rodents. Data from *Apc*$^{Min+/-}$ mouse studies may also be clinically relevant since the *Apc* gene is commonly affected in human disease, both in sporadic cases and in patients with FAP.

A limitation of the model is that the tumours occur in the small intestine rather than the colon as in human disease. However, data from these animals can still have clinical relevance. The chemopreventive efficacy of aspirin in *Apc*$^{Min+/-}$ mice (Mahmoud *et al.* 98) for example, translated into clinical success and was demonstrated to significantly reduce adenoma incidence in patients with previous colorectal cancer (Sandler *et al.* 03). Similarly, the efficacy of celecoxib as a colon chemopreventive agent was initially demonstrated in *Apc*$^{Min+/-}$ mice (Swamy *et al.* 06), before being confirmed in clinical studies of FAP patients (Steinbach *et al.* 00, see Section 1.1.3.2).

#### 1.1.3.4 Dietary components influencing tumour burden in animal models of colorectal cancer

As discussed above, both genetic and environmental factors have been proposed to contribute to colorectal carcinogenesis. Gene therapy however, is not yet available and therefore preventive efforts have concentrated in targeting the environmental risk factors, including the role of obesity. Interest in this field stems from epidemiological data, with one study of 368277 individuals showing that abdominal obesity, as reflected by waist to hip ratio, significantly increased the risk of colorectal cancer with a risk ratio of 1.5 in men and women ($p<0.001$, Pischon *et al.* 06). Another trial suggested that it may not be obesity *per se*, but rather the metabolic disturbances occurring as a result which increased the risk of colorectal cancer. A study of 2392 individuals showed that elevated fasting glucose, rather than waist circumference, was associated with a significantly increased risk of metachronous colorectal cancer development (OR 1.46, Ashbeck *et al.* 09). These results, taken together with the prevalence of obesity in the US (Singh *et al.* 10), could mean that obesity is a significant risk factor for non-familial colorectal cancer cases in the Western World.

As a consequence of these clinical associations, the effects of dietary manipulation have been investigated in animal models of colorectal carcinogenesis. Administration of high fat diets to $Apc^{Min+/-}$ mice, defined as 15-21 % fat by weight compared to control diets of 3-5 % fat, increased tumour burden and decreased survival in two studies (Wasan *et al.* 97, Baltgalvis *et al.* 09). In these cases, the fat source was corn oil or not specified. The pro-carcinogenic effects of dietary fat was thought to be mediated by inflammation and immunosuppression as demonstrated by measurement of plasma haptoglobin, an acute phase protein secreted by the liver in response to

inflammation, increased peripheral white blood cell count and increased spleen size. Exercise did not reduce the tumour burden in these animals (Baltgalvis *et al.* 09), nor in another study of *Apc*$^{Min+/-}$ mice on a standard diet (Colbert *et al.* 03).

Other studies in *Apc*$^{Min+/-}$ mice however, have shown that the relationship between tumour burden and dietary fat is more complicated. A high fat diet containing 20 % butter and vegetable oils (w/w) increased adenoma numbers in *Apc*$^{Min+/-}$ mice, compared to control animals receiving 7 % fat, but only in the distal intestine and only if administered concurrently with beef protein (Mutanen *et al.* 00). Mechanistic studies could not identify the pathways involved. Other studies have reported that high fat diets either caused no change or even decreased the adenoma burden in *Apc*$^{Min+/-}$ mice (van Kranen *et al.* 98, Yu *et al.* 01). The authors suggested that these results, in contradiction to other published data, may be due to different types of fat used or differences in other dietary ingredients, for example calcium and vitamin D. The underlying mechanisms accounting for this effect on tumour load were not investigated.

High sucrose diets have also been implicated in increasing adenoma burden in *Apc*$^{Min+/-}$ mice. Animals on a diet containing 52 % sucrose as the sole carbohydrate source, compared to corn starch in control mice, experienced increased body weight and proximal intestinal tumour burden (Wang *et al.* 09). The exact mechanisms of action involved were unclear but the high sucrose diet also increased serum glucose and insulin levels, and liver Insulin Growth Factor 1 (IGF1) mRNA concentrations as well as altering the gene expression of the intestinal epithelium (Wang *et al.* 09a, Wang *et al.* 09b).

The effects of dietary fat on colorectal carcinogenesis were not exclusive to the *Apc*$^{Min+/-}$ mouse model. In wild type rodents, a high dietary fat intake increased the tumour burden of metastatic colorectal cancer cells inoculated into the spleen (Van Saun *et al.* 09), as well as primary colon cancers induced by AOM or DMH (Rodriguez *et al.* 88, Kiunga *et al.* 04). This tumour promoting effect was evident if the fat source was beef tallow, corn oil or fish oil (Kiunga *et al.* 04). Immunohistochemistry analysis showed that dietary fat increased hepatic infiltration by inflammatory cells, potentially promoting metastatic carcinoma deposits by altering the stromal environment and increasing cytokine levels. In primary colorectal tumours, the pro-carcinogenic effects of dietary fat may involve bile acids, as cholecystectomy augmented the increase in tumour burden (Rodriguez *et al.* 88). Examination of faeces confirmed that dietary fat significantly increased faecal bile acid content, which concurred with an increased colonic epithelial proliferation rate as assessed by immunohistochemistry (Bianchini *et al.* 89). In another study where NMU was used to induce colon cancers in F344 rats, dietary fat increased tumour burden only if iron was administered concurrently, in the form of haemoglobin (Sawa *et al.* 98). Mechanistic studies suggested that lipid peroxidation was involved, generating free radicals which caused DNA cleavage, with this process enhanced in the presence of iron.

### 1.1.4 Prostate carcinogenesis and its chemoprevention

Prostate cancer is a disease with significant mortality and morbidity, accounting for 30,000 deaths per year in the US alone (Jemal *et al.* 05). Current methods for prostate cancer detection and diagnosis include measurement of serum prostate specific antigen (PSA), digital examination and prostate biopsies. These methods are

suboptimal, resulting in under-diagnosis as well as over-diagnosis in men who have clinically insignificant cancers, as the progression of the disease is very slow for low grade tumours (Johansson *et al.* 89). Chemoprevention strategies for prostate cancer have been hampered by these issues, as tumours may not be clinically significant for years even without any intervention. There is therefore uncertainty in defining the optimal demographic group for inclusion in studies, the treatment duration and definition of clinically significant trial endpoints.

Epidemiological data on prostate cancer have shown a significant environmental contribution to its pathogenesis, with migrants adopting the risk of their new country regardless of the age of emigration, suggesting that environmental factors may be responsible for both disease initiation and progression (Shimizu *et al.* 91). The molecular pathogenesis of prostate cancer has been postulated to include infection, inflammation and carcinogen exposure as initiation events, with genetic and hormonal changes involved in the progression stage of carcinogenesis (Nelson *et al.* 07).

#### 1.1.4.1 Environmental factors

Prostatitis is a disease of unclear aetiology and includes symptoms of dysuria and pelvic discomfort. It may be due to chemical irritation from chronic urine reflux, increased levels of environmental or developmental oestrogen exposure, dietary factors such as the consumption of red meat and fats or immunological in origin (De Marzo *et al.* 07). Prostatitis has been linked to prostate cancer in epidemiological studies (Platz and de Marzo 04) but there are potential confounding factors, as the symptoms of the two illnesses are similar and symptomatic patients are more likely to be investigated.

Sexually transmitted diseases have also been implicated in the pathogenesis of prostate cancer. A positive association has been noted with gonorrhoea and syphilis in a case control study involving 2300 men (Hayes *et al.* 00), where a previous history of infection increased the odds ratio of prostate cancer development to 1.6. A large prospective case-control study of 1400 men followed up to 7 years (Sutcliffe *et al.* 07) however, showed no association between prostate cancer and *Chlamydia trachomatis*, Human Papilloma Virus (HPV) 16, HPV-18 or HPV-33. In contrast, a significant inverse association was found with Human Herpes Virus (HHV) 8 antibody seropositivity (OR 0.70).

To date, chemopreventive strategies for prostate cancer have been targeted against inflammation rather than specific infectious agents. The efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) has been equivocal with no benefit in a case-control study involving 2392 participants (Bosetti *et al.* 06), and slight benefit in a cohort study of 69,810 men with aspirin use of at least 325 mg/day (RR 0.81) (Jacobs *et al.* 07). Dietary modulation to increase antioxidant intake has also been investigated, both in terms of encouraging a high fruit and vegetable diet as well as oral vitamin supplements. The Selenium and Vitamin E Cancer Prevention Trial (SELECT) study, involving 33,500 healthy men with normal digital examination and low serum PSA levels, showed that supplementation with vitamin E and selenium for a median follow up of 5.46 years did not protect against the development of prostate cancer (Lippman *et al.* 09). There was indeed the suggestion these vitamin supplements non-significantly increased the risk of prostate cancer and type 2 diabetes mellitus.

### 1.1.4.2 Genetic factors

Twin studies have shown that up to 42 % of all prostate cancer may have a genetic component (Lichtenstein *et al.* 00) but no specific genetic loci have been identified. There has also been debate with regard to the degree of contribution of genetic versus environmental factors in cases of familial prostate cancer.

Three types of genetic alterations have been identified which increase the risk of prostate cancer development. The first group consists of inactivating alleles of immune system genes which were first identified in prostate cancer families, resulting in a decreased host inflammatory response to infection. These include genes encoding for ribonuclease I (RNASEL) at chromosome 1q24-26 and macrophage scavenger receptor 1 (MSR1) at chromosome 8p22-23 (De Marzo *et al.* 07). The second is somatic fusion of genes, specifically an androgen-regulated gene (TMPRSS2) and the ETS family of transcription factors (Tomlins *et al.* 07). This alters androgen signalling and may account for the hormone dependency of prostate cancer cells. The third type of genetic alteration is epigenetic changes which are the most frequent genomic alterations seen in prostate cancer. Genetic transcription is silenced or activated by hypermethylation or hypomethylation of the CpG islands respectively. Silencing of the glutathione S-transferase (GST) gene for example, prevents detoxification of carcinogen and reactive chemical species (Lee *et al.* 94). More than 40 affected genes have been identified to date with different genes inactivated at different stages of carcinogenesis.

Although no strategy exists to prevent somatic fusion of the TMPRSS2/ETS genes, chemoprevention has been investigated by hormonal manipulation instead. 5α-

Reductase converts testosterone to the more potent 5α-dihydrotestosterone in the prostate. Two 5α-reductase inhibitors, finasteride and dutasteride, have been investigated as chemopreventive agents in the Prostate Cancer Prevention Trial (PCPT) and Reduction by Dutasteride in Prostate Cancer Study (REDUCE) respectively. The PCPT study involved 18,000 men who were treated with finasteride for 7 years. Prostate cancer prevalence, measured as mean percentage of positive cores, reduced significantly from 24 % in the placebo group to 18 % with finasteride (Thompson *et al.* 03). There was however, a significant increase in high grade tumours, defined as Gleason score ≥7 at biopsy, in patients who underwent prostatectomy from 25.4 % in placebo group to 42.7 % in the finasteride group (Lucia *et al.* 07). The authors have postulated that the increase in high grade tumours was due to improvement in diagnosis, but finasteride has not been widely adopted as a chemopreventive agent.

In the REDUCE study, dutasteride was administered to 6729 men over a 4 year period. There was no difference in overall tumour incidence, but sub-analysis of biopsies occurring during years 3 and 4 showed that dutasteride significantly decreased the incidence of tumours with Gleason scores of 8-10 (Andriole *et al.* 10). Dutasteride however, also increased the incidence of cardiac failure compared to the placebo group.

## 1.2 Resveratrol

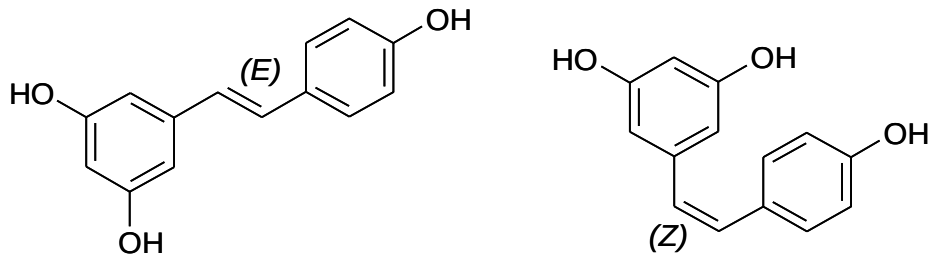
### 1.2.1 Occurrence and properties

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a naturally occurring phytoalexin, an antifungal and antibacterial chemical produced by plants, which is found in a variety of foods and drink including peanuts and red wine (Fremont *et al.* 00). It has been shown in preclinical studies to be active against different types of tumours *in vivo* and *in vitro* (Aggarwal *et al.* 04). Interest in this compound was initially precipitated by research into the "French Paradox", the epidemiological observation that despite a high fat diet there is a disproportionately low incidence of coronary atherosclerosis in the south of France. This phenomenon was postulated to be due to regular consumption of red wine (Renaud and de Lorgeril 92). Although ethanol has been shown in rat hepatocytes to inhibit lipid oxidation (Ontko *et al.* 73), a pathway implicated in cardiovascular disease, ethanol *per se* was not felt to be the cardioprotective agent as alcohol could also promote heart disease (Snow *et al.* 09). Other components of red wine, namely the phenolic compounds isolated after removal of alcohol by distillation, were therefore investigated as potential mediators of this cardioprotective effect and they were indeed shown to be potent antioxidants *in vitro* (Frankel *et al.* 93).

Following these observations, resveratrol was specifically isolated and its antioxidant activity confirmed (Fauconneau *et al.* 97). Oxidative damage also plays a role in the carcinogenic process (Valko *et al.* 04) and so it was postulated that resveratrol could be protective against malignancies. Interest in resveratrol as a chemopreventive agent intensified after publication of a seminal paper by Jang *et al.* in 1997, which showed that this compound was active against all three stages of carcinogenesis *in vitro* (Jang

*et al.* 97, see Section 1.1.1). Epidemiological evidence also suggested that although alcohol consumption increased the risk of colorectal cancer, the type of alcohol was important with wine conferring a protective effect compared to beer (Ferrari *et al.* 07, Pederson *et al.* 03).

Resveratrol can occur as either *cis* or *trans* isomeric forms naturally (Fig. 1.3). Resveratrol exists predominantly in nature as a *trans*-isomer and since this is the more stable form sterically, most studies have administered and analysed the *trans*-isomer selectively. Only trace amounts of the *cis*-isomer were detected in plasma of rodents (Yu *et al.* 02, Asensi *et al.* 02) after administration of *trans*-resveratrol. The two isomers can be separated by chromatographic behaviour and identified by spectrophotometric UV absorption as well as nuclear magnetic resonance spectroscopy (NMR). Resveratrol also exists in plants in the form of resveratrol 3-*O*-*β* glucoside which is termed piceid.




***trans* (*E*) resveratrol** ***cis* (*Z*) resveratrol**

**Figure 1.3 Geometric isomers of resveratrol**

### 1.2.2 Absorption, Distribution, Metabolism and Excretion (ADME) of resveratrol

#### 1.2.2.1 Metabolism *in vitro* and *ex vivo*

Two *ex vivo* experiments of resveratrol in isolated rat small intestine models showed that absorption was associated with metabolism, mainly to glucuronides (Andlauer *et al.* 00, Kuhnle *et al.* 00). Vascular uptake of the luminally administered resveratrol was 20 %. A study in human intestinal Caco-2 cell monolayers however, showed sulphates were the main metabolites found (Kaldas *et al.* 03). This latter study also noted extensive accumulation of resveratrol in the Caco-2 cells.

Resveratrol has been shown to also undergo extensive glucuronidation and sulphation when incubated with human liver microsomes, with both processes inhibited by quercetin (de Santi *et al.* 00a, de Santi *et al.* 00b). No phase 1 metabolites were identified in human liver microsomes, human hepatocytes or rat hepatocyte preparations *in vitro,* with *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulphate being the most abundant metabolites formed (Yu *et al.* 02). Glucuronidation has been shown to occur via the 1A family of UDP-glucuronosyltransferases (UGT) in experiments using recombinant human enzymes (Aumont *et al.* 01).

Phase I metabolites of resveratrol were identified in other *in vitro* studies. Resveratrol was metabolised to piceatannol, 3,5,3’,4’-tetrahydroxystilbene in human liver microsomes via cytochrome P450 enzymes CYP1A2 (Piver *et al.* 04), and in human lymphoblasts via CYP1B1 (Potter *et al.* 02). Piceatannol has been shown to induce apoptosis in leukaemic cells *in vitro* (Wieder *et al.* 01) with actions on a multitude of tyrosine kinases. The structure of the major resveratrol metabolites are shown in Fig

1.4.

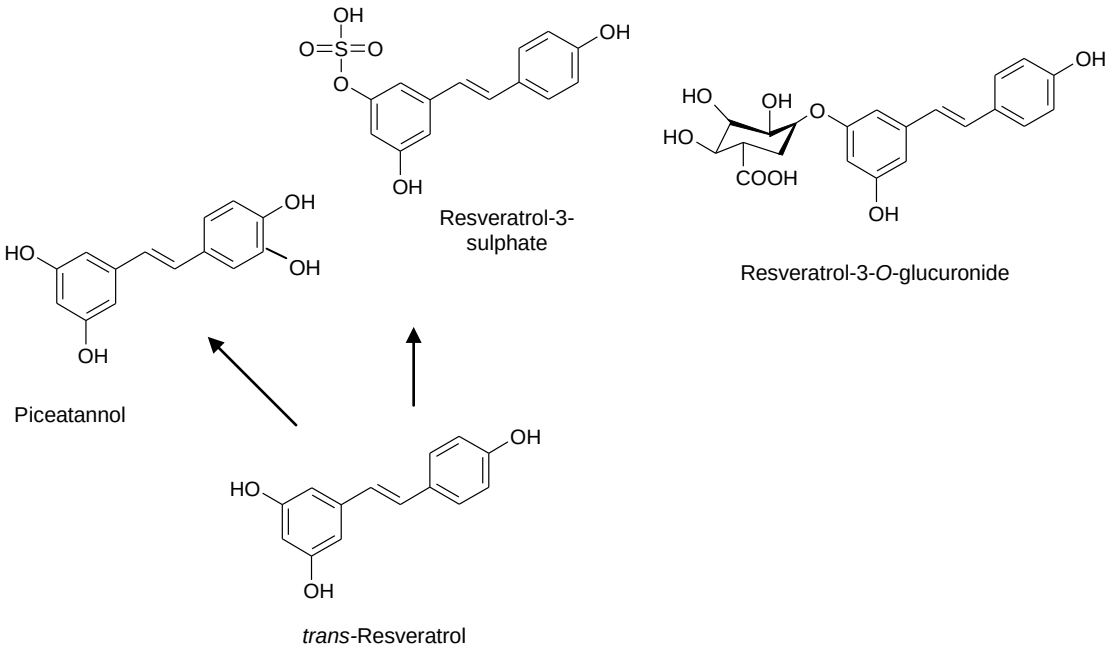



**Figure 1.4 Structures of the major resveratrol metabolites identified in humans, animals and *in vitro* studies**

### 1.2.2.2 ADME of resveratrol in animals

Resveratrol has been administered as single doses to rats, mice and rabbits in pharmacokinetic studies at doses from 2-60 mg/kg orally, intravenously or intraperitoneally (Yu *et al.* 02, Asensi *et al.* 02, Juan *et al.* 02a, Marier *et al.* 02). Resveratrol was rapidly absorped with peak plasma levels at <10 min post dose and short half lives of <15 min in all species.

The distribution of resveratrol was investigated in a study of oral [$^{14}$C]-resveratrol in Balb/c mice (Vitrac *et al.* 03). The highest levels of radioactivity were found in the gastrointestinal tract, liver and kidney as the organs of absorption and elimination. Radioactivity was also detected in brain and testicular tissue. Other studies also

confirmed the high tissue concentrations of resveratrol in the liver and kidneys (Bertelli *et al.* 96). However, the organ levels were similar to the plasma levels, suggesting no extravascular resveratrol accumulation (Asensi *et al.* 02).

No phase I metabolites of resveratrol have been detected *in vivo* in animal studies, but resveratrol has been shown to undergo rapid phase II metabolism within 15 min of dosing (Yu *et al.* 02). One study of [$^{3}$H]-labelled gastrically administered resveratrol in rats showed that a non-specified glucuronide was the major metabolite at 2 h post dose in the plasma, whereas parent resveratrol was the main form identified in liver, heart, lung and brain tissue at 18 h post dosing (El-Mohsen *et al.* 06). In this study, resveratrol was therefore retained in tissue as the parent compound. In the colon, dihydroresveratrol was found to be the most abundant compound after oral administration and was thought to be formed by intestinal bacteria (Alfaras *et al.* 10). Resveratrol glucuronides were present in higher concentrations in the colonic content than the parent compound or sulphates (Alfaras *et al.* 10). The plasma clearance of resveratrol glucuronide was much slower in rats (CL/Fm 0.7 L/h/kg) than the parent compound (CL/Fm 32.4 L/h/kg), leading to a 7 times higher systemic exposure as measured by Area Under Curve (AUC, Marier *et al.* 02). A linked-rat model demonstrated that 25 % of oral resveratrol and 24 % of resveratrol glucuronide underwent enterohepatic recirculation (Marier *et al.* 02).

Excretion studies have shown that urinary levels of resveratrol as a parent compound are virtually undetectable in animals after administration of a single dose (Asensi *et al.* 02), but phase II metabolites were detected. With chronic dosing for 8 weeks in rats, the resveratrol dose seems to affect the route of excretion. At 50 mg/kg/day, 15

% and 13 % of resveratrol was recovered in the urine and faeces respectively, but these figures were 54 % and 17 % for rats administered a much higher dose of 300 mg/kg/day (Wenzel *et al.* 05). The resveratrol metabolites found in the urine also differed with dose, with mainly glucuronides noted at the lower dose whilst sulphates were more abundant at the higher dose. The authors proposed that these effects were due to the elimination of resveratrol occurring via a two-rate reaction of phase II conjugation enzymes. Resveratrol was postulated to undergo sulphation only if the parent compound reached a certain concentration, whereas glucuronidation occurred after administration of both low and high doses.

#### 1.2.2.3 ADME of resveratrol in humans

All clinical trials of resveratrol conducted to date are summarised in Table 1.1. Most of these studies have investigated resveratrol given orally in its natural matrices within food and drink and as discussed in Section 1.1.1, conclusions are difficult to draw from these trials as many other chemicals were co-administered. These studies have been useful however, to demonstrate that the extent of absorption of resveratrol was unaffected by the matrices used, namely red wine, white wine, sparkling wine (Zamora-Ros *et al.* 06), vegetable juice or grape juice (Goldberg *et al.* 03). Absorption of resveratrol was not altered by concomitant meals of varying lipid content or fasting state (Vitaglione *et al.* 05). A recent study conducted at the Universities of Leicester and Michigan revealed that the pharmacokinetic profile of resveratrol after multiple doses is similar to that after a single dose (Brown *et al.* 10).

**Table 1.1 Clinical trials of oral resveratrol administered as a single agent or in food matrices**

| Trial reference | Study population | Form of resveratrol | Resveratrol dose | Study outcome |
|---|---|---|---|---|
| Walle *et al.* 04 | 3 men<br>3 women | Single agent as [$^{14}$C]-resveratrol orally and intravenously | 25 mg single dose | Pharmacokinetic and metabolite profile |
| Boocock *et al.* 07a | 22 men<br>18 women | Single agent as capsules | 0.5 g, 1g, 2.5 g or 5 g single dose | Pharmacokinetic and metabolite profile |
| Zern *et al.* 05 | 44 women | Lyophilised grape powder | 58 µg per day for 4 weeks | Decreased plasma lipid and oxidative stress in pre and postmenopausal women |
| Meng *et al.* 04 | 3 men | Single agent in whisky and water | 0.5 or 1mg/kg single dose, then 0.03 mg/kg single dose 3 days later | Pharmacokinetic and metabolite profile |
| Meng *et al.* 04 | 3 men | Grape juice | 1.7 mg, 3.5 mg, 7.0 mg or 13.9 mg single dose | Pharmacokinetic and metabolite profile |
| Vitaglione *et al.* 05 | 14 men<br>11 women | Red wine in fasting state or with different lipid content meals | 246 µg, 480 µg or 1.92 mg single dose | Resveratrol bioavailability was not influenced by food or lipid content |
| Goldberg *et al.* 03 | 12 men | Single agent in white wine, grape juice or vegetable juice | 25 mg/ 70 kg single dose | Resveratrol absorption was similar in all 3 matrices |
| Zamora-Ros *et al.* 06 | 10 men<br>10 women | 200 mL red wine, 300 mL sparkling wine or 200 mL white wine per day for 4 weeks | 0.357 mg, 0.398 mg and 2.56 mg per day for sparkling, red and white wine respectively | Urinary resveratrol metabolites maybe useful biomarkers of wine intake. |
| Lekakis *et al.* 05 | 30 men with coronary heart disease | Red grape extract in water | 0.9 mg single dose | Improved endothelial function as per flow-mediated dilatation of brachial artery |
| Burkon *et al.* 08 | 9 men | Single agent dissolved in ethanol and milk | Single oral dose of 85.5 mg piceid | Pharmacokinetic and metabolite profile |
| Chow *et al.* 10 | 11 men<br>31 women | Single agent as capsules | 1 g per day for 4 weeks | Resveratrol altered the activity of enzymes in the cytochrome P450 pathway |

**Table 1.2 Summary of pharmacokinetic data from human trials of single dose oral resveratrol**

| Pharmacokinetic parameter | Boocock *et al.* 07a | Walle *et al.* 04 | Meng *et al.* 04 | Goldberg *et al.* 03 |
|---|---|---|---|---|
| Resveratrol dose administered | 0.5, 1, 2.5 or 5 g | 25 mg | 0.5, 1 or or 0.03 mg/kg | 25 mg/70 kg weight |
| $C_{max}$ | | | | |
| Total resveratrol (free and protein bound) | 1982-7853 ng/mL | 491 ng/mL | 2.7 mg in 3.6 L | 471 µg/L |
| Free resveratrol | 73-539 ng/mL | < 5 ng/mL | Not specified | 8.5 µg/L |
| $T_{max}$ | 1.5 h | 1 h | Not specified | 0.5 h |
| Clearance | 9198-22226 L | Not specified | Not specified | Not specified |
| $t_{1/2}$ | 2.85-8.52 h | 7-14 h | Not specified | Not specified |
| Sulphate concentration | $C_{max}$ 1135-4294 ng/mL | 11-31 % of dose excreted in urine Maximum plasma level 124 ng/mL | Not specified | Not specified |
| Glucuronide concentration | $C_{max}$ 405-1285 ng/mL and 370-1735 ng/mL for two different monoglucuronides | 9-16 % of dose excreted in urine Plasma level not measured | Main urinary metabolite but not quantitated | Not specified |
| Systemic bioavailability | Low | Almost zero | Not specified | Not specified |
| Urinary excretion | <0.04 % unchanged resveratrol in 24 h 77 % excreted <4 h post dose 11% of dose excreted as sulphate | 53 – 85 % of resveratrol dose recovered in 72 h collection | Most excreted <10 h post dose. 52 and 26 % of resveratrol dose excreted for 0.03 and 1 mg dose respectively | 16-17 % of resveratrol dose recovered in 24 h collection |
| Faecal excretion | 0-23 µg resveratrol/g of dry faeces. Negligible metabolites detected. | 0.3-38 % of resveratrol dose recovered in 72h collection | Not measured | Not measured |

$C_{max}$ maximal plasma concentration; $T_{max}$ time of maximal plasma concentration; V/F apparent volume of distribution, $t_{1/2}$ half life

A second potential limitation of these trials is that the resveratrol content of red wine is only between 0.1 and 42 mg/L (Fremont *et al.* 00). The doses used in the human trials however, have often been much higher than what would be consumed from a daily intake of 1-2 glasses of wine per day, challenging the application of these results to primary chemoprevention, as long term administration of agents at supra-dietary doses to healthy individuals may prove unacceptable due to potential toxicity. Use of supra-dietary doses also conflicts with any epidemiological evidence that consumption of low doses in red wine could confer molecular changes as consistent with protection against cardiovascular disease or carcinogenesis.

Resveratrol has been investigated in clinical trials by two other routes of administration. Topical resveratrol has been applied in unpublished studies for its anti-viral effects (Investigator's Brochure form Royalmount Pharma, Montreal) and one study administered resveratrol intravenously (Walle *et al.* 04). Walle *et al.* administered [$^{14}$C]-labelled resveratrol to volunteers orally and intravenously in ethanol at least a week apart, using both liquid scintillation counting as well as conventional Liquid Chromatography/Mass Spectrometry (LC/MS) methods to detect the presence of resveratrol and its metabolites in plasma and urine samples. Results were similar to the studies of resveratrol administered orally as shown in Table 1.2.

Pharmacokinetic data referring to oral resveratrol only will be discussed as this is the method of administration in this study and is the only suitable route for the long term administration for cancer prevention. Four pharmacokinetic studies of oral resveratrol have been carried out in human volunteers and showed high inter-individual differences in pharmacokinetic parameters, as summarised in Table 1.2. In general,

resveratrol has a short plasma half life ($t_{1/2}$) of 3-14 h. It was rapidly absorped with peak plasma concentrations ($C_{max}$) of the parent compound or its metabolites occurring at 30-90 min post dose ($T_{max}$). Resveratrol has a very low oral systemic bioavailability in all studies, suggesting that the concentrations typically required for activity using *in vitro* systems (>5 μM) are therefore not achievable in humans.

The poor bioavailability of resveratrol is a consequence of its extensive rapid phase II metabolism, with all studies identifying plasma metabolites confirmed by mass spectrometry and/or enzyme hydrolysis within 30 minutes post dose and at levels much higher than that of the parent compound. Indeed, one study was able to detect the presence of resveratrol metabolites at only 5 min post dose (Boocock *et al.* 07a). Free resveratrol accounted for <2 % of the total resveratrol concentration in one trial (Goldberg *et al.* 03), whilst resveratrol-3-sulphate and resveratrol monoglucuronides had AUC values up to 23 times greater than those of resveratrol in another study (Boocock *et al.* 07a). Given the extremely low plasma concentrations, it is possible that resveratrol may exert effects via its metabolites or as a result of higher local target tissue concentrations. Alternatively, low concentrations may prove efficacious in humans with repeat dosing.

In volunteers who were administered [$^{14}$C]-resveratrol, 2 peaks of plasma radioactivity were noted at 1 h and 6 h post dose, suggesting enterohepatic recirculation may occur (Walle *et al.* 04). This hypothesis was supported by further data from the same study, which showed that the elimination phase was not well characterised due to observed concentration increase in the terminal portion of the profile, and faecal excretion was less than urinary excretion. Based on these

elimination data, absorption of oral resveratrol was >70 % (Walle *et al.* 04), confirming the hypothesis that the low bioavailability of resveratrol was due to rapid metabolism rather than poor absorption. In another study, urinary resveratrol levels were minimal but high levels of its metabolites were identified within 4 h post dose. A variable amount of the administered dose (16 – 85 %) was excreted in the urine and the matrix did not seem to affect the proportion of resveratrol excreted (Goldberg *et al.* 03).

### 1.2.3 Anti-tumour effects of resveratrol

#### 1.2.3.1 Anti-tumour effects *in vitro*

The *in vitro* anti-tumour efficacy of resveratrol has been demonstrated in a wide range of malignant cell lines including leukaemia, breast, colon, pancreatic, gastric, prostate, melanoma, lung, thyroid, head and neck, ovarian and endometrial cancer cells. Although these data do not reflect chemopreventive efficacy *per se*, the biological effects of resveratrol on established cancer cells are similar to the actions required to halt or reverse the process of carcinogenesis. These *in vitro* data of resveratrol in the therapeutic setting are therefore also valuable in understanding its purported chemopreventive role. The resveratrol levels needed for effect in these therapeutic studies were at least 5 μmol/L. Resveratrol affected a large number of cellular pathways including:

(a) Antiproliferation by inhibition of Protein Kinase C (PKC) to induce cell cycle arrest (Atten *et al.* 01).

(b) Induction of apoptosis by activating the caspase-9/mitochondrial pathway (Dorrie *et al.* 01), redistribution of Tumour necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) death ligands (Delmas *et al.* 04), inhibition of the

Rb-E2F/DP (Adhami *et al.* 01), induction of p53/Bax gene expression (Lu *et al.* 01) and inhibition of Bcl-2 expression (Surh *et al.* 99).

(c) Suppression of inflammation by inhibition of COX-2 (Subbaramaiah *et al.* 98); suppression of lipid peroxidation, Tumour Necrosis Factor (TNF), Nuclear Factor kappa-beta (NF-κB) and AP-1 (Manna *et al.* 00); antioxidation by scavenging reactive oxygen species (Miura *et al.* 04).

(d) Inhibition of iron catalysed lipid peroxidation (Tadolini *et al.* 00).

(e) Hormonal effects including suppression of androgen receptors (Benitez *et al.* 07), oestrogenic and anti-oestrogenic activity (Bhat *et al.* 01).

(f) Suppression of Mitogen Activated Protein Kinase (MAPK) and Epidermal Growth Factor (EGF) activation (Shih *et al.* 04).

(g) Suppression of angiogenesis by inhibition of fibroblast growth factor induced proliferation (Lee *et al.* 06a).

(h) Inhibition of CYP gene expression (Berge *et al.* 04) and stimulation of phase II detoxifying enzymes (Jang *et al.* 97), thereby potentially altering metabolism of carcinogens like B[*a*]P.

(i) Suppression of mutagenesis by DMBA in mouse mammary gland culture (Jang *et al.* 97).

(j) Sensitisation of cancer cell lines to radiotherapy and chemotherapy (Garg *et al.* 05).

(k) Immunomodulatory effects with inhibition of interleukin-2 (IL-2), IL-12, interferon gamma (IFNγ) and TNF production (Gao *et al.* 01).

### 1.2.3.2 Anti-tumour effects in animals

Resveratrol has demonstrated chemopreventive and therapeutic efficacy in an impressive portfolio of animal models of malignancy, among them lung cancer and melanoma (see Tables 1.3). Although it lacked activity for certain tumour types, resveratrol was particularly promising in its effect on colorectal cancer, inhibiting both genetic as well as chemical-induced animal models (Sengottuvelan *et al.* 06, Schneider *et al.* 01). There are also contradictory reports of its effects on the same model of malignancy. Five week old $Apc^{Min+/-}$ mice were administered 0.01 % (equivalent to ~12 mg/kg/day) resveratrol in their drinking water for 7 weeks, which resulted in 70 % reduction in tumour formation (Schneider *et al.* 01). In contrast, another study of 6 week old $Apc^{Min+/-}$ mice treated for the same duration with resveratrol in the diet at 4, 20 or 90 mg/kg body weight per day showed no changes in colorectal tumourigenesis (Ziegler *et al.* 04). A third study of dietary resveratrol in $Apc^{Min+/-}$ mice also demonstrated a reduction in adenoma load by 24 %, but the dose was much higher at 0.2 % (equivalent to 240 mg/kg/day) and treatment duration was longer at 10-14 weeks (Sale *et al.* 05).

**Table 1.3 Anti-tumour effects of resveratrol in animal models of cancer**

| Route of resveratrol administration | Cancer type | Animal | Reference |
|---|---|---|---|
| Topical | DMBA, PMA and UVB-induced skin cancers | Mouse | Jang *et al.* 97 |
| Intragastric | NMU-induced breast cancer | Sprague Dawley rats | Bhat *et al.* 01 |
| Oral | DMH-induced colorectal cancer | Wistar rats | Sengottuvelan *et al.* 06 |
| Oral | AOM-induced colorectal cancer | F344 rats | Tessitore *et al.* 00 |
| Oral or i.p. | NMBA-induced oesophageal cancer | F 344 rats | Li *et al.* 02 |
| Oral | colorectal cancer | $Apc^{Min/+}$ Mice | Sale *et al.* 05 |
| Oral | colorectal cancer | $Apc^{Min/+}$ Mice | Schneider *et al.* 01 |
| Oral | colorectal cancer | $Apc^{Min/+}$ Mice | Ziegler *et al.* 03 **No effect** |
| Oral | DMH-induced colorectal cancer | Sprague Dawley rats | Alfaras *et al.* 10 |
| Intraperitoneal | Yoshida AH-130 hepatoma cells | Wistrar rats | Carbo *et al.* 99 |
| Intraperitoneal | H22 liver cancer cells inoculated into liver | Balb/c mice | Wu *et al.* 05 |
| Oral | Lewis lung cancer | Mice | Lee *et al.* 06b |
| Oral | Prostate adenocarcinoma | TRAMP mice | Harper *et al.* 07 |
| Oral | BL6 melanoma inoculated into the footpad | Mice | Asensi *et al.* 02 **No effect** |
| Oral | BL6 melanoma inoculated into the spleen | Mice | Asensi *et al.* 02 |
| Oral | T241 fibrosarcoma | C57B16/J mice | Brakenhielm *et al.* 01 |

Resveratrol inhibited tumour growth in all models tested except for the two indicated.

AOM = azoxymethane, DMBA = 7,12-Dimethylbenzoic Acid, DMH = 1,2-Dimethylhydrazine, NMBA = *N*-nitrosomethylbenzylamine NMU = *N*-methyl-*N*-nitrosourea, PMA = Phorbol Myristate Acetate, UVB = ultraviolet B light.

A potential limitation of the animal data is that the resveratrol doses used in most studies may not be appropriate to chemoprevention, as they were significantly higher than what would be consumed from a normal diet or would be clinically relevant, in terms of toxicity or the number of capsules that could feasibly be consumed over a prolonged duration. Exceptions include a study of AOM-induced colorectal cancer in rats where resveratrol was administered at 200 µg/kg/day for 100 days; this translates into 2 mg/day for the average adult by body surface area conversion (Reagan-Shaw *et al.* 08), and was able to significantly reduce mean aberrant crypt foci/colon to 25.7 compared to 39.4 in control animals (Tessitore *et al.* 00). This is a dietary relevant dose, but definitive conclusions are difficult to draw from a single trial.

### 1.2.4 Safety of resveratrol

Single dose studies of up to 2000 mg/kg resveratrol did not cause any clinical or histological toxicities in rats (Crowell *et al.* 04). Repeat dose studies also showed no toxicity in rats administered resveratrol by gavage at doses <100 mg/kg/day (equivalent to 1.12 g/day in humans) for 28 days (Juan *et al.* 02b, Sengottuvelan *et al.* 06). However when given for 28 days by gavage at a higher dose of 1000 mg/kg/day (equivalent to 11.2 g/day in humans), animals experienced reduced body weight, laboured breathing, dehydration and increased white cell count (Crowell *et al.* 04). Even higher doses of 2000 and 3000 mg/kg/day (equivalent to 22.68 g and 34.02 g/day in humans respectively) for 28 days resulted in increased liver weight, severe renal pathologies including hydronephrosis, and abnormal blood results including anaemia. There was also 40 % mortality rate at the 5000 mg/kg/day dose due to physical obstruction of the gastrointestinal tract by resveratrol (Horn *et al.* 07).

*In vitro*, resveratrol at 1 µM appeared clastogenic in the mouse lymphoma assay (Schmitt *et al.* 02) and at 22 µM, it induced sister chromatid exchanges in a Chinese hamster lung cell line (Matsuoko *et al.* 01). Although the 1 µM concentration used in the former study may have clinical relevance, an *in vivo* study administering resveratrol at extremely high doses, up to 4000 mg/kg/day (equivalent to 22.68 g/day in humans) for 26 weeks by gavage to p53 heterozygous (+/-) mice, showed no evidence of carcinogenicity (Horn *et al.* 07).

There have been no significant adverse events in human trials of resveratrol administered orally or topically (unpublished data from Royalmount Pharma, Montreal), as a single agent or in food matrices. The maximum dose and duration administered to humans to date was 5 g per day orally for 28 days (Brown *et al.* 10). This was on the whole well tolerated except for mild (Common Toxicity Criteria, CTC, grade 1-2) gastrointestinal side effects including nausea and diarrhoea.

## 1.3 Mechanisms of actions of resveratrol

### 1.3.1 The effects of resveratrol on the Metabolic Syndrome

Published studies of the effects of resveratrol, other than its anti-tumour activity, have focused mainly on its activity in The Metabolic Syndrome. This term was first defined in 1998 by the WHO (Alberti *et al.* 98) and more recently clarified to refer to diabetes mellitus, hypertension, hyperlipidaemia and obesity (Alberti *et al.* 09). These conditions increase the risk of cardiovascular disease as well as carcinogenesis, with diabetes mellitus and obesity for example being risk factors for the development of colorectal cancer (Pischon *et al.* 06, Ashbeck *et al.* 09).

The effects of resveratrol on the Metabolic Syndrome from published animal studies are summarised in Table 1.4. These data show that resveratrol has an overall beneficial effect *in vivo* to improve insulin sensitivity, decrease plasma glucose levels, normalise body weight and improve plasma lipid profile. Resveratrol may therefore confer protection against cardiovascular disease and carcinogenesis. Multiple mechanisms of action have been identified *in vivo* and *in vitro*, but the hierarchy of importance between these different pathways is unclear, with resveratrol activating different intracellular messengers under different experimental conditions.

#### 1.3.1.1 Mechanisms of the effects of resveratrol on the Metabolic Syndrome

Like insulin, resveratrol has been shown to promote glucose uptake in rat myocytes *in vitro* by stimulating the translocation of intracellular GLUT4 transporters to the plasma membrane (Breen *et al.* 08, Park *et al.* 07, Chi *et al.* 07, Deng *et al.* 08). Mechanistic data showed that different pathways were activated at different times, leading to biphasic glucose uptake at 1 h and then at 6-14 h post dose (Park *et al.* 07, Deng *et al.* 08). Resveratrol mediated these effects both on a local level, by up-regulating GLUT4 activity, as well as on a systemic level by increasing insulin secretion. 5' Adenosine monophosphate-activated protein kinase (AMPK) for example, was activated by resveratrol (Breen *et al.* 08) and this enzyme is central to the homeostasis of energy, as its activity is controlled by the Adenine monophosphate (AMP):adenine triphosphate (ATP) ratio. AMPK has been shown to modulate pancreatic insulin secretion, increase GLUT4 mRNA levels by phosphorylating the GLUT4 enhancer factor transcription factor (Holmes *et al.* 05), as well as stimulate GLUT4 translocation (Yamaguchi *et al.* 05).

In other insulin-sensitive tissues however, resveratrol cannot be simply classified as an insulin agonist or antagonist, since its actions differed depending on the experimental conditions. In mouse adipocytes for example, resveratrol at concentrations of 1-100 μM inhibited glucose uptake (Floyd *et al.* 08) but in rat adipocytes extracted from diabetic animals, resveratrol at 0.01-1 μM exerted the opposite effects (Su *et al.* 06). This discrepancy may be due to the difference in species, in tissue source in terms of *in vitro* and *ex vivo*, or in the resveratrol dose. Similarly in rat pancreatic β-islet cells, resveratrol at 1-100 μM attenuated insulin secretion stimulated by both physiological and diabetic glucose concentrations (Szkudelski *et al.* 06, Szkudelski *et al.* 07). In malignant murine insulinoma cells however, insulin secretion was promoted by similar concentrations of resveratrol and glucose (Chen *et al.* 07).

#### 1.3.1.2 Effects of resveratrol on diabetes mellitus *in vivo*

In healthy rats and in rats with diabetes mellitus induced by chemical-induced pancreatic β cell damage, a single dose of resveratrol at 0.5 mg/kg via gavage decreased plasma glucose levels, both at basal state and following stimulation by the glucose tolerance test (Su *et al.* 06, Chi *et al.* 07). Analysis of the soleus muscle from the diabetic rats *ex vivo* showed that resveratrol increased GLUT 4 expression (Chi *et al.* 07).

With repeated dosing, resveratrol exposure for 7-30 days at 0.5-5 mg/kg/day similarly decreased plasma glucose levels and increased plasma insulin concentrations in healthy and diabetic rats (Su *et al.* 06, Chi *et al.* 07, Palsamy *et al.* 08). The improvement in glycaemic control in the diabetic animals was mirrored by reduced

hyperglycaemia-induced renal vasculitis, as indicated by decreased creatinine and uric acid levels (Su *et al.* 06, Palsamy *et al.* 08). Levels of glycosylated haemoglobin, another indicator of long term diabetic control as glycosylation only occurs with chronic hyperglycaemia, was also reduced (Palsamy *et al.* 08). Resveratrol improved the fasting plasma triglyceride levels (Su *et al.* 06) and protected against the development of diabetic peripheral and central neuropathy, as assessed by thermal hyperalgesia and the degree of lipid peroxidation in brain tissue respectively (Kumar *et al.* 07, Ates *et al.* 07).

Although resveratrol improved glycaemic control in diabetic rats with chemical-induced pancreatic β cell damage, resveratrol did not protect healthy rats from developing hyperglycaemia and hypertriglyceridaemia induced by 10 % fructose in drinking water, as compared to control animals (Miatello *et al.* 05). Resveratrol did however, prevent fructose-induced hypertension and increase in plasma lipid peroxidation levels, as measured by thiobarbituric acid-reactive substances (TBARS).

#### 1.3.1.3Effects of resveratrol on hyperlipidaemia and obesity *in vivo*

The effects of resveratrol have been investigated in genetic and diet-induced animal models of obesity and hyperlipidaemia. KKAy mice and Zucker rats are two genetic animal models of obesity, where mutations of the agouti and the leptin neuropeptide gene respectively result in central appetite stimulation (Leibel *et al.* 97, Zucker *et al.* 65). In both models, resveratrol decreased fasting glucose levels and improved insulin sensitivity (Lagouge *et al.* 06, Lekli *et al.* 07, Rivera *et al.* 09). In addition, resveratrol normalised body weights and reduced the blood pressure of the Zucker rats (Rivera *et al.* 09). Resveratrol reduced fat deposit and hepatic steatosis by promoting

AMPK inhibition of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), to increase fatty acid oxidation and decrease fatty acid synthesis respectively (Rivera *et al.* 09). Confirmation of the role of AMPK was demonstrated in a study where resveratrol protected wild type mice from obesity and impaired glucose tolerance induced by a high fat diet, but failed to do so in transgenic animals with mutations in the AMPK catalytic subunits (Um *et al.* 09).

In wild type rodents exposed to a high fat diet, concomitant administration of resveratrol at a dose of 1 or 22.4 mg/kg/day (Baur *et al.* 06, Rocha *et al.* 09) did not affect body weight, but administration at 100, 200 and 400 mg/kg/day reduced obesity (Shang *et al.* 08, Pearson *et al.* 08, Lagouge *et al.* 06). There was a similar dose response in terms of hyperlipidaemia, with resveratrol decreasing plasma lipid levels at a dose of 200 mg/kg/day (Pearson *et al.* 08), but there was no effect at 1 or 22.4 mg/kg/day (Baur *et al.* 06, Rocha *et al.* 09). Resveratrol at all doses either decreased fasting glucose levels and/or improved insulin sensitivity. The AMPK/ACC/FAS pathway identified in Zucker rats was also found to be activated by resveratrol in the hepatic tissue of these wild type animal models (Baur *et al.* 06, Shang *et al.* 08, Rocha *et al.* 09). Histological, microarray and protein analysis of hepatic and skeletal muscle tissues showed that resveratrol may induce weight loss by increasing basal metabolic rate, by enhancing mitochondrial numbers and enzyme function via a transcription factor, PPARγ coactivator 1α (Baur *et al.* 06, Lagouge *et al.* 06).

**Table 1.4 The effects of resveratrol on the Metabolic Syndrome from published *in vivo* data**

| Effect | Healthy mice/rats on standard diet (Su *et al.* 06, Chi *et al.* 07) | Diabetic rats with chemical-induced pancreatic damage (Su *et al.* 07, Chi *et al.* 07, Palsamy *et al.* 08) | Healthy rats, 10 % fructose in water (Miatello *et al.* 05) | KKAy mice and Zucker rats (Lagouge *et al.* 06, Leckli *et al.* 07, Rivera *et al.* 09) | Wild type mice/rats on high fat diet (Baur *et al.* 06, Rocha *et al.* 09) | Hyper-cholesterolaemic animal models (Deng *et al.* 08, Miura *et al.* 03) |
|---|---|---|---|---|---|---|
| Decrease basal glucose levels | X | X | | X | X | |
| Improve insulin sensitivity as per GTT[1] | X | | | X | X | X |
| Increase insulin levels | X | X | | | | |
| Decrease symptoms of diabetes mellitus[2] | | X | | | | |
| Decrease complications of diabetes mellitus[3] | | X | | | | |
| Decrease blood pressure | | | X | X | | |
| Decrease lipid peroxidation | | X | X | | | |
| Decrease body weight | | | | X | X | |
| Decrease hyperlipidaemia | | X | | | | X |
| Decrease atherosclerosis | | | | | | X |

[1] GTT = glucose tolerance test
[2] Decreased water and food intake, increased body weight.
[3] Decreased uric acid and creatinine, decreased central and peripheral neuropathy.

Rodent models of hyperlipidaemia without obesity have also been established, by administering a high cholesterol and a high fructose diet (Deng *et al.* 08), or by injection with hepatoma cells (Miura *et al.* 03). The pathophysiology underlying the latter model is unclear but hypercholesterolaemia in hepatoma patients is a recognised clinical paraneoplastic syndrome, possibly due to the tumour increasing cholesterol efflux (Hirayama *et al.* 2006). In both models, resveratrol improved plasma lipid levels by increasing phosphorylated oestrogen receptor protein levels (Deng *et al.* 08), and promoting excretion of bile acids and sterols into the faeces (Miura *et al.* 03).

### 1.3.2 The IGF1 axis

Activity of the IGF axis is mediated by interaction of three ligands, namely insulin, IGF1 and IGF2, and their respective receptors as shown in Fig 1.5. Most published data have concentrated on the role of IGF1 and IGF1 receptors (IGF1R) in carcinogenesis, as IGF2-stimulated IGF2R lacks tyrosine kinase activity and their function therefore remains ambiguous.

IGF1 is released principally from the liver, but also from other tissues for endocrine and paracrine functions respectively. It is secreted physiologically on stimulation by growth hormone released from the pituitary and in response to high calorific intake. Highest IGF1 levels occur at puberty but concentrations can also be altered by body mass index, gender, alcohol, fasting, diet and race (DeLellis *et al.* 04, Moller *et al.* 09, Katz *et al.* 02, Crowe *et al.* 09). Most circulating IGF1 exists as a ternary complex, bound to one of the seven high affinity IGFBP and an acid labile subunit (ALS), a liver derived glycoprotein (Sridhar *et al.* 09). IGFBP3 is the main binding protein and regulates IGF1 activity by preventing its proteolysis and receptor binding.

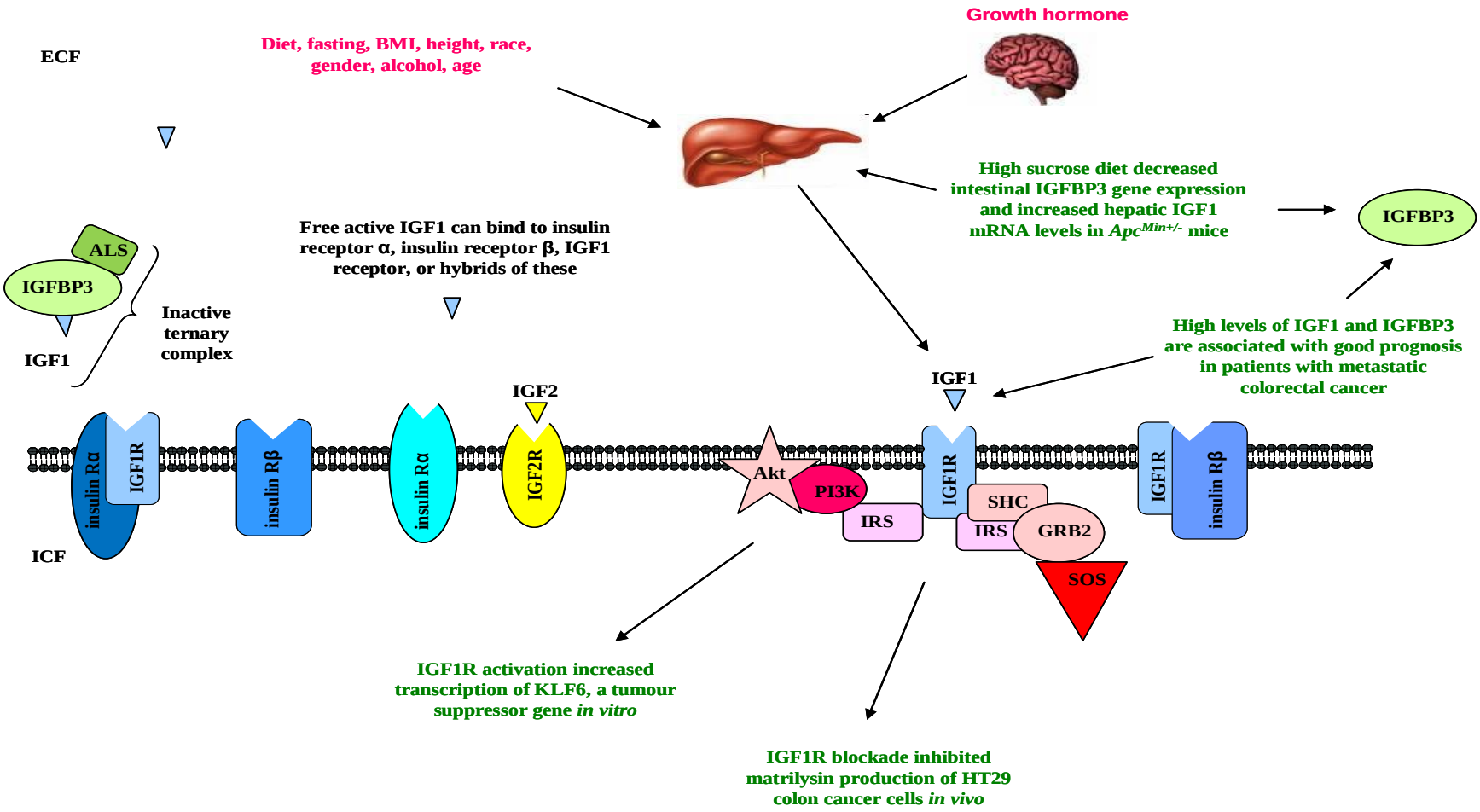



**Figure 1.5 Alterations of the IGF1 axis by physiological stimuli, as well as its role in colorectal carcinogenesis.** Up-regulation of the IGF1 axis has been implicated in carcinogenesis, but the published data are conflicting with some studies showing instead that up-regulation may be associated with chemoprevention, or with no effect. ALS - acid labile subunit, ECF - extracellular fluid, ICF - intracellular fluid, IGF - insulin growth factor, IGFBP - insulin growth factor binding protein, IGFR - insulin growth factor receptor, IRS - insulin related substrate, KLF - Krüppel like finger.

The insulin axis overlaps with the IGF axis at several stages, including involvement of the same intracellular messengers (the PI3K/Akt and MAPK pathways), structural homology between insulin and IGF ligands, and binding to the same receptors albeit with different affinities. Despite these similarities, the two axes have distinct functions. The insulin axis is involved in anabolic metabolism, whilst the IGF axis is involved primarily in growth and development, including hormone synthesis and immune cell recognition (Werner *et al.* 08).

#### 1.3.2.1 The role of the IGF1 axis in colon carcinogenesis

The IGF1 axis has been implicated in colon cancer, with down-regulation associated with chemoprevention. In transgenic LID mice with hepatic specific IGF1R knockout for example, AOM induced a lower tumour burden compared to control animals, suggesting that the IGF1 axis was involved in the pro-carcinogenic actions of AOM by unclear mechanisms (Ealey *et al.* 08, Olivo-Marston *et al.* 09). Similarly in nude mice injected subcutaneously with HT29 human colon cancer cells, administration of an IGF1R tyrosine kinase inhibitor or transfection with a recombinant adenovirus vector expressing a truncated IGF1R reduced xenograft invasion to the underlying muscle layer (Adachi *et al.* 09). Tumour immunohistochemistry analysis and western blot assays showed that IGF1 blockade inhibited production of matrilysin, an endopeptide involved in the degradation of extracellular matrix and therefore implicated in tumour invasion and metastases.

The IGF1 axis has also been suggested to be involved in the mechanisms through which some diets can alter colon tumour burden. Mice injected with colon MC38 cancer cells and exposed to caloric restriction for example, exhibited the longest time to

palpable tumour and the lowest serum IGF1 levels compared to control animals (Wheatley *et al.* 08). In another study a high sucrose diet, compared to a high corn starch diet, increased tumour numbers in *Apc*$^{Min+/-}$ mice. Mechanistic data showed that this tumour promotion was associated with decreased intestinal epithelium IGFBP3 gene expression and increased hepatic IGF1 mRNA levels (Wang *et al.* 09a, Wang *et al.* 09b).

Another study however, showed that up-regulation of the IGF1 axis can also result in chemoprevention. In HCT116 colon cancer cells, IGF1 increased transcription of Krüppel-like factor 6 (KLF6), a tumour suppressor gene shown to be mutated in human colon and prostate tissue samples (Bentov *et al.* 08). Clinical data are similarly conflicting for the role of IGF1 in colon cancer. In a study of patients with metastatic colorectal cancer, a higher baseline IGFBP3 concentration, but not IGF1 level, was predictive for response to chemotherapy (Fuchs *et al.* 08). High levels of both proteins were associated with a better prognosis. In patients with non-metastatic colorectal cancer however, mortality was inversely associated with IGFBP1 levels and there was no relationship to IGF1 or IGFBP3 concentrations (Wolpin *et al.* 09).

#### 1.3.2.1 Effects of resveratrol on the IGF1 axis

The published literature on the effects of resveratrol on the IGF1 axis is limited. In HCT116 and HT29 colon cancer cells, resveratrol concentrations of 10-150 μM decreased IGF1R protein levels (Majumdar *et al.* 09, Vanamala *et al.* 10). In MCF7 breast cancer cells, resveratrol at 5-10 μM decreased IGF1R mRNA expression as measured by real time PCR (Lu *et al.* 99, Serrero *et al.* 01). In another breast cancer cell line, resveratrol at 10-20 μM decreased MDA-MB 435 cells migration induced by

IGF1, by inhibiting expression of MMP-2 and via the PI3K/Akt pathway (Tang *et al.* 08). The role of resveratrol on the IGF1 axis has not been investigated in prostate cancer cells *in vitro*.

*In vivo*, there are no data for the effects of resveratrol on the IGF1 axis in animal models of colon cancer. In TRAMP mice, resveratrol at 625 mg/kg of diet (equivalent to 530 mg/day in humans) decreased IGF1 and increased IGF1R protein levels in the dorsolateral prostate, with no effect on IGFBP3 concentrations (Harper *et al.* 07). These results were not seen in the ventral prostate, leading the authors to suggest that physiological differences between the prostate lobes were responsible.

## 1.4 Optimising clinical trial design: methods for developing pharmacokinetic and pharmacodynamic biomarkers of resveratrol efficacy

As discussed in Section 1.1.2.1, surrogate efficacy biomarkers are extremely useful in clinical chemoprevention studies in an effort to decrease the treatment duration necessary to arrive at a valid endpoint, and therefore to speed up the clinical development process. In this chapter, three different methods used in this project to develop pharmacokinetic and pharmacodynamic biomarkers of resveratrol efficacy are discussed, namely accelerator mass spectrometry, microarray analysis and proteomic analysis.

### 1.4.1 Accelerator mass spectrometry (AMS)

Although the ADME of resveratrol at supra-dietary doses has been investigated in multiple studies as discussed in Section 1.2.2, its pharmacokinetic profile at dietary dose is unknown. It is unclear as to whether alteration in dose could affect its

metabolism, or its distribution including its ability to reach its target tissues. The methods routinely employed for the analysis of resveratrol and its metabolites, High Performance Liquid Chromatography (HPLC) and LC-MS/MS, lack the necessary sensitivity to investigate the pharmacokinetic profile of resveratrol at low dietary doses. A more sensitive technique of accelerator mass spectrometry was therefore employed.

A variety of methods exist for detecting radiolabelled molecules, with liquid scintillation counting the most commonly used. This measures radioisotope decay events to indirectly predict the number of nuclei present. Beta particles emitted from the radioisotope excite fluor molecules present in liquid scintillation fluid, resulting in light emission which is then detected. This method is relatively inefficient and can be inaccurate, as it depends on the half life of the isotope, the sample size and the duration of counting. Accelerator mass spectrometry is an alternative method which is $10^3$-$10^9$ times more sensitive than decay counting, depending on the isotope, as it measures the number of radioactive nuclei directly and requires much smaller samples (Brown *et al.* 06).

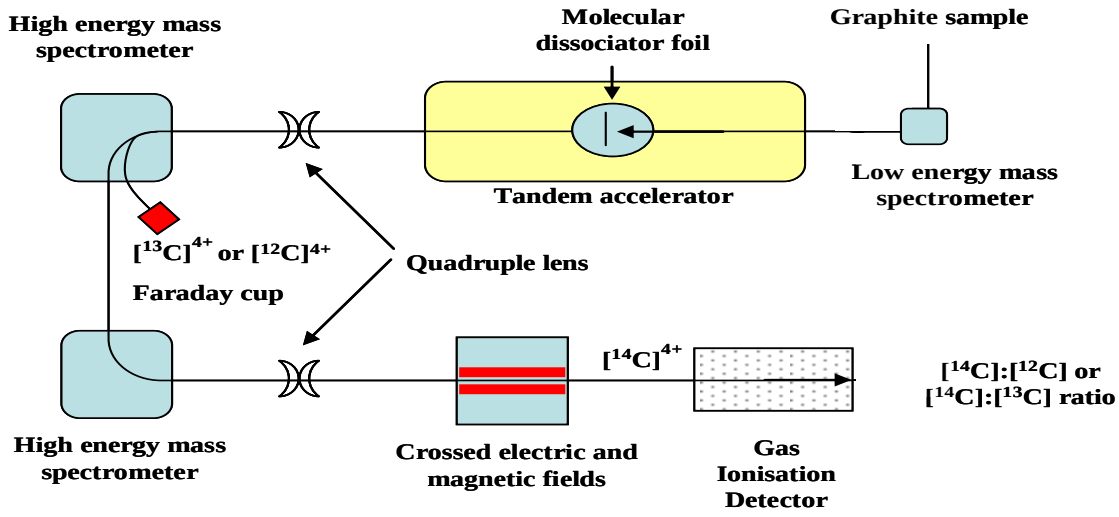



**Figure 1.6 [$^{14}$C] tandem accelerator AMS system (adapted from Brown *et al.* 06).** Samples for analysis are converted to elemental carbon by graphitisation which then become positively charged ions by the removal of electrons by the molecular dissociator foil. Subsequent analyser magnets and mass spectrometers select [$^{14}$C], [$^{13}$C] and [$^{12}$C] ions based on their mass and energy. The more abundant [$^{13}$C] or [$^{12}$C] ions are measured in faraday cups, whilst the rarer [$^{14}$C] ions are detected in a gas ionisation chamber. The results are then expressed as a ratio of [$^{13}$C] or [$^{12}$C]:[$^{14}$C].

Figure 1.6 is a diagrammatic representation of a tandem accelerator mass spectrometry system. The schematic illustrates the analysis of [$^{14}$C] since this is the isotope of interest in this study. Organic samples for analysis (e.g. DNA tissue or HPLC fraction) must be converted to a form compatible with the ion source of the AMS instrument, which is elemental carbon in most cases. The organic samples are oxidised to $CO_2$ and then heated to reduce the elemental carbon to graphite.

The samples are then bombarded with positive caesium ions, resulting in negatively charged ions which are then separated by a low energy mass spectrometer and accelerated towards the positive terminal in the middle of the tandem accelerator. The negative ions are converted to positive ions by passing through a molecular dissociator foil, carbon sheets which strip off electrons and remove interfering molecular isobars, which are unstable with high positive charges. The magnitude of the positive charge is dictated by the size of the accelerator, and the charge required depends on the concentration of the [$^{14}$C] in the sample. A charge state of +1 or +2 may be sufficient if the samples contain a high level of [$^{14}$C] above background, as is the case for many biomedical samples, but +4 charge is needed if the [$^{14}$C] level is low, for example in radiocarbon dating. These now positive ions accelerate again in the second half of the tandem accelerator as they are repelled by the positive source. The ions are then separated by their mass to charge ratio by passing through two high energy magnetic analysers.

For each sample, the amount of rare radioisotope [$^{14}$C] is expressed as a ratio to a more abundant non-radioactive isotope, in this case [$^{12}$C] or [$^{13}$C], which can be measured as a current in a Faraday cup. Ion transport fluctuation through the AMS system is

corrected by measuring ions as ratios and the results are quantitated by comparing Australian National University sucrose standards. Each sample is typically analysed 3 times or up to a maximum of 7 times until the measurement variation is less than 3 %.

Sample preparation is the rate limiting step in AMS (Ognibene *et al.* 03). Samples should be screened by liquid scintillation counting prior to analysis to ensure the level of [$^{14}$C] does not exceed the upper limit of measurement by AMS. This is traditionally in the order of 100 Modern (1350 dpm/g of carbon), with 1 Modern being the background atmospheric level of [$^{14}$C] in 1950s. The limit of detection of AMS is for ratios of $^{14}$C:$^{12}$C or $^{13}$C in the order of $10^{-18}$ to $10^{-21}$ M range. In biomedical research, the common radioisotope ions analysed by AMS are [$^{14}$C] and [$^{3}$H] as these elements have >1 stable isotope, the radioactive isotope has low natural abundance, there are no interfering nuclear isobars and most organic compounds of interest can be synthesised containing these isotopes.

#### 1.4.1.1 AMS use in clinical trials

The sensitivity of AMS means that minute doses of radiation can be safely administered to human subjects, including children (Gunnarsson *et al.* 02) and repeat dosing is potentially possible (Lappin *et al.* 06). Published clinical AMS trials for mass balance and metabolism studies have administered radiation doses in the order of 10 nCi/subject, whilst DNA binding studies require higher levels and have employed radiation doses of 50 µCi/subject. AMS can detect DNA adducts at levels of $1/10^{12}$ nucleotides which is <1 modification per human cell, making it one of the most sensitive technique for DNA adduct detection and quantification.

The main use of AMS in biomedical science to date has been for pharmacokinetic and metabolism studies of synthetic drugs (Skerjanec *et al.* 03), nutrients (Clifford *et al.* 98) and carcinogens (Buchholz *et al.* 99, Dingley *et al.* 99). Only small amounts of biological material are needed for analysis, enabling long term studies to be performed; indeed, one volunteer underwent blood sampling over a 209 day period for a study of β-carotene metabolism (Dueker *et al.* 00). The use of AMS in microdosing studies is a relatively new area of research, whereby doses of drugs are administered to humans at $<1/100^{th}$ of that calculated to be a pharmacologically active dose and at an absolute dose of <100 µg. This has allowed pharmacokinetics and bioavailability to be assessed in humans without toxicity, in order to select drugs for future development at an early stage (Lappin *et al.* 06).

The sample preparation steps described above mean that although AMS can provide quantitative measurements of the number of carbon atoms present, it does not provide structural information. If characterisation of the $^{14}C/^{3}H$ species is required, then fractionation by HPLC or gel electrophoresis is necessary before AMS analysis. In this way, metabolites can be identified on the basis of chromatographic properties and compared to authentic standards. The distribution and metabolism of PhIP for example, was investigated in rats in this manner (Mauthe *et al.* 98). PhIP is a potential carcinogen found in cooked meats but as the levels consumed are very low, AMS analysis of [$^{14}C$] labelled PhIP was required in order to administer a dietary relevant dose.

### 1.4.2 Principles of genomic analysis

Genomic analysis is defined as the study of the genome and ranges from specific

analysis of certain genes by PCR, to profiling of the whole genome by microarray. Microarray was developed in 1995 by Stanford University as "a high capacity system to monitor the expression of many genes in parallel" (Schena *et al.* 95) by quantitatively measuring messenger RNA (mRNA) or micro RNA (miRNA). The principles of microarray analysis are shown in Fig 1.7. MicroRNA (MiRNA) are a family of regulatory RNAs, 21-23 nucleotides long, identified initially in *C. elegans* (Lagos-Quintana *et al.* 01), which have subsequently been found in humans also. Their main function is to bind to the 3'-untranslated regions (3'-UTRs) of specific mRNA targets and inhibit their translation (Jackson *et al.* 07). The targets of many miRNAs identified to date have not been clarified but they have been shown to be involved in colonic carcinogenesis (Michael *et al.* 03, Xi *et al.* 06).

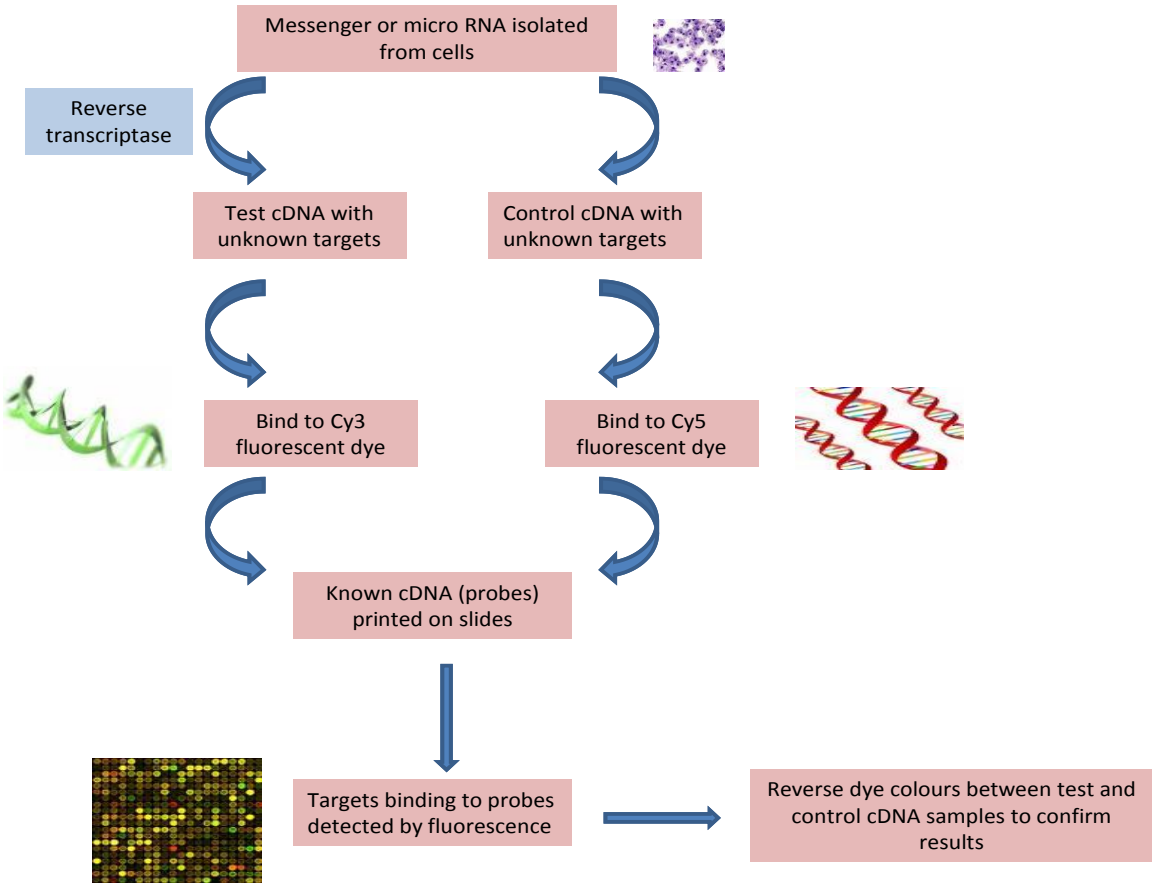



**Figure 1.7 Principles of cDNA microarray.** mRNA or microRNA is converted to complimentary (cDNA) by reverse transcriptase. The resulting cDNA is then attached to a fluorescent dye and it binds specifically to oligonucleotide probes printed on glass slides. The amount of cDNA is quantitatively assessed by the resulting colour intensity.

The advent of microarray has allowed the identification of genes altered by carcinogenesis, for example by comparing the genetic profile of colonic tumour cells to normal epithelial cells in $Apc^{Min+/-}$ mice (Chen *et al.* 08). Agents which alter the genomic profile towards benign or malignant genotype may therefore have the potential to act as chemopreventive or pro-carcinogenic agents. For compounds with known chemopreventive properties, microarray has been used to elucidate the underlying mechanisms of action. Sulforaphane from broccoli and dibenzoylmethane from liquorice for example, were administered orally for 10 weeks to $Apc^{Min+/-}$ mice. These agents decreased tumour burden and analysis of intestinal tissue showed that the underlying mechanisms included changes in the expression of cell proliferation and signal transduction genes (Shen *et al.* 07).

Microarray has also enabled identification of potential new chemopreventive agents by comparing the effects they have on the genomic profiles of target cells, to the actions of known chemoprevention drugs. Sulindac for example, has been clinically proven to decrease colonic polyps in FAP patients (Giardiello *et al.* 93), but it had unwanted toxicities as a non-steroidal anti-inflammatory. Butyrate, a short chain fatty acid produced by colonic microbial fermentation of dietary fibre, altered the expression profiles of genes in a similar manner to sulindac to induce cell cycle arrest and apoptosis in SW620 colonic epithelial cells, suggesting it could also be a chemopreventive agent (Mariadason *et al.* 00). Administration of butyrylated-resistant starch subsequently confirmed its chemopreventive efficacy *in vivo* where it decreased the burden of AOM-induced tumours in rats (Clarke *et al.* 08). A study in 9 FAP patients for 1-2 weeks with butyrate however, showed no changes in cell proliferation as measured by incorporation of [$^3$H]-thymidine (Tonelli *et al.* 95).

**Table 1.5 Published data for the effects of resveratrol on genomic profile *in vitro* or *in vivo***

| Reference | Resveratrol dose, cell type | Effects of resveratrol |
|---|---|---|
| Narayanan *et al.* 02 | 10 µM for 48 h in LNCaP prostate cancer cells | Resveratrol affected genes in multiple pathways including apoptosis, differentiation, transcription, cell cycle and growth factors. |
| Yang *et al.* 03 | 50 µM for 6-48 h in ovarian cancer PA-1 cells | NAD(P)H quinine oxidoreductase 1, involved in p53 regulation, was up-regulated. |
| Narayanan *et al.* 03 | 10 µM for 24-48 h to LNCaP prostate cancer cells | Resveratrol down-regulated PSA and androgen receptor co-activator ARA24 and NFkB p65. |
| Jones *et al.* 05 | 75 or 150 µM for 0-60 h in LNCaP prostate cancer cells | Affected androgen pathway, lipid metabolism, vesicle formation, protein trafficking and stress response. Inverse dose response relationship for apoptosis and cell proliferation genes. |
| Whyte *et al.* 07 | 25 µM for 48 h in A549, NCI H460 and NCI H23 lung cancer cells | 6000 genes affected using 1.2 fold change cut off affecting cell cycle checkpoints, apoptosis and TGFβ pathways |
| Golkar *et al.* 07 | 25-100 µM for 3-24 h in S2-013 and CD18 pancreatic cancer cells | Resveratrol up-regulated macrophage inhibitory cytokine (MIC-1), a member of the TGFβ superfamily and histone genes. |
| Pan *et al.* 08 | 70 µM, duration not specified, to *S cerevisaie* yeast cells | Resveratrol altered genes involved in methionine metabolism, mitochondrial functions, drug detoxification and transcription. |
| Nicholson *et al.* 08 | 0.1 µM for 24 h to human umbilican vein endothelial cells | Resveratrol increased the expression of genes encoding endothelial NO synthase, and decreased expression of endothelin-1. |
| Kweon *et al.* 10 | 10-100 µM for 24h to doxorubicin resistant acute myeloid leukaemia cells | Resveratrol down regulated expression of the Multiple Drug Resistance Protein 1 (MRP1) gene which has been shown to mediate doxorubicin resistance. |
| Wong *et al.* 10 | Resveratrol added to drinking water in ageing hybrid mice for 6 months. Resveratrol concentration not specified but equivalent to 1.5-2.27 mg resveratrol/kg body weight | Resveratrol downregulated CD72 gene expression, which was thought to mediate some of the immunological changes seen with ageing. |
| Wen *et al.* 10 | 100 µM for 8 h to UW228-2 and UW 228-3 medulloblastoma cells | NF-κB impair resveratrol-induced apoptosis by up-regulating BCl-2 expression in medulloblastoma cells. |
| Labbe *et al.* 10 | 0.04% supplement in the diet to a murine model of Werner Syndrome, a disorder of premature ageing. Treatment started from weaning until 9 months of age. | Resveratrol decreased expression of genes involved in lipogenesis. Resveratrol increased expression of genes involved in the insulin signalling pathway and glutathione metabolism. |

Microarray data for the chemopreventive effects of resveratrol are limited and summarised in Table 1.5. Published studies have been carried out mainly in prostate cancer cells (Narayanan *et al.* 02, Jones *et al.* 05, Narayanan *et al.* 03), with no results reported for colorectal cancer cells *in vitro* or *in vivo*. The clinical relevance of these data is unclear, as all studies were carried out for short term durations only and at supra-dietary concentrations. There are no published studies of the effect of resveratrol on miRNA expression, but there has been one study of a resveratrol analogue CAY10512 in human neuronal CC-2599 cells (Lukiw *et al.* 08). Microarray data for the non-chemopreventive effects of resveratrol have come from *in vivo* studies of *C. elegans* (Viswanathan *et al.* 05), and C57BL/6NIA mice on a high calorie diet (Baur *et al.* 06, Pearson *et al.* 08) or a normal diet (Barger *et al.* 08).

### 1.4.3 Principles of proteomic analysis

The term "proteome" was first coined in 1996 to denote the set of proteins encoded by the genome (Wilkins *et al.* 96). Changes in gene expression however, do not always correlate to changes in protein expression due to controls at both the transcriptional and translational levels. Proteomics is the study of the proteome and can range from specific assessment of a protein of interest by Western blotting, to global profiling of the whole proteome by mass spectrometry. There have been numerous studies investigating the effects of resveratrol on the proteome. It is not possible to list these exhaustively due to their large number but as discussed in Section 2.1.3, this project focused specifically on the effects of resveratrol on the levels of proteins involved in the apoptotic pathway in colon cancer cells. Published studies pertinent to this subject have therefore been summarised in Table 1.6.

**Table 1.6 Published data of the effect of resveratrol on colon cancer cell apoptosis *in vitro***

| Reference | Resveratrol dose | Cell type | Proteins activated or levels increased unless stated otherwise |
|---|---|---|---|
| Schneider *et al.* 00 | 25 μM | Caco-2 cells | Ornithine decarboxylase |
| Walter *et al.* 01 | 200 μM | Caco-2 cells | Caspase 3, cyclin E and cyclin A levels increased. Cyclin D1 and Cyclin Dependent Kinase 4 (cdk4) levels decreased. Cdk2, cdk6 and proliferating cell nuclear antigen were unaltered. |
| Mahyar-Roemer *et al.* 02 | 100 μM | HCT116 cells | Caspases 3 and 9 |
| Delmas *et al.* 03 | 10-100 μM | SW480 cells | Multiple caspases activated, no change in Fas or FasL |
| Delmas *et al.* 04 | 30-100 μM | HT29, HCT116, SW480 and SW620 cells | Redistribution of Fas (CD95), DR4 and DR5 death receptors. |
| Lee *et al.* 06 | 50-200 μM | HCT 116 cells | Fragmentation of lamin A/C protein, increased ribosomal protein P0, decreased dUTPase and decreased stathmin 1. |
| Mohan *et al.* 06 | 25-100 μM | HCT116 cells | Caspase 2 |
| Park *et al.* 07 | 20 - 100 μM | HT 29 cells | CCAAT/enhancer-binding protein-homologous protein (CHOP) and Glucose-Regulated Protein 78 |
| Woo *et al.* 07 | 20-100 μM | HT29 cells | CCAAT/enhancer-binding protein-homologous protein (CHOP) |
| Hope *et al.* 08 | 10 μM | RKO cells | Decreased beta catenin levels in nucleus. |
| Cosan *et al.* 09 | 25-100 μM | Caco-2 cells | Bak and FADD |
| Kim *et al.* 09 | 50-250 μM | HCT 116 cells | P53, Bax, DR4, Fas (CD95) and caspase 8 |
| Majumdar *et al.* 09 | 10 μM | HCT 116 cells | Inhibited NF-kappaB, EGFR and IGF1R. |
| Vanamala *et al.* 10 | 100-150 μM | HT 29 cells | Decreased IGF1R protein levels and inhibited Akt/Wnt pathway. |

## 1.5 Aims of Research

The overall aim of this project was to investigate strategies for optimising the clinical development of dietary chemopreventive agents, by using the available preclinical data

to facilitate the translation of these agents from the laboratory into early clinical trials. Resveratrol, a polyphenol present in grapes and peanuts, was chosen as a model compound because of the extensive published preclinical data available which suggested that it could exert chemopreventive actions (see Chapter 1.2.3).

This project focused specifically on the question of how to determine the appropriate dose for clinical studies, selecting two doses for investigation. This approach taken in this research could subsequently be utilised as a paradigm to aid drug development for chemoprevention. The first dose was 5 mg of resveratrol per day in an adult human, which is equivalent to the amount of resveratrol that may be consumed in a normal diet and is termed throughout this thesis as the "dietary dose". The second, higher dose, was 1 g per day in an adult human, which was selected as its safety for use has already been demonstrated in a published clinical trial (Boocock *et al.* 07a). This higher dose will be referred to as the "pharmacological dose" in this thesis. The *in vitro* data were collected by exposing colon cancer cells to the estimated human plasma concentrations after oral dosing. Published data suggest that resveratrol does not exert anti-tumour effects at such low concentrations, so the potential for interaction between resveratrol and other dietary agents was investigated as a possible mechanism for efficacy at dietary levels. The doses used for the *in vivo* studies were calculated to result in similar target tissue concentrations in murine and human organs.

The specific aims in this project were:

● To investigate the potential for interaction between resveratrol and malvidin-3-*O*-glucoside, when incubated with HCA7 colon cancer cells at dietary and pharmacological concentrations. Malvidin-3-*O*-glucoside was chosen as this occurs in

the same foods as resveratrol and so would be consumed at the same time, possible contributing to the benefits reported in epidemiological studies.

● To investigate genomic and/or proteomic changes induced by long term exposure of HCA7 colon cancer cells to dietary or pharmacological concentrations of resveratrol, at 0.01 and 1.4 μM, for 3 months as assessed by microarray and proteomic analysis (see Chapter 1.4.2 and 1.4.3). The prolonged treatment duration was selected to mimic the chronic exposure of colon cells to dietary agents *in vivo*. These results were envisaged to be useful to guide the choice of efficacy biomarkers for analysis in the subsequent $Apc^{Min+/-}$ mice study.

● To investigate the pharmacokinetic and tissue distribution of [$^{14}$C]-labelled resveratrol in F344 rats, after administration of a single dietary or pharmacological dose by gavage. This was done to identify tissues with high concentrations of [$^{14}$C]-resveratrol equivalents, as these may be potential target organs for the chemopreventive effects of resveratrol.

● To investigate the pharmacodynamic effects of resveratrol administered to C57BL/6J $Apc^{Min+/-}$ mice in the diet, at 0.7 or 143 ppm, for up to 13 weeks post weaning with either a standard or a high fat diet. The high fat diet was selected to represent the dietary composition now commonly consumed in the Western world, which has been implicated as a risk factor for colorectal cancer development (Ma *et al.* 07). It is unclear how dietary fat may promote colorectal carcinogenesis, but resveratrol has been shown to protect against the metabolic insults exerted by a high fat diet (see Chapter 1.3.1). It was therefore hypothesised that resveratrol may mediate its chemopreventive

actions via normalisation of the metabolic profile. Blood and multiple organs were collected from *Apc*$^{Min+/-}$ mice to determine intestinal tumour load, metabolic changes as well as identifying plasma and tissue efficacy biomarkers.

● To investigate the pharmacokinetic profiles of [$^{14}$C]-resveratrol at dietary and pharmacological doses in patients with colorectal cancer or prostate cancer, since preclinical data suggested that these may be target organs for the chemopreventive effectiveness of resveratrol (see Chapter 1.2.3). Confirmation that the levels achievable in human tissue are similar to efficacious concentrations *in vitro,* or the levels *in vivo,* would support further clinical development of resveratrol as a cancer chemopreventive agent. Colon tissue samples were also analysed for changes in putative efficacy biomarkers identified in the *Apc*$^{Min+/-}$ mice study discussed above. This is the first clinical study to investigate tissue levels of resveratrol species in the prostate, and the first study to compare the tissue levels in the colon after dietary and pharmacological dosing. Accelerator mass spectrometry was used, rather than other standard analytical techniques like HPLC or LC-MS/MS, as its greater sensitivity allowed the detection of [$^{14}$C]-resveratrol species in plasma and tissues of patients despite the administration of very low chemical and radiation doses.

● To investigate the pharmacokinetic profile of [$^{14}$C]-resveratrol after a single oral dose at 5 mg or 1.005 g in healthy volunteers. These were analysed together with the patient tissue results to complete the clinical pharmacokinetic profile of resveratrol. This is the first comprehensive analysis of the clinical pharmacokinetic profile of resveratrol after administration of a dietary dose, and its comparison to the data after administration of a pharmacological dose.

# 2. Preclinical Materials and Method

# 2. Preclinical Materials and Methods

## 2.1 Preclinical Materials and Methods *in vitro* studies

All cell culture plastic-ware was purchased from Greiner Bio-One (Stonehouse, UK) and all chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless stated otherwise. The resveratrol used throughout the preclinical part of this project was of 99.9 % purity and supplied by Shanghai Novanet Co. Ltd, Shanghai, China.

### 2.1.1 Cell culture

HCA7 cells were obtained from the European Collection of Cell Culture (ECCC), Wiltshire, UK. The culture medium used throughout was Dulbecco's Modified Eagle's Medium containing 4.5 g/L glucose, supplemented with 10 % (v:v) fetal bovine serum (Invitrogen Ltd, Paisley, UK).

#### 2.1.1.1 Choice of doses for *in vitro* studies

The dietary and pharmacological concentrations of resveratrol were determined from published literature as an estimate of the likely resveratrol concentrations attained in human plasma after oral administration. Resveratrol has been administered as a single dose at 1 g and 25 mg in two studies to healthy volunteers (Boocock *et al.* 07, Goldberg *et al.* 04), with plasma $C_{max}$ values of 1.33 µM and 0.04 µM respectively. Presuming a linear relationship, back extrapolation to a 5 mg dose would yield a $C_{max}$ value in the region of 0.007 µM from both studies. Based on these plasma values and tissue levels from Section 5.2.2, concentrations of 1.4 µM and 0.01 µM were chosen as the resveratrol levels to correspond to the 5 mg and 1 g doses respectively in the clinical study.

The dietary concentration of malvidin-3-*O*-glucoside used was chosen based on the natural ratio of resveratrol to anthocyanin in red wine. This ratio was in the order of 40 as HPLC analysis of pinot noir (data not shown), with a resulting dietary dose calculated as 0.4 μM. This concentration of total anthocyanins has also been proven to be achievable in the colon tissue of cancer patients after oral consumption of an anthocyanin mixture (Thomasset *et al.* 09). The natural ratio of resveratrol to anthocyanin was not used to calculate the pharmacological dose as this would be so high (56 μM) that it would have no clinical relevance and would probably be too cytotoxic to the cells. The pharmacological concentration of malvidin-3-*O*-glucoside was therefore selected as 10 μM, as the likely colon tissue level from clinical trials of other chemopreventive agents, for example silibinin from milk thistle (Hoh *et al.* 07).

#### 2.1.1.2 Short term experiment to investigate interactions of resveratrol and malvidin-3-*O*-glucoside on HCA7 cell proliferation rate

For the short term experiment to investigate the interactions of resveratrol and malvidin-3-*O*-glucoside, HCA7 cells (passage 5 to 7) were plated at 5 x $10^4$ cells per well in 6 well plates and incubated for 24 h at 37 °C with 5 % $CO_2$ prior to dosing. Previous optimisation experiment showed that this seeding density would result in confluence after one week. To minimise the possibility that well position may influence outcome, treatments were randomly assigned. Resveratrol was prepared fresh each day by dissolving in dimethyl sulphoxide (DMSO). The DMSO concentration in all treatment groups was 0.09 %. Malvidin-3-*O*-glucoside chloride (Extrasynthese, Genay Cedex, France) was dissolved in sterile water as a stock solution of 0.5 mg/ml and stored in aliquots at -20 °C. Aliquots were defrosted daily for administration. Malvidin-3-*O*-glucoside chloride was shown to be stable under such conditions for 4

weeks (M Laborderie, Leicester University, unpublished data).

Experiments were performed on 3 separate occasions and in quadruplicate for each dose (Table 2.1). Cells were harvested with 10x trypsin (25 mg/mL, Invitrogen Ltd, Paisley, UK) after washing with phosphate buffer saline (PBS 0.01 M, Oxion Ltd, Hampshire, UK). Daily cell counts were carried out using a Coulter counter after dilution with Coulter Isoton II diluent (Beckman Coulter UK, Buckinghamshire.)

**Table 2.1 Concentrations of resveratrol and malvidin-3-*O*-glucoside used to treat HCA7 colon cancer cells**

| | Resveratrol dose | Malvidin-3-*O*-glucoside dose |
|---|---|---|
| Media only negative control (Group 1) | 0 | 0 |
| Vehicle only negative control (Group 2) with 0.09% DMSO in media | 0 | 0 |
| Dietary resveratrol dose (Group 3) | 0.01 µM | 0 |
| Dietary malvidin-3-*O*-glucoside dose (Group 4) | 0 | 0.4 µM |
| Dietary resveratrol + malvidin-3-*O*-glucoside dose (Group 5) | 0.01 µM | 0.4 µM |
| Pharmacological resveratrol dose (Group 6) | 1.4 µM | 0 |
| Pharmacological malvidin-3-*O*-glucoside (Group 7) | 0 | 10 µM |
| Pharmacological resveratrol + malvidin-3-*O*-glucoside dose (Group 8) | 1.4 µM | 10 µM |
| Positive control (Group 9) | 25 µM | 0 |

### 2.1.1.3 Long term experiment to investigated the effects of reseveratrol treatment on genomic and proteomic profiles of HCA7 colon cancer cells

For the long term experiment to investigate the genomic and proteomic changes induced by resveratrol after chronic treatment, 4 x $10^5$ HCA7 cells (passages 10 to 11) were seeded in 175 $cm^2$ flasks and incubated at 37 °C with 5 % $CO_2$). Comparison of

results between Group 1 and Group 2 of the short term experiment showed that the presence of 0.09 % DMSO did not alter HCA7 cell proliferation rate. The long term study to investigate the effects of resveratrol on the genomic and proteomic profile of HCA7 colon cancer cells therefore had only one control arm consisting of vehicle treated cells only. Cells were treated twice weekly, and passaged and counted once per week for 10 passages. The frequency of treatment was selected based on the stability data of resveratrol in cell media at 37 °C (personal communication by K Patel, Leicester University). Resveratrol was prepared fresh for each treatment by dissolving in DMSO. DMSO concentration did not exceed 0.09 % and previous optimisation data showed that the presence of vehicle or resveratrol in the media did not impair cell adherence. Experiments were performed on 3 separate occasions and in duplicates for each dose group.

For the last passage, cells were expanded to enable collection of media for measurement of IGF1 levels, and cell pellets for microarray and proteomic analyses. Cell pellets were harvested on ice with 10x trypsin, washed twice with PBS and the number of cells in each pellet was measured with a Coulter Z2 particle analyser following dilution with Coulter Isoton II diluent. The medium was centrifuged for 3000 *x g* for 20 min at 4 °C and the supernatant removed for IGF1 analysis. All samples were stored at -80 °C.

### 2.1.2 Microarray analysis of resveratrol treated colorectal cancer cells

#### 2.1.2.1 RNA extraction

The cell pellet from each flask was mixed thoroughly with 500 µL of TRI reagent and incubated at room temperature for 5 min. 1-Bromo-3-chloro-propane (100 µL) was

then added, the sample shaken vigorously for 15 s and vortexed for 5 min. The sample was incubated at room temperature for 2 min before being added to a Maxtract High Density 2 mL tube (Qiagen, Crawley, UK) which had been previously centrifuged at 12000 *x g* for 30 s. The tube containing the sample was then centrifuged at 12000 *x g* for 14 min at 4 °C and the upper aqueous layer containing RNA was carefully removed into a fresh eppendorf without disturbing the gel layer at the interphase. Isopropanol was added in a 1:1 ratio, mixed well and incubated at room temperature for 10 min. The sample was then centrifuged at 12000 *x g* for 10 min at 4 °C to precipitate the RNA pellet, which was then washed twice with 1 mL 75 % ice cold ethanol and centrifuged (12000 *x g* for 5 min). The RNA pellet was placed in the SPD1010 Speedvac (Thermo Savant, Massachusetts, US) for 5 min at room temperature to remove all traces of ethanol. It was then dissolved in 20-40 µL of diethyl pyrocarbonate (DEPC) treated water and the concentration measured using a NanoDrop1000 spectrophotometer. The purity for all RNA samples, determined by the $\lambda_{260/280}$ ratio, was between 1.93 to 2.00. RNA samples from the same cell flasks were used for both the mRNA and the miRNA microarray analyses.

#### 2.1.2.2 Conversion of mRNA to cDNA

cDNA microarrays were imprinted on aldehyde slides (Genetix, New Milton, UK) using a Stanford type microarray spotter. Targets were the Human Exonic Evidence Based Oligonucleotide (HEEBO) set developed by Stanford University (http://www.microarray.org/sfgf/heebo.do) and manufactured by Invitrogen (Paisley, UK).

Targets were printed from a 10 µM solution in 1.5 M Betaine / 3x SSC (saline sodium

citrate) and the slides stored in the dark at room temperature. On the day prior to use, the slides were washed twice in 0.2 % SDS (sodium dodecyl sulphate) for 2 min and then twice in double deionised water for 2 min. They were then dried by centrifugation at 1000 *x g* for 4 min and stored in the dark. The cover slips were washed in 1 % SDS for 30 min then underwent 5x5 minute washes in deionised water. They were similarly dried by centrifugation at 1000 *x g* for 4 min and stored in the dark.

cDNA for mRNA microarray analysis was transcribed from total RNA using a DNA Engine tetrad PTC-225 Peltier Thermal Cycler (Bio-Rad, Hercules, California, US). RNA (10 µg) in 13 µL of 0.1 % DEPC water was added to 1 µL of anchored oligo $dT_{23}N_2$ (8µg/µL) and 1 µL of pentadecamers (10 nmol/µL). The mixture was heated to 95 °C for 5 min, then 70 °C for 10 min before being cooled on ice for 1 min. Labelling mix (14 µL, see Table 2.2) and 1 µL of Superscript III RT (Invitrogen, Paisley, UK) were added to the sample which was further incubated at 50 °C for 3 h.

Any untranscribed RNA was hydrolysed by addition of 10 µL 0.5 M ethylenediaminetetraacetic acid (EDTA) and 10 µL of 1 M NaOH, followed by incubation at 65 °C for 15 min. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 25 µL of 1M) was added and the resulting cDNA was purified using Microcon 30 filters as follows: 375 µL of 0.1 % DEPC water and the sample were added to the filter and centrifuged at 12000 *x g* for 7 min. The flow-through was discarded and this step was repeated twice more with 450 µL of 0.1 % DEPC water each time. The column was then inverted into a new collection tube and centrifuged for 12000 *x g* for 2 min. The collected filtrate concentrated to dryness under vacuum and stored overnight at -20 °C.

**Table 2.2 Content of labelling mix for cDNA transcription from total RNA for mRNA microarray**

| Nucleotide | Stock | Volume | Final concentration in reaction |
|---|---|---|---|
| dATP | 100 mM | 15 µL | 0.5 mM |
| dGTP | 100 mM | 15 µL | 0.5 mM |
| dCTP | 100 mM | 15 µL | 0.5 mM |
| dTTP | 100 mM | 6 µL | 0.2 mM |
| aadUTP | 50 mM | 18 µL | 0.3 mM |
| First Strand Buffer | 5X | 600 µL | 1X |
| Dichloro-Diphenyl-Trichloroethane | 0.1 M | 300 µL | 0.01 mM |
| 0.1 % DEPC water | not applicable | 431 µL | not applicable |

### 2.1.2.3 cDNA labelling and hybridisation with microarray slides

cDNA was re-suspended in 3.5 µL of 0.1 % DEPC water. DMSO (4 µL) was used as a solvent for the dried Alexa Fluor dye powder and 2 µL of this was added to the reconstituted cDNA, together with 0.5 µL of 1 M carbonate buffer (titrated to pH 9.0 with concentrated HCl). Each test RNA sample was labelled with Alexa Fluor 555 and Alexa Fluor 647 dyes (Invitrogen, Paisley, UK) for forward and reverse slides, whilst the control samples were labelled in the opposite manner. The samples were incubated in the dark at room temperature for 1 h, after which 4.5 µL of 4 M hydroxylamine was added to each sample and further incubation was carried out in the dark at room temperature for 15 min. The corresponding pairs of test and control RNA samples were then pooled and 35 µL of 0.1 M sodium acetate (titrated to pH 5.2) was added to each pair.

The labelled cDNA was then purified using a Qiagen Polymerase Chain Reaction (PCR) purification kit (Qiagen, Crawley, UK). Buffer PB (250 µL) and each RNA

sample were placed in a Quick Spin PCR column, centrifuged (1 min, 1300 *x g*) and the flow-through discarded. This was repeated with 750 µL of Buffer PE and the column then centrifuged to dryness. Buffer EB (30 µL) was added, incubated at room temperature for 1 min and then centrifuged at 13000 rpm for 1 min over a new collection tube. The eluent containing the dye-labelled cDNA could then be quantified using a NanoDrop1000 spectrophotometer.

An aliquot (1 µL) of tRNA (4 mg/mL, Invitrogen, Paisley, UK), 60 µL of 2x formamide-based hybridization buffer (Genisphere, Philadelphia, US) and 30 µL of 0.1 % DEPC water were added to the cDNA. The mixture was denatured at 100 °C for 5 min then incubated at 42 °C for 30 min. An aliquot (35 µL) of the cDNA / hybridisation mixture was pipetted around the edges of the cover slip placed over each of the 3 HEEBO slides, allowing the sample to disperse by capillary action. DEPC water (0.1 %) was added to each hybridisation chamber (Genetix, New Milton, UK) for humidification and the slides incubated at 42 °C overnight or over the weekend.

**Table 2.3 Washing sequence for mRNA microarray slides prior to scanning**

| Wash (made up to 1 L in total with double deionised water) | 20 % SSC | 10 % SDS | Time |
|---|---|---|---|
| 1 | 50 mL | 3 mL | 5 min |
| 2 | 10 mL | Nil | 3 min |
| 3 | 2.5 mL | Nil | 3 min |

On removal from the hybridisation chamber, slides were washed as in Table 2.3 and dried by centrifugation at 1000 *x g* for 4 min. The slides were stored in the dark and scanned using an Axon 4200A scanner and Genepix 5.10.19 (Molecular Devices Coop, California, US) within 48 h. Scanning was at 70 % power, 0.05 % saturation and the

PMT gain for 635/532 set automatically. The array dots on each slide were fitted individually prior to analysis.

#### 2.1.2.4 Analysis of microarray slides

mRNA microarray data were analysed by MRCstats-200807 software developed by SD Zhang (Zhang *et al.* 04). Data underwent *logarithmic* transformation and were normalised by the Lowess method (Zhang *et al.* 04). The forward and reverse slides were analysed by the student t test. The functions of significantly altered genes and the biological pathways they are involved in were identified by the following websites:

http://bioinfo.vanderbilt.edu/webgestalt/

http://www.genecards.org/

Pathways were identified as being significant if at least 2 genes were involved with $p<0.05$. The quality of the microarray slides was analysed with the arrayQuality package from Stanford University (http://www.bioconductor.org/).

#### 2.1.2.5 Real time Polymerase Chain Reaction

Real time PCR was performed to confirm the microarray findings that expression of the baculoviral inhibitor of apoptosis repeat-containing gene (BIRC3), ankyrin repeat + SOCS box-containing gene (ASB6), tumour necrosis factor receptor super family 10b gene (TNFRSF10b) and mitogen activated protein kinase 10 gene (MAPK10) genes were altered by resveratrol concentrations of 0.01 μM. Primer Express software v2.0 (Applied Biosystems, Warrington, UK) was used to design primers to include exon-exon boundaries for increased specificity. Primers were only used if the sequence was present in all splice variants and did not form secondary structures or dimers as per information from Sigma-Aldrich on ordering. Specificity of the primer for the gene

tested was confirmed using *Basic Local Alignment Search Tool* (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

**Table 2.4 Content of master mix for cDNA synthesis from total RNA for PCR**

| Reagent | Stock concentration | Volume |
|---|---|---|
| PCR buffer (Invitrogen, Paisley, UK) | 10x | 100 µL |
| $MgCl_2$ | 50 mM | 50 µL |
| dATP | 100 mM | 10 µL |
| dTTP | 100 mM | 10 µL |
| dCTP | 100 mM | 10 µL |
| dGTP | 100 mM | 10 µL |
| Random hexamers | 9000 U/mL | 9.9 µL |
| DTT | 100 mM | 10 µL |
| Water | not applicable | 615.1 µL |

cDNA for PCR analysis was transcribed as below. RNA (0.45 µg) was added to 0.5 µL of d(N)6 (Amersham Biosciences, Little Chalfont, UK) and heated to 90 °C for 3 min before being cooled on ice of master mix (28.5 µL, see Table 2.4), 1 µL RNAsin, 2 µL superscript III and 4 µL 0.1 % DEPC water were added and the mixture incubated at 23 °C for 10 min, 50 °C for 45 min, 95 °C for 10 min then snapped cool on ice.

The sequences (forward primer/reverse primer) were TGGACCGGCCCAGTTATGT / CGTTGCTCACGCCTTCCT for ASB6; TGTGGGTAACAGTGATGATGTCAA / TACTCACACCTTGGAAACCACTTG for BIRC3; GACTCACCTTCCCCAAACTCTTC / GCTTGGCTGGCTTTGAGTTTA for MAPK10; and GACCTAGCTCCCCAGCAGAGA / AGGTGGACACAATCCCTCTGA for TNFRSF10B.

Primer solutions (5 pM and 50 pM) were made with 0.1 % DEPC water for use in optimisation and sample analysis respectively. For all PCR analyses, the cDNA product was amplified with SYBR Green Mastermix (Applied Biosystems, California, US) using an ABI PRISM 7700 RT-PCR Sequence Detection System. Optical 96-well fast thermal cycling plates (Applied Biosystems, Warrington, UK) and the comparative cycle threshold method was used (Applied Biosystems, User Bulletin no 2, 1997) for each pair of primers. PCR amplification occurred at 50 °C for 2 min, then 95 °C for 10 min, then 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. The final dissociation step involved 95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s. Each sample was tested in triplicate with each well containing 1 µL of cDNA or water as control.

Optimisation with dummy cDNA was carried out to establish the concentrations of the forward and reverse primer that would result in the lowest cycle threshold level. Concentrations used were as below in Table 2.5:

**Table 2.5 Different concentrations for optimisation of PCR primers**

| Test concentration | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | 50 nM | 50 nM | 50 nM | 300 nM | 300 nM | 300 nM | 900 nM | 900 nM | 900 nM |
| Reverse primer | 50 nM | 300 nM | 900 nM | 50 nM | 300 nM | 900 nM | 50 nM | 300 nM | 900 nM |

Expression levels were normalised to TLN1 (Talin 1) which encodes a cytoskeleton protein. This endogenous gene was identified due to its high FDR and its structural function, which was unlikely to be changed by resveratrol. Its suitability as an

endogenous gene was confirmed by PCR which showed that its expression was not altered by resveratrol as shown in Fig 2.6. The sequences (forward primer/reverse primer) were AGACGATCATGGTGGATGACTCT / AATGCGGGCACAGATGGT for TLN1. The optimal concentrations of forward and reverse primers, defined as resulting in the lowest cycle threshold values, were 900 nM each for TLN1, BIRC2, MAPK10 and TNFRSF10B. The optimal concentrations for ASB6 were 300 nM for the forward primer and 900 nM for the reverse primer.

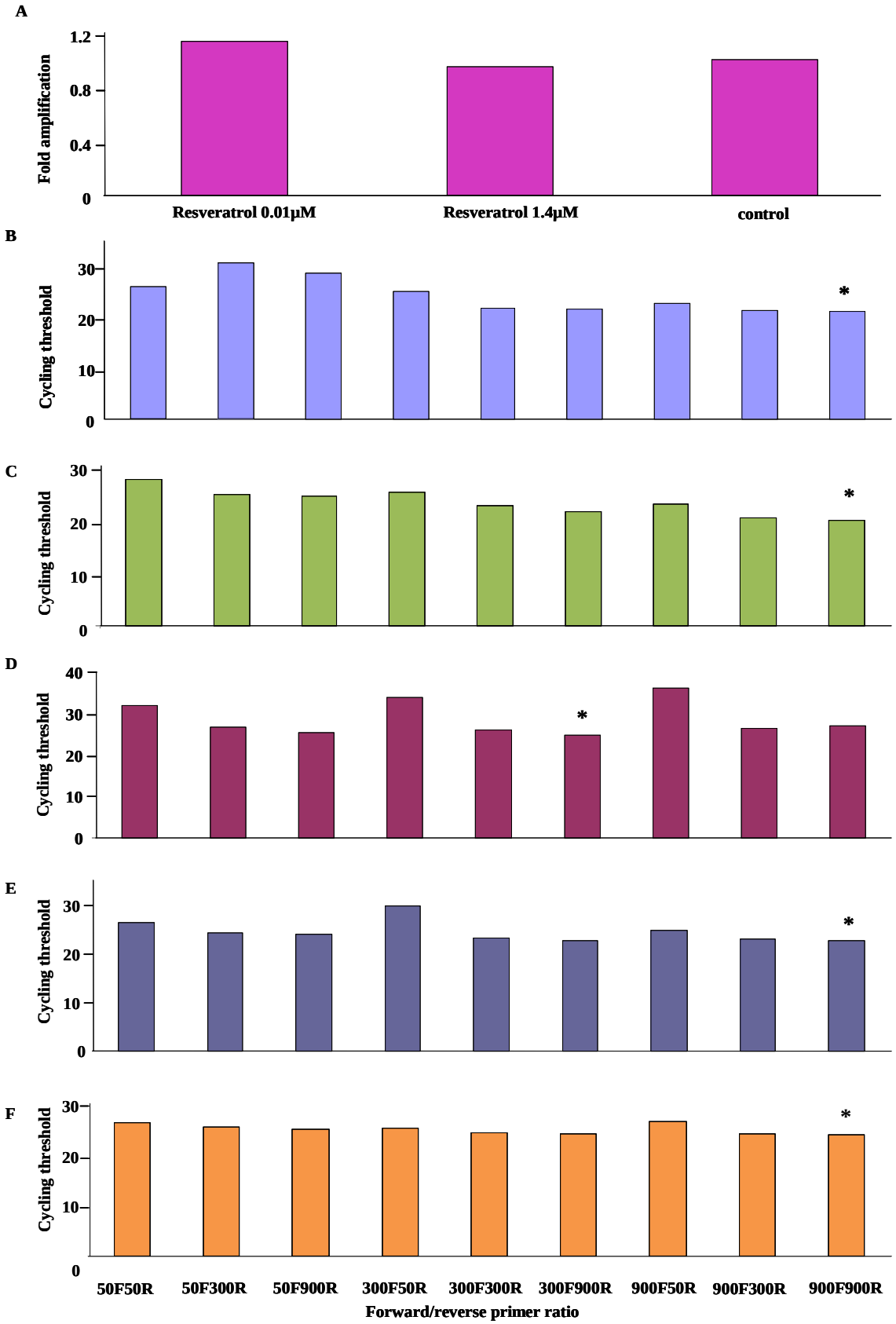



**Figure 2.6 Confirmation of TLN1 gene as a suitable endogenous gene for PCR.** TLN1 expression was not significantly altered in any of the treatment groups (**A**). The optimum ratio (denoted by *****) for forward (F) and reverse (R) primers for PCR analyses, as defined by the lowest cycling threshold achievable, was then established for TLN1, BIRC3, ASB6, TNFRSF10b and MAPK10 (**B-F**).

### 2.2.2.3 microRNA profiling

cDNA microarrays were imprinted on aldehyde slides (Genetix, New Milton, UK) using a Stanfordtype microarray spotter. Targets were the Exiqon miRCURY ready to probe set (Exiqon, Vedbaek, Denmark) and were printed from a 20 µM solution in 1.5 M Betaine / 3x SSC. The slides were stored in the dark at room temperature and were washed, with the cover slips, as per the mRNA microarray slides on the day prior to use.

cDNA for miRNA microarray analysis was transcribed from total RNA as follows: all reagents were from the NCode labelling kit (Invitrogen, Paisley, UK) and were used at stock concentrations except the ATP solution, which was diluted 1:500 with 0.1 % DEPC water. Total RNA (1.5 µg) was added to 0.75 µL of 10x miRNA reaction buffer, 0.75 µL of $MnCl_2$, 0.5 µL diluted ATP solution and 0.5 µL poly(A) polymerase enzyme. These were mixed and then incubated at 37 °C for 15 min. Each test RNA sample was then labelled with 2 µL of 6x Alexor Fluor 3 or 6 Rapid Ligation Mix for forward and reverse slides, whilst the control samples were labelled in the opposite manner. T4 DNA ligation solution (1 µL) was added to poly(A) tail reaction and the samples incubated at room temperature for 30 min. The reaction was terminated by addition of 1.25 µL Stop Solution and the appropriate pairs of samples pooled. Bovine serum albumin (2.5 µL) and 26 µL of enhanced cDNA hybridisation buffer were added to the mixture and heated to 65 °C for 10 min. Arrays were loaded onto the slides as for mRNA microarray analysis and incubated in a humidified chamber at 52 °C overnight or over the weekend.

On removal from the chamber, the slides were washed (see Table 2.6) and dried by centrifugation at 1000 rpm for 4 min. The slides were stored in the dark and scanned using an Axon 4200A scanner and Genepix 5.10.19 within 48 h at 70 % power, with saturation at 0.05 % and photomultiplier (PMT) gain for 635/532 set automatically. They were scanned twice, with the PMT gains of the 2nd scan determined by adding 200 to the automatically set PMT values in order to detect significant gene changes whilst excluding background noise. The array dots on each slide were fitted individually prior to analysis.

**Table 2.6 Washing sequence for miRNA microarray slides prior to scanning**

| Wash (heated on full power in microwave for 2 min to approximately 52 °C) | 20 % SSC | 10 % SDS | Time |
|---|---|---|---|
| 1 | 50 mL | 5 mL | 2 min |
| 2 | 25 mL | Nil | 2 min |
| 3 | 5 mL | Nil | 2 min |

miRNA microarray data were analysed by MRCstats-200807 software developed by SD Zhang (Zhang *et al.* 04). Data underwent *logarithmic* transformation and were normalised by the lowess method. The forward and reverse slides were analysed as technical two labelling samples by the student T test. The functions of significantly altered genes were identified using the following websites:

http://www.ensembl.org/index.html

http://www.targetscan.org/

### 2.2.3 Proteomic analysis for the effects of resveratrol on the apoptosis pathway in colorectal cancer cells

Proteome Profiler® kits for the apoptosis pathway were purchased from R&D Systems (Minnesota, US). One set of cell pellets from each experiment, i.e. n=3 for each treatment group, was resuspended in lysis buffer and solubilised by placing on a rocking platform for 30 min at 4 °C. The lysate was then microcentrifuged (14000 *x g*, 5 min) and the supernatant removed. The protein concentration for each lysate was determined using the Bradford protein assay as follows (Bradford *et al.* 76): bovine serum albumin was dissolved in distilled water and serially diluted to produce solutions ranging from 2-20 μg/mL. Each standard (800 μL) was then added to 200 μL of Biorad reagent and samples were analysed on a Varian Cary 50 Bio UV-visible spectrophotometer (Oxford, UK) to construct a standard curve. Each cell lysate sample (2 μL) was added to 998 μL of distilled water and the concentration determined from this standard curve.

The cell lysates were then analysed according to the kit protocol. Primary antibodies raised against the proteins in Table 2.7 were spotted in duplicate onto nitrocellulose membranes which were placed on a rocking platform with a blocking buffer from the kit for 1 h at room temperature. Positive control spots consisting of antibodies to irrelevant proteins and negative control spots for PBS were included on each membrane.

**Table 2.7 Protein targets in the apoptosis pathway analysed**

| | | | |
|---|---|---|---|
| Bad | Catalase | TRAILR1/DR4 | HSP27 |
| Bax | cIAP-1 | TRAILR2/DR5 | HSP60 |
| Bcl-2 | cIAP-2 | FADD | HSP70 |
| Bcl-x | claspin | Fas/TNFSF6 | p21/CIP1/CDNK1A |
| Pro-caspase-3 | clusterin | HIF-1α | p27/kip1 |
| Cleaved capsase-3 | cyctochrome c | HO-1/HMOX1/HSP32 | phospho-p53 (S15) |
| HTRA2/omi | Livin | HO-2/HMOX2 | phospho-p53 (S46) |
| SMAC/Diablo | Survivin | TNFR1/TNFRSF1A | phospho-p53 (S302) |
| phospho-rad17 (S365) | XIAP | | |

Protein (500 μg) from each cell lysate was then added to the nitrocellulose membrane and incubated overnight at 4 °C. Excess proteins were removed by washing (3x10 minutes) with a wash buffer prior to the addition of biotin-conjugated secondary antibodies for 1 h at room temperature. Excess antibody was removed before addition of horse radish peroxidase-linked streptavidin for further incubation (30 min, room temperature). Any excess enzyme was again washed off prior to addition of enhanced luminol-based chemiluminescent (ECL) detection reagents. The membranes were exposed to X-ray film for 1-5 min, developed using the Curix 60 automated developing system (Agfa, Middlesex, UK) and the resulting films scanned by a Gene Genius Bio-imaging machine (Syngene, Cambridge, UK) for quantification. The pixel density from each spot was normalised to the positive control spots on that membrane. The normalised results from the test membranes were then expressed as ratios to the control membranes, and significance was calculated using the student t test.

## 2.2 Preclinical Materials and Methods: *in vivo* studies

All chemicals were purchased from Sigma-Aldrich (Poole, UK) unless stated otherwise.

### 2.2.1 Single dose [$^{14}$C]-resveratrol study in F344 rats

Male F344 rats at 6-7 weeks old were purchased from Harlan Laboratories (Bicester, UK). They were acclimatised for 7 days prior to study commencement and randomised into groups of 3. Female rats were excluded as the prostate was an organ of interest. [$^{14}$C]-resveratrol powder (23.5 μCi/mg) (Quotient Ltd., Northampton, UK) was used in the dietary dose study, but was radiodiluted in the pharmacological dose study to a specific activity of 0.3525 μCi/mg in an effort to limit the radiation exposure of the animals.

[$^{14}$C]-Resveratrol dosing solutions were made up fresh for each study in 1 % methylcellulose and administered via gavage. The dietary and pharmacological doses were determined by weight conversion as being equivalent to a 5 mg or 1 g dose of resveratrol in a 70 kg human, i.e. 0.07 or 14.29 mg of resveratrol/kg body weight respectively. Rats were selected for this study rather than mice to increase the amount of tissue available for analysis, and resveratrol has been demonstrated in published papers to be extensively metabolised in mouse, rat and humans (see Section 1.2.2). The animals were culled at 0.25, 0.5, 1, 2, 4, 6, 8, 10 and 24 h post dose. Brain, heart, lung, stomach, kidney, colon, spleen, prostate and blood samples were collected from each animal and snap frozen in liquid nitrogen, then stored in a -80 °C freezer until time of analysis.

#### 2.2.1.1 Liquid scintillation counting (LSC) of tissues

Liquid scintillation counting technique was performed according to a protocol provided by Perkin Elmer Ltd. *(las.perkinelmer.com/content/.../TCH_LSCSamplePrepSolubilization.pdf)* and all reagents in section 3.2.1.1 were purchased from them. Solid tissue samples (~100 mg) were homogenised, added to Soluene-350 solubiliser (1 mL) and incubated at 60 °C for up to 4 h in a shaking water bath. The samples were then cooled to room temperature before adding Optiphase HiSafe II Liquid Scintillation Cocktail (10 mL) for analysis on an LS 6500 Liquid Scintillation Counter (Beckman Coulter Ltd., California, US). Samples were analysed repeatedly until the values were stable. Blood samples were directly added to Optiphase HiSafe II Liquid Scintillation Cocktail without the solubiliser step. They were serially diluted with PBS until the quench values were within the range of acceptability.

### 2.2.2 Long term administration of resveratrol at dietary and supra-dietary doses in C57BL/6J A*pc*$^{Min+/-}$ mice on a high fat diet

Male C57BL/6J *Apc*$^{Min+/-}$ mice were obtained from an established colony in Leicester University, UK, and their genotypes confirmed by PCR of ear clips. Female mice were excluded from this study as there is evidence that oestrogen may alter the metabolic changes induced by obesity (Riant *et al.* 09). The male *Apc*$^{Min+/-}$ mice were randomised at 3-4 weeks old to 6 different chows (IPS Product Supplies Ltd., London, UK) as detailed in Table 2.8. Resveratrol was mixed in with the feed at 0.7 mg/kg (0.00007 % dose) or 14.3 mg/kg feed (0.0143 % dose), which is equivalent to 0.5 mg or 100 mg per day in 70 kg man based on body surface area conversion (see Appendix 8.2 for detailed constituents of these diets). The standard fat diets contained 16 % of calories from fat, whilst 60 % of calorific value in the high fat diets was from coconut oil. Coconut oil

was chosen as this fat source at such a high concentration has previously been shown to be tolerable for mice for long term administration (Baur *et al.* 06). Resveratrol was mixed in by serial dilution prior to the addition of coconut oil to ensure its even distribution within the chow. The identities of the different diets were confirmed by HPLC, using a standard method for the analysis of resveratrol (Boocock *et al.* 07b) by Dr H Cai (Cancer Biomarkers and Prevention Group, Leicester University).

The mice were randomised into groups of 12 for each diet group, with separation of pups from the same litter to decrease any confounding genetic factors. Diets were changed on a weekly basis and weighed to estimate the amount of food eaten. This study received local ethical review board approval and was compliant with the activities permitted by the departmental animal license.

**Table 2.8 Control and test diets for C57BL/6J *Apc*$^{Min+/-}$ mice**

| Treatment group | Description |
|---|---|
| Group 1 | Standard AIN-93 diet |
| Group 2 | Standard AIN-93 diet and 0.00007 % resveratrol |
| Group 3 | Standard AIN-93 diet and 0.0143 % resveratrol |
| Group 4 | High fat AIN-93 diet |
| Group 5 | High fat AIN-93 diet and 0.00007 % resveratrol |
| Group 6 | High fat AIN-93 diet and 0.0143 % resveratrol |

At 17 or 14 weeks of age for the animals in Groups 1-3 and Groups 4-6 respectively, mice were fasted overnight prior to exsanguination by cardiac puncture under anaesthesia. The mice were culled at different times as the high fat diet decreased their survival. The alimentary canal was flushed through with PBS and opened up for counting and measurement of all adenomas. Intestinal mucosa, adenoma, heart, liver,

pancreas, lungs, spleen, kidneys, skeletal muscle from the hind legs and prostate tissue were either snap frozen in liquid nitrogen and stored at -80 °C, or preserved in formalin prior to analysis. Blood samples were collected in lithium heparin tubes, centrifuged (13000 rpm, 10 min, 4 °C) and the plasma supernatant removed for storage at -80 °C until time of analysis. Weights of livers and spleens were recorded as alterations may be found in steatosis or inflammatory infiltration respectively.

#### 2.2.2.1 Plasma glucose assay

A commercially available plasma glucose assay from Cayman Chemical (Tallinn, Estonia) was used for the analysis of plasma glucose levels; whilst it is not specific for murine blood, the company has confirmed its compatibility. Blood was collected in lithium heparin tubes and the plasma samples incubated with glucose oxidase, horseradish peroxidise, 3,5-dichloro-2-hydroxybenzenesulphonic acid and 4-aminoantipyrine at 37 °C for 10 min. Any glucose present was oxidised to δ-gluconolactone with concomitant reduction of glucose oxidase. The reduced glucose oxidase was re-oxidised by atmospheric oxygen to generate hydrogen peroxide, which in turn reacted with 3,5-dichloro-2-hydroxybenzenesulphonic acid and 4-aminoantipyrine to generate a pink colour. Horseradish peroxidase was present as a catalyst to this reaction. The colour intensity was quantified at 514 nm on a FLUOstar OPTIMA plate scanner (BMG Labtech, Offenberg, Germany) as a measure of plasma glucose concentration. Published data suggested that fasting mouse plasma glucose levels were in the order of 100 mg/dL for normal mice and 200-300 mg/dL for diabetic mice (Mordes *et al.* 80). The standard curve for this assay was therefore designed to range from 0-400 mg/dL.

### 2.2.2.2 Tissue and plasma IGF1 assay

A commercial ELISA kit for the measurement of murine IGF1 from R&D Systems (Minnesota, US) was used which consisted of a 96 well plate pre-coated with monoclonal antibodies to IGF1. Protein extracts for analysis were made from *Apc*$^{Min+/-}$ mouse tissue samples by mechanical homogenisation and incubation with Complete lysis-M buffer, Complete mini protease inhibitor cocktail and PhosSTOP phosphastase inhibitor cocktail (Roche Applied Science, Burgess Hill, UK) at a ratio of 100 mg tissue: 1 mL buffer mixture for 10 min at room temperature. The homogenate was centrifuged (13000 *x g*, 10 min, 4 °C) and supernatant removed. then the protein concentration determined against the Bradford standard curve as per Section 2.2.3. The protein extract was stored at -80 °C until time of IGF1 analysis.

An Assay Diluent buffer was added to each well in addition to plasma, tissue samples or the standards, which were present in duplicates. The plate was incubated in the dark at room temperature on a rocking platform for 2 h to allow any IGF1 present to bind to the immobilised antibodies, before being washed 5 times to remove excess reagents. A horseradish peroxidase-linked polyclonal antibody specific to IGF1 was then added prior to incubation again in the dark on a rocking platform at room temperature for 2 h. The plate was again washed 5 times to remove excess reagents before hydrogen peroxide and tetramethylbenzidine were added. The plate was incubated at room temperature in the dark for 30 min to allow colour development in proportion to the level of IGF1. The reaction was terminated by the addition of diluted hydrochloric acid solution, concentration not stated in the kit. The plate was immediately scanned on a FLUOstar OPTIMA plate scanner (BMG Labtech, Offenberg, Germany) at 450 nm and 540 nm to measure the pixel intensity. Absorbance values from 450 nm were

subtracted from values at 540 nm for analysis, as the former wavelength reflects background levels.

Method optimisation using colon and prostate tissue samples from wild type male C57BL/6J mice showed that 100 µg of tissue per well should give values falling within the standard curve. Plasma samples were diluted 500 fold with Calibrator Diluent prior to analysis as per the kit instructions. Interplate validation data from 3 plates for prostate tissue and plasma samples from wild type and $Apc^{Min+/-}$ mice showed that the variation was <20 % as shown in Appendix 8.3. The accuracy of each experiment was confirmed by the value of the IGF1 kit control being within the specified range.

#### 2.2.2.3 Plasma insulin assay

Ultrasensitive mouse insulin ELISA kits were purchased from Mercodia (Uppsala, Sweden) and analysis carried out according to the manufacturer's instructions.

#### 2.2.2.4 Plasma cholesterol and triglyceride assays

Triglyceride and cholesterol assay kits were purchased from Cayman Chemical (Tallinn, Estonia) and the assays were carried out according to the manufacturer's instructions. For the cholesterol assay, test plasma samples were incubated in duplicate at 37 °C for 30 min with an enzyme mixture. This contained cholesterol esterase to convert any cholesterol esters present into cholesterol for analysis, and cholesterol oxidase which oxidised cholesterol to produce ketones and hydrogen peroxide as a byproduct. The hydrogen peroxide was then measured by a colourimetric reaction, by the addition of horseradish peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine. The plate was immediately scanned on a FLUOstar OPTIMA plate scanner (BMG Labtech,

Offenberg, Germany), with fluorescence measured using excitation wavelengths of 565-580 nm and emission wavelengths of 585-595 nm.

For the triglyceride assay, plasma samples were incubated in duplicate at room temperature for 15 min with an enzyme mixture. This contained lipoprotein lipase which hydrolysed any triglycerides present into glycerol and fatty acids, glycerol kinase converted phosphorylate glycerol into glycerol-3-phosphate, and glycerol phosphate oxidase which oxidised glycerol phosphate and produced hydrogen peroxide as a byproduct. The hydrogen peroxide was then measured by a colourimetric reaction, by the addition of 4-aminoantipyrine and *N*-ethyl-*N*-(3-sulfopropyl)-*m*-anisidine. The plate was immediately scanned on a FLUOstar OPTIMA plate scanner (BMG Labtech, Offenberg, Germany), with fluorescence measured at 540 nm.

#### 2.2.2.5 Histological analysis

Haematoxylin and eosin stained sections were obtained from liver, pancreas, spleen, kidney, lungs, skeletal muscle and heart for examination by light microscopy by Dr Peter Greaves, Consultant Histopathologist, University of Leicester. Qualitative data were collected, with abnormalities noted in a descriptive manner.

# 3. Preclinical Results and Discussion

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## 3.1 The effect of dose on the molecular changes induced by resveratrol *in vitro*

Published studies have shown that resveratrol has preclinical activity in models of chemoprevention at concentrations of >5 μM and although little is known at levels lower than this, it is assumed that resveratrol is probably inactive below this threshold. Given that a 1 g oral dose generates plasma levels of 1.4 μM and a dietary intake can be estimated to produce concentrations in the region of 0.01 μM, it was hypothesised that repeated exposure might produce an effect and that even if resveratrol does not inhibit proliferation as a mechanism for chemoprevention, effects may become apparent when another agent is added.

In this chapter, changes in proliferation rate of human HCA7 colon cancer cell caused by resveratrol and malvidin-3-*O*-glucoside at dietary and pharmacological concentrations were investigated. As a chemopreventive rather than a chemotherapeutic agent however, the protective effects of resveratrol against carcinogenesis may be more subtle than the reduction of tumour cell proliferation. A long term study was therefore also carried out where HCA7 cells were subjected to resveratrol treatment at 0.01 μM or 1.4 μM for 3 months, to determine whether these low doses can elicit changes in the genomic profile or the expression of proteins in the apoptosis pathway, and whether the pattern of changes is similar at each dose.

### 3.1.1 Effect of resveratrol and *malvidin-3-O-glucoside on colon cancer cell proliferation*

HCA7 colon cancer cells were incubated in the presence of resveratrol and malvidin-3-*O*-glucoside, which were added fresh daily for 5 days, alone or concurrently, at dietary and pharmacological concentrations. Dietary concentrations of 0.01 µM and 0.4 µM, for resveratrol and malvidin-3-*O*-glucoside respectively, were estimated to be the levels achievable from dietary intake. Pharmacological concentrations of 1.4 µM and 10 µM, for resveratrol and malvidin-3-*O*-glucoside respectively, were chosen based on the plasma levels achievable from previous clinical trials. Oral administration of resveratrol at 1 g resulted in average plasma levels of 1.4 µM (Boocock *et al.* 07a) and this concentration was used in this investigation, as colon concentrations were unknown when this study was designed. For malvidin-3-*O*-glucoside, plasma and colon concentrations after oral administration are unknown, so the estimated 10 µM selected for use in this study was based on colon concentrations achievable from trials of other dietary agents (Hoh *et al.* 06).

Exposure to resveratrol and malvidin-3-*O*-glucoside alone, at dietary and pharmacological concentrations, did not alter the proliferation rate of human HCA7 colon cancer cells as shown in Fig 3.1. Furthermore, there was no evidence that combining the two compounds had any effect on cell proliferation. These data suggest that if resveratrol was active as a chemopreventive agent at dietary doses, these effects may be mediated by resveratrol metabolites rather than the parent compound, as the former has been shown in rodents to be present in the plasma at higher concentrations (El-Mohsen *et al.* 06) and with a slower rate of clearance (Marier *et al.* 02). There is however, no published literature where the chemopreventive effects of resveratrol

metabolites have been studied, and instead investigators have always administered the parent compound to both *in vitro* and *in vivo* cancer models.

Another possibility is that measurable inhibition of cell growth would only be evident if resveratrol, at these low doses, was administered concurrently with other active dietary constituents. Analysis with Calcusyn software for example, has shown that curcumin at 1 μM behaved in a synergistic manner with resveratrol, also at 1μM, in the inhibition of HCT116 colon cancer cell growth *in vitro* (Majumdar *et al.* 09). Similarly in rat glioma cells, apoptosis was induced by incubation with resveratrol at 50 μM for 24 h but at 10 μM, this effect was only seen if the cells were concomitantly exposed to quercetin at 25 μM and if treatment duration was increased to 72 h (Zamin *et al.* 09). Low dose resveratrol was therefore an active chemopreventive agent in rodent glioma cell lines, but only under certain experimental conditions. The underlying mechanisms of action were unclear as although resveratrol and quercetin both reduced phosphorylation of Akt, administration of a PI3 kinase inhibitor showed that this alone was not responsible for the pro-apoptotic effects seen.

Quercetin is a polyphenol that, like resveratrol, is found in grapes and berries. Curcumin, another polyphenol, is found in different food types compared to resveratrol, namely tumeric, but it could also be easily consumed concurrently in the diet. These examples highlight the difficulty, based on dietary data, in identifying the correct combinations of compounds to be administered in order to investigate the hypothesis of additive effects, as we consume so many different food types. Alternatively, combinations may be selected based on similar mechanisms of action, with the hypothesis that multiple agents would augment the overall effect if they all acted on the

same pathway. Most dietary agents, however, have been identified to have multiple mechanisms of actions from preclinical data (Bhat *et al.* 01, Shih *et al.* 04), but the mechanistic hierarchy is not well understood. As discussed in Section 1.1.2.1, this lack of understanding has hindered the pursuit of efficacy biomarkers but it has also impaired the search for potentially efficacious dietary combinations.

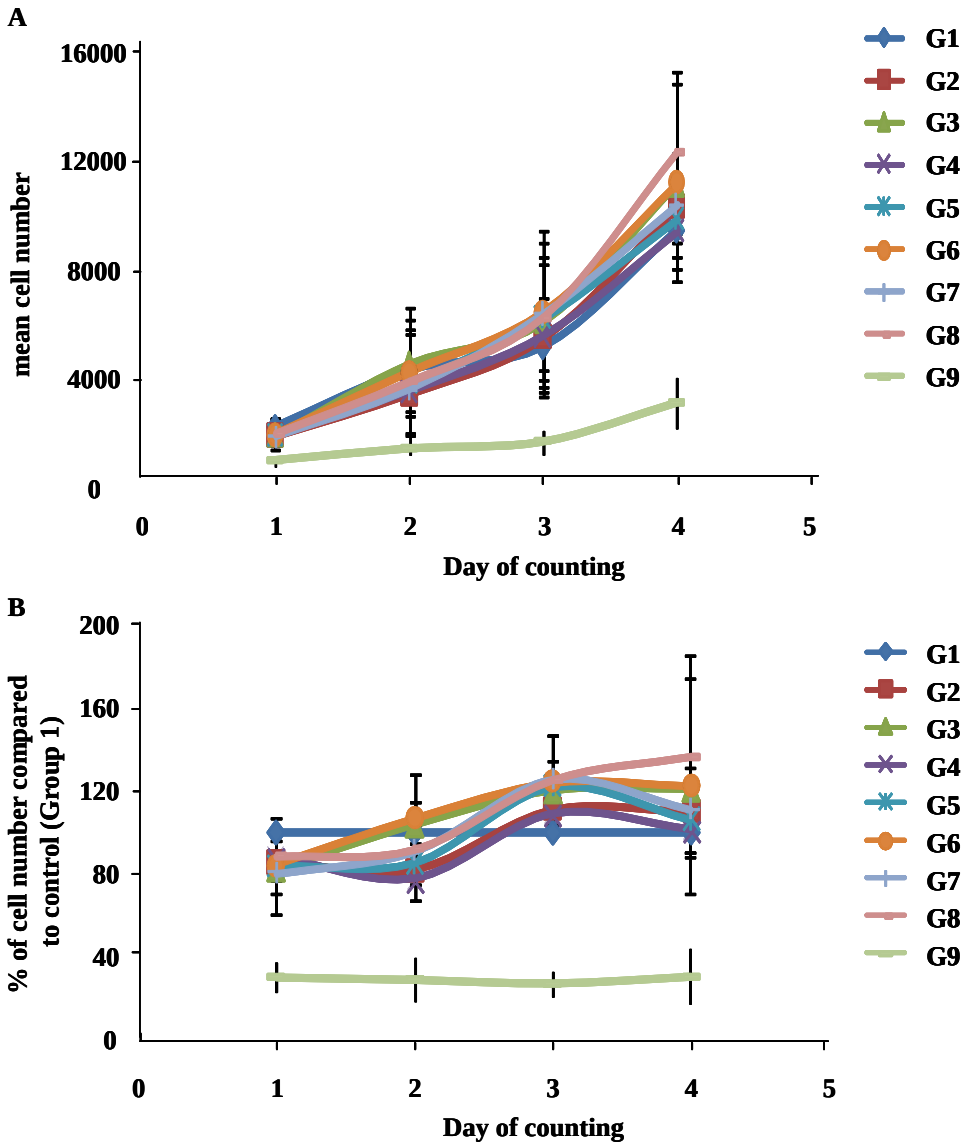


**Figure 3.1 Effects of resveratrol and *malvidin-3-O-glucoside*, alone or in combination, on HCA7 proliferation.** Cells were dosed daily for 5 days. Negative controls were Group 1 = media alone, and Group 2 = vehicle (DMSO) alone. Positive control was Group 9 = 25 µM resveratrol, a concentration of resveratrol which was known from method development data to inhibit HCA7 cell growth. Treatment groups were Group 3 = 0.01 µM resveratrol, Group 4 = 0.4 µM malvidin, Group 5 = 0.01 µM resveratrol + 0.4 µM malvidin. Group 6 = 1.4 µM resveratrol, Group 7 = 10 µM malvidin, Group 8 = 1.4 µM resveratrol + 10 µM malvidin. Average cell number (**A**) and % of cell number compared to Group 1 control (**B**) are shown, with error bars denoting standard deviation (n=4).

As suggested above, the chemopreventive activity of resveratrol at dietary doses may be evident only after prolonged dosing as consistent with the clinical scenario, where resveratrol administration would be long term. The difficulty in applying this to the design of *in vitro* studies is that exposure for months or years, as would occur clinically and *in vivo*, is difficult to practically replicate *in vitro*. In the findings discussed above from Zamin *et al.*, short term exposure was defined as 24 h whereas long term treatment was defined as 72 h. In another study, the actions of resveratrol at 29 nM on endothelial progenitor cell number and intracellular nitric oxide levels were similarly time-dependent. In this experiment by Balesterieri *et al.* however, there was no effect at 72 h but treatment needed to continue for 20 days in order to elicit significant changes (Balestrieri *et al.* 08). Although the aim of this study was to explore the role of resveratrol in cardiovascular disease, the nitric oxide pathway examined could also be relevant in chemoprevention.

It can be seen from these examples that in *in vitro* experiments, it is unclear what the appropriate treatment duration is in order for the results to reflect the *in vivo* mechanisms of actions. An appropriate treatment duration for one study, defined as the duration needed to elicit significant changes, may be too short for another experiment. Although the exposure of cancer cells for days may be sufficient to elicit the same changes *in vitro* as would have been seen after longer treatment durations, this is by no means certain. If treatment duration is designed to be for weeks then it may still be the case that further changes could occur after months, and this is all balanced against the practical difficulties of long term *in vitro* experiments. Although the optimum treatment duration is unknown, *in vitro* effects have certainly been observed *in vivo*, suggesting that some cellular data are relevant in reflecting what happens in animal

models of cancer (Howells *et al.* 07). Based on the hypothesis that longer *in vitro* treatment durations would reflect more accurately what occurs in animal chemoprevention models on a cellular level, an experiment was designed to expose HCA7 colon cancer cells to 3 months of continuous resveratrol treatment. This treatment duration is far longer than any published study and will hopefully yield new information to help determine the mechanistic hierarchy of resveratrol.

### 3.1.2 Effect of resveratrol on the genomic and proteomic profiles of colon cancer cells after chronic treatment

HCA7 human colon cancer cells were continuously exposed to resveratrol at 0.01 µM and 1.4 µM for 3 months. Resveratrol did not alter cell proliferation rate or cell morphology at either concentration, as monitored during weekly cell passage (Fig 3.2). Treatment with resveratrol for months may have, however, altered cellular components or target molecules/pathways, and so this was further investigated by microarray and protein analysis to establish any genomic or proteomic changes.

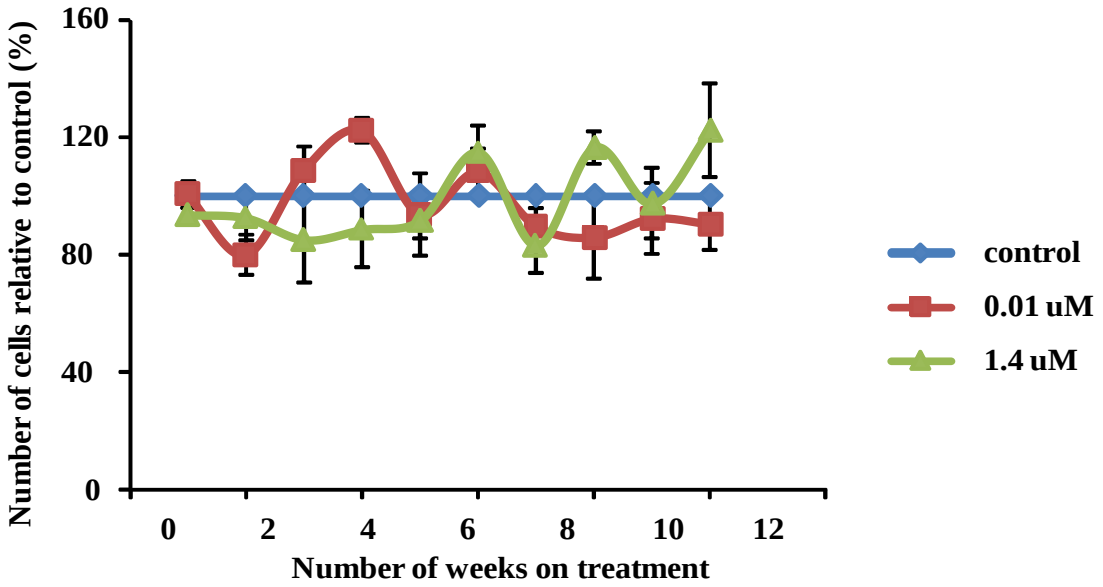


**Fig 3.2 The effect of prolonged resveratrol treatment on HCA7 cell count.** Mean values relative to controls are shown with bars denoting standard deviation (n=6). Resveratrol, at 0.01 µM or 1.4 µM, did not alter the mean cell count after 10 weeks of treatment.

### 3.1.2.1 Genomic changes induced by resveratrol in colon cancer cells

Different methods may be employed to determine significance in the analysis of microarray data. Based on fold changes in transcription levels, no gene was identified as being significantly altered by resveratrol in terms of reduced expression by >50 %, or increased by >100 %. It has been recognised however, that the selection of significance based on fold change alone can be misleading (Jain *et al.* 03). Small fold changes may still be of biological significance if there are gene interactions which amplify the individual changes, or if the genes altered were all involved in the same biological pathway. In an *in vivo* study of all known genes in *Drosophila melanogaster* for example, it was found that ageing induced fold changes in expression by only 20-50 % (Jin *et al.* 01).

An alternative criterion to define significance is to use the t-test, which is a single analysis between two groups of values to determine the likelihood that any change noted was real or due to chance. Using a significance level of $p < 0.05$, 2669 genes in the 0.01 µM group and 935 gene in the 1.4 µM group were deemed to be significantly altered by resveratrol. Although a 5 % false positive detection rate is low, interpretation of microarray data actually requires multivariate analysis and a 5 % false positive detection rate of all tests can result in a large number of false positives. The false discovery rate (FDR) has therefore been advocated as a more stringent criterion for assessing significance, when multiple tests are carried out on a set of genetic data (Chen *et al.* 10). An FDR of 0.05 for example, means that 5 % of significance tests are false positives rather than 5 % of all tests, as would be reflected by $p=0.05$.

Using a criterion of FDR $\leq 0.20$, 11 genes were identified as being significantly altered

by treatment with resveratrol at 0.01 μM. This false discovery rate meant that 9 genes were correctly identified as being significantly altered, but 2 genes were falsely identified. In contrast, using this criterion no genes were altered by exposure to the higher concentration of 1.4 μM. With an FDR level of ≤0.30, 51 genes were significantly altered by dietary resveratrol concentration, increasing the correctly identified genes to 36 and falsely identifying 15. Again, no genes were altered by treatment with resveratrol at a pharmacological concentration. FDR ≤0.3 was selected as the level of significance which resulted in a reasonable number of genes being falsely identified, without excluding a large number of significant genes. It should be noted however, that it is not possible to identify which of the 15 genes out of the 51 genes selected are false positives. This level of FDR has been deemed as acceptable by other authors (Dozmorov *et al.* 08, Raponi *et al.* 04). The fold changes induced by treatment with resveratrol at 0.01 μM were small, with alterations in gene transcription of only ~10-30 %.

An example of an mRNA microarray slide is shown in Fig 3.3. Each dot represents one probe cDNA visualised after hybridisation with target cDNA, with the colour indicating whether the expression of that gene has been increased, decreased or unchanged by resveratrol. The functions of 24 of the genes identified as being significantly altered in this study were unknown, as per the Genecards version 3 database (http://www.genecards.org/). For the remainder, the functions identified included roles in apoptosis, glucose transportation, protein transportation, cell adhesion and DNA repair as shown in Table 3.1. Some of these genes exerted multiple functions and so computational analysis was carried out to evaluate the relative importance of each gene in each pathway, using the following programs:

http://www.ensembl.org/index.html

http://www.targetscan.org/

Three pathways were subsequently identified as being significantly altered by resveratrol treatment at 0.01 μM, namely apoptosis, glucose transportation and cell adhesion ($p < 0.05$).

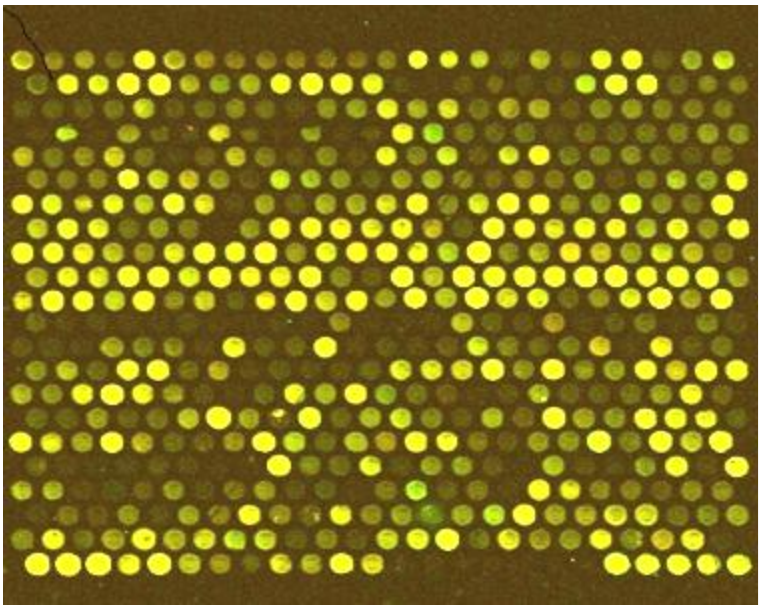

**Figure 3.3 Example of an mRNA microarray slide.** Thousands of cDNA probes have been printed on the slide, each visualised as one circle after hybridisation with the target cDNA which has been labelled with red or green fluorescent dyes, for the control and the test sample respectively. Genes which were over or under expressed were shown by green or red/brown spots, whilst the yellow dots represented genes whose expression were unchanged with equal balance of the green and red dyes. The colours used to label the control and test cDNA were then reversed to confirm the results seen in this slide. Data from two slides, termed the forward and the reverse slide, were then analysed together to identify genes whose expression was altered

| Table 3.1 Gene expression significantly altered (FDR ≤0.30) by resveratrol treatment of HCA7 colon cancer cells at 0.01 μM for 3 months | | | |
|---|---|---|---|
| Pathway | Gene name | Function | Fold change |
| Glucose transport | ASB6 (Ankyrin repeat + SOCS Box-containing) | Involved in protein ubiquitination and the translocation of the GLUT4 sodium-independent glucose receptor, from its intracellular location to the plasma membrane to increase glucose uptake. | 1.26 |
| | RHOQ (Ras Homolog gene) | Encodes TC10 which is involved in insulin-stimulated glucose uptake by aiding translocation of the GLUT4 sodium-independent glucose transporter to the plasma membrane. TC10 also controls intracellular vesicle transport by its effects on actin polymerisation. | 0.90 |
| | SLC2A8 (Solute Carrier, family 2) | Encodes the GLUT8 hexose bidirectional transporter which is mainly expressed in testis and brain tissues. | 0.90 |
| Apoptosis | TNFRSF10B (Tumour Necrosis Factor Receptor Super Family) | Encodes DR5 (Death Receptor) which is activated by TRAIL (TNF-related Apoptosis Inducing Ligand), with downstream effects on FADD (Fas-associated protein with Death Doman) and caspase 8 to result in apoptosis in tumour cells. DR5 activation increase T cell selectivity and cytotoxicity. | 1.28 |
| | TNFRSF8 (Tumour Necrosis Factor Receptor Super Family) | CD30 protects against autoimmunity by limiting the proliferative potential of autoreactive CD8 effector T cells. The CD30 receptor activates NFκβ to regulate BIRC3 to inhibit apoptosis. | 1.31 |
| | TRPM4 (Transient Receptor Potential cation channel, subfamily M) | Causes membrane depolarisation, with the subsequent increase in intracellular calcium level resulting in insulin release from pancreatic β cells, apoptosis, IL-2 production and cardiac arrhythmias. | 0.85 |
| | BIRC3 (Baculoviral Inhibitor of apoptosis Repeat- Containing) | Inhibit apoptosis by binding to TRAF1 and TRAF2 (TNF Receptor Associated Protein). Involved in B cell MALT lymphoma, myeloma, cervical, prostate and lung cancers. Gene expression controlled by TNFRSF8 (Tumour necrosis factor receptor super family). | 1.13 |
| | SPRY1 (Sprouty homolog 1) | SPRY1 is an upstream antagonist of RAS and has been suggested to have tumour suppressor function, with mutations commonly occurring in childhood embryonal rhabdomyosarcoma (Schaaf 10). | 0.88 |
| | MAPK10 (Mitogen-Activated Protein Kinase) | Mediates neuronal apoptosis. | 1.17 |

| Table 3.1 continued | | | |
|---|---|---|---|
| Pathway | Gene name | Function | Fold change |
| Cell adhesion | CDH26 (Cadherin-like) | Calcium dependent cell to cell adhesion in solid tissues. | 0.78 |
| | UBXD5 (Ultrabiothorax Domain-containing) | Affects protein ubiquitination and in the promotion and inhibition the RHO family of GTPases which regulate actin function. | 0.91 |
| Protein transport | GORASP2 (Golgi Reassembly Stacking Protein) | Affects the stacked structure of the Golgi apparatus to control the sorting and modification of proteins exported from the endoplasmic reticulum. | 0.87 |
| | GPIAP1 (GPI-Anchored membrane Protein) | Encodes glycolipid to direct proteins to the endoplasmic reticulum and subsequent release to the plasma membrane. | 1.03 |
| | PPIL3 (Peptidylprolyl isomerase, Cyclophilin-like) | Act as either catalysts or molecular chaperones in protein-folding events. | 0.72 |
| | RAB10 (Ras oncogene family member) | Controls protein transport. | 0.86 |
| DNA repair | ACTL6B (Actin-like) | Chromatin-mediated transcription repression, vesicular transport and spindle orientation. | 0.82 |
| | RAD51L3(Rad51-Like gene) | Catalyses homologous pairing between single and double-stranded DNA in recombinational repair. | 0.83 |
| | RASSF1 (Ras Association domain family) | Similar to the RAS effector proteins with tumour suppressor functions. RASSF1 interacts with XPA, a DNA repair protein, and cyclin D1. | 0.85 |
| Others | DLEC1 (Deleted in Lung and Oesophageal Cancer) | Unclear but thought to be involved in cell cycle control. Aberrant transcription in lung, oesophagus and kidney cancers. | 1.34 |
| | PRCC (Papillary Renal Cell Carcinoma) | PRCC gene translocation occurs in renal cancers to encode a mitotic checkpoint protein (MAD2B). | 1.24 |

MicroRNA microarray analysis identified one gene as being significantly increased using a criterion of FDR ≤0.30 after exposure to resveratrol at 0.01 μM, but no effect at the pharmacological concentration of 1.4.μM. The miRNA identified was hsa-miR-27b*. (During transcription, miRNA exist transiently as duplex intermediates. One of these strands is denoted a * symbol to differentiate between them and it remains unclear how the cell selects one strand for degradation.) MiR-27b* has been purported to control the ST14 (suppressor of tumourigenicity 14) gene to influence breast cancer cell proliferation and migration (Wang *et al.* 09c). MiR-27b* has also been shown to alter DNA methylation in metastatic melanoma cells (Costa *et al.* 09). In benign cell lines, miR-27b* altered pathways that may be involved in carcinogenesis, namely metalloproteinase 13 (Akhtar *et al.* 10) which could influence tumour cell invasion, and peroxisome proliferator-activated receptor gamma (Jennewein *et al.* 10) which is involved in inflammation. The dose response pattern from these miRNA data is similar to the mRNA findings, suggesting that resveratrol truly exerted different effects at different doses. This pattern has also been noted in the published literature, with an *in vitro* study in human fibroblasts showing that resveratrol at 0.2 μM and 1 μM for 46 days significantly altered the expression of the INK4a gene with an inverse dose response (Stefani *et al.* 07). Expression of the INK4a gene has been shown to increase in many organs with ageing, and the authors proposed that resveratrol may impede ageing *in vivo* via this mechanism.

The effects of resveratrol on the microarray profile of colon cells, benign or malignant, have never been previously investigated *in vitro* or *in vivo*. The potential biological significance of the transcriptional changes seen in this study is unclear. Some of the glucose transporters affected, for example, were bidirectional and so it is difficult to

conclude whether resveratrol would increase or decrease intracellular glucose levels *in vivo*. There are no published data on the actions of resveratrol on glucose uptake into colon cancer cells, but the available information suggests that it can inhibit glucose uptake in other types of human cancer cells including leukaemia, lymphoma and ovarian tumour cells at 20-120 μM (Park *et al.* 01, Faber *et al.* 06, Kueck *et al.* 07). The mechanisms of actions identified included decreased phosphorylation of the downstream enzymes of the insulin pathway, as well decreased transcription of some glycolytic enzymes. By decreasing glucose availability, resveratrol may mediate its cancer chemopreventive actions by reducing cancer cell growth and activity.

The published literature also suggests that resveratrol can exert different effects on the same cell type, and that its actions on glucose transportation may be specific to the experimental conditions. In rodent hepatocytes isolated from diabetic animals *ex vivo* for example, resveratrol at 0.01-1.7 μM mimicked the actions of insulin, promoting glucose uptake and glycogen synthesis by unclear mechanisms (Su *et al.* 06). In contrast, resveratrol inhibited the activity of insulin when incubated concomitantly with rat hepatocytes *ex vivo* (Zhang *et al.* 06). At a similar concentration of 1 μM, resveratrol decreased phosphorylation and mRNA levels of Akt and MAPK, two intracellular messengers in the insulin effector pathway. These different actions on the same cells *ex vivo*, from healthy and diabetic animals, suggest that resveratrol elicits different responses in healthy and diabetic cells, and that diabetes mellitus induces changes in cell behaviour that persist despite the interruption to the intact insulin axis by culling. It is unknown as to whether the behaviour of immortalised cell lines is similar to that of healthy or diabetic cells, and therefore it is difficult to use *in vitro* findings, from immortalised cell lines, to predict the effects of resveratrol on glucose

uptake *in vivo*.

The expression of genes encoding both pro and anti-apoptotic proteins in this study were altered by resveratrol, but it is difficult to conclude whether the overall effect was to promote or inhibit apoptosis. Although the total cell count was unaffected by resveratrol exposure, apoptosis may still have been increased if there was an accompanying increase in proliferation rate. It was hypothesised that proteomic analysis may detect more subtle changes in apoptotic protein levels. This was therefore investigated using antibodies specific to proteins in the apoptotic pathway, as discussed in Section 3.1.2.2.

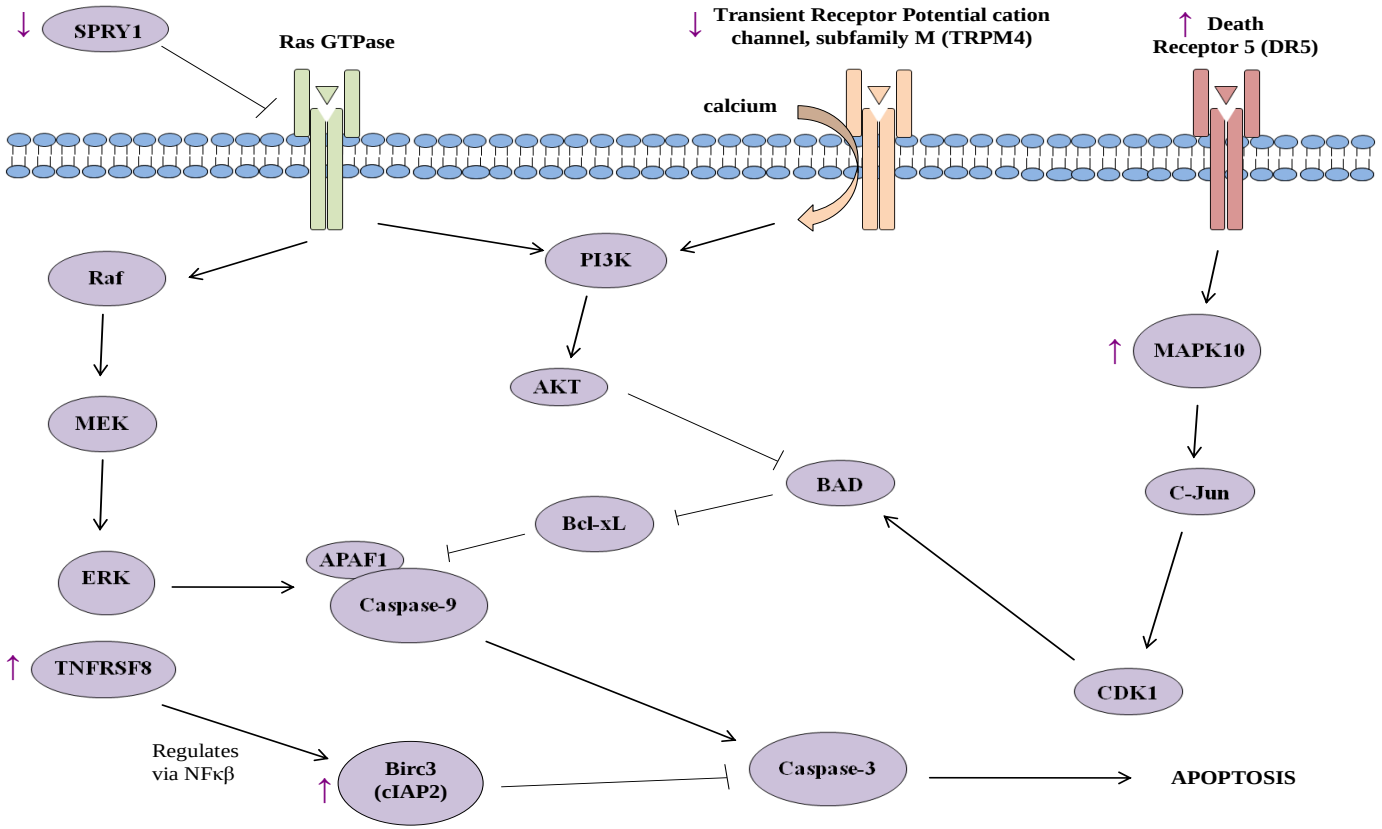


**Figure 3.4 Effectors in the apoptotic pathway altered in HCA7 cells by resveratrol.** HCA7 cells were treated with 0.01 µM resveratrol for 3 months and mRNA extracted for microarray analysis. The purple arrows indicate whether gene expression was found to be increased (↑) or decreased (↓) by resveratrol. The black arrows indicate stimulatory effects (→), whilst the ------| symbol indicates inhibitory effects. APAF = Apoptotic protease activating factor 1, BAD = bcl2-associated death promoter, BIRC = Baculoviral IAP repeat-containing protein, CDK = cyclin dependent kinase, ERK = extracellular signal-regulated kinase, MAPK = mitogen-activated protein kinase, PI3K = Phosphoinositide 3-kinases, SPRY1 = sprouty homolog 1, TNFRSF = tumour necrosis factor super famil

### 3.1.2.2 Proteomic changes induced by resveratrol in colon cancer cells

Apoptosis is the process of programmed cell death which occurs as part of normal cell growth. Morphologically, the cells undergo changes including blebbing, breakdown of the cytoskeleton, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation. Resistance to apoptosis is one of the classical characteristics of a cancer cell which differentiates it from a benign cell (Hanahan *et al.* 00). Resveratrol treatment at 0.01 µM or 1.4 µM for 3 months did not significantly alter the levels of apoptotic proteins in HCA7 colon cancer cells as shown in Fig 3.5. This contradicts the microarray data which suggested that relevant to solvent treated control cells, the transcription of some apoptotic proteins as shown in Fig 3.4 were altered at a genomic level by exposure to resveratrol at 0.01 µM.

It is not unusual for genomic data to be discordant with proteomic results. In a clinical study of lung adenocarcinoma samples for example, correlating protein level changes were seen in only 21 % of the genes identified as being significantly altered compared to benign tissue (Chen *et al.* 02). One possible reason for this discrepancy may be post-translational regulation, where proteolysis could occur to control the levels of active protein, thereby resulting in disproportionately low protein concentrations compared to mRNA levels (Chen *et al.* 02). Alternatively, an *in vitro* study in MCF7 breast cancer cells showed that the discordance between mRNA and protein levels was due to alternative splicing (Bitton *et al.* 08). This is a process where mRNA, which has been spliced to remove the non-coding introns, is reconnected in different manners, resulting in one gene encoding multiple protein isoforms or indeed multiple proteins.

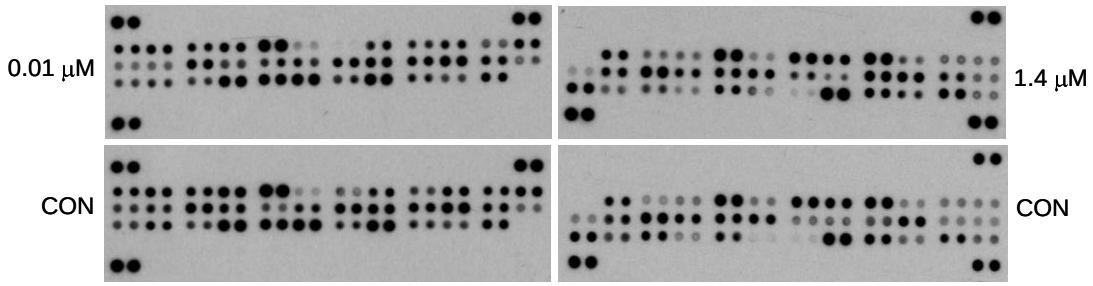


**Figure 3.5 Spot blot analysis for the effects of resveratrol on apoptotic protein levels in the HCA7 cells.** Antibodies to the different proteins were attached to the membrane in duplicates as per Table 2.7, with the density of each spot reflecting the amount of protein present. The blots on the left are after exposure to 0.01 μM of resveratrol for 3 months compared to the control group of solvent only, whilst the blots on the right are for the 1.4 μM group versus control. Resveratrol did not alter the protein levels at either concentration as per analysis of the pixel densities.

Although there are multiple studies showing that resveratrol affects the apoptotic pathway in colon cancer cells (see Table 1.7), the results presented here do not contradict the published literature as the experimental designs were different. All other investigators have used far higher concentrations of resveratrol, sometimes up to 25000 times higher than the dietary concentration in this project (Kim *et al.* 09). The treatment durations in the published literature were also much shorter at only hours or days, although the purpose of such a prolonged exposure in this study was hypothesised to result in a positive effect, rather than a negative outcome. Since the mechanisms of action of resveratrol are many and of unclear hierarchy and relevance, it is theoretically possible that a shorter treatment duration could have resulted in a positive effect which was then lost with time.

This idea, however, is not usually investigated as once a positive outcome is noted and the hypothesis has been confirmed, investigators would not usually increase the treatment period to explore the duration of the effect. Circumstantial data from the literature, however, support that this could be a theoretical possibility in that the

characteristics of immortalised cell lines have been shown to change with passaging. Human LNCaP prostate cancer cells for example, respond differently *in vitro* to androgen and steroids depending on their passage number (Esquenet *et al.* 97). These differences in genotypic and phenotypic expression of transformed cell lines are thought to evolve over time, due to the stress of being cultured under artificial conditions. This and other studies however, defined low passage numbers as ~25 and high passage numbers as ~80. These results are therefore not applicable to the current project, and the negative data from this experiment are likely to be a genuine discordance between genomic and proteomic findings.

In conclusion, the *in vitro* data presented above suggested that resveratrol at 0.01 µM exerted different effects on human HCA7 colon cancer cells after 3 months of treatment, compared to the higher concentration of 1.4 µM. Low dose resveratrol did not affect tumour cell proliferation rate but may alter glucose transport, apoptosis and cell adhesion based on microarray data. Changes in the expression of genes involved in these pathways may contribute to the purported chemopreventive effects of resveratrol in colorectal carcinogenesis. Proteomic data, however, have not confirmed the involvement of the apoptosis pathway but this does not necessarily negate the relevance of the genomic findings. Discordance may for example, be due to alternative splicing of mRNA where exons are spliced and reconnected, resulting in a single gene encoding for multiple proteins which may be different to the ones that were measured.

### 3.2 The effect of dose on the pharmacokinetic profile and the pharmacodynamic actions of resveratrol *in vivo*

The pharmacokinetic (PK) profiles and tissue distribution of resveratrol in F344 rats was investigated after administration of a single dietary or pharmacological dose by gavage. This study was carried out to identify potential target organs for the chemopreventive actions of resveratrol, as determined by tissues with high concentrations. A second *in vivo* study was designed based on the *in vitro* data from this project, suggesting that resveratrol may exert effects on the glucose transportation, apoptosis and cell adhesion pathways in colorectal cancer cells. The ability of resveratrol to exert efficacy *in vivo* and inhibit adenoma formation was investigated in $Apc^{Min+/-}$ mice, after long term administration in the diet at either dietary or pharmacological dose, with either standard or high fat diet. A high fat diet was selected as this is known to be a risk factor for the development of diabetes mellitus, and it is clinically relevant since such a diet is commonly consumed in the Western World (Ma *et al.* 07).

#### 3.2.1 The effect of a single dose on the pharmacokinetic profile of [$^{14}$C]-resveratrol in F344 rats after administration by gavage

[$^{14}$C]-Resveratrol was administered to F344 rats via gavage as a single dose and animals were culled at 8 time points post dose. Gavage was chosen as the route of administration to mimic the oral intake of resveratrol, as would be necessary for the long term treatment needed in the chemopreventive setting. The doses administered were chosen to potentially result in the same tissue levels as in the clinical trial. Blood and major organs were harvested and the total [$^{14}$C]-resveratrol equivalent concentrations were calculated after subtraction of background levels of [$^{14}$C] in control

animals, which were administered vehicle only. The limits of detection by liquid scintillation counting, determined as the mean of blank samples + 2 Standard Deviations (SD), were 0.006 and 0.85 pmol resveratrol/mg tissue at 0.07 mg/kg and 14.3 mg/kg respectively. The pharmacokinetic profiles of [$^{14}$C]-resveratrol in various tissues are shown in Fig 3.6. Maximum plasma concentration ($C_{max}$) and area under the plasma concentration-time curve (AUC) values for each organ at the two doses are shown in Fig 3.7.

Overall, considering all organs together, AUC and $C_{max}$ increased linearly with dose, with a 200 fold increase in resveratrol dose resulting in increases in $C_{max}$ and AUC of 50-180 and 50-150 fold respectively. The pattern of organ distribution for total [$^{14}$C]-resveratrol equivalent concentrations was similar at both doses, with high [$^{14}$C]-resveratrol equivalent levels found in the prostate, gastrointestinal tract, liver and kidney. The highest $C_{max}$ values were found in stomach tissue, as expected since resveratrol was administered via gavage, with levels of 0.5 and 56.8 pmol resveratrol/mg tissue at the dietary and pharmacological dose, respectively. Lower [$^{14}$C]-resveratrol equivalent concentrations were found in the lung, heart and spleen at the pharmacological dose, but levels in these tissues were actually below the limit of detection at the dietary dose. These findings are consistent with other pharmacokinetic studies of resveratrol or [$^{14}$C]-resveratrol, administered intravenously or orally as single or multiple doses in rodents, where the highest concentrations were found in these organs of absorption, metabolism and excretion (Asensi *et al.* 02, Vitrac *et al.* 03, Wenzel *et al.* 05, Juan *et al.* 10).

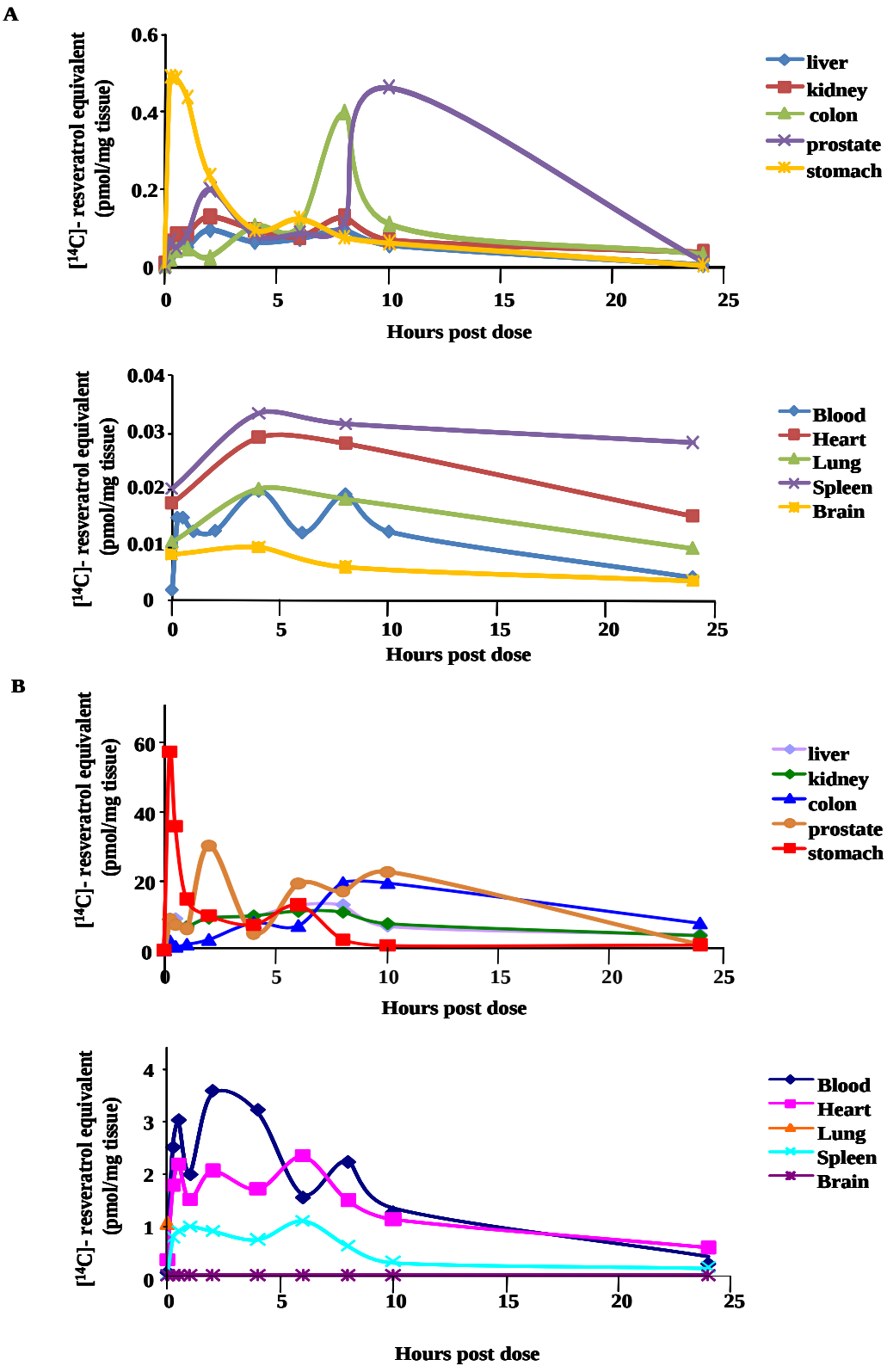


**Figure 3.6 Mean total [$^{14}$C]-resveratrol equivalent concentrations in organs of F344 rats.** A single dose of [$^{14}$C]-resveratrol at 0.07 mg/kg (**A**) or 14.3 mg/kg (**B**) was administered via gavage. Total [$^{14}$C]-resveratrol equivalents were measured in blood and major organs by liquid scintillation counting, which included the parent compound and all metabolites. Mean values only are shown above for clarity (n=3). The standard deviations for each organ are given in Section 8.1 of Appendix.

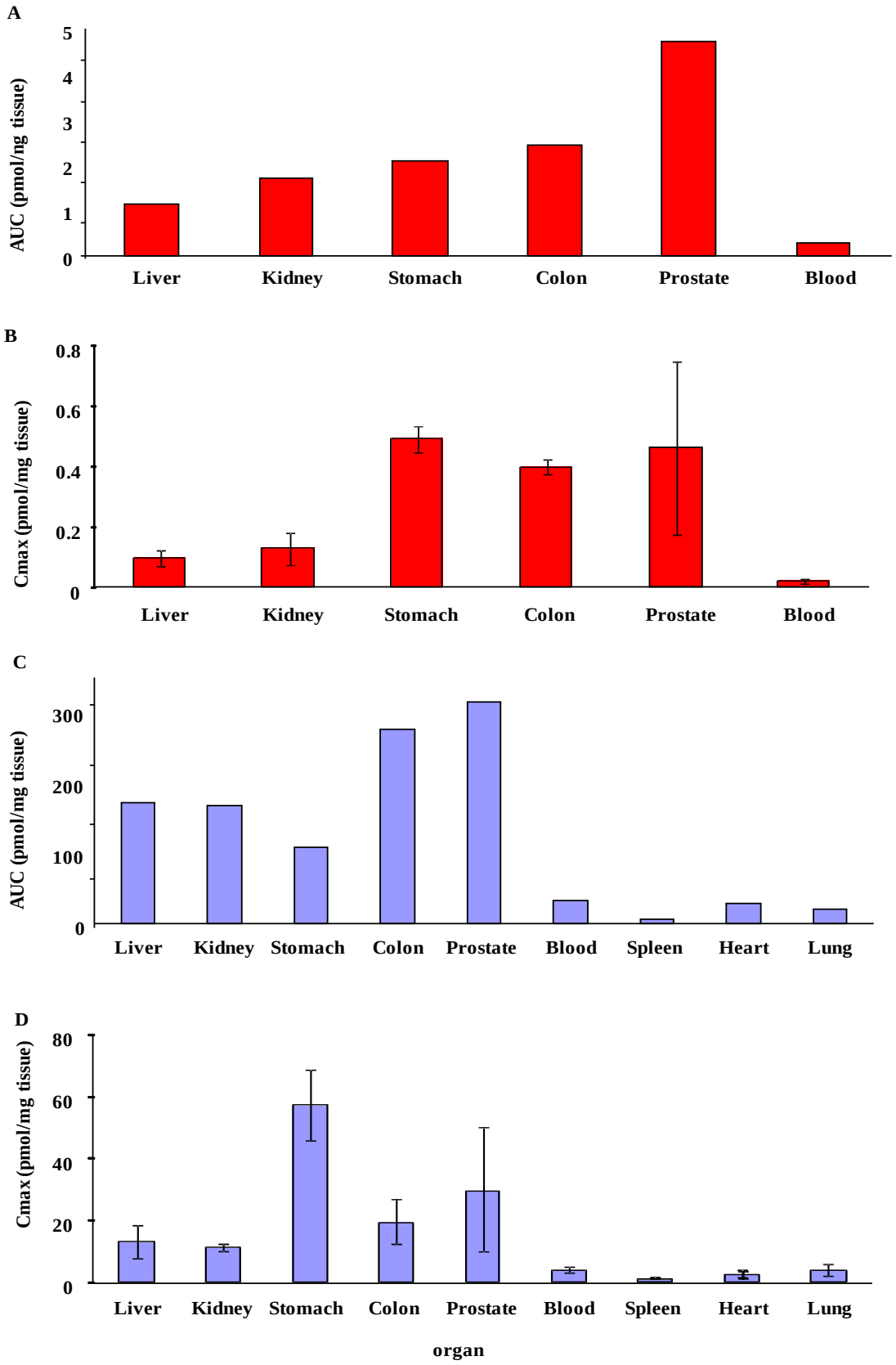


**Figure 3.7 Mean AUC and $C_{max}$ values of total [$^{14}$C]-resveratrol equivalent concentrations for each organ in F344 rats.** A single dose of [$^{14}$C]-resveratrol at 0.07 mg/kg (**A, B**) or 14.3 mg/kg **C, D**) was administered via gavage (n=3). Error bars cannot be calculated for AUC as 3 different animals were sacrificed at each time point to construct the curve.

[$^{14}$C]-Resveratrol equivalent concentrations were noted in this study to exhibit a bimodal distribution in multiple organs analysed at both doses. The timings of the first and second peaks were variable between the different organs, but were similar in the same organs at different doses. These results are consistent with published studies and some authors have postulated that this is due to enterohepatic recirculation (Goldberg *et al.* 03).

[$^{14}$C]-Resveratrol equivalent concentrations were below the limit of detection in brain tissue in this study at both doses. Other studies however, have reported that resveratrol and its metabolites were detectable in the brain, although levels were the lowest amongst all organs analysed (Asensi *et al.* 02, Juan *et al.* 10). This difference may be due to experimental designs differing in terms of the route of administration, or the higher doses used in the published studies. Asensi *et al*. also investigated earlier post dose time points, with levels detectable only at 5 and 10 min. At 30 min post dose, resveratrol was no longer detectable which is consistent with the results from this study. These data from the published studies and from this project suggest that the blood brain barrier impedes, but does not completely prevent, the movement of resveratrol and its metabolites into the central nervous system. The ability of resveratrol to permeate the blood brain barrier has also been inferred from efficacy studies *in vivo*, where orally administered resveratrol impaired the development of Parksinon's disease induced by 6-hydroxydopamine directly injected into rat brain tissue (Jin *et al.* 08).

In this study, high [$^{14}$C]-resveratrol equivalent levels were also found in prostate tissue where there was a bimodal distribution at both doses, with peak concentrations at 2 h and 10 h post dose. Indeed, prostate tissue AUC values were the highest for all the

organs analysed, at 4.7 and 314.8 pmol resveratrol/mg tissue/hr for the dietary and the pharmacological dose respectively. There have been no published data for the levels of resveratrol or its metabolites in prostate tissue, or any investigations as to the method of uptake or metabolism in this organ. The novel results from this study suggest that prostate may be a target organ for the chemopreventive actions of resveratrol, and that resveratrol may be bound to proteins in prostate tissue as demonstrated by high concentrations persisting even after plasma levels had decreased.

This hypothesis is supported by published data showing that resveratrol binds to proteins in hepatic cells and in serum. In hepatic HepG2 cells, resveratrol uptake was localised to the cytoplasm and the nucleolus by the use of an attached fluorescent dye (Lancon *et al.* 04). This specific localisation suggests that resveratrol may have been bound to intracellular proteins. In another study, HPLC analysis of resveratrol in cell-free culture medium showed that 50 % was bound to serum proteins after 2 h, and all was bound after 24 h of incubation (Jannin *et al.* 04). This binding was augmented by the presence of fatty acids, and binding affinity was greater to lipoproteins than lipoprotein-free proteins.

Extravascular accumulation of resveratrol has also been suggested by higher AUC values in gastrointestinal, hepatic and renal tissue as compared to blood [$^{14}$C]-resveratrol equivalent levels. This is consistent with the published data and although the presence of resveratrol and its metabolites was not differentiated in this study, other rodent investigations suggest that this extravascular accumulation is due to metabolites rather than the parent compound (Wenzel *et al.* 05, Juan *et al.* 10). This pattern was found in all organs analysed except for brain tissue, where parent resveratrol was the

dominant form. This difference in metabolic profile between organs could be due to different resveratrol metabolism in brain tissue, or differences in the permeability of resveratrol and its metabolites through the blood brain barrier.

The plasma [$^{14}$C]-resveratrol equivalent levels detected in this study on the other hand, are likely to be related to the parent compound immediately post dose and then due to the presence of metabolites thereafter, as demonstrated in multiple published rodent studies utilising HPLC separation. These studies showed that plasma resveratrol levels were only detectable for minutes post dose, whereas glucuronides and sulphates were detectable for hours (Yu *et al.* 02, Asensi *et al.* 02, Juan *et al.* 10). The slow clearance of the metabolites from the plasma again suggested that there may be extravascular pools of metabolites in the tissues to account for their persistence.

### 3.2.2 The effect of dose on the pharmacodynamic actions of resveratrol in male *Apc$^{Min+/-}$* mice on standard or high fat diet

Male *Apc$^{Min+/-}$* mice were exposed from weaning to resveratrol, at 0.07 or 143 parts per million (ppm), added into the diet with 7 % or 60 % of calories derived from fat. Initial studies showed that the high fat intake decreased animal survival as consistent with the existing literature (Wasan *et al.* 97, Baltgalvis *et al.* 09), so treatment duration was selected to be 13 and 10 weeks for the standard and the high fat diet groups respectively, in order to ensure that all animals from the same group could be culled at the same time. Intestinal tumour burden was noted, and blood and tissue samples were harvested for biomarker analysis.

### 3.2.2.1 Effects of dietary modulation on food intake and animal weight

Animal weights across all groups were similar at the beginning of the study as demonstrated in Fig 3.8. As the experiment progressed however, weights for mice in Groups 4 (high fat) and 5 (high fat and dietary dose resveratrol) increased at a slower rate then significantly decreased beyond week 12 compared to the other groups, as shown in Fig 3.8B.

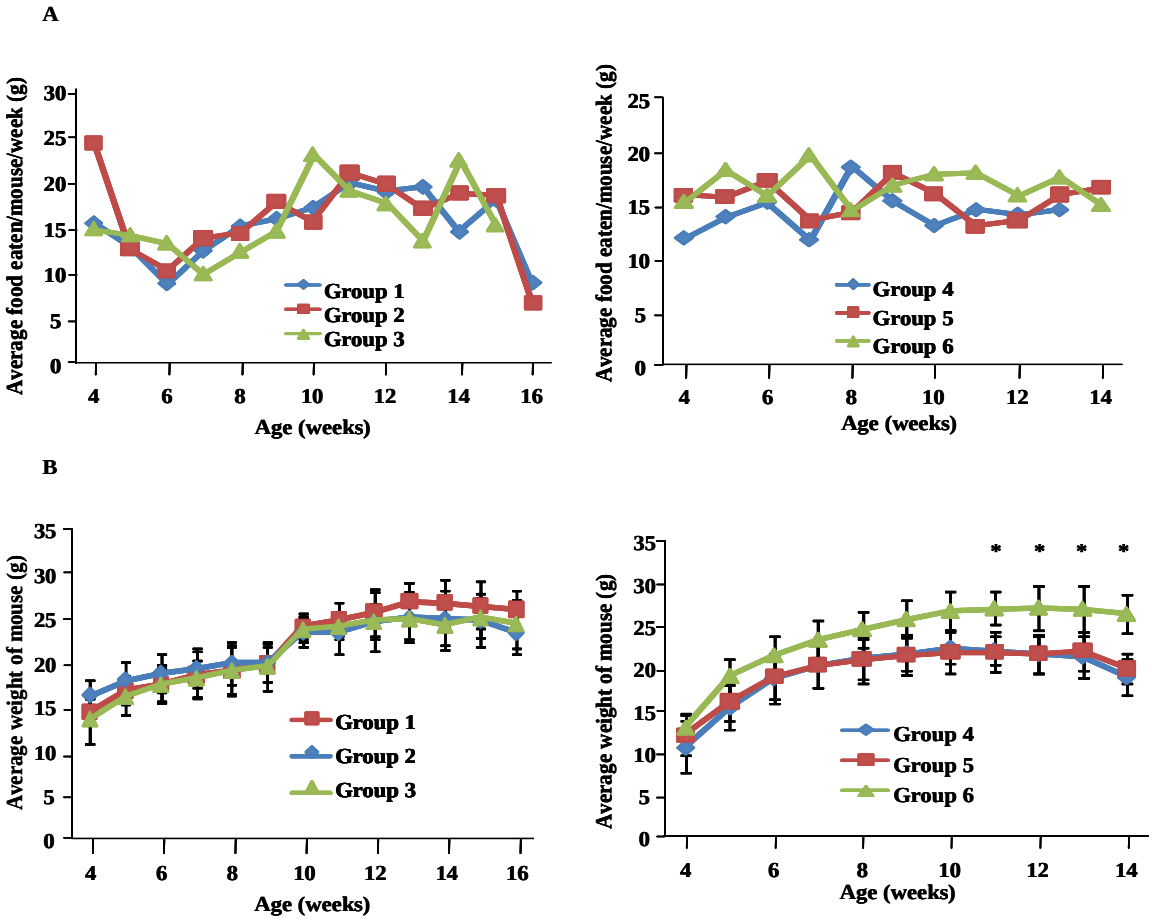


**Figure 3.8 Average food intake (A) and animal weights (B) of C57BL/6J *Apc*$^{Min+/-}$ mice.** Standard chow (G1), standard chow + dietary dose resveratrol (G2), standard chow + pharmacological dose resveratrol (G3), high fat chow (G4), high fat chow + dietary dose resveratrol (G5), and high fat chow + pharmacological dose resveratrol (G6), n=12 per group. These data are divided into the standard fat or high fat groups for clarity, with error bars denoting standard deviations. Significant differences between the groups are denoted with an asterisk (*).

Measurement of tumour burden showed that this weight loss was not due to increased adenoma volume or numbers (see Section 3.2.2.2, Fig 3.9). It is also difficult to attribute the weight loss to the high dietary fat intake, as animals in Group 6 (high fat

and pharmacological dose resveratrol) were not affected. One possibility for this anomaly may be that within the high fat groups, high dose resveratrol increased consumption of high fat diet, thereby maintaining body weights and possibly contributing to the increased tumour burden seen in Group 6, if the dietary fat acted as a pro-carcinogen (see Section 3.2.2.2, Fig 3.9). This hypothesis is supported by the fact that although there is no overall significant difference in food intake between the treatment groups (Fig 3.8A), there is a trend for animals in Groups 4 and 5 to consume slightly less food than mice in Group 6.

### 3.2.2.2 Effects of resveratrol and high fat diet on tumour burden

Tumour volume and tumour numbers for *Apc*$^{Min+/-}$ mice treated with standard or high fat diet, with or without resveratrol at different doses are shown in Fig 3.8. Tumour burden was similar between animals in Groups 1 (standard diet) and 4 (high fat diet). However, the difference in treatment duration between these groups was a significant confounding factor when drawing conclusions as to the role of dietary fat in this animal model of colonic carcinogenesis. Mice in the high fat groups were on the study diet for only 10 weeks as they had to be culled early due to weight loss, but it is highly likely that their tumour burden would have increased if treatment was for 13 weeks, as was the case for animals in the low fat groups.

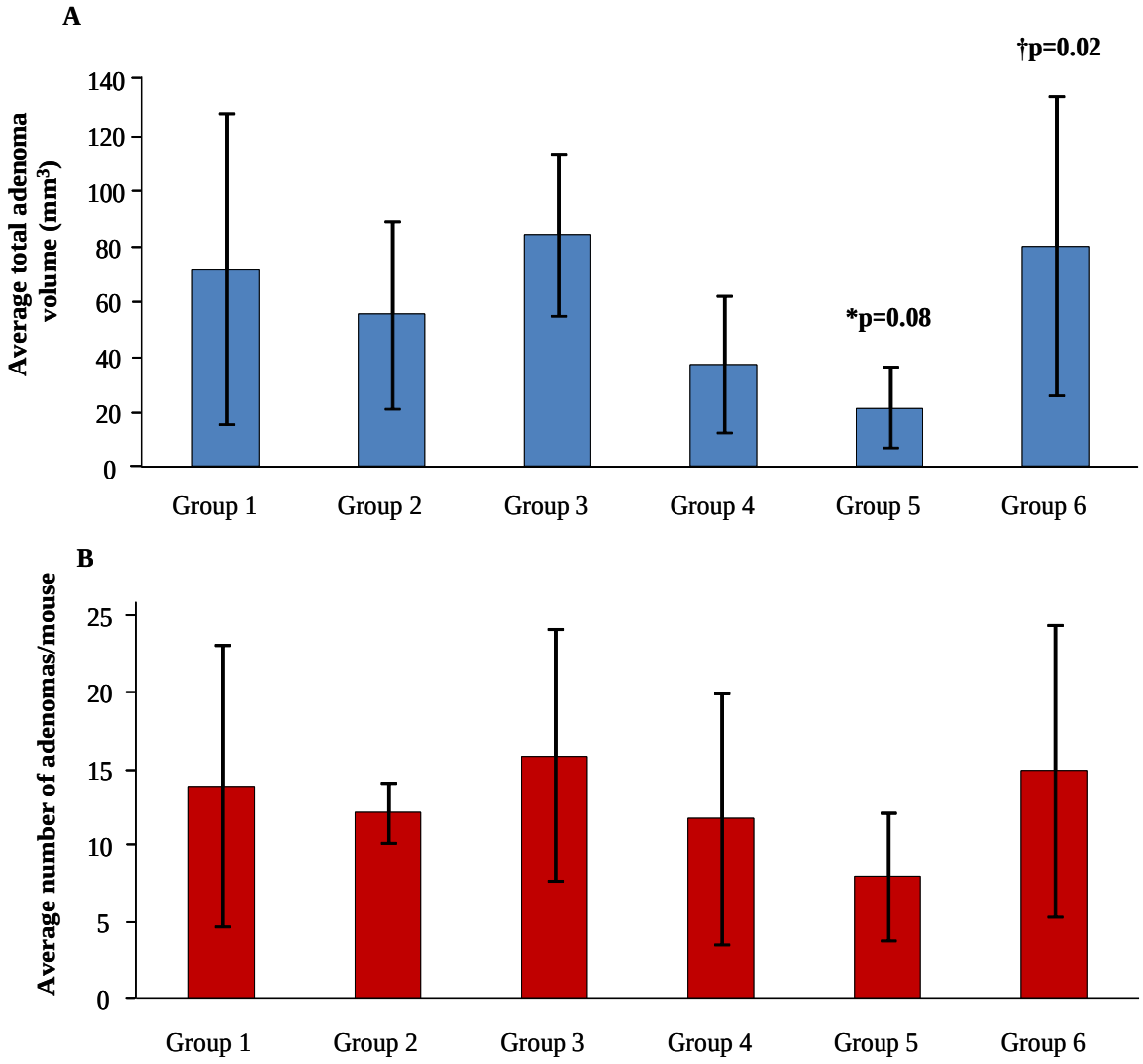


**Figure 3.9 Average tumour volume (A) and adenoma numbers (B) in C57BL/6J *Apc*$^{Min\ +/-}$ mice.** Standard chow (G1), standard chow + dietary dose resveratrol (G2), standard chow + pharmacological dose resveratrol (G3), high fat chow (G4), high fat chow + dietary dose resveratrol (G5), and high fat chow + pharmacological dose resveratrol (G6), n=12 per group**.** Bars denote standard deviations. The differences between the groups are not of statistical significance except for a trend towards significance between groups 4 and 5 (***** p=0.08), and between groups 4 and 6 († p= 0.02) as assessed by the T test.

The inter-individual differences in tumour burden led to wide standard deviations in this study, with this variable phenotypic expression also noted by other investigators of *Apc*$^{Min+/-}$ mice (Sale *et al.* 05). Despite this, the results from this study suggest that resveratrol at different doses exerted different actions. In animals on high fat diet, dietary dose resveratrol decreased mean tumour number and burden by 34 % and 43 % respectively. Pharmacological dose resveratrol on the other hand, increased mean

tumour number and burden by 25 % and 120 % respectively. These effects of resveratrol were also seen in animals on a standard fat diet but not to a statistically significant level, where dietary and pharmacological dose decreased and increased mean tumour burden by 23 % and 16 % respectively.

There have been three published studies of the effects of resveratrol on intestinal carcinogenesis in *Apc*$^{Min+/-}$ mice, where it was administered either in food or in drinking water but always with standard dietary fat intake. The doses administered were all higher than the dietary dose used here of 0.07 mg/kg, although two studies administered doses similar to the pharmacological dose of 14.3 mg/kg (Ziegler *et al.* 03, Schneider *et al.* 01). The published results are, however, conflicting, with one study citing chemopreventive efficacy at 0.01 % resveratrol in the drinking water (equivalent to resveratrol consumed as 15 mg/kg in the diet, Schneider *et al.* 01), whilst another study reported no effect at 20 mg/kg body weight (Ziegler *et al.* 03).

The data from this study cause further controversy by providing a third outcome, namely that resveratrol can augment tumour burden. There is limited evidence in the published literature to support the hypothesis that resveratrol can be pro-carcinogenic. In MDA-MB-435s breast cancer cells for example, resveratrol was pro-carcinogenic both *in vitro* and *in vivo*, at 5 μM and 16.5 mg/kg respectively, when cells were transplanted into nude mice (Fukui *et al.* 10). At a higher *in vivo* dose of 33 mg/kg however, resveratrol inhibited tumour cell growth. Mechanistic data showed that resveratrol promoted tumour cell growth by activating the NF-κB pathway at lower doses. *In vitro*, resveratrol at 5-10 μM also stimulated the growth of DU145 prostate cancer cells but it had no effect on breast MCF-7 and MDA-MB-231 cancer cells

(Fukui *et al.* 10). It therefore seems that the actions of resveratrol may be specific to the experimental conditions, and that it cannot be simply classified as a pro or anti-carcinogen.

This biphasic activity of resveratrol has also been demonstrated in a murine model with indomethacin-induced gastric ulcers. Resveratrol promoted ulcer healing at 2 mg/kg but at 10 mg/kg, resveratrol promoted dyspepsia (Dey *et al.* 09). Mechanistic data showed that at low doses, resveratrol promoted eNOS expression whereas at higher doses, resveratrol inhibited COX-1 expression to mediate these differing outcomes. Although this study was not in an animal model of cancer, the pathways activated can also be involved in the process of carcinogenesis.

The pro-carcinogenic effects of resveratrol seen in this study may however, be spurious due to the inter-individual differences in tumour burden in the $Apc^{Min+/-}$ mice. In this study for example, the adenoma numbers in animals in groups 3, 4 and 6 tended to decrease with time as shown in Fig 3.10, possibly reflecting slow genetic change in the breeding colony. Since pups were not housed in individual cages for welfare reasons, recruitment into the different groups occurred at different rates at different times, thereby potentially influencing the overall data. Increasing the number of animals recruited into each group as well as speeding up the recruitment process would potentially decrease these inter-individual differences. Another possible explanation for the difference between these data and published studies is the difference in resveratrol exposure, with treatment duration in this study of 13 weeks being nearly double that of the two published studies where comparable doses were administered, but only for 7 weeks.

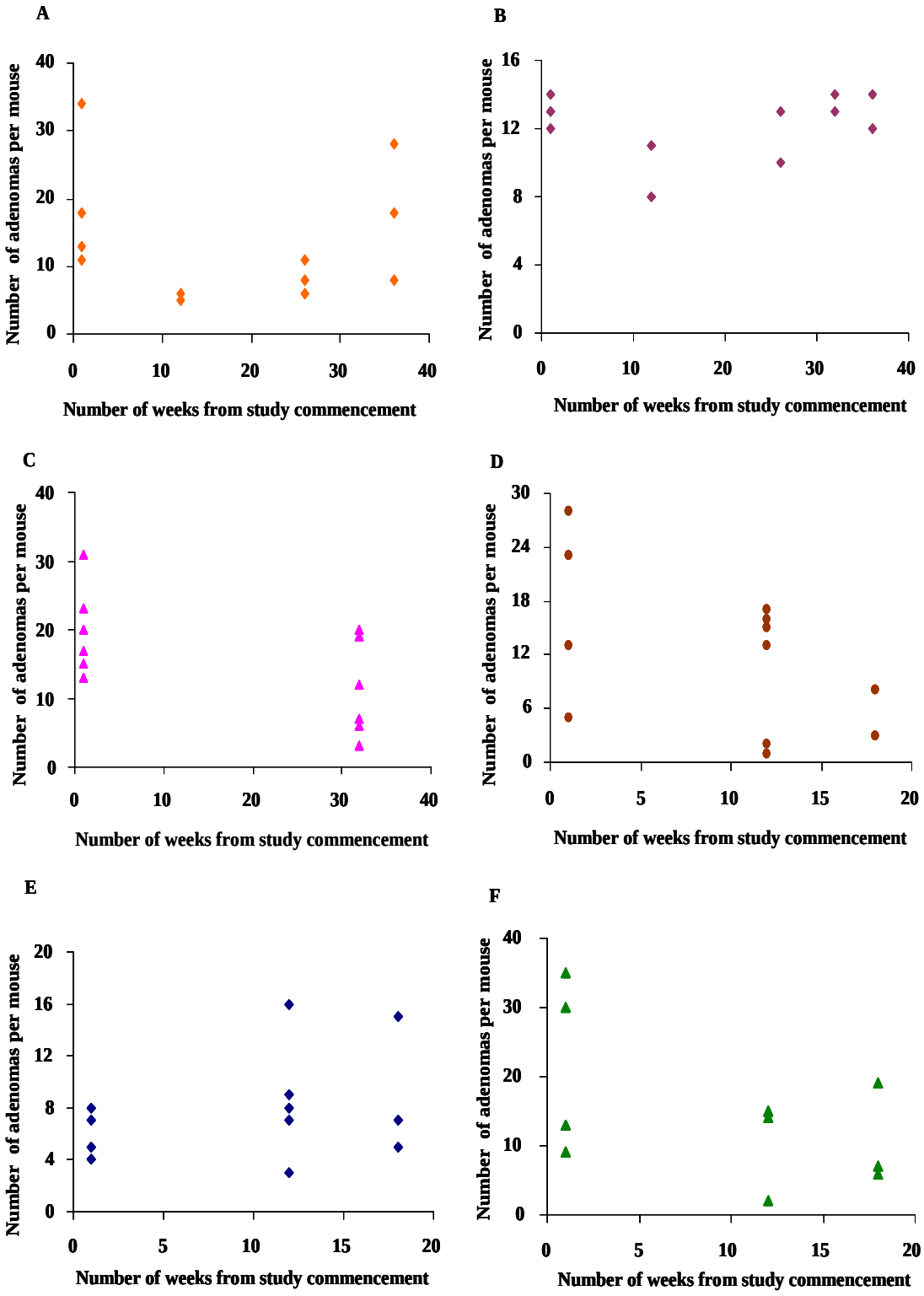


**Figure 3.10 Scatter plot of the adenoma numbers in C57BL/6J *Apc*$^{Min\ +/-}$ mice against time from commencement of study.** Animals were administered standard chow (**A**), standard chow + dietary dose resveratrol (**B**), standard chow + pharmacological dose resveratrol (**C**), high fat chow (**D**), high fat chow + dietary dose resveratrol (**E**), and high fat chow + pharmacological dose resveratrol (**F**), n=12 per group. The adenoma numbers decreased in groups 3, 4 and 6 with time, independent of study intervention, likely to be caused by gradual genetic changes in the breeding colony.

The results from this study of resveratrol administered at a dietary dose, with either a standard or a high fat diet, are novel and cannot be compared to the existing literature for support or rebuttal. The published data do however, circumstantially support these results in that dietary dose resveratrol has been shown to be active *in vivo*, albeit at slightly higher doses than that administered here, and in a cardiovascular rather than in a chemopreventive setting. Resveratrol administered orally at 0.1 mg/kg for 2 weeks to diabetic rats, equivalent to 0.2 mg/kg in mice by surface area conversion, increased plasma insulin levels and decreased plasma triglyceride concentrations (Su *et al.* 06). This improvement in diabetic control was mirrored by decreased symptoms of diabetes mellitus, with increasing body weight and decreased water consumption. Similarly a single dose of resveratrol by gavage at 0.5 mg/kg, equivalent to 1 mg/kg in mice, decreased plasma glucose levels at 90 min post dose, both at basal state and following stimulation by intravenous or oral glucose tolerance tests (Su *et al.* 06, Chi *et al.* 07). In another study in rats with diet-induced hyperlipidaemia, resveratrol exposure at 1 mg/kg/day orally for 15 days improved the plasma lipid profile (Deng *et al.* 08).

Other published literature further supports the findings in this study in that high dietary fat intake, as well as its sequelae of diabetes mellitus and hyperlipidaemia, have all been purported as carcinogenic risk factors. If resveratrol can protect against the development of some cardiovascular and metabolic consequences of a high fat diet, it may be that resveratrol can also protect against the pro-carcinogenic effects of such a diet. A high fat diet for example, promoted tumour burden in both genetic and chemical-induced animal models of colorectal carcinogenesis as discussed in section 1.1.3.4 (Wasan *et al.* 97, Baltgalvis *et al.* 09, Van Saun *et al.* 09, Rodriguez *et al.* 88, Kiunga *et al.* 04). Similarly, insulin has been shown to augment AOM-induced

colorectal tumours in F344 rats, compared to control animals which were injected with saline (Tran *et al.* 96).

In this study, dietary dose resveratrol altered tumour burden to a greater extent than tumour number, suggesting that adenoma size was limited more than the initiation of their formation. This suggestion that resveratrol has greater effect during the post initiation stage of carcinogenesis, rather than at the initiation phase, was also reported in a DMH-induced rodent model of colorectal cancer (Sengottuvelan *et al.* 06). Resveratrol was shown to exert greater chemopreventive efficacy when administered after exposure to DMH, compared to resveratrol treatment occurring before and during the carcinogenic insult. Resveratrol was however, effective in both schedules, with greatest efficacy seen if administration commenced before, and continued during and after the carcinogenic exposure has ceased. Although the authors discussed how inhibition of the promotion phase may be more useful than inhibition of the initiation stage, as the former occurs slowly over years thereby allowing time for intervention, it was unclear why resveratrol should possess this property. Some pharmaceutical colorectal chemoprevention agents, however, demonstrate similar characteristics. Aspirin or difluoromethylornithine and sulindac, for example, were more effective in reducing the recurrence of large adenomas (>1 cm) than smaller ones in patients with a history of histologically documented adenomas (Baron *et al.* 03, Meyskens *et al.* 08).

Overall, the results from this study are inconclusive as to whether resveratrol, at 0.07 or 14.3 mg/kg body weight, can alter the tumour burden in *Apc*$^{Min+/-}$ mice. Published data suggest that resveratrol is active at both doses, although not necessarily in a beneficial manner nor in the chemopreventive setting. One major variable in this study was that

adenoma numbers decreased over time in each generation of the breeding colony, thereby leading to large error bars when interpreting significance. Despite the lower adenoma numbers however, dietary dose resveratrol still appeared to exert chemopreventive actions in *Apc*$^{Min+/-}$ mice, suggesting that this effect is real.

This study has therefore been repeated using a different recruitment model, aiming to breed and recruit all animals in 3 large batches so that genetic variations over time will no longer be a confounding factor. Preliminary results of this second study are available only for mice in the high fat groups (n=7-11 per group). These data confirmed that dietary dose resveratrol decreased adenoma volume and number by 70 % (p=0.13) and 39 % (p=0.001) respectively, compared to control animals on high fat diet only. Again, the effects of resveratrol on tumour volume were greater than that on tumour number. Surprisingly, pharmacological dose resveratrol exerted similar actions with decrease in adenoma volume and number by 51 % (p=0.006) and 38 % (p=0.004). Although this study is incomplete, these findings do confirm the chemopreventive activity of resveratrol at the doses investigated when administered concurrently with a high fat diet.

### 3.2.2.3 Effects of resveratrol and high fat diet on potential molecular markers of efficacy

Increased dietary fat intake or administration of resveratrol did not alter hepatic or splenic weights in *Apc*$^{Min+/-}$ mice as shown in Fig 3.11A, suggesting that there was no significant fat or inflammatory infiltration, respectively. This finding was supported by histological analysis as shown in Table 3.2, where examination of haematoxylin and eosin stained slides of these organs by light microscopy showed that they were mostly within normal limits.

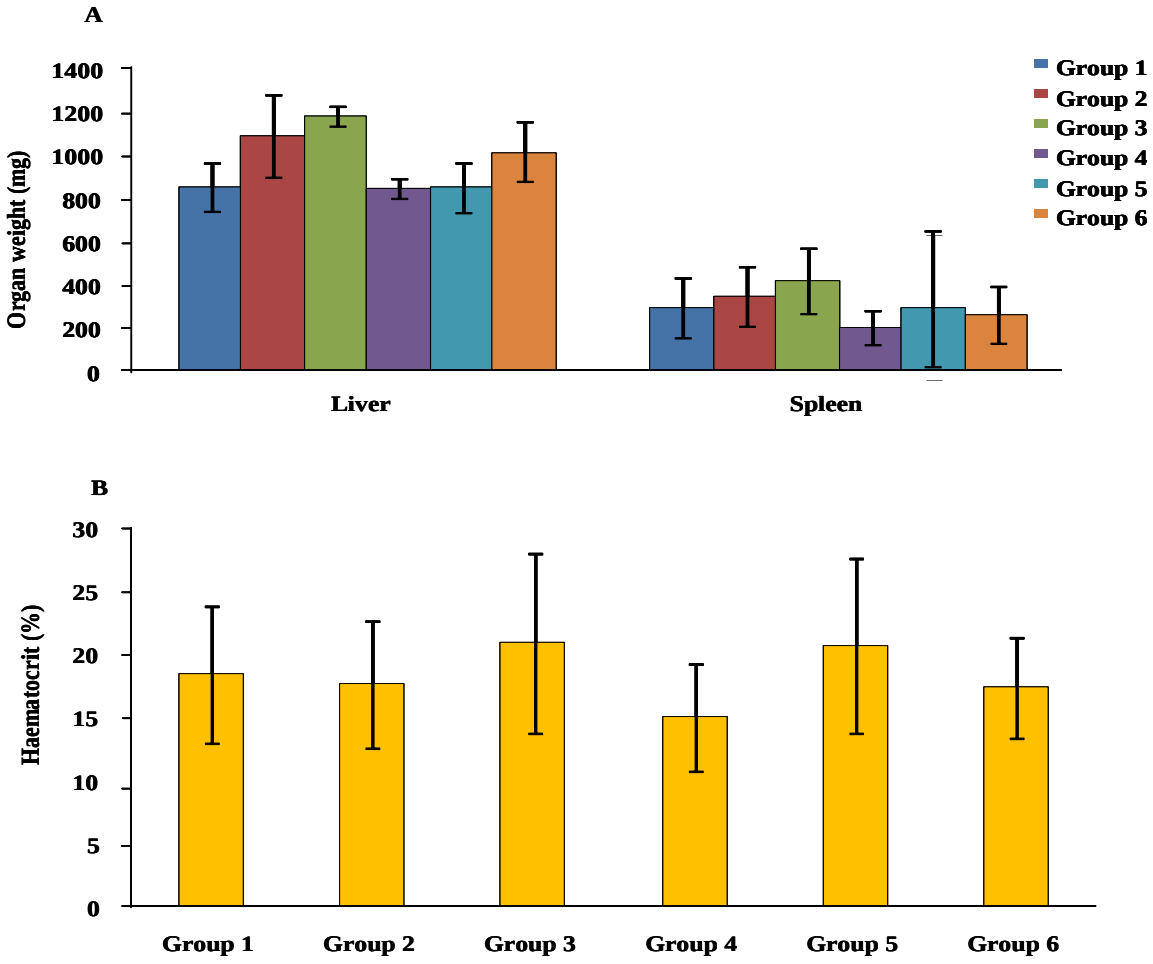


**Figure 3.11 Average weights of spleen (A), liver (A) and haematocrit (B) of *Apc*$^{Min+/-}$ mice.** Standard chow (Group 1), standard chow + dietary dose resveratrol (Group 2), standard chow + pharmacological dose resveratrol (Group 3), high fat chow (Group 4), high fat chow + dietary dose resveratrol (Group 5), and high fat chow + pharmacological dose resveratrol (Group 6). Spleen and liver, n=3-8 mice per group. Haematocrit, n= 8-11 per group due to limited sample availability. Error bars denote standard deviations. T-test analysis showed no significant differences between the treated animals compared to the appropriate control groups.

Despite the alterations in adenoma burden by the addition of resveratrol, there were no corresponding differences seen in the haematocrit levels as demonstrated by Fig 3.11B, either in the animals with higher or lower adenoma burden relative to the controls. This is surprising as adenomas are known to cause gastrointestinal bleeding, with often an increase in haematocrit noted with interventions that reduce adenoma burden, such as curcumin (Perkins *et al.* 02). Anaemia however, is also a non-specific marker of illness and may reflect other pathophysiological processes.

**Table 3.2 Histological abnormalities noted in organs of *Apc*$^{Min+/-}$ mice by light microscopy**

| | Cardiac slide insufficient for analysis | Focal chronic myocardial inflammation | Endocardial thrombosis | Other |
|---|---|---|---|---|
| Group 1 (standard diet) | | n=2 | n=2 | |
| Group 2 (standard diet + dietary dose resveratrol) | | n=1 | n=1 | |
| Group 3 (standard diet + pharmacological dose resveratrol) | | n=1 | | |
| Group 4 (high fat diet alone) | n=2 | n=3 | n=2 | Large islets of Langerhans (n=1) |
| Group 5 (high fat diet + dietary dose resveratrol) | n=1 | | n=1 | Focal chronic hepatic inflammation (n=1) |
| Group 6 (high fat diet + pharmacological dose resveratrol) | | | | Large islets of Langerhans (n=1)<br>Focal chronic hepatic inflammation (n=1)<br>Extramedullary haematopoiesis (n=1) |

Histological analysis with haematoxylin and eosin staining for example, showed that in addition to the development of adenomas, histological changes occurred in other organs (see Table 3.3). These included myocardial inflammation, endocardial thrombosis (Fig 3.12) and hepatic inflammation, but the incidence of cardiac abnormalities may have been higher than shown as some histological sections were incomplete. Cardiac thrombosis occurred in all groups except for mice on the pharmacological dose of resveratrol. The numbers are however, too small for any definitive conclusions to be made as to whether this dose of resveratrol was protective.

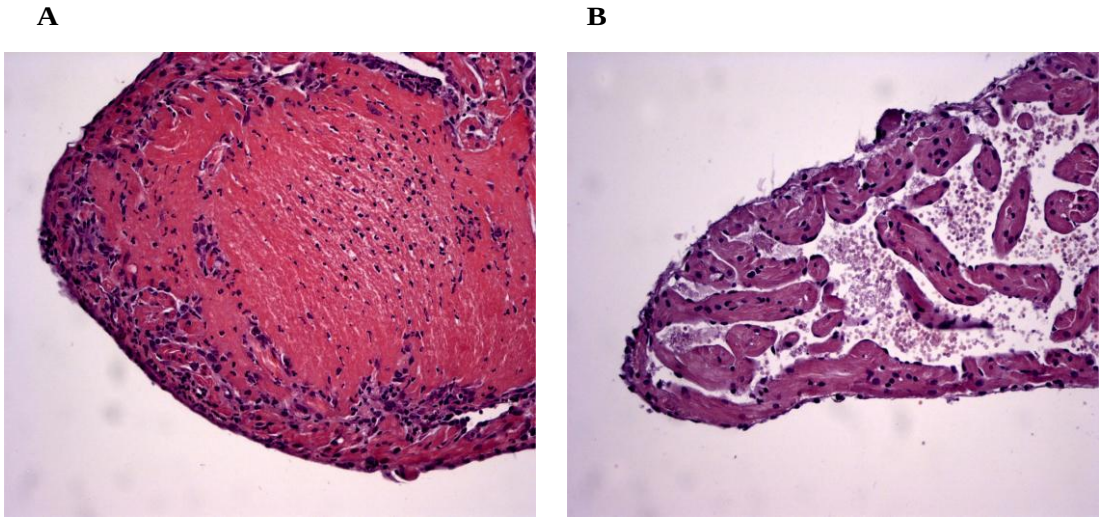


**Figure 3.12 Haematoxylin and eosin stained sections from the cardiac atria of an *Apc*$^{Min+/-}$ mouse as examined by light microscopy.** **A** demonstrates the presence of a large endocardial thrombus compared to **B**, which shows normal cardiac tissue.

Published studies showed that endocardial thrombosis is rare in wild type mice on a standard diet, but this can be induced by a high dietary fat intake (Yoshizawa *et al.* 05). These animals also developed concurrent anaemia which was thought to be linked to the pathophysiology of thrombosis, since administration of erythropoietin was protective against both (Van Vleet *et al.* 86). In this study, endocardial thrombosis occurred at similar frequency in mice on the standard as well as the high fat diet. This

does not however, necessarily contradict the published literature as this study recruited *Apc*$^{Min+/-}$ mice rather than wild type animals. Although the cardiac consequence of the *Apc* gene mutation has not been previously investigated in mice, a clinical study showed that the Apc pathway was involved in cardiac development, with different isoforms of the Apc protein found in foetal cardiac tissue compared to the adult heart (Rezvani *et al.* 00). These results suggest that *Apc*$^{Min+/-}$ mice, even if given a standard diet, may be at greater risk of developing cardiac abnormalities than wild type mice.

Potential metabolic biomarkers were also analysed in the *Apc*$^{Min+/-}$ mice after overnight fasting. Increased fat intake did not alter fasting plasma insulin, glucose or triglyceride levels, but cholesterol levels were significantly increased when comparing animals in Group 1 (control diet) versus Group 4 (high fat diet, Fig 3.13). This difference was not noted when comparing mice on standard versus high fat diet which also received resveratrol. The mean plasma cholesterol values of animals in the high fat resveratrol groups however, were still higher than mice in the standard fat resveratrol group. It therefore seemed likely that the lack of difference in the plasma cholesterol levels between animals on high fat resveratrol diets versus standard fat resveratrol diets was not due to resveratrol exerting a protective effect, but rather that the wide standard deviations resulted in a non-significant increase.

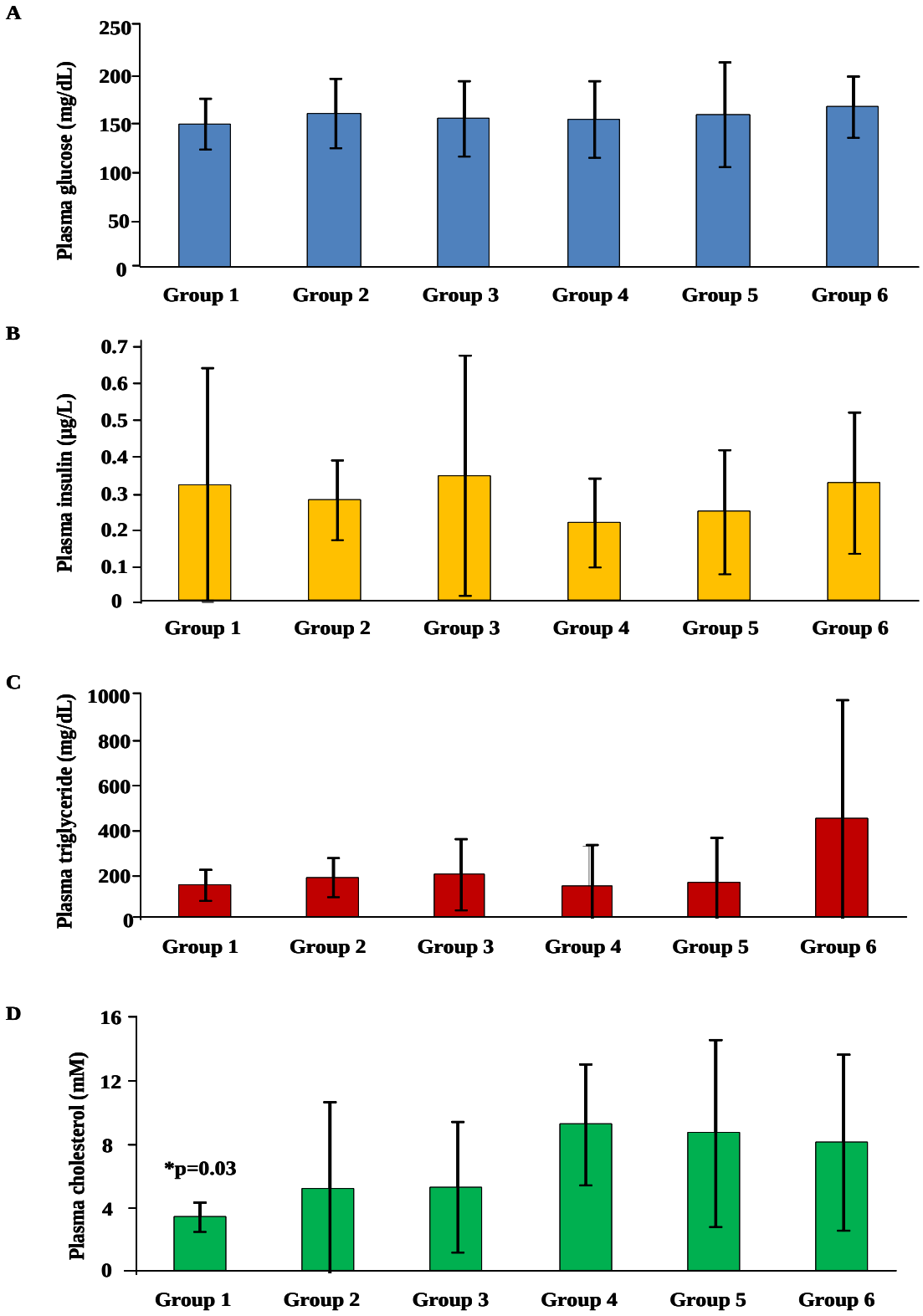


**Figure 3.13 Average fasting plasma glucose (A), insulin (B), triglyceride (C) and cholesterol (D) in C57BL/6J *Apc*$^{Min+/-}$ mice.** Standard chow (G1), standard chow + dietary dose resveratrol (G2), standard chow + pharmacological dose resveratrol (G3), high fat chow (G4), high fat chow + dietary dose resveratrol (G5), and high fat chow + pharmacological dose resveratrol (G6), n=6 per group. Error bars denote standard deviations. Plasma cholesterol levels were significantly lower for mice in Group 1 versus Group 4 (*) as assessed by t-test. All other markers were not significantly altered by diet or resveratrol.

Coconut oil consists of 85 % saturated fat and these results are similar to published animal and clinical data, where high coconut oil intake also resulted in hypercholesterolaemia which is sometimes, but not always, associated with hypertriglyceridaemia (Baur *et al.* 06, Muller *et al.* 03, Rivellese *et al.* 03). Cholesterol and triglycerides are both produced endogenously as well as consumed in the diet. Although dietary restrictions can decrease plasma concentrations, this reduction is limited as acetate, the raw material for production of both types of lipids, is also available from the catabolism of dietary carbohydrates and proteins (Engelberg *et al.* 62). Hypercholesterolaemia has been associated with high dietary saturated fat intake (Keys *et al.* 57), whereas hypertriglyceridaemia has been associated with high carbohydrate intake (Coulston *et al.* 89). The hypercholesterolaemia seen in this study is therefore as expected from the published data. Both lipids are digested into fatty acids and bound to lipoproteins for transportation, as their hydrophobicity would otherwise render them insoluble in the plasma. Triglycerides must be transported with cholesterol, as the latter is a constituent of lipoproteins. Cholesterol on the other hand, can be transported without triglyceride and this characteristic is thought to account for why hypertriglyceridaemia is usually accompanied by hypercholesterolaemia, whereas hyerpcholesterolaemia can occur without hypertriglyceridaemia (Engelberg *et al.* 62).

In healthy volunteers exposed to a high coconut oil diet, plasma glucose and insulin levels were not measured (Muller *et al.* 03, Rivellese *et al.* 03). In wild type mice, plasma triglyceride levels were not altered by a high coconut oil diet but isolated hypercholesterolaemia was reported (Baur *et al.* 06). These effects were unchanged by the addition of resveratrol which is consistent with the current data. The published findings, however, showed that a high fat diet increased fasting plasma glucose levels

and decreased fasting insulin levels, both of which were corrected by the concurrent administration of resveratrol. Although the diets administered in the published and the current studies were similar, there were other study designs which could have accounted for these differences in plasma glucose and insulin levels. The published results were for example, from mice which were exposed to the study diet and 22.4 mg/kg resveratrol from adulthood for 1 year. The current study, on the other hand, exposed animals to lower doses of resveratrol from weaning for 10-13 weeks. In addition to these differences, different strains of mice were also used. Although the effect of the *Apc* gene mutation on glucose metabolism has not been directly investigated *in vivo* nor in their clinical counterparts, FAP patients, the pro-carcinogenic effect of insulin in preclinical and clinical studies (Tran *et al.* 96, He *et al.* 10) suggests that this genetic mutation may also be an important confounding factor when comparing the data from the two studies.

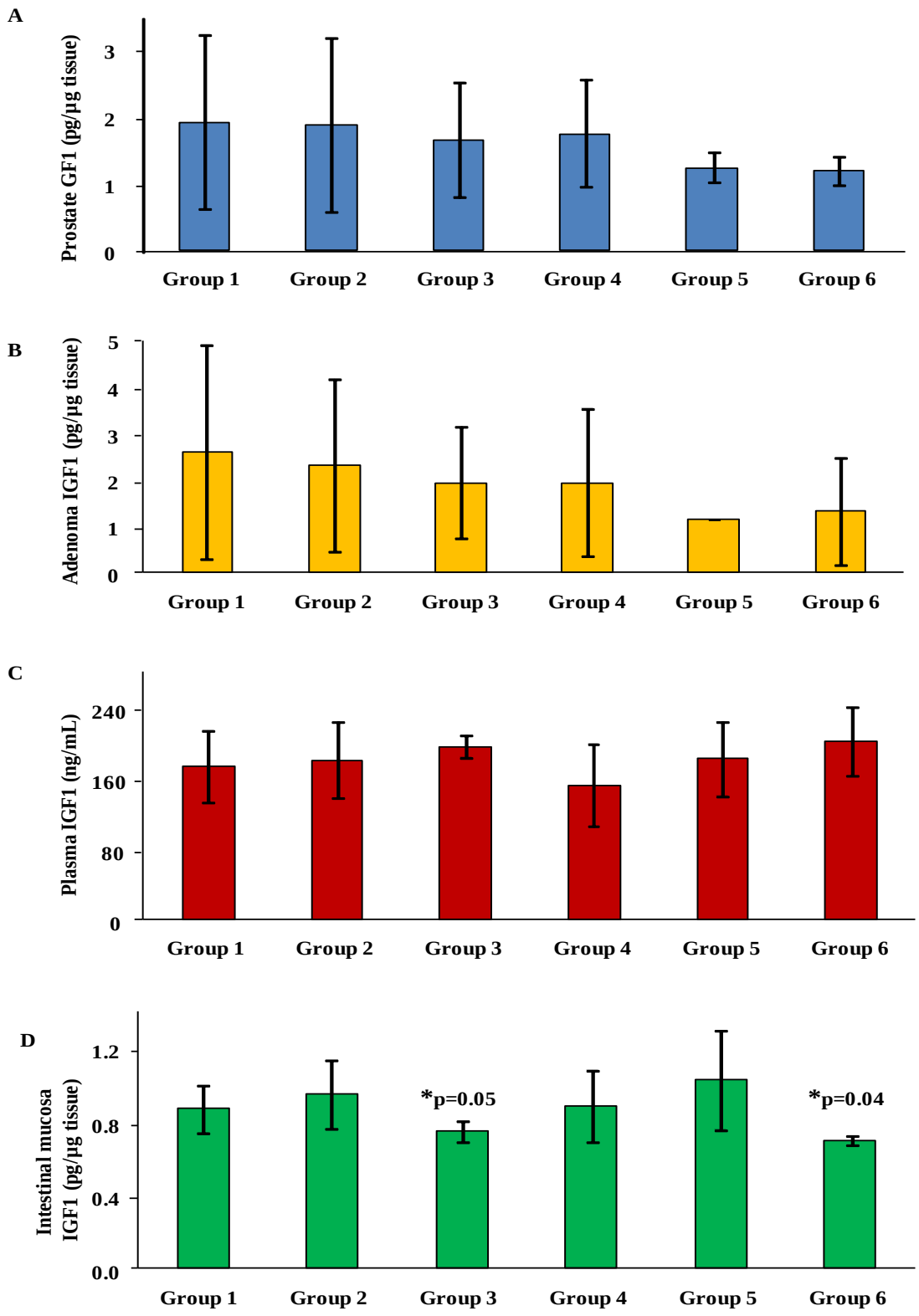


**Figure 3.14 Average plasma and tissue IGF levels in C57BL/6J *Apc*$^{Min+/-}$ mice.** Standard chow (G1), standard chow + dietary dose resveratrol (G2), standard chow + pharmacological dose resveratrol (G3), high fat chow (G4), high fat chow + dietary dose resveratrol (G5), and high fat chow + pharmacological dose resveratrol (G6). Animals n=6 per group except for adenoma analysis in Group 5, where there was only sufficient tissue in 1 mouse due to the low tumour burden. IGF1 levels were measured in prostate tissue (**A**), intestinal adenomas (B), plasma (**C**) and intestinal mucosa (**D**). Error bars denote standard deviations. Intestinal mucosa IGF1 levels were significantly decreased by pharmacological dose resveratrol treatment (*****). All other markers were unchanged by resveratrol.

Plasma, prostate and intestinal adenoma IGF1 levels were unchanged by the addition of dietary fat or resveratrol as shown in Fig 3.14. All samples from the same organ of origin were analysed on the same plate for valid comparison between the treatment groups. The assessment of adenoma IGF1 concentration was dictated by tissue availability, with 6 animals analysed in each group except Group 5 where tissue was only available in 1 animal due to the low tumour load. There are many *in vitro* and *in vivo* examples of how IGF1 promotes colon tumour growth. Cultured colon cancer cells with high IGF1R expression for example, demonstrated a faster proliferation rate, resisted serum deprivation-induced apoptosis and possessed greater migratory capabilities both in a wounding assay and when transplanted into nude mice (Sekharam *et al.* 03a, Sekharam *et al.* 03b). Similarly, *in vivo* administration of antibodies against IGF1, IGF2 or IGF1R inhibited HT29 colon cancer xenograft growth in SCID and nude mice (Bauer *et al.* 07, Miyamoto *et al.* 05). These effects were mediated by inhibition of apoptosis and angiogenesis, as assessed by immunohistochemistry and Vascular Endothelial Growth Factor (VEGF) mRNA levels (Bauer *et al.* 07, Miyamoto *et al.* 05).

Analysis of the correlation between intestinal IGF1 concentrations and adenoma burden in this study however, suggest that this marker does not reflect chemoprevention with no difference in levels between animals from Group 4 (high fat diet alone) and Group 5 (high fat and dietary dose resveratrol). Pharmacological dose resveratrol on the other hand, significantly decreased IGF1 levels in intestinal mucosa (Fig 3.14D) but not in adenomas (Fig 3.14B), both in the context of standard and high fat diet. Although it is possible that as a chemopreventive agent, resveratrol may exert greater effects in benign mucosa than in pre-neoplastic adenoma tissue, these results are still difficult to interpret since the actions of resveratrol at pharmacological dose on tumour burden is

unclear. As discussed in Section 3.2.2.2, the pro-carcinogenic actions of resveratrol at pharmacological doses as shown in Fig 3.8 may in fact be spurious, with a false statistical significance noted due to a slow phenotypical change in the breeding colony with time. An incomplete repeat study has certainly not supported these original findings, with preliminary data suggesting that pharmacological dose resveratrol decreases tumour burden when concomitantly administered with a high fat diet.

In this study, decreased intestinal mucosal IGF1 levels cannot be considered as being associated with chemoprevention since this was not noted with dietary dose resveratrol, nor can it be correlated to carcinogenesis since the actions of pharmacological dose resveratrol is unclear. Although there has been no other investigation into the effects of resveratrol on the IGF1 axis in colon cancer cells *in vitro* or *in vivo,* some of the published literature does question the precise role of the IGF1 axis in colon carcinogenesis. Histological data for example, showed that elevated IGF1R gene expression in human colon cancer tissue was clinically associated with a poorer prognosis, in terms of venous invasion and the presence of liver metastasis (Oshima *et al.* 08). In this same study however, colon tumour tissue actually exhibited lower IGF1 gene expression than benign mucosa. Similarly, some epidemiological studies showed that elevated plasma IGF1 levels were associated with increased risks of colon cancer and new adenoma development in healthy volunteers and acromegalic patients respectively (DeLellis *et al.* 04, Renehan *et al.* 04, Jenkins *et al.* 00). Other trials however, showed no correlation (Max *et al.* 08).

Prostate tissue levels were measured in this study even though the *Apc*$^{Min+/-}$ mouse is a model of colorectal carcinogenesis, as the rodent pharmacokinetic data from Section 3.2.1 showed that there is concentration of resveratrol and its metabolites in prostate tissue, suggesting that this maybe a chemopreventive target organ. Despite these pharmacokinetic data, resveratrol at either dose was unable to alter prostate IGF1 levels, suggesting that this may not be an appropriate exposure biomarker of resveratrol either. The effect of resveratrol on the IGF1 axis in prostate cancer has been investigated in one published study, where the Simian Virus-40 T-antigen targeted (SV40 Tag) probasin promoter transgenic rat model was used (Harper *et al.* 07). Resveratrol at 250 mg/kg diet (equivalent to 560 mg/day in man based on surface area conversion) inhibited prostate carcinogenesis, together with decreased cell proliferation index and increased apoptosis as indicated by immunohistochemistry. Prostatic tissue IGF1 levels were decreased as measured by Enzyme-Linked ImmunoSorbent Assay (ELISA), but the significance of this is unclear as these changes were also noted at a resveratrol dose of 83 mg/kg diet which had no effect on tumour load.

Other preclinical data are similarly conflicting with regard to the role of the IGF1 axis in prostate carcinogenesis. IGF1 was pro-carcinogenic in DU145 and PC3 prostate cancer cells, inhibiting apoptosis by increasing survivin protein levels (Vaira *et al.* 07) and promoting cell migration via the alphavbeta3 integrin and PI-3K/Akt signalling pathways (Marelli *et al.* 06). Similarly, treatment of SCID mice with antibodies to IGF1 or injections of IGFBP3, which binds to and lowers free active IGF1 levels, inhibited the growth of implanted human MDA PCa 2b and CaP prostate cancer cell xenografts (Goya *et al.* 04, Liu *et al.* 07). These effects were replicated by the use of an IGFBP3 analogue which did not bind to IGF1, suggesting that IGFBP3 has its own

independent chemopreventive activity via unclear mechanisms (Liu *et al.* 07). In another study of SCID mice however, over expression of IGF1 by viral vectors had no effect on the growth of implanted C4-2 and PC3 human prostate cancer cells (Rubin *et al.* 06). Similarly in a screening study of asymptomatic men, measurement of plasma IGF1, IGF2, IGFBP2 or IGFBP3 did not enhance specificity of prostate cancer detection beyond using PSA alone (Oliver *et al.* 04).

In conclusion, the data presented in this chapter suggest that the prostate and the colon may be potential chemopreventive targets of resveratrol, as resveratrol and its metabolites were found in these organs after a single oral dose by gavage in F344 rats. This extravascular accumulation persisted after plasma levels had decreased, suggesting resveratrol and its metabolites may be bound to tissue proteins. In $Apc^{Min+/-}$ mice, long term exposure to a high fat diet resulted in hypercholesterolaemia which was not reversed by the concomitant administration of resveratrol, at either 0.07 or 14.3 mg/kg body weight. Resveratrol did however, decrease adenoma burden when administered concurrently at 0.07 mg/kg with a high fat diet. The effect of resveratrol at a dose of 14.3 mg/kg is unclear and a repeat study is underway for clarification. The mechanisms of action by which resveratrol mediated the chemopreventive actions seen at the dietary dose are unknown. Fasting plasma glucose, insulin, cholesterol and triglyceride levels were not altered by resveratrol treatment. Similarly, plasma and adenoma IGF1 levels did not reflect tumour burden. Intestinal mucosa IGF1 levels were significantly decreased by exposure to pharmacological dose resveratrol, but the significance of this is unclear as the effect of this dose on tumour burden awaits clarification, and IGF1 levels were not altered by dietary dose resveratrol.

# 4. Clinical Materials and Method

# 4. Materials and Methods

## 4.1 Clinical trial protocol summary

A clinical trial was carried out involving the administration of [$^{14}$C]-resveratrol capsules to healthy volunteers, colon patients and prostate patients for comparison of the pharmacokinetic profiles at dietary versus pharmacological doses. A summary of the trial is outlined below and the clinical protocol is attached as Appendix 8.3. The dietary dose (5 mg) of resveratrol in this study was calculated based on a resveratrol content of 0.1-4.2 mg/L red wine (Fremont *et al.* 00) and would be equivalent to 1-2 glasses of red wine per day. The pharmacological dose was chosen to be 1 g as this has previously been given as a single dose to volunteers without significant toxicity (Boocock *et al.* 07a). Furthermore, a study of oral resveratrol at doses of up to 5 g per day for 28 days in healthy volunteers has shown that 1 g is the maximum dose that could be taken long term without gastrointestinal side effects (Brown *et al.* 10).

### 4.1.1 Study in healthy volunteers

Healthy volunteers were given a single oral dose of [$^{14}$C]-resveratrol at dietary or pharmacological doses for pharmacokinetic sampling. Blood samples were taken just before administration to provide a control sample for background [$^{14}$C] levels, then at 9 time points over the 24 h post dose period. The pharmacokinetic time points in healthy volunteers were chosen based on previous studies showing rapid resveratrol uptake and metabolism (See Section 1.2.2.3).

### 4.1.2 Study in patients with colorectal cancer or prostate disease

Patients with colorectal cancer or prostate disease took resveratrol orally for 7 or 7-14 days prior to surgery respectively. For each arm of the study, patients were divided into three groups: dietary dose arm, pharmacological dose arm and control arm with no intervention. Both benign and malignant colorectal and prostate tissues were sampled. Full thickness sections of benign colon tissues were obtained and the inner mucosal lining, the muscle layer, any polyps which were present and any adherent peritoneal fat were dissected where possible, and analysed separately as shown in Fig 4.1. It was not possible to obtain full thickness sections of malignant tissue in an effort to preserve the specimen for clinical staging, and therefore only luminal shavings were taken.

The duration of resveratrol administration for prostate and colorectal patients was chosen to be as long as possible to maximise the chance of any pharmacodynamic changes occurring without delaying surgery, whilst allowing participants sufficient time to consider the study. In the University Hospitals of Leicester (UHL), there is usually 2 weeks after a patient has been given their diagnosis of colorectal malignancy prior to surgery. Seven days was therefore determined as a feasible duration for resveratrol administration. For prostate cancer patients, there is usually a waiting list of 2-4 weeks for transrectal prostatic biopsies as the biology of prostate cancer is slow. For those with benign disease, the wait is 6 months for transurethral prostatic resection as this is treatment for benign disease. Patients in the latter group are not notified of their surgery date until a month before. Two weeks was therefore determined as a feasible duration for resveratrol administration in prostate patients as a whole before their operations.

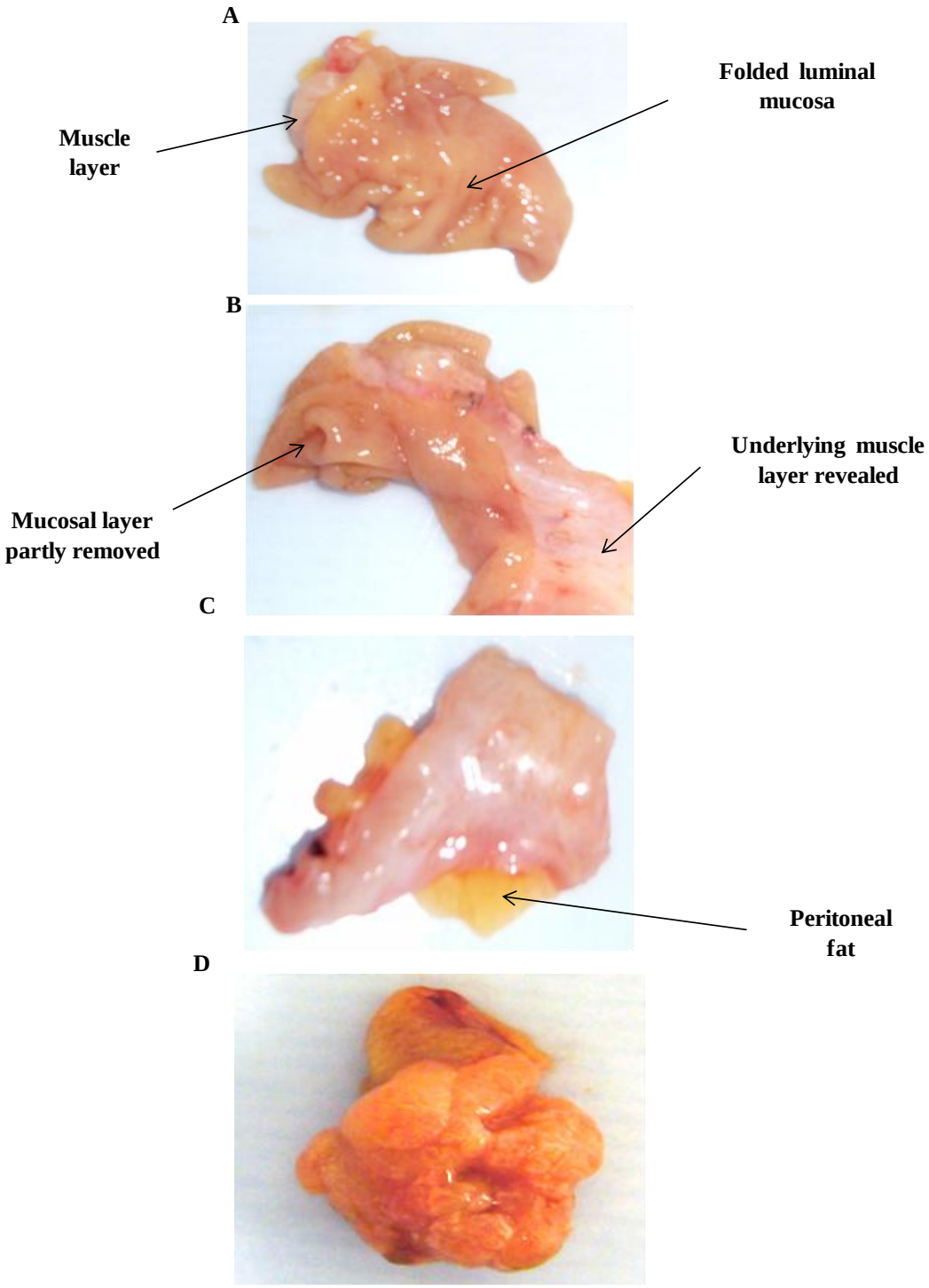


**Figure 4.1. Dissection of human colon tissue.** **A** shows a full thickness section of benign human colon tissue with the luminal mucosal layer facing upwards. This mucosal layer was removed and analysed separately to the underlying muscle layer as shown in **B**. Any peritoneal fat attached (**C**) was also analysed separately. **D** is an example of malignant colon tissue and demonstrates how it is macroscopically different to benign tissue.

The timing of [$^{14}$C]-resveratrol capsule administration was determined by two factors. Previous clinical studies have shown that the plasma half life of resveratrol was in the order of 3-14 h (Boocock *et al.* 07a, Walle *et al.* 04) and this was used as an estimate of the time for agent delivery to target tissue. Due to the radioactivity the [$^{14}$C]-resveratrol capsule could not be taken off hospital premises for safety and regulatory reasons. For colorectal and prostate patients, the [$^{14}$C]-resveratrol capsules were administered before their operations at a practically feasible time to minimise patient inconvenience. For colorectal patients, they were admitted the night before their surgery and so the [$^{14}$C]-resveratrol capsules were administered at least 12 h before their operation. For prostate patients, they would normally arrive a few hours before the procedure and the [$^{14}$C]-resveratrol capsules would be administered then. Preliminary analysis of samples from the first few recruited patients were used as guidance to optimise the timing of the [$^{14}$C]-resveratrol dosing, which could potentially be adjusted if necessary.

### 4.2 Ethics, regulatory and local site approvals

Clinical trial approval was received from the Liverpool Adult Ethics Committee, the Medicines and Healthcare products Regulatory Authority, UHL Research and Development Department and the Administration of Radioactive Substances Advisory Committee.

Three major and one minor amendment were submitted during the course of this trial. The major amendments were submitted due to a commercial takeover of the companies involved, problems due to the resveratrol powder interfering with the capsule shell locking mechanisms, and discrepancies between the HPLC method and the [$^{14}$C]-resveratrol capsule specifications in the Investigational Medicinal Product Dossier and

the Batch Manufacturing Records.

Although the received radiation dose per study participant was clinically insignificant, certain precautions were taken in the design of the protocol to maximise safety. Lactating and pregnant women were excluded and all participants had to use barrier contraception for the study duration. Only the last dose of resveratrol administered to colorectal and prostate patients contained [$^{14}$C] to minimise radiation exposure. Local, national and international experts at the Institute of Cancer Research and Lawrence Livermore National Laboratory (California, US) were consulted in the design of this study.

### 4.3 Extraction of [$^{14}$C]-resveratrol species from plasma, colon and prostate tissue

Whole volunteer plasma, colon tissue and prostate tissue were subjected to liquid scintillation counting (see Section 2.2.1.1) to verify that radiation levels were below the limit of detection, prior to being submitted for AMS analysis at Lawrence Livermore National Laboratory to quantify total [$^{14}$C]-resveratrol equivalent levels.

Resveratrol and its metabolites were extracted from volunteer plasma, colon tissue and prostate tissue by protein precipitation. The plasma protein precipitation method used was the same as that from a published paper (Boocock *et al.* 07b) and consisted of 1 mL of plasma acidification with 17.5 μL of concentrated hydrochloric acid, prior to the addition of 1mL of methanol and vortexing for 1 minute. The sample was then placed at -20 °C for 10 minutes, centrifuged for 13000 rpm for 15 minutes at 4 °C and the supernatant removed. The centrifuge used for all clinical sample preparation was a Beckman Coulter Allegra 6KR centrifuge. The supernatant was dried down under

vacuum and reconstituted in 50:50 MeOH:water. The sample was again centrifuged at 13000 rpm for 15 minutes at 4 °C, and the supernatant removed for HPLC analysis.

For colon and prostate samples, the protein precipitation method used was modified from an established in-house method (Patel *et al.* 10). This modified method involved sequential extraction with methanol followed by acetone, which was necessary to maximise recovery from the small amount of clinical tissue available. The extraction efficiency of the modified method, as determined from using control colon samples spiked with known quantities of resveratrol, was in the order of 60 % which is similar to that of the original method. The modified method was developed in the hope of improved recovery of resveratrol metabolites, which was not determined.

The modified method involved first homogenising 10 mg of tissue in 100 µL of pre-mixed buffer (400 µL of potassium HEPES buffer and 7 µL of concentrated hydrochloric acid). MeOH (100 µL) was then added to the tissue homogenate and the samples placed at -20 °C for 10 minutes for protein precipitation. The samples were centrifuged at 13000 rpm for 15 minutes at 4 °C and the supernatant removed into a clean eppendorf. The pellet was resuspended in 100 µL of acetone, vortexed and placed at -20 °C for 10 minutes. This sample was also centrifuged as above and the supernatant added to the supernatant from the MeOH extraction. The combined extracts were then concentrated to dryness under vacuum and reconstituted in 50:50 MeOH:water. The sample was again centrifuged for 13000 rpm for 15 minutes at 4 °C, and the supernatant removed for HPLC analysis.

## 4.4 Optimisation of resolution and separation of resveratrol and its metabolites by HPLC

All chemicals were purchased from Sigma-Aldrich Company Limited (Poole, Dorset, UK) and Fisher Scientific (Loughborough, Leicestershire, UK) unless otherwise stated. Resveratrol (100 % purity) was a gift from Royalmount Pharma, Montreal, Canada. Resveratrol-3-sulphate, resveratrol-4'-sulphate, resveratrol-3-glucuronide and resveratrol-4'-glucuronide were gifts from Dr Rob Britton, Leicester University, UK (all of 99-100 % purity).

A published HPLC method (Boocock *et al.* 07b) was optimised to improve separation between the metabolites of resveratrol. One minute fractions were collected from each test run for AMS analysis, and blank runs in between were also analysed to ensure that there was no cross contamination. There was an estimated 37 s delay between the chromatograms of the test samples compared to the standards due to the length of tubing used for fraction collection, as the standards were detected by an on-line UV-detector whereas the [$^{14}$C]- species were detected by AMS.

Chromatographic separation was accomplished using an HPLC system consisting of a Waters 1525 binary pump, 717+ autosampler with a refrigeration unit, 2487 dual wavelength UV visible detector and in-line vacuum degasser (Waters, Elstree, UK). The HPLC system and detectors were controlled and the results calculated by the Empower 1.3 chromatography manager software (Waters, Elstree, UK). A Waters Atlantis 4.6 mm x 150 mm, 3-µm $C_{18}$ column was used in combination with a Waters Atlantis 4.6 mm x 20 mm 5-µm $C_{18}$ guard column. Column oven was set at 35 $^{\circ}$C. Method development was conducted using 5 mM ammonium acetate with 2 %

isopropanol (mobile phase A), and methanol with 2 % isopropanol (mobile phase B), whilst the needle wash was acetonitrile:deionised water:methanol:isopropanol (1:1:1:1). All mobile phases and needle wash were stored at room temperature. The UV wavelength for detection was 325 nm.

Test plasma samples collected in sodium EDTA tubes were obtained 150 min post dose from a volunteer who was administered a single 5 g dose of resveratrol orally. Control plasma was obtained from the blood bank of Leicester Royal Infirmary (UK) for use as control and for spiking in standards. Resveratrol and its metabolites were extracted from human plasma by the plasma precipitation method as described in Section 4.3. Exposure to light was limited as practically feasible to avoid resveratrol isomerisation.

Altering the isopropanol content or the pH of the mobile phases A and B using ammonium hydroxide or acetic acid respectively did not improve chromatographic separation. The flow rate was not increased as although this would probably decrease the run time, it is likely to decrease the separation between metabolite peaks which is not conducive to fraction collection prior to AMS analysis, where peaks must be completely resolved. Similarly, the temperature was not increased as this would also decrease the run time. The original gradient elution system, termed the Atlantis Method, is shown in Table 4.1 and a typical HPLC chromatogram for analysis of test plasma, using this method, is shown in Fig 4.3.

| Table 4.1 Gradient elution for original Atlantis method | | |
|---|---|---|
| **Time** | **% mobile phase A** | **% mobile phase B** |
| 0.01 | 100.0 | 0.0 |
| 4.00 | 80.0 | 20.0 |
| 7.00 | 80.0 | 20.0 |
| 16.00 | 45.0 | 55.0 |
| 18.00 | 45.0 | 55.0 |
| 18.50 | 5.0 | 95.0 |
| 23.50 | 100.0 | 0.0 |

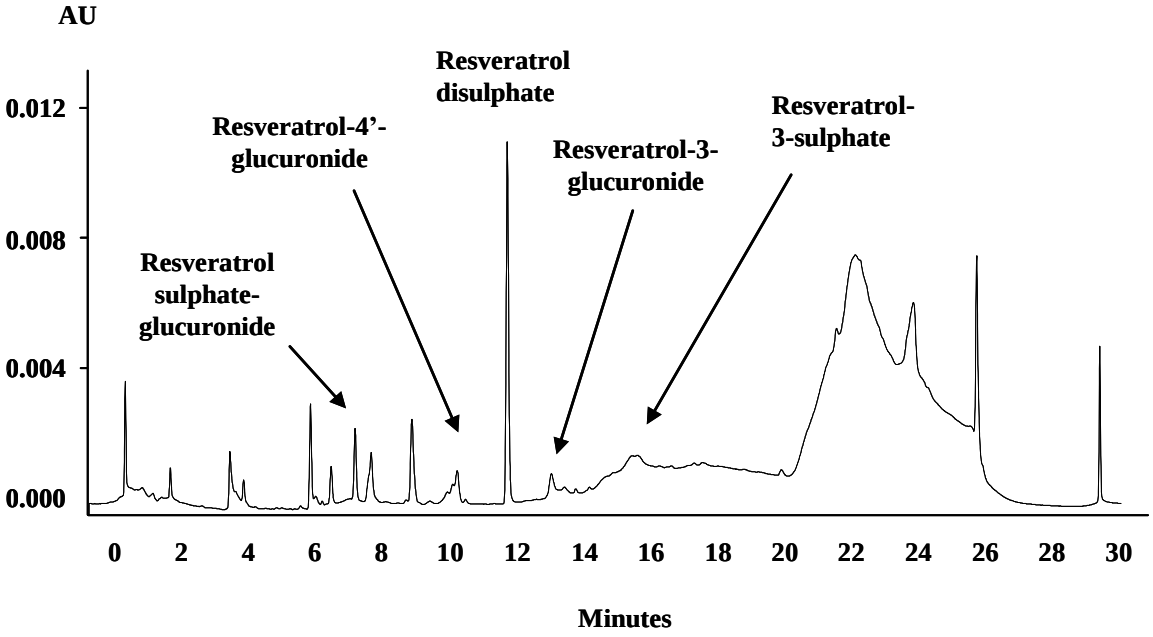


**Figure 4.2 HPLC chromatogram of resveratrol and its metabolites using the Atlantis Method.** Resveratrol and its metabolites were extracted from the plasma of a volunteer 150 min after a single oral 5 g dose of resveratrol. The peaks were identified from the use of standards or by mass spectrometry (Boocock *et al.* 07b). Parent resveratrol was not identified in this chromatogram due to extensive metabolism.

The optimal gradient elution system developed for fraction collection for AMS analysis, termed the AMS Method, is shown in Table 4.2 and has a run time of 38 min. Retention times of resveratrol and resveratrol sulphate were identified by using standards of these two compounds, with the resulting HPLC traces shown in Fig 4.2.

The test plasma was then spiked with resveratrol and resveratrol sulphate standards (15 µL of test plasma mixed with 5 µL of standard). The peaks due to resveratrol and resveratrol sulphates in the test plasma increased after spiking with the standards as shown in Fig 4.3 (panels 3 and 4), confirmed by the appropriate retention times. Typical HPLC chromatograms from analysis of test plasma (Fig 4.3, panel 2, black chromatogram) were also compared to that of control plasma (Fig 4.3, panel 2, red chromatogram) to confirm the peaks seen were due to resveratrol and its metabolites. Some peaks are unidentified (peaks A-D, Fig 4.3, panel 2, black chromatogram) as standards were available only for the parent drug and the 3' and 4' sulphate metabolites. This separation was considered satisfactory and the best that can be achieved with the column and mobile phases used.

**Table 4.2 Optimal gradient elution for fraction collection for AMS analysis (AMS Method)**

| Time | % mobile phase A | % mobile phase B |
|---|---|---|
| 0.01 | 100.0 | 0.0 |
| 4.00 | 90.0 | 10.0 |
| 7.00 | 87.0 | 13.0 |
| 11.00 | 87.0 | 13.0 |
| 14.00 | 85.0 | 15.0 |
| 23.00 | 84.0 | 16.0 |
| 27.00 | 60.0 | 40.0 |
| 30.00 | 30.0 | 70.0 |
| 36.00 | 5.0 | 95.0 |
| 38.00 | 100.0 | 0.0 |

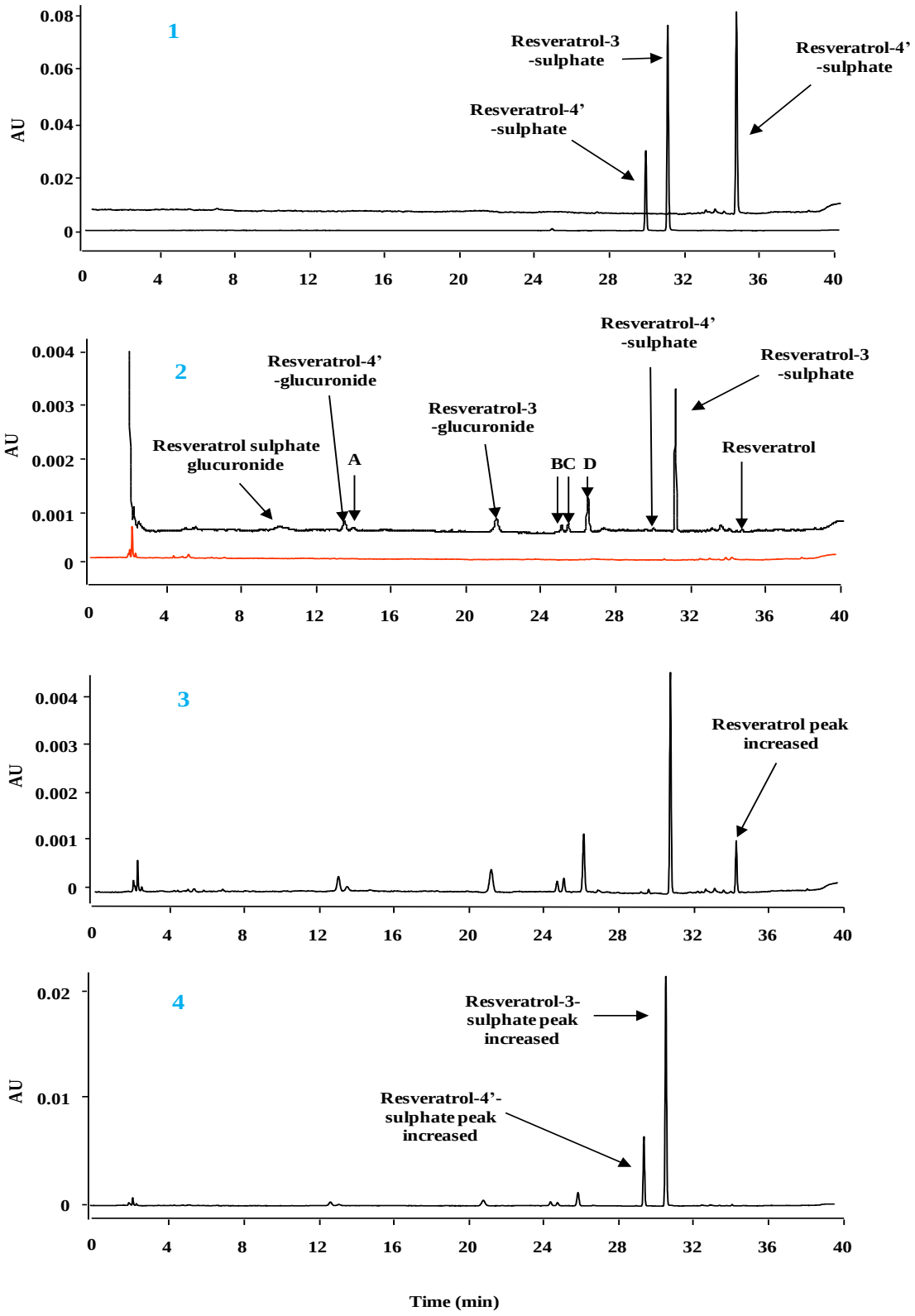


**Figure 4.3 HPLC chromatograms of resveratrol and its metabolites with AMS method.** Test plasma was obtained from a volunteer 150 min after a single oral 5 g dose of resveratrol. Retention times for resveratrol and resveratrol sulphate were identified by use of standards (1), confirmed by increase in peak size in test plasma after spiking with standard (3 and 4). Unidentified peaks in the test plasma (**A-D** in black trace, 2) were confirmed as due to resveratrol and its metabolites by comparison to analysis of control plasma (red trace in 2).

## 4.5 AMS analysis of samples

Equipments were swabbed and reagents were analysed to ensure all were free from [$^{14}$C] contamination before study commencement. Only HPLC grade water was used. Great care was taken to prevent cross contamination between samples, with control tissues handled first followed by treated tissue in increasing order of radioactivity. Disposable equipment was used where possible including frequent glove changes in between each sample. Control and treated tissues were stored in separate boxes or double bagged.

HPLC fractions were reconstituted in 200 µL of 50:50 water:MeOH overnight prior to analysis. Tissue, plasma and HPLC fractions were converted to elemental carbon using standard protocols, by combustion to $CO_2$ and then reduction to filamentous graphite (Ognibene *et al.* 03). The resulting graphite was then packed into individual sample holders for AMS analysis as per Section 1.3.1. For whole plasma, colon and prostate tissue samples, the carbon content in each sample was sufficient for efficient graphitisation but for HPLC fractions, 1 µL of tributyrin (TB) was added to each sample to provide 615 µg of extra carbon. Tributyrin is used routinely to supplement the carbon contents of samples for AMS analysis as it is derived from petroleum, therefore its [$^{14}$C] content is extremely low due to the extent of radioactive decay over time.

### 4.5.1 Calculation of AMS results

For whole tissue and plasma samples

Tributyrin carrier was not necessary as the carbon levels in each sample were sufficient for efficient graphitisation. The carbon content for each sample was therefore calculated based on the amount of tissue analysed and the percentage of carbon for each tissue, namely 3.4 %, 9.87 % and 8.75 % for plasma, colon and prostate tissue respectively. The proportion of carbon was determined previously by elemental analysis of tissues and plasma (Ted Ognibene, Lawrence Livermore Laboratory, personal communication).

$[^{14}C]$ level~~s~~ above background = total $[^{14}C]$ level~~s~~ in test sample - $[^{14}C]$ level~~s~~ in control sample

To convert the fraction modern values to total attomole of $[^{14}C]$/mg tissue (presuming 1 mL plasma = 1 mg):

1 fraction modern = $9.8 \times 10^{-8}$ attomole $[^{14}C]$/mg carbon (based on $[^{14}C]$ concentration in the air in 1950)

Attomole $[^{14}C]$/mg carbon = fraction modern x $(9.8 \times 10^{-8})$

Attomole $[^{14}C]$/mg tissue = fraction modern x $(9.8 \times 10^{-8})$ x carbon % in tissue (i.e. 0.034 for plasma, 0.0987 for colon and 0.0875 for prostate)

Attomole resveratrol/mg tissue = fraction modern x $(9.8 \times 10^{-5})$ x carbon % in tissue x fraction labelling of $[^{14}C]$-resveratrol (i.e. 0.0881 % for 5 mg dose and 0.00044 % for 1 g dose)

The above calculation was carried out for all test and control samples. Only 1 control colon and 1 control prostate sample was analysed due to the cost of analysis and limited supply of tissue. This is an acceptable protocol since background radiocarbon concentrations are highly consistent and variation is not expected amongst individuals that have not been exposed to $[^{14}C]$.

For HPLC fractions

Tributyrin (TB) carrier was added to each sample (1 μL/sample, containing 0.615 mg carbon/μL) to increase the carbon content for efficient graphitisation. The carbon content was therefore deemed to be 615 μg/sample, since the contribution from the HPLC solvents were negligible as they were volatile and completely removed under vacuum. Total amount of [$^{14}$C] in 1 μL of tributyrin carrier:

$$\frac{\text{Fraction modern x mass of carbon in carrier (μg) x No of [}^{14}\text{C] atoms/μg total carbon equal to 1 modern}}{\text{No of [}^{14}\text{C] atoms equal to 1 fmole}}$$

$$\text{Therefore, fmoles [}^{14}\text{C] in 1 μL tributyrin} = \frac{\text{fraction modern x 615 x 59000}}{6.023\text{x}10^{8}}$$

Four tributyrin samples were analysed with each batch of samples and the mean calculated as the [$^{14}$C] contribution by the carrier. To convert the fraction modern values to total attomole [$^{14}$C] in HPLC fractions:

1 fraction modern = $9.8\text{x}10^{-8}$ attomole [$^{14}$C]/mg carbon (based on [$^{14}$C] concentration in the air in 1950)

Attomole [$^{14}$C]/mg carbon = fraction modern x ($9.8\text{x}10^{-8}$)

Total mass of carbon in each fraction = 0.615 mg as the carbon content in 1 μL tributyrin

Total attomole [$^{14}$C] in sample= 0.615 x attomole [$^{14}$C]/mg total carbon

= 0.615 x fraction modern x ($9.8\text{x}10^{-8}$)

The above calculation was carried out for all test and control samples.

To measure the [$^{14}$C] levels above background:

[$^{14}$C] levels above background = total [$^{14}$C] levels in test sample - [$^{14}$C] levels in control - TB [$^{14}$C] levels

## 4.6 Colon tissue IGF1 analysis

Commercial ELISA kits for the measurement of human IGF1 from R&D Systems (Minnesota, US) were used. Tissue IGF1 level was identified as a possible efficacy biomarker of resveratrol from the *Apc*$^{Min}$ data in Section 3.4. It was not possible to measure IGF1 concentrations in the prostate samples as the amount of tissues obtained from biopsies was insufficient, but larger amounts were obtained from colon patients as open operations were performed.

The IGF1 assay was validated with volunteer plasma, as this was carried out prior to obtaining control colon tissue samples. The assay was carried out as per manufacturer's instructions. Blood was taken from 3 volunteers in EDTA tubes, centrifuged at 3000 *x g* for 15 min at 4 °C and stored as aliquots of plasma at -80 °C to avoid repeated freeze-thaw cycles. The plasma samples were pretreated with an acidic buffer to release IGF1 from its binding proteins. Colon samples for analysis were not pre-treated, as in-house method development by Dr L Howells showed that pre-treatment did not affect IGF1 analysis (unpublished data, Leicester University). Colon tissue was homogenised and proteins extracted as per murine intestinal tissue as described in Section 2.2.2.2. Method optimisation was limited by the concentration of the colon lysate being too dilute, but at least 750 µg of protein per well was needed for the values to be within the standard curve.

The test samples and an Assay Diluent buffer were then added in duplicate to a 96 well plate pre-coated with monoclonal antibodies specific for IGF1. The plate was incubated in the dark at 2-8 °C for 2 h to allow any IGF1 present to bind to the immobilised antibodies, before being washed 4 times to remove excess reagents. A

horseradish peroxidase-linked polyclonal antibody specific to IGF1 was then added prior to incubation again in the dark at 2-8 °C for 2 h. The plate was again washed 4 times to remove excess reagents before hydrogen peroxide and tetramethylbenzidine were added. The plate was incubated at room temperature in the dark for 30 min to allow colour development in proportion to the level of IGF1. The colour reaction was terminated by the addition of sulphuric acid. The plate was then immediately scanned on a FLUOstar OPTIMA plate scanner (BMG Labtech, Offenberg, Germany) at 450 nm and 540 nm to measure the pixel intensity. Absorbance values from 450 nm were subtracted from values at 540 nm for analysis, as the former wavelength reflected background levels. A commercially available Quality Control (QC) sample was used with each plate to confirm the values obtained were within the expected range.

Interplate validation was carried out on 3 plates, with interplate variability demonstrated to be <10 % as shown in Fig 4.4A. One plate was therefore deemed to be sufficient for subsequent storage studies. Intraplate variations were much lower at <5 %. Serial dilution to 80 %, 55 %, 40 % and 20 % of neat plasma with the Calibrator Diluent buffer from the commercial kit was carried out to confirm parallelism as shown in Fig 4.4B. The parallelism demonstrated that the antibody-binding characteristics of the analyte (IGF1) in the plasma samples are the same as those in the standard reference samples. This finding confirms that it is appropriate to determine the concentrations of IGF1 in plasma samples using the standard curve, as the difference in matrices did not alter the antigen-antibody specificity. Storage data showed that storage at -80 °C for 6 weeks or 4 months did not significantly decrease plasma IGF1 levels as shown in Fig 4.4C.

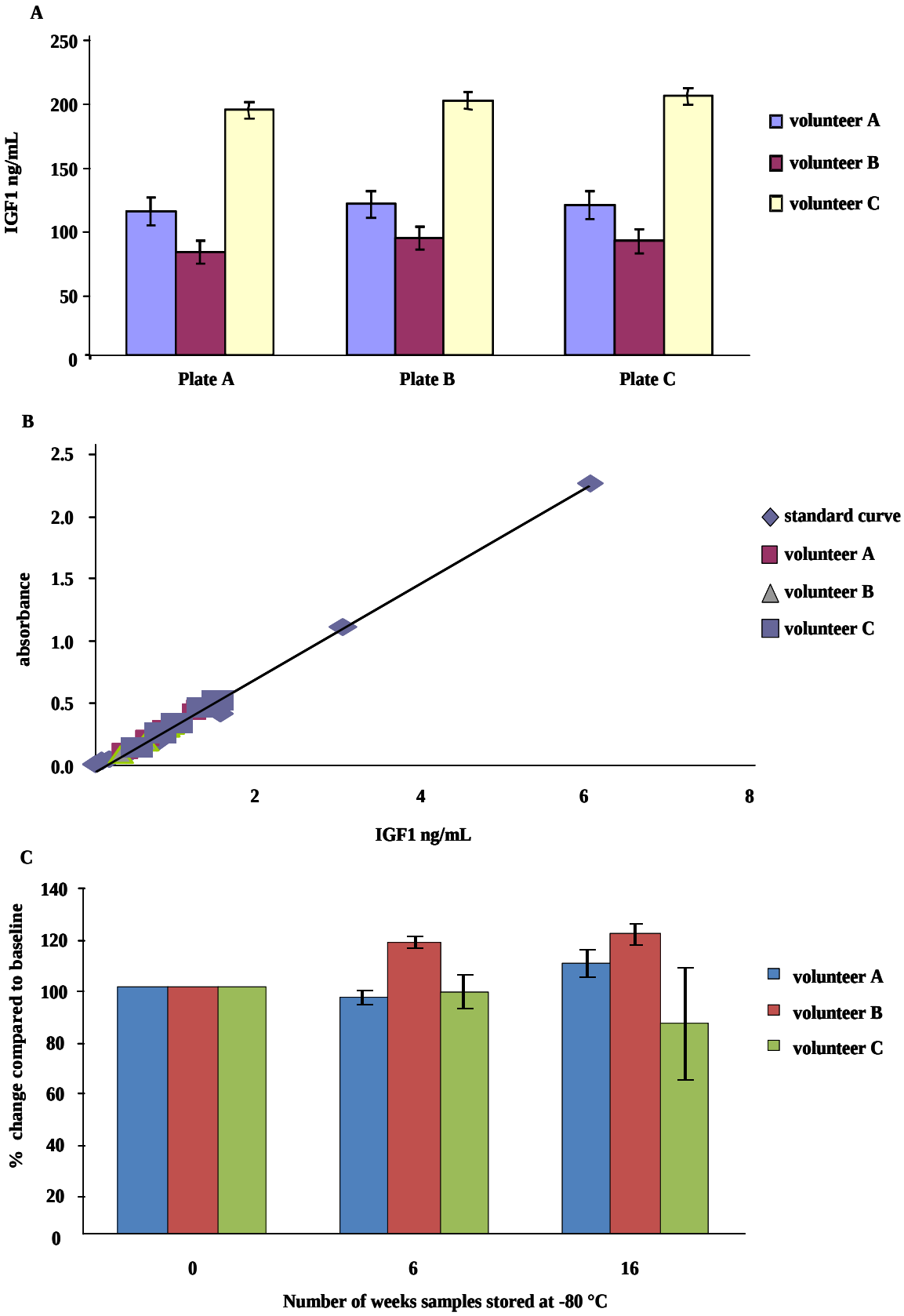


**Figure 4.4 Validation data for the human plasma IGF1 assay.** Interplate (**A**) and parallelism (**B**) validation with plasma from 3 volunteers analysed on 3 plates are shown. Storage stability data at baseline, 6 weeks and 4 months with plasma from 3 volunteers stored at -80 °C are shown in **C**.

# 5. Clinical Results and Discussion

# 5. Clinical Results and Discussion

In this chapter, the effect of dose on the pharmacokinetic distribution of resveratrol and its metabolites in plasma, colon tissue and prostate tissue was investigated. Colon tissue samples were also analysed for IGF1 as a possible efficacy biomarker of resveratrol. Biomarker analysis of prostate tissue was not possible due to the small volumes of tissue available.

## 5.1 Effect of dose on the plasma pharmacokinetic profile of resveratrol and its metabolites in healthy volunteers

Healthy volunteers were administered a single dose of [$^{14}$C]-resveratrol orally and blood samples were collected at 9 time points post dose for analysis of total [$^{14}$C]-resveratrol equivalent concentrations by AMS. The metabolite profile was also compared in a selected number of patients by HPLC-AMS analysis.

The demographic data of the healthy volunteers are shown in Table 5.1. There were no apparent differences in the demographics of volunteers in the 5 mg and 1 g dose groups. All subjects completed the study with the only adverse event being fainting in a single person in the 5 mg dose group. This volunteer has always had a dislike of needles and indeed fainted during a previous acupuncture session. She recovered quickly and declined the offer to come off study. Her pharmacokinetic profile was therefore complete except for the 1 h time point. There were no serious adverse events.

**Table 5.1 Demographic data of healthy volunteers**

| | 5 mg dose group (mean, range) | 1 g dose group (mean, range) |
|---|---|---|
| Age (years) | 31.0 (22-49) | 31.7 (23-40) |
| Gender | 4 female 6 male | 5 female 5 male |
| Ethnicity | 8 Caucasian 1 Indian 1 Chinese/Caucasian | 9 Caucasian 1 Indian |
| Body surface area ($m^2$) | 1.92 (1.63 - 2.11) | 1.85 (1.58 - 2.28) |

The pharmacokinetic profiles of the 5 mg and the 1 g dose groups are shown in Fig 5.1 and 5.2 respectively. At both dose levels, a bimodal distribution of plasma [$^{14}$C]-resveratrol equivalents was evident in some volunteers with peaks at about 1 h and 6 h post dose. Plasma levels of [$^{14}$C]-resveratrol equivalents were linear to dose as demonstrated by comparing the AUC and the $C_{max}$ levels as shown in Fig 5.3.

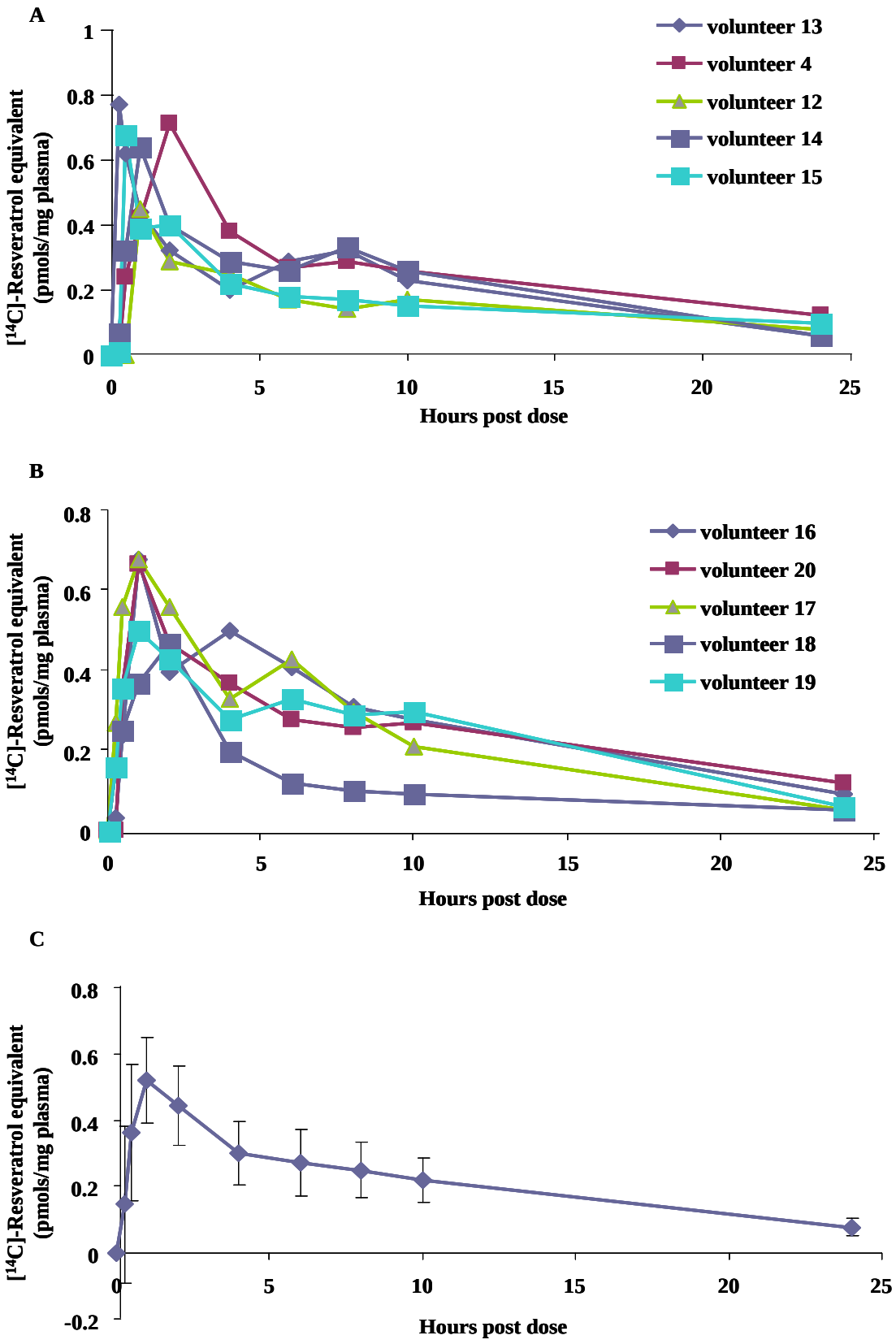


**Figure 5.1 Plasma [$^{14}$C]-resveratrol equivalents levels in healthy volunteers after administration of a single oral 5 mg dose.** **A** and **B** show the individual PK profiles of the 10 volunteers, which are presented on 2 charts for clarity. **C** shows the mean plasma [$^{14}$C]-resveratrol equivalents levels in all 10 volunteers with error bars indicating the standard deviation range.

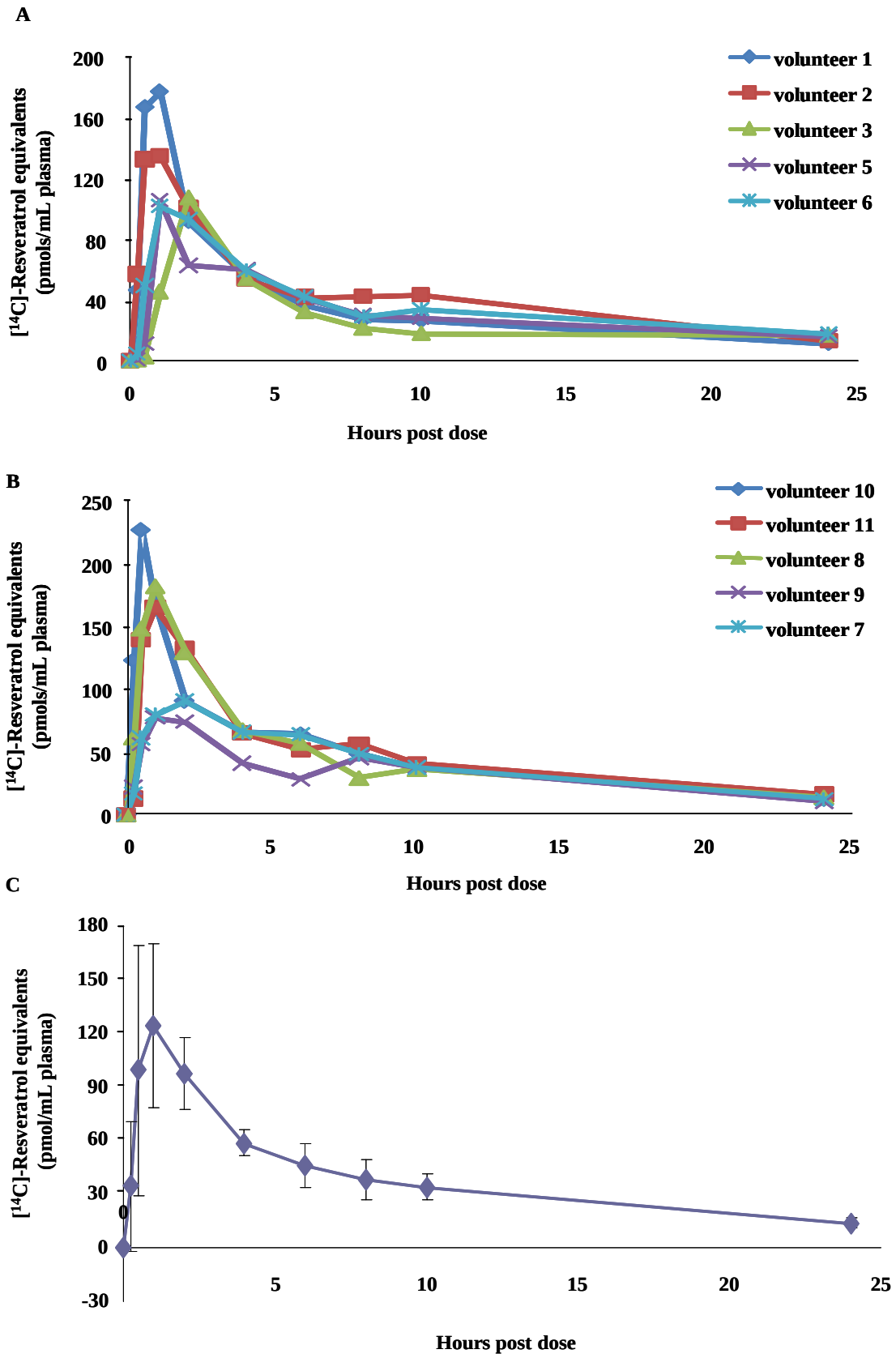


**Figure 5.2 Profiles of plasma [$^{14}$C]-resveratrol equivalents levels in healthy volunteers after administration of a single oral 1 g dose.** **A** and **B** show the individual PK profiles of the 10 volunteers, which are presented on 2 charts for clarity. **C** shows the mean plasma [$^{14}$C]-resveratrol equivalents levels in all 10 volunteers with error bars indicating the standard deviation range.

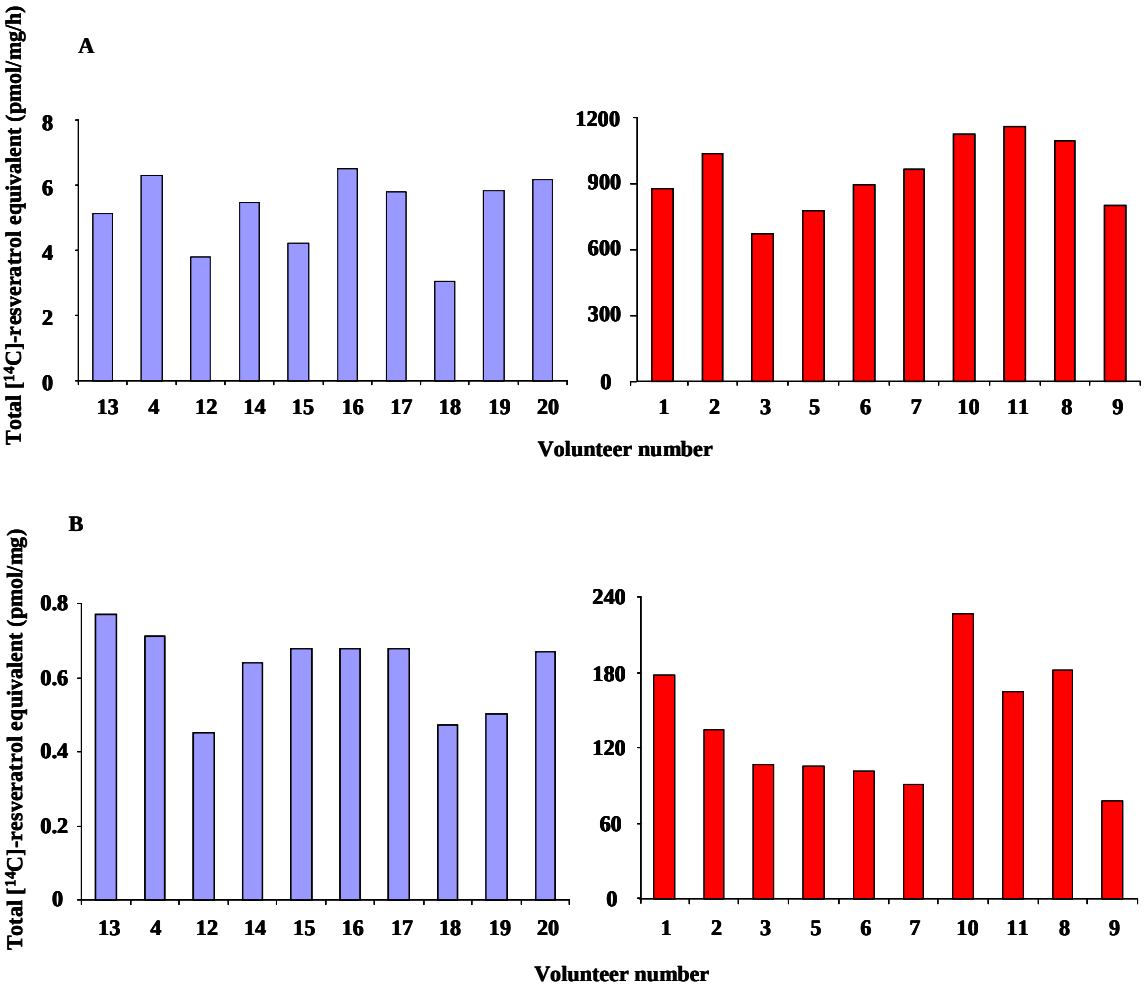


**Figure 5.3 AUC (A) and $C_{max}$ (B) of plasma [$^{14}$C]-resveratrol equivalents in healthy volunteer after oral administration of 5 mg or 1 g dose.**

HPLC fractions were collected from plasma samples taken at 30 min from volunteer 10 in the 1 g group, and at 1 h from volunteer 20 in the 5 mg group. These 2 samples were selected for analysis as they were near the $C_{max}$ values for each group and therefore it was likely that [$^{14}$C] levels would be detectable in the HPLC fractions. The plasma sample from volunteer 10 at 0 min before ingestion of resveratrol was used as the control to determine background levels of [$^{14}$C] in HPLC fractions.

Chromatograms from analysis of these samples and standards are shown in Fig 5.4. Peaks were detectable by UV at the 1 g dose (see Fig 5.4C) but this was for a $C_{max}$ sample, and it is very unlikely that the full spectrum of metabolites would be detectable at later time points by this method alone, whereas this would be achievable by HPLC-AMS. The metabolic profile of resveratrol was unchanged by dose, with metabolite concentrations being higher than that of the parent compound. This is consistent with published clinical pharmacokinetic trials of resveratrol (Boocock *et al.* 07a). Resveratrol-3-sulphate was the most prominent metabolite formed at these time points. Two peaks visible on the radioactivity trace at retention times of 9 and 20 min at both doses were assigned as sulphate-glucuronide and disulphate derivatives respectively, according to published analysis of resveratrol metabolites by mass spectrometry using a similar chromatographic system (Boocock *et al.* 07b). Standards of these were not available to demonstrate their retention time by HPLC, but identities were concluded based on order of elution relative to known metabolites where standards were available, as shown in Fig 5.4B. No additional peaks of radioactivity that may be due to novel resveratrol metabolites were identified.

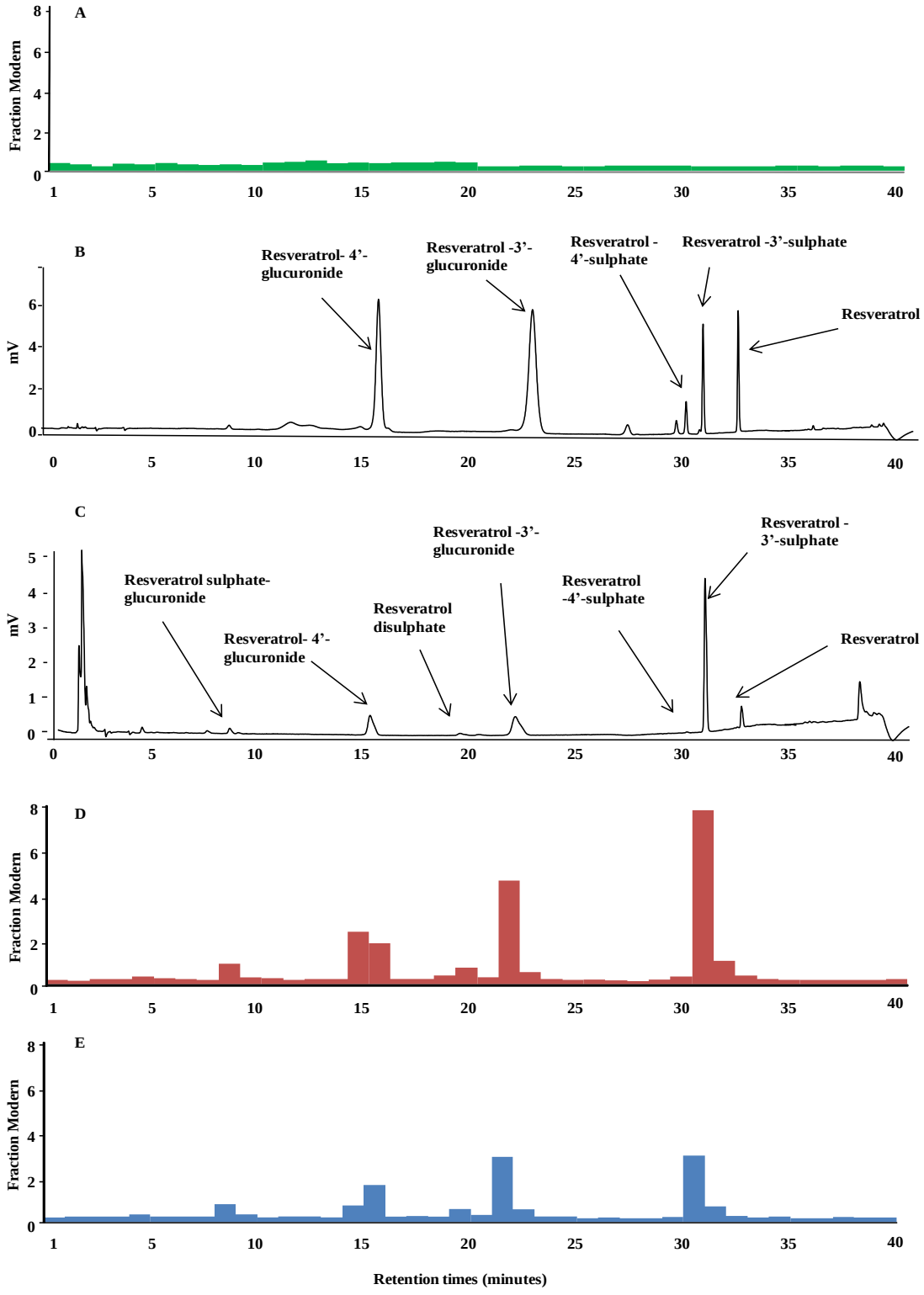


**Figure 5.4 Analysis of resveratrol and its metabolites in plasma of healthy volunteers by HPLC-AMS.** Resveratrol and its metabolites were extracted from plasma at one time point for one volunteer in each dose group. A 37 s delay in retention times was calculated between UV and AMS detection due to the length of tubing used for fraction collection. This explains why the peaks in the HPLC-AMS chromatogram are slightly offset. AMS analysis of plasma from the 1 g (**D**) and 5 mg (**E**) groups showed peaks of radioactivity which were absent in the control plasma (**A**). HPLC chromatograms from the 1 g group (**C**) showed corresponding peaks in the UV spectrum, but analysis of plasma from the 5 mg group was below the limit of UV detection. The identities of these peaks were confirmed by comparison of retention times to those of synthetic standards run at the same time (**B**).

## 5.2 Effect of dose on the pharmacokinetic profile and pharmacodynamic actions of resveratrol in colorectal cancer patients

### 5.2.1 Demographic and toxicity data of colorectal patients

The demographic data of the colon cancer patients and a summary of the types of tissues obtained are shown in Table 5.2. There were no apparent differences in the demographic data between the 5 mg and the 1 g dose groups. Benign tissue was obtained from all patients, but it was not possible to obtain malignant tissue in some due to the small tumour size and therefore the risk of disturbing the clinical staging by sampling. Peritoneal fat and colonic polyps were also obtained where available. One patient was diagnosed preoperatively with metastatic colorectal cancer to the ovary from radiological appearances, and the operating surgeon confirmed clinically the presence of a Krukenberg tumour and the colonic primary. Histologically however, it transpired that this patient had a primary ovarian adenocarcinoma which had metastasised to the colon. With the exception of this patient, all other patients were confirmed histologically to have a primary colorectal adenocarcinoma.

**Table 5.2 Demographic data and tissues obtained from colorectal patients**

| | Control group (mean, range, SD) | 5 mg dose group (mean, range, SD) | 1 g dose group (mean, range, SD) |
|---|---|---|---|
| Number of patients | 11 recruited<br>9 completed<br>1 incomplete<br>1 patient from 1 g group [A] | 10 recruited<br>8 completed<br>2 incomplete | 10 recruited<br>7 completed as 1 g dose, 2 incomplete,<br>1 completed as control [A] |
| Age (years) | 66.3<br>range 44-80<br>SD 10.9 | 72.7<br>range 45 - 88<br>SD 12.3 | 69.1<br>range 43 - 85<br>SD 11.5 |
| Gender | 5 male<br>6 female | 8 male<br>2 female | 8 male<br>2 female |
| Ethnicity | All Caucasians | All Caucasians | All Caucasians |
| Performance status (PS) | PS0 = 7<br>PS1 = 3<br>PS2 = 1 | PS0 = 5<br>PS1 = 4<br>PS2 = 1 | PS0 = 6<br>PS1 = 4 |
| Previous chemoradiotherapy | 4 | 0 | 1 |
| Interval between [$^{14}$C]-resveratrol capsule and surgery commencing | Not applicable | 11.7<br>range 3.5-17<br>SD 5.4 | 14.9 [B]<br>range 6-19.6<br>SD 5.2 |
| Number of days treated with resveratrol | not applicable | 7 days (n = 9)<br>8 days (n = 1) | 7 days (n = 9)<br>0 days ( n = 1)[A] |
| Benign colon tissue collected | n = 10 | n = 8 | n = 8 |
| Primary malignant colon tissue collected | n = 7 | n = 7 | n = 6 |
| Secondary malignant colon tissue collected | | n = 1[C] | |
| Colonic polyp collected | n = 0 | n = 1 | n = 0 |
| Peritoneal fat collected | n = 3 | n = 4 | n = 3 |
| Malignant ovarian tissue collected | n = 0 | n = 1[C] | n = 0 |

[A] One patient in the 1 g group was operated on before she commenced the resveratrol capsules due to a cancellation slot becoming available. Her tissue was therefore collected as a control patient.
[B] Data excluded one patient whose tissue was collected 6 days post administration of [$^{14}$C]-resveratrol, due to surgery cancellation because of lack of beds.
[C] One patient was recruited where pre-operatively, the radiological and clinical diagnosis was metastatic colorectal cancer with an ovarian Krunkenberg tumour deposit. Histologically however, it transpired that this patient had metastatic ovarian carcinoma to colon. The malignant colonic tissue was therefore actually ovarian in origin.

The interval between the administration of the [$^{14}$C]-resveratrol capsule and surgery commencing was dependent on bed availability and local hospital policy. Patients on an afternoon theatre list should arrive and therefore be dosed at 8 am on the day of their operation, whilst patients on a morning theatre list should arrive and therefore be dosed the evening before. It was not possible to recruit only patients on morning or afternoon theatre lists in an effort to ensure the timings between [$^{14}$C]-resveratrol capsule administration and surgery were constant, since theatre list orders were not determined until the day before surgery. One patient in the 1 g dose group was dosed on time the evening before his surgery, but his operation was cancelled the following morning due to a lack of intensive care beds for postoperative care. His tissue was still collected at his operation which was rescheduled for 6 days later, but this long interval between dosing and surgery was taken into account for data analysis.

Two patients in the control group did not complete the study, as their operations were cancelled due to unexpected brain metastases and viscus perforation respectively. These were the only two serious adverse events noted. A patient in the 1 g dose group was then substituted into one of these cancelled theatre slots by the surgeon before she commenced her course of resveratrol. Her tissue was therefore collected as control samples, but she could not be replaced as her capsules had already been dispensed to her by pharmacy. Three patients, two from the 5 mg and one from the 1 g dose group, did not complete the study as their tumours were found to be inoperable at laparotomy.

Toxicity data were collected on an intention to treat basis. Resveratrol was on the whole well tolerated and all patients completed the seven day course. The adverse events noted in the colorectal patients are summarised in Table 5.3, with only one

toxicity, dyspepsia, judged by the Principal Investigator as possibly due to the trial medication since all other events were present at baseline. Most of these adverse events were mild at CTC severity grade 1-2, except two patients with grade 3 anaemia at baseline due to their underlying malignancy.

**Table 5.3 Adverse events noted in the colorectal patient group**

| Adverse event (date of onset) | Number of patients (dose group, severity as per CTC grading) | Diagnosis | Causality |
|---|---|---|---|
| Anaemia (baseline) | n = 1 (5 mg, CTC grade 1)<br>n = 2 (5 mg, CTC grade 2)<br>n = 1 (control, CTC grade 3)<br>n = 1 (1 g, CTC grade 3) | Due to underlying colorectal tumour | Not caused by resveratrol |
| Hyperbilirubinaemia (baseline) | n = 2 (control, CTC grade 2) | Gilbert's Syndrome | Not caused by resveratrol |
| Heartburn | n = 1 (1 g, CTC grade 1) | Dyspepsia | Possibly caused by resveratrol |

### 5.2.2 Pharmacokinetic profile of [$^{14}$C]-resveratrol in colorectal patients

All tissue samples were verified to be below the limit of detection by LSC before analysis by AMS. Samples that were above the limit of detection were analysed by LSC alone, which was only applicable for tissue from patient C034. The total [$^{14}$C]-resveratrol equivalent levels in tissues from colorectal patients are summarised in Tables 5.4 and 5.5, and Fig 5.5. These absolute values have not been presented as averages as there were wide inter-individual differences in levels, due to the variable timing between administration of the [$^{14}$C]-dose and the commencement of surgery, with shorter intervals resulting in generally higher concentrations. Despite these wide inter-individual differences, the pharmacokinetic profile seems to be linear to dose with ~200 fold difference in the concentrations detected as shown in Fig

**Table 5.4 Total [$^{14}$C]-resveratrol equivalent levels in 5 mg dose group**

| Patient ID | Total [$^{14}$C]-resveratrol equivalent levels (pmols/mg tissue) | | | | | Levels relative to mucosal concentration | | | | | Time between [$^{14}$C] dose and surgery commencement (h) | Right or left sided tumour | Pathological tumour stage (Tumour, Node, Metastasis, TNM) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mucosa | Muscle | Peritoneal fat | Malignant | Other | Mucosa | Muscle | Peritoneal fat | Malignant | Other | | | |
| C027 | 0.19 | 0.06 | | 0.15 | | 1 | 0.3 | | 0.8 | | 17 | right | T3N0M0 |
| C032 | 0.05 | 0.01 | 0.02 | 0.04 | | 1 | 0.2 | 0.5 | 0.7 | | 11.5 | left | TisN0M0 |
| C033 | 1.03 | 0.17 | | 0.47 | | 1 | 0.2 | | 0.5 | | 15 | right | T3N0M0 |
| C034 | 6.19 | 2.77 | | 6.26 | polyp 7.17 | 1 | 0.4 | | 1.0 | polyp 1.2 | 6 | 2 tumours transverse and left colon | T3N0 transverse T3N1 left |
| C065 | 0.097 | 0.084 | | 0.154 | | 1 | 0.9 | | 1.6 | | 16 | left | T4N2M0 |
| C077 | 0.064 | 0.086 | 0.001 | 0.037 | | 1 | 1.3 | 0.02 | 0.6 | | 7.5 | right | T3N0M0 |
| CO79 | 6.375 | 1.208 | 1.662 | 7.906 | | 1 | 0.2 | 0.3 | 1.2 | | 3.5 | right | T2N2M0 |
| C080[A] | 0.360 | 0.237 | | 0.248 | outer ovary 0.219 inner ovary 0.236 | 1 | 0.7 | | 0.7 | outer ovary 0.6 inner ovary 0.7 | 17 | right | NA as primary ovarian tumour |

[A] Patient was found to have a primary ovarian tumour with secondary colorectal deposits.

**Table 5.5 Total [$^{14}$C]-resveratrol equivalent levels in 1 g dose group**

| Patient ID | Total [$^{14}$C]-resveratrol equivalent levels (pmols/mg tissue) | | | | Levels relative to mucosal concentration | | | | Time between [$^{14}$C] dose and surgery commencement (h) | Right or left sided tumour | Pathological tumour stage (Tumour, Node, Metastasis, TNM) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mucosa | Muscle | Peritoneal fat | Malignant | Mucosa | Muscle | Peritoneal fat | Malignant | | | |
| C040 | 560.24 | 717.72 | | 376.07 | 1 | 1.3 | | 0.7 | 6 | right | T3N1M0 |
| C043 | 7.81 | 8.39 | | 7.61 | 1 | 1.1 | | 1.0 | 12 | left | T3N0M0 |
| C048 [A] | 0.11 | below LoD | | below LoD | 1 | not applicable | | not applicable | 138 | left | T4N1M0 |
| C056 [B] | 33.47 | 2.33 | 2.13 | | 1 | 0.1 | 0.1 | | 15 | rectal | T4N1M0 |
| C058 | 145.86 | 6.60 | 6.88 | 5.17 | 1 | 0.05 | 0.05 | 0.04 | 17.5 | right | T4N1M0 |
| C059 | 4.46 | 3.43 | 0.33 | 2.84 | 1 | 0.8 | 0.1 | 0.6 | 19.6 | left | T3N1M1 |
| C072 | 4.68 | 4.53 | 0.30 | 5.17 | 1 | 1.0 | 0.06 | 1.1 | 19.5 | left | T3N1M0 |
| C081 | 170.10 | 26.92 | 11.42 | 41.26 | 1 | 0.2 | 0.07 | 0.2 | 18.5 | right | T4N2M0 |

LoD = limit of detection

[A] Surgery was delayed due to lack of postoperative intensive care bed. There was therefore a six day gap between administration of [$^{14}$C]-resveratrol capsule and surgery.

[B] Patient underwent preoperative concurrent chemoradiotherapy

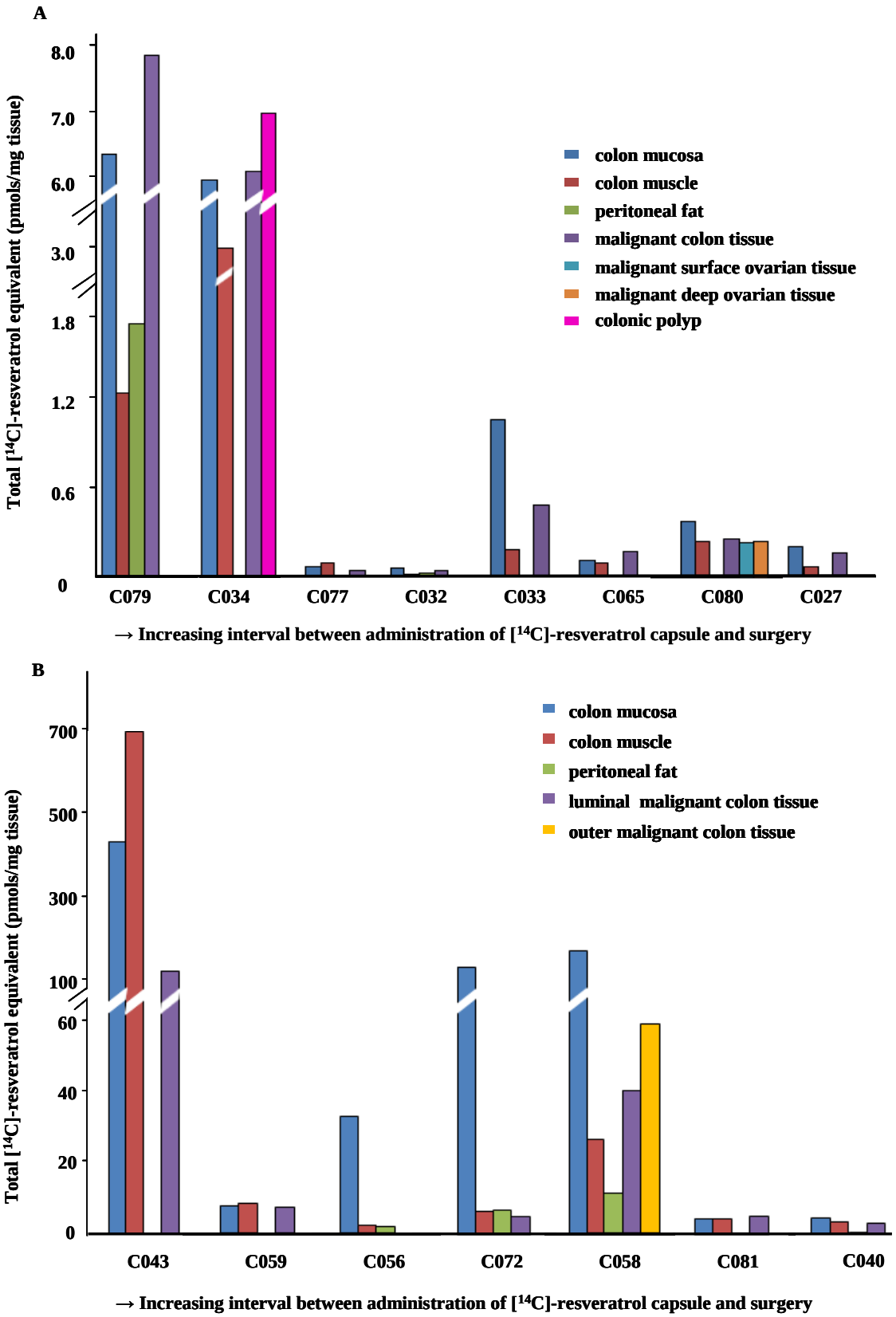


**Figure 5.5 Relationship between total [$^{14}$C]-resveratrol equivalent levels in tissues from colorectal patients, as measured by AMS, and timing of surgery.** Results from patient C048 are not shown as levels were too low to be represented with the other data, since surgery was delayed for 1 week after administration of the [$^{14}$C]-resveratrol capsule. Interruption of the y axis was necessary in order to represent all the data on the same chart due to the wide range of values.

Despite the inter-individual variability, there are conclusions that can be drawn from these data. Firstly, the total [$^{14}$C]-resveratrol equivalent levels in the benign mucosa, malignant tissue and colonic polyps were on the whole higher than those in the colonic muscle layer, peritoneal fat and ovarian tissue as shown in Fig 5.6. It is difficult to comment on whether this concentration gradient also occurred in malignant tissue, as usually only luminal shavings were taken in an effort to preserve the specimen for clinical staging. In patient C081 however, the tumour was large enough for malignant tissue to be taken as a slice extending from the lumen down to a depth of 8 mm. Analysis of these samples, labelled as inner and outer malignant colon tissue in Fig 5.6, showed that total [$^{14}$C]-resveratrol equivalent levels were similar.

It is unclear why [$^{14}$C]-resveratrol equivalent concentrations in the luminal tissue should be higher than that of outer samples. Although resveratrol was administered orally and therefore levels would have been higher in the lumen, presumably resveratrol would have passively diffused outwards along its concentration gradient into the colonic muscle layer. One possibility for the higher mucosal levels seen is that although all tissues were washed with water prior to analysis, the luminal samples were contaminated with colonic content containing resveratrol. Another possibility is that the transport of resveratrol across colonic tissue was active, with the actions of transporters outweighing the effects of passive diffusion.

A second observation is that the total [$^{14}$C]-resveratrol equivalent levels between malignant and benign mucosa were similar, with concentrations determined more by the duration between dosing and surgery than by tumour staging. Although this is an interesting observation, its significance is unclear until the levels of resveratrol and its

metabolites necessary to achieve chemopreventive efficacy have been established.

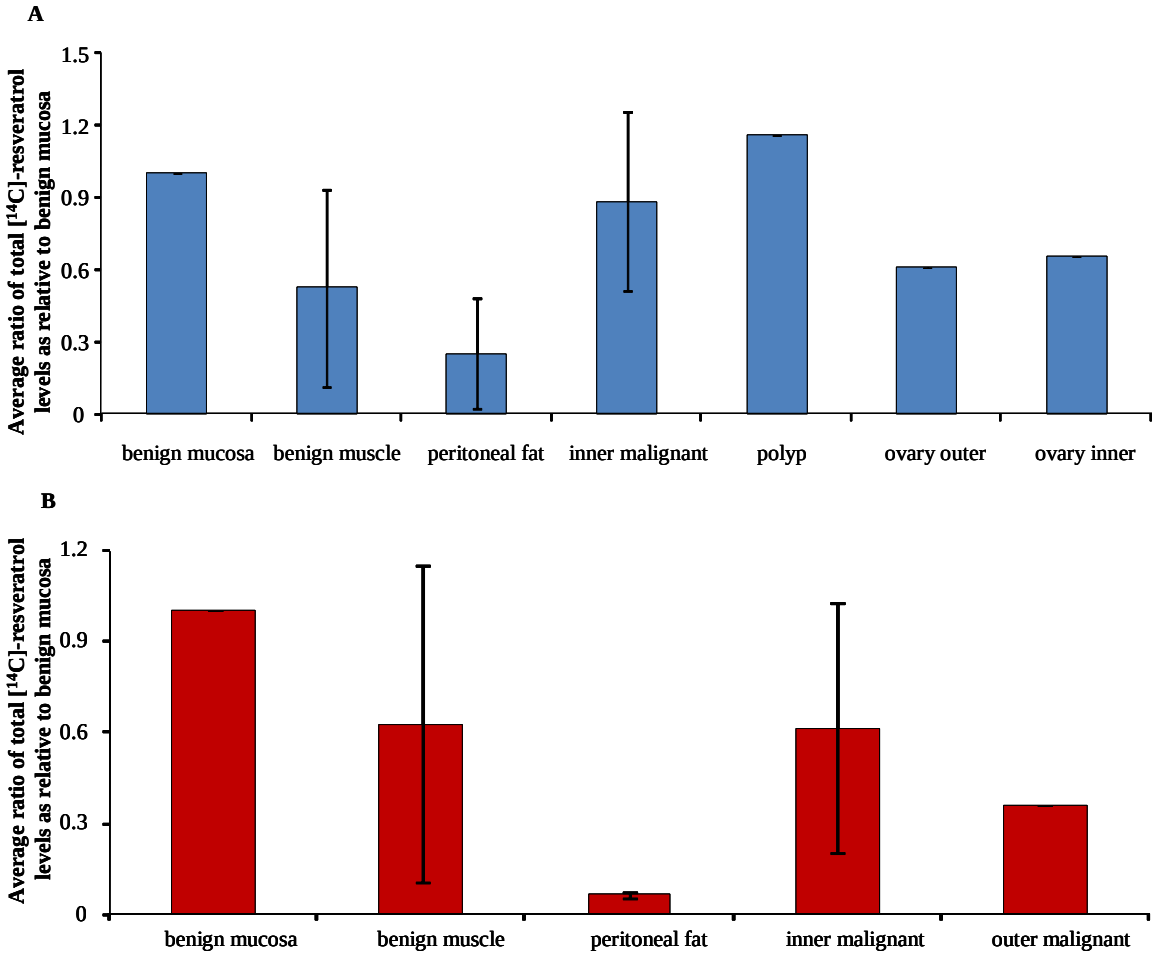


**Figure 5.6 Comparison of total [$^{14}$C]-resveratrol equivalent levels in different tissue types from colorectal patients.** Samples of benign colonic mucosa, benign colonic muscle layer, peritoneal fat, polyp, malignant colonic tissue and metastatic colorectal tumour deposit in ovarian tissue were analysed by AMS. Averages of the ratio of each tissue group compared to levels in benign colonic mucosa are shown with standard deviation for patients in the 5 mg (**A**) and the 1 g (**B**) dose group.

A third conclusion from these data is that the tumour anatomical site influenced total [$^{14}$C]-resveratrol equivalent levels. Colonic tumours may be present in the right ascending colon, the transverse colon or the left descending colon as shown in Fig 5.7, with faecal transit following this order. Total [$^{14}$C]-resveratrol equivalent concentrations were higher in right compared to left sided colonic samples, with this pattern seen in both luminal and outer tissue. This anatomical difference persisted in samples taken at similar post dose intervals, suggesting that resveratrol delivery is

partly topical since if distribution was solely via the bloodstream, [$^{14}$C] levels in all parts of the colon should have been similar at a specified time. These findings are supported by data from another study which included a patient with two synchronous tumours, where levels of resveratrol and its metabolites were higher in the tumour in the ascending colon than the one in the descending colon (Patel *et al.* 10).

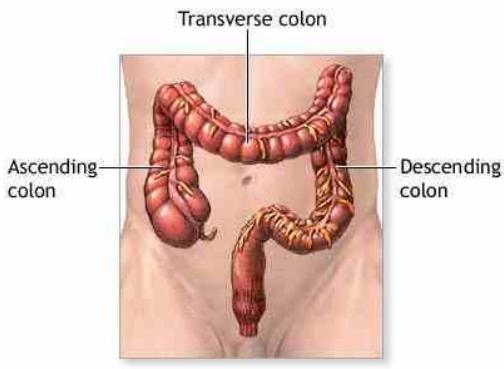


**Figure 5.7 Anatomical distribution of the human colon (adapted from educational website www.healingdaily.com.)**

Published studies have shown that oral administrations of other compounds, for example quinine and 5-aminosalicylic acid, also resulted in higher levels in the right compared to the left side of the colon (Hebden *et al.* 99, Thorpe *et al.* 09). One possible reason is that faecal transit is slower in the ascending colon, with a median transit time of 31 h compared to 19 h in the descending colon, as demonstrated by ingestion of radio-opaque markers by healthy volunteers (Pomerri *et al.* 09). Orally administered compounds may therefore have greater contact time with the mucosa on the right side of the colon than the left, resulting in higher tissue levels.

Another possible cause is that the luminal pH of the ascending colon is more acidic than the descending colon (Nugent *et al.* 01). Resveratrol is itself a mild acid and would be in a more ionic form in the alkali conditions of the left colon, thereby possibly

impeding its absorption. Luminal pH may also affect the activity of transporters if they were required for the uptake of resveratrol into the mucosal cells. The Breast Cancer Resistance Protein (BCRP) transporter for example, has been shown to transport resveratrol into canine renal MDCKII cells at pH 6.0 but not at pH 7.4 (Breedveld *et al.* 07). Anatomically, the mucosal layer of the left side of the colon is also thicker than the right, which could potentially impair the uptake of luminal compounds (Pullan *et al.* 94). Physiologically, faecal matter in the ascending colon is more liquid than that in the descending colon. The uptake of resveratrol and its metabolites in solution may be greater than their uptake in solid form.

A fourth point of interest arising from these clinical data refers to the samples of patient C048, whose operation was cancelled due to a lack of postoperative intensive care beds. Despite a significant delay of six days between administration of the [$^{14}$C] dose and surgery, total [$^{14}$C]-resveratrol equivalent concentrations were still detectable in the benign mucosal lining. Levels were however, below the limit of detection in the malignant tissue and the benign colonic muscle layer. The prolonged retention of resveratrol and/or its metabolites in colonic cells may be due to enterohepatic recirculation as demonstrated in animal models (Marier *et al.* 02, Section 1.2.2.2), or they may be bound to intracellular proteins accounting for their slow clearance. This latter hypothesis is supported by preclinical data which showed that resveratrol is bound to plasma proteins and also to have specific intracellular locations after uptake (Jannin *et al.* 04, Lancon *et al.* 04, Section 3.2.1). A clinical pharmacokinetic study similarly demonstrated that after administration of a single oral dose of 85.5 mg of piceid (*trans*-resveratrol-3-*O*-β-D-glycoside), up to 50 % of resveratrol plasma metabolites were non-covalently bound to proteins (Burkon *et al.* 08).

*In vitro* in human colonic Caco-2 cells, resveratrol transport at 150-300 μM was shown to be by passive diffusion as it was unaffected by ATP depletion (Henry *et al.* 05). This study also demonstrated that intracellular resveratrol levels plateau after 5 min of incubation, which may be due to transport saturation as increasing resveratrol concentration did not promote uptake. The plateau seen was not due to the formation of metabolites as shown by HPLC analysis. The addition of an inhibitor to the multidrug resistance-associated protein 2 (MRP2) however, abolished this plateau effect, suggesting that the limit in intracellular resveratrol concentrations was due to the activity of this apical active transporter establishing an equilibrium between influx and efflux. Another study in Caco-2 cells confirmed the activity of MRP2, but suggested that there may also be an active transporter on the basolateral side, namely MRP3 (Kaldas *et al.* 03). This study administered resveratrol at 5-40 μM and showed that resveratrol was metabolised to sulphates in colon cells.

Active uptake of resveratrol has also been demonstrated in other cell lines. In human hepatocytes for example, resveratrol uptake was found to be greater at 37 °C than at 4 °C, suggesting that active transporters were present (Lancon *et al.* 04). Furthermore, this study showed that concomitant exposure to *cis*-resveratrol inhibited uptake of the *trans*-isomer, suggesting competition at a transporter level. Some resveratrol uptake however, was still demonstrated at 4 °C, suggesting passive diffusion was also possible. *In vivo*, involvement of the MRP3 and breast cancer resistance protein (BCRP) in resveratrol metabolism was demonstrated in knockout mice (van de Wetering *et al.* 09). MRP3 was shown to be an active transporter of resveratrol-3-glucuronide, whilst BCRP was an active transporter of resveratrol-3-glucuronide, resveratrol-3-sulphate and resveratrol-4’-sulphate.

Resveratrol and its metabolites were extracted from the luminal and the outer benign colon samples of patients C033 and C058, in the 5 mg and the 1 g groups respectively, and analysed by HPLC-AMS. The resulting HPLC chromatograms are shown in Fig 5.8, with the peaks identified from the retention times of standards, as well as using information on order of elution and knowledge of the metabolite profile in plasma determined previously using a similar system (Boocock *et al.* 07b). Samples from these two patients were selected for analysis as they had high total [$^{14}$C] levels, and were therefore most likely to allow for the detection of metabolites even if present in small amounts. Benign tissue was chosen to allow for the comparison of the pharmacokinetic profiles of resveratrol in luminal tissue with that in outer samples.

Although samples were analysed from only one patient per group, tentative conclusions can be drawn from these data. At the 1 g dose, the pharmacokinetic profiles of resveratrol were similar between luminal and outer tissues as shown in Fig 5.8E-F. From UV data, sulphate levels were higher than that of glucuronides with resveratrol-4'-sulphate being the predominant metabolite according to peak area, and present at a slightly lower concentration than the parent compound. This is different to the profile seen in plasma samples from healthy volunteers as shown in Fig 5.4, where the level of resveratrol-3-sulphate was higher than that of the parent compound.

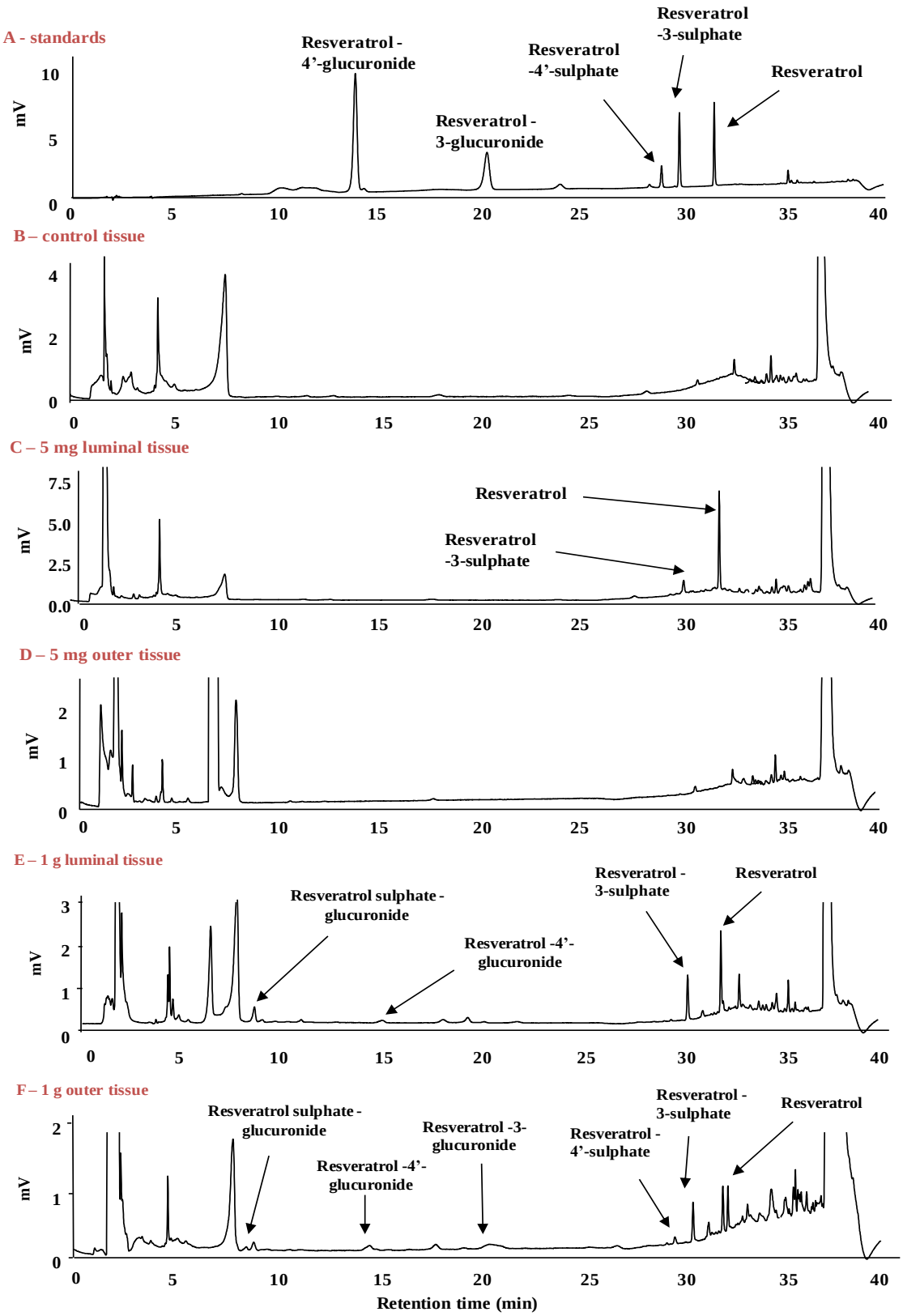


**Figure 5.8 Analysis of resveratrol and its metabolites in colorectal tissue of cancer patients by UB-HPLC.** Resveratrol and its metabolites were extracted from the benign colon samples of patients in the control (**B**), 5 mg (**C**=luminal, **D**=outer) and 1 g (**E**=luminal, **F**=outer) groups, and analysed by UV-HPLC. The peaks on the chromatograms were identified from the retention times of standards (**A**), as well as using information on order of elution and knowledge of the metabolite profile in plasma determined previously using a similar system (Boocock *et al.* 07b).

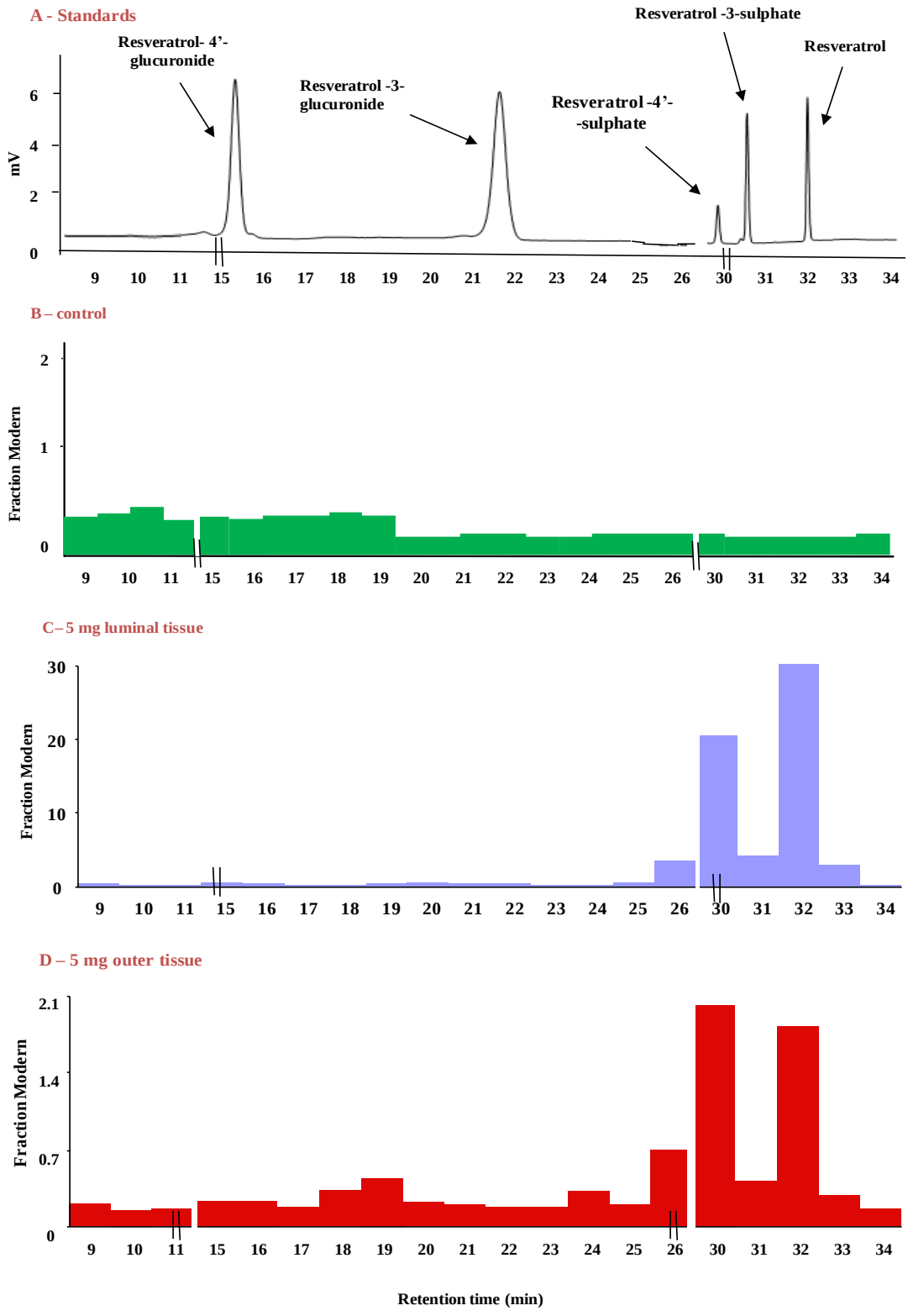


**Figure 5.9 Analysis of resveratrol and its metabolites in colorectal tissue of cancer patients by HPLC-AMS.** One minute HPLC fractions were analysed by AMS for the luminal (**C**) and outer (**D**) tissue of a patient in the 5 mg group, with background [$^{14}$C] levels defined by values from the control patient (**B**). The peaks on the chromatograms were identified from the retention times of standards (**A**), as well as using information on order of elution and knowledge of the metabolite profile in plasma determined previously using a similar system (Boocock *et al.* 07b). The retention times on the AMS traces were 37 s later than the corresponding HPLC chromatograms, due to the length of the fraction collecting tube beyond the UV detector.

At the 5 mg dose, the pharmacokinetic profile was incomplete from UV-HPLC analysis alone due to the low concentrations of parent compound and metabolites, as shown in Fig 5.8C-D. Fractions were therefore selected for analysis by AMS, based on the retention times of the parent compound and known metabolites. The resulting peaks of radioactivity (Fig 5.9) showed that the pharmacokinetic profile of the luminal tissue was again similar to that of the outer tissue, and the profile at the 5 mg dose was also similar to that at the 1 g dose. The radioactivity peaks of the resveratrol-3-sulphate (0.138 and 0.014 pmols/mg tissue for luminal and outer tissue respectively) were similar to that of resveratrol (0.206 and 0.012 pmols/mg tissue for luminal and outer tissue respectively), but the UV-HPLC chromatograms suggested that resveratrol was the major species. This difference is due to the two having different extinction coefficients, with the sulphate peak being ~3 times smaller than resveratrol for an equivalent concentration.

In addition, an unknown metabolite was identified at a retention time of 26 min for both luminal and outer tissue samples, which is unlikely to be due to contamination as this peak was seen at both doses. Additional metabolites may have been present in colon tissue compared to plasma due to metabolism of resveratrol by gastrointestinal bacteria, which has been demonstrated to occur *in vitro* and in rodents (Jung *et al.* 09, Alfaras *et al.* 10). The identity of this unknown peak can be determined by mass spectrometry, after first increasing the metabolite concentration by amalgamating fractions collected at this time point after extraction from multiple tissue samples.

In conclusion, the pharmacokinetic data from the colon samples showed that the levels of resveratrol and its metabolites were generally higher in luminal than outer tissue,

with the latter including intestinal muscle layer as well as peritoneal fat. Delivery of orally administered resveratrol to the colon is by direct luminal transport as well as via the systemic route. The pharmacokinetic profile of resveratrol in luminal and outer tissues were similar, and the profile at the 5 mg dose was also similar to that at the 1 g dose. Resveratrol is metabolised to a lesser extent in colon tissue than in the plasma, but the most abundant metabolite in both was resveratrol-3-sulphate.

### 5.2.3 Effect of resveratrol on IGF1 levels in colon tissue

Administration of resveratrol for 7 days did not alter IGF1 levels in benign colonic mucosa as shown in Fig 5.10. IGF1 levels were measured in samples from only half of the patients selected at random as a preliminary test and due to the negative findings, further analysis was not carried out in the remaining tissue.

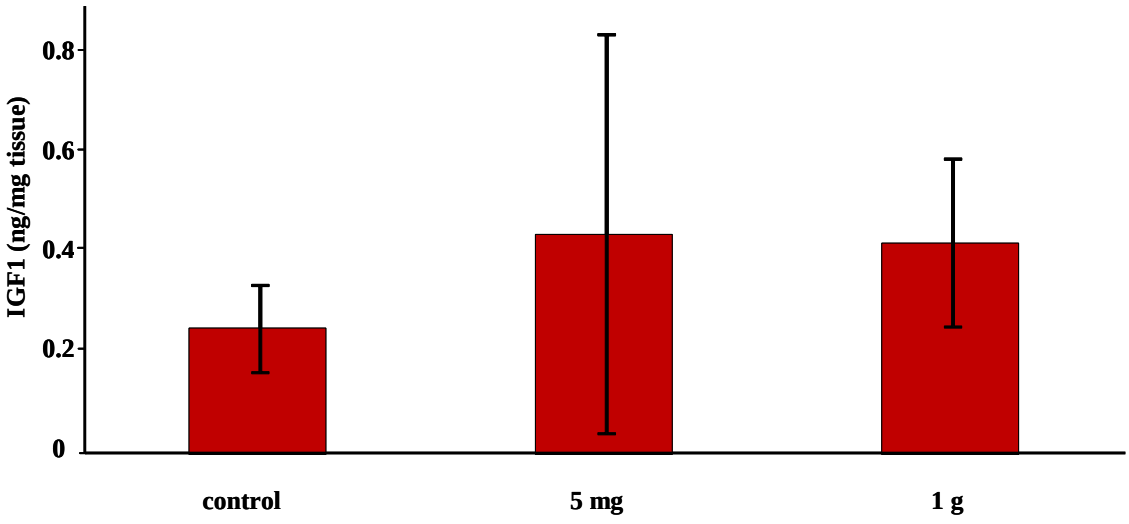


**Figure 5.10 Analysis of IGF1 levels in colorectal tissue of cancer patients by ELISA.** Benign colonic mucosal samples were used from patients in the control, 5 mg and 1 g groups (n=4-5). Averages are shown in the figure above with error bars denoting standard deviations.

## 5.3 Effect of dose on the pharmacokinetic profile of resveratrol in prostate tissue

### 5.3.1 Demographic and toxicity data of prostate patients

The demographic data of the prostate patients are shown in Table 5.6. There were no apparent differences in the demographic data between the 5 mg and the 1 g dose groups.

**Table 5.6 Demographic data of prostate patients**

| | Control group (mean, range, SD) | 5 mg dose group (mean, range, SD) | 1 g dose group ( mean, range, SD) |
|---|---|---|---|
| Number in group | 10 recruited<br>10 completed | 10 recruited<br>9 completed | 10 recruited<br>10 completed |
| Age in years | 66.3<br>range 56-83<br>SD 8.0 | 63.4<br>range 52-78<br>SD 7.2 | 60.9<br>range 51-68<br>SD 6.0 |
| Ethnicity | 9 Caucasian<br>1 Indian/Caucasian | 10 Caucasian | 10 Caucasian |
| Performance status (PS) | PS 0 = 8<br>PS 1 = 1<br>PS 2 = 1 | PS 0 = 7<br>PS 1 = 1<br>PS 2 = 1 | PS 0 = 10 |
| Previous radiotherapy | 0 | 0 | 0 |
| Final histological diagnosis | Benign (n = 3)<br>PIN (n = 1)<br>Gleason 6 (n = 2)<br>Gleason 7 (n = 2)<br>Gleason 9 (n = 1)<br>Gleason 10 (n = 1) | Benign (n = 1)<br>Gleason 6 (n = 5)<br>Gleason 7 (n = 2)<br>Gleason 8 (n = 1) | Benign (n = 2)<br>PIN (n = 1)<br>Gleason 6 (n = 4)<br>Gleason 7 (n = 2)<br>Gleason 9 (n = 1) |
| Time between oral [$^{14}$C]-resveratrol capsule and tissue sampling (h) | Not applicable | 2.35<br>range 1.5-3<br>SD 0.71 | 2.85<br>range 2.33-4<br>SD 0.54 |
| Number of days treated with resveratrol | Not applicable | 13.3<br>range 10-15<br>SD 3.8 | 10.9<br>range 7-14<br>SD 2.6 |

PIN = Prostate *in-situ* neoplasia

There were no serious adverse events. All patients completed the study except one, in the 5 mg dose group, who stopped after two doses due to migraines. This patient was known to suffer from migraines and was already on regular medication for this, but he felt that the frequency of his headaches increased due to the resveratrol. The treatment duration was determined by the interval between patients being identified as requiring prostate biopsy and their allocated operation date. All patients, except the one with migraine mentioned above, completed their full course of resveratrol as practical timing would allow. Patients who received less than 14 doses of resveratrol therefore did not stop their capsules prematurely due to toxicity.

**Table 5.7 Adverse events noted in prostate patients**

| Adverse event (date of onset) | Number of patients (dose group, severity as per CTC grading) | Diagnosis | Causality |
|---|---|---|---|
| Diarrhoea | n = 1 (1 g, CTC grade 1) | Side effect of resveratrol | Possibly caused by resveratrol |
| Abdominal pain | n = 1 (1 g, CTC grade 1) | Side effect of resveratrol | Possibly caused by resveratrol |
| Cough | n = 1 (5 mg, CTC grade 1) | Viral infection | Not caused by resveratrol |
| Hyperbilirubinaemia (baseline) | n = 1 (1 g, CTC grade 1)<br>n = 1 (control, CTC grade 1) | Gilbert's Syndrome | Not caused by resveratrol |
| Elevated alanine transaminase (baseline) | n = 1 (5 mg, CTC grade 1) | Exercise | Not caused by resveratrol |
| Hyperkalaemia (baseline) | n = 1 (control, CTC grade 2) | Normal physiological variation | Not caused by resveratrol |
| Headache | n = 1 (5 mg, CTC grade 2) | Migraine | Probably not related |

Toxicity data were collected on an intention to treat basis and the adverse events noted are summarised in Table 5.7, all of which were CTC grade 1-2 in severity. Only adverse events occurring in one patient, diarrhoea and abdominal pain, was felt by the Principal Investigator as being possibly caused by the trial medication.

### 5.3.2 Pharmacokinetic profile of [$^{14}$C]-resveratrol in prostate patients

Total [$^{14}$C]-resveratrol equivalent concentrations for prostate patients are shown in Table 5.8. Samples were all taken at similar post dose intervals with no correlation between levels and tumour staging. Average levels measured in prostate samples from treated patients (n=6-7 per group) and from controls (n=3) are shown in Table 5.9. The pharmacokinetic profile to dose ratio was linear, with the average tissue levels at the 1 g dose being ~280 fold higher than that at the 5 mg dose, which is comparable to the ratio between the 2 doses administered (1:200).

**Table 5.8 Total [$^{14}$C]-resveratrol equivalent levels in human prostate tissue**

| 5 mg dose group | | | | 1 g dose group | | | |
|---|---|---|---|---|---|---|---|
| Patient ID | Total [$^{14}$C]-resveratrol equivalent levels (pmols/mg) | Interval between [$^{14}$C] dose and biopsy (h) | Pathological stage | Patient ID | Total [$^{14}$C]-resveratrol equivalent levels (pmols/mg) | Interval between [$^{14}$C] dose and biopsy (h) | Pathological stage |
| P030 | 0.094 | 2.75 | Gleason 7 | P038 | 24.77 | 2.67 | Gleason 7 |
| P035 | 0.062 | 2 | Gleason 6 | P049 | 29.72 | 4 | Gleason 9 |
| P054 | 0.052 | 2.33 | Benign | P051 | 34.35 | 3 | Gleason 7 |
| P067 | 0.048 | 3 | Gleason 8 | P052 | 11.07 | 2. | Gleason 6 |
| P029 | 0.069 | 3 | Gleason 6 | P055 | 21.89 | 2.5 | Benign |
| P031 | 0.153 | 3 | Gleason 6 | P061 | 16.67 | 2.67 | Prostate *In-situ* neoplasia |
| | | | | P063 | 16.17 | 2.33 | benign |

**Table 5.9 Comparison of average total [$^{14}$C]-resveratrol equivalent levels in human and rat prostate tissue and plasma**

| | 5 mg dose group (n = 6) | 1 g dose group (n = 7) |
|---|---|---|
| Average total [$^{14}$C]-resveratrol equivalent levels in human prostate tissue | 0.079 pmols/mg tissue (SD 0.039) | 22.09 pmols/mg tissue (SD 8.18) |
| Estimated human plasma levels at the time of biopsy | 0.4 pmols/μL | 70 pmols/μL |
| Estimated total [$^{14}$C]-resveratrol equivalent levels in rat prostate tissue at same time point post dose | 0.18 pmols/mg tissue | 19 pmols/mg tissue |
| Estimated rodent plasma levels at same time point post dose | 0.013 pmols/μL | 3.4 pmol/μL |

Further analysis was carried out by estimating the total plasma [$^{14}$C]-resveratrol equivalent levels at the time of prostate biopsy from the healthy volunteer data in Section 5.1, and the plasma and prostate concentrations from the rodent study in Section 3.2.1. Comparisons between these values were made based on the assumption that 1 μL of plasma is equivalent to 1 mg of tissue in weight. As shown in Table 5.9 and Fig 5.11, prostate levels at both doses were lower than the corresponding plasma levels in humans, but the reverse was true in rodents where tissue concentrations were higher than plasma levels. Total prostate [$^{14}$C]-resveratrol equivalent concentrations were similar in both species, but human plasma levels were much higher than rodent plasma.

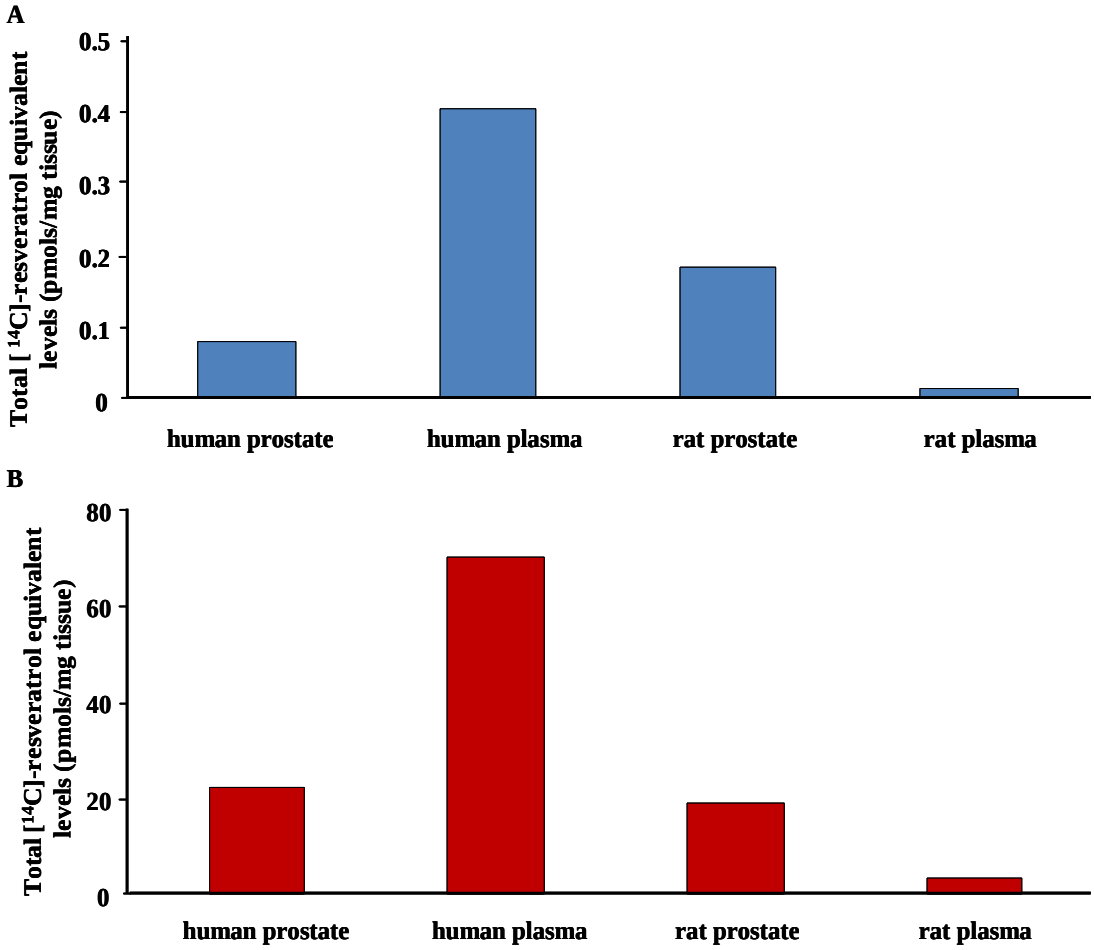


**Figure 5.11 Comparison of total [$^{14}$C]-resveratrol equivalent levels in prostate tissue and plasma samples from humans and rats.** Prostate patients were administered resveratrol at 5 mg (A) or 1 g (B) per day for 7-14 days before tissue sampling was carried out. Healthy volunteers were administered a single dose and plasma samples taken at 9 different time points post dose (see Section 5.1). These human data were then compared to results from the single dose rodent study in Section 3.2.1 at the same time point post dose.

It is difficult to draw definite conclusions from these comparisons between species as the human volunteer plasma and the rodent values were from single dose studies, whereas the human prostate tissue levels were from a multiple dose schedule. In house clinical data from healthy volunteers however, suggest that the pharmacokinetic profile of resveratrol after repeated dosing is similar to that after a single dose, when given once per day at the same dose (Brown *et al.* 10).

The total [$^{14}$C]-resveratrol equivalent levels in this study suggest that the optimum method for dose conversion between species may be different depending on the specific compound in

question, and that the empirical use of a formula based on surface area as suggested by the FDA may not be always suitable (Reagan-Shaw *et al.* 08). Data from this study showed that dose conversion by body weight for example, was appropriate as the target organ concentrations achieved were similar in both species. The results also demonstrated that plasma and target tissue levels may be significantly different. Plasma values should not therefore be used as surrogate markers for tissue concentrations, despite the fact that this may be an attractive extrapolation due to the obvious difficulty in obtaining human target tissue. It should not be presumed either that the ratio between the plasma and target tissue levels in humans is the same as in rodents, since this ratio can be completely different between species as shown in this study. The ultimate proof that a dose conversion calculation was appropriate between species would be the confirmation that similar target tissue levels were obtained.

It is interesting to note that in rodent prostate tissue, total [$^{14}$C]-resveratrol equivalent levels were higher than the corresponding plasma concentrations, suggesting perhaps protein binding for this selective extravascular accumulation. This did not seem to occur in humans at the same post dose time point. Such comparison between species is however, fraught with difficulties as there are many variables that cannot be taken into account. The clinical study for example, did not allow calculation of AUC as levels were available only in a short time window post dose. The rate of resveratrol uptake and metabolism may be different between species, and therefore it may not have been appropriate to compare the same single time point post dose between rat and man as peak plasma levels may have occurred at different times. The types of metabolites formed may also have been different between species, accounting for different rates of excretion of [$^{14}$C] levels. A pharmacokinetic study in rats and mice for example, showed that the proportion of sulphates and glucuronides formed after administration of resveratrol was different in rat and mouse serum and urine (Yu *et al.* 02).

Resveratrol and its metabolites were also extracted from the prostate tissue of one patient from each of the control, 5 mg and 1 g groups for separation by HPLC. The resulting HPLC chromatograms are shown in Fig 5.12, with the peaks identified from the retention times of standards, as well as using information on order of elution and knowledge of the metabolite profile in plasma determined previously using a similar system (Boocock *et al.* 07b). Large unknown peaks were seen in both the control and the 5 mg patient UV chromatograms at different retention times compared to the standards. These are likely to be due to other materials present, for example concurrent medications taken by these patients. Resveratrol and its metabolites were not visible on the HPLC-UV chromatogram from the patient in the 5 mg group (Fig 5.12C), but were seen in the extract from the 1 g patient (Fig 5.12D). One minute fractions were therefore selected for AMS analysis, based on the retention times of the known standards, to compare the metabolite profiles at the 5 mg and 1 g dose.

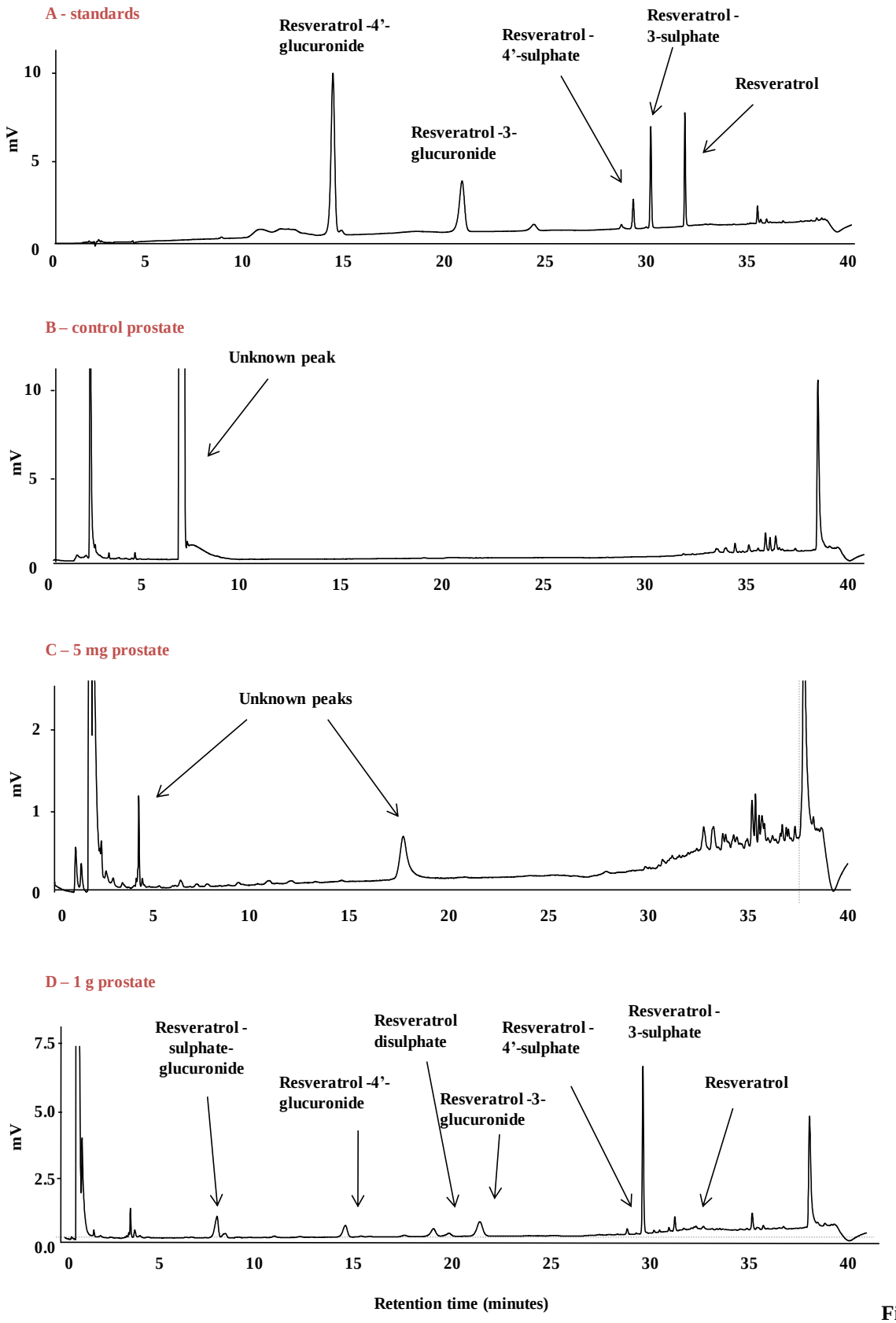


**Figure 5.12 Analysis of resveratrol and its metabolites in prostate tissue of cancer patients by HPLC-AMS.** Samples were analysed from one patient in the control (B), 5 mg (C) and 1 g (D) groups. The peaks on the chromatograms were identified from the retention times of standards (**A**), as well as using information on order of elution and knowledge of the metabolite profile in plasma determined previously using a similar system (Boocock *et al*. 07b).

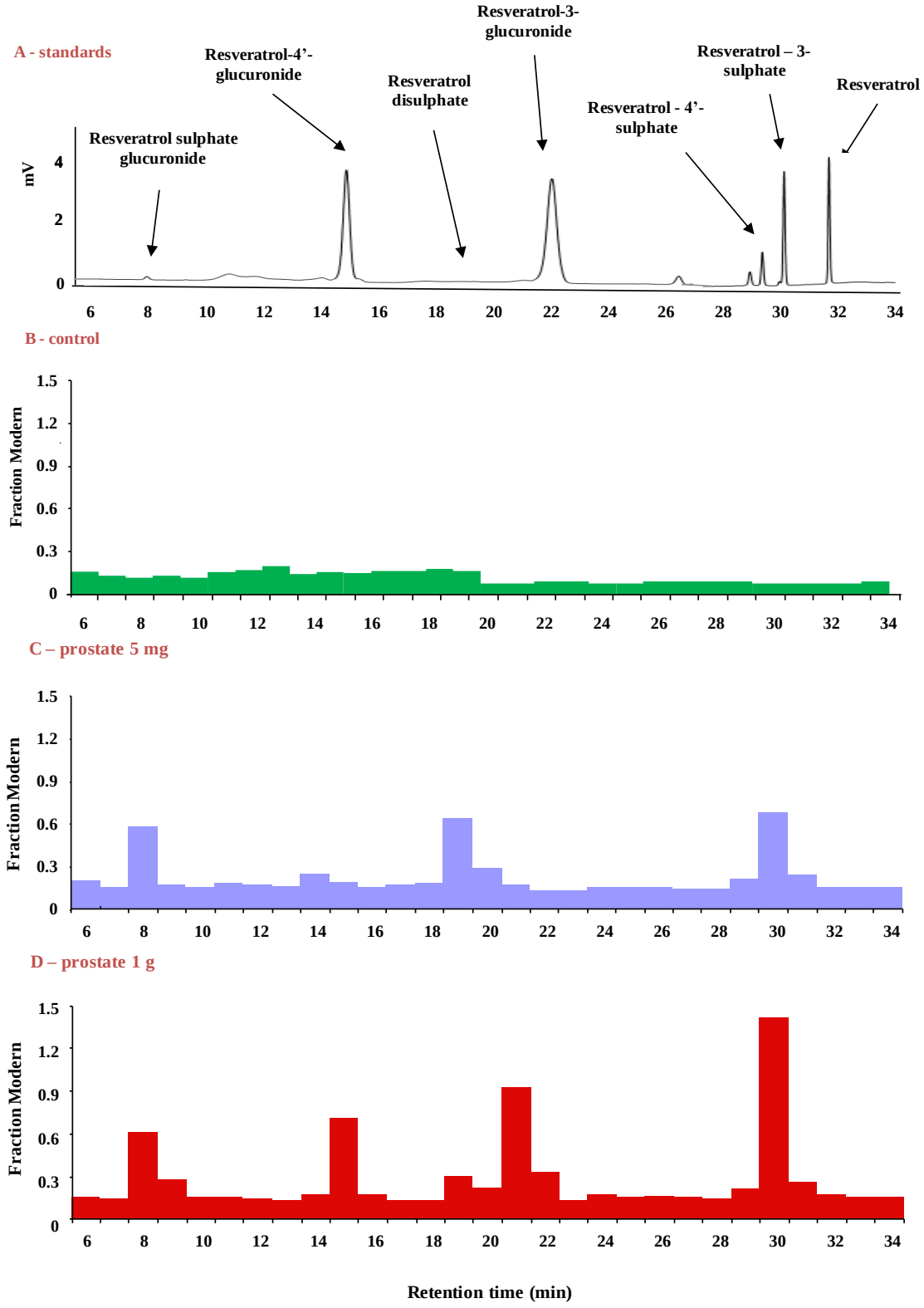


**Figure 5.13 Analysis of resveratrol and its metabolites in prostate tissue of cancer patients by HPLC-AMS.** One minute HPLC fractions were analysed by AMS for the 5 mg (C) and the 1 g (D) patient, with background [$^{14}$C] levels defined by values from the control patient (B). The AMS retention times were 37 s later than the corresponding HPLC retention times, due to the length of the fraction collecting tube beyond the UV detector. The peaks on the chromatograms were identified from the retention times of standards (**A**), as well as using information on order of elution and knowledge of the metabolite profile in plasma determined previously using a similar system (Boocock *et al* 07b).

Total metabolite levels at both doses were higher than that of the parent compound. This extensive metabolism was also seen in plasma as shown in Fig 5.4 but unlike the plasma data, resveratrol-3-sulphate was not the only predominant metabolite in prostate tissue. Similar levels of resveratrol glucuronides, resveratrol disulphate and resveratrol glucuronide-sulphate were also found as shown in Fig 5.13C-D and Table 5.10.

**Table 5.10 Concentrations of resveratrol metabolites in prostate tissue after oral administration of 5 mg or 1 g of [$^{14}$C]-resveratrol (n = 1)**

| Resveratrol species | 5 mg dose | 1 g dose |
|---|---|---|
| Resveratrol sulphate-glucuronide | 0.010 pmols/mg tissue | 2.072 pmols/mg tissue |
| Resveratrol-4'-glucuronide | 0.003 pmols/mg tissue | 2.392 pmols/mg tissue |
| Resveratrol-3-glucuronide | 0.010 pmols/mg tissue | 3.166 pmols/mg tissue |
| Resveratrol-3-sulphate | 0.012 pmols/mg tissue | 4.816 pmols/mg tissue |

The data presented in this chapter shows for the first time the pharmacokinetic profile of resveratrol investigated in prostate tissue in man. By using an extremely sensitive technique such as AMS, these results are also unique in demonstrating that resveratrol and its metabolites are detectable in multiple organs in humans after oral administration of only dietary doses. Although resveratrol was extensively metabolised in plasma, colon and prostate tissue, the metabolic profile differed between the different organs. The clinical significance of these different metabolic profiles is uncertain as it is unclear whether the active chemopreventive agent is resveratrol, its metabolites or both.

In plasma and colon tissue, the predominant metabolites were sulphates whereas in prostate tissue, glucuronides were found in equal abundance. Parent resveratrol was found at higher concentrations relative to the levels of metabolites in colon tissue compared to plasma and prostate samples, suggesting that enzymes may be present in the intestine to convert

metabolites back to the parent compound. (van de Wetering *et al.* 09) Alternatively, this finding may be because orally administered resveratrol was delivered to the colon via the lumen as well as via the bloodstream, with the topical route of delivery being unique to the gastrointestinal tract and therefore perhaps resulting in different metabolism, or lack of metabolism until it is absorbed systemically. *In vitro* and rodent data for example, have shown that up to 20 % of colonic luminal resveratrol can be metabolised by intestinal bacteria into piceid and dihydroresveratrol (Jung *et al.* 09, Alfaras *et al.* 10).

Despite these differences, the pharmacokinetic profiles in each organ were similar at both dietary and supra-dietary doses, suggesting that the mechanisms and enzymes involved in the pharmacokinetics of resveratrol were not saturated at dietary doses. Administration of a supra-dietary dose could therefore lead to far higher levels of both parent compound and metabolites, which may then lead to different effects compared to that of a dietary dose. Resveratrol, at 1 g per day, has certainly been demonstrated to be pharmacologically active in man in terms of inducing or inhibiting the activity of different enzymes in the cytochrome P450 pathway (Chow *et al.* 10). This study administered resveratrol orally for only 4 weeks, whereas the treatment duration for chemoprevention purposes could be for years. This is therefore a clinically important finding which needs to be considered in the design of future chemoprevention trials. Resveratrol at pharmacological doses could potentially alter the deactivation of carcinogens but could also alter the efficacy of concomitant therapeutic drugs, including anticancer agents such as tamoxifen.

In conclusion, the results from this project confirm that non-toxic doses of resveratrol after oral administration in patients can reach both prostate and colon tissues, suggesting that these organs may be targets for its purported chemopreventive activity. It is as yet unclear if the

levels detected are biologically active clinically as such low doses have not been previously investigated, but preliminary data from the *in vitro* and *in vivo* work presented here suggest that resveratrol can exert changes on the colorectal carcinogenesis pathway.

# 6. Conclusions

## 6. Conclusions

Epidemiological data suggest that cancer chemopreventive agents found in the diet may be active at dietary doses, whilst preclinical studies have shown that supra-dietary doses can also be efficacious. This project therefore investigated the effect of dose on the pharmacokinetic profile and cancer chemopreventive actions of resveratrol, a polyphenol found in grapes and peanuts, *in vitro*, *in vivo* and in man, comparing a dietary and a clinically relevant supra-dietary dose. The dietary dose was determined as 5 mg per day since this was the amount that could be consumed in a normal diet, whilst the supra-dietary dose was determined to be 1 g per day as this has previously been demonstrated to be safe in clinical trials (Boocock *et al.* 07a, Brown *et al.* 10). The equivalent doses for murine administration were determined based on body weight conversion, and were shown to result in the same intestinal tissue levels as those found in man. The 5 mg and 1 g doses in man were found to be equivalent to murine doses of 0.07 mg/kg and 143 mg/kg body weight respectively. F344 rats were used to determine the appropriate animal doses, rather than $Apc^{Min+/-}$ mice, as more tissue was then available for analysis. [$^{14}$C]-Resveratrol was used to allow the detection of resveratrol species by AMS, as the dietary dose administered was below the limit of detection by other standard techniques, for example HPLC.

Pharmacokinetic data from this project in healthy volunteers and F344 rats showed that plasma and tissue levels of total [$^{14}$C]-resveratrol equivalents were detectable at 24 h after administration of a single oral dose, suggesting that once daily dosing may be sufficient. High concentrations were found in tissues from the gastrointestinal tract and the prostate, leading to the hypothesis that these organs are potential chemopreventive targets. Resveratrol was then administered once daily for 1-2 weeks, at 5 mg or 1 g per day, to colon and prostate cancer patients preoperatively. As expected, resveratrol was well tolerated at both doses with

no related adverse events occurring in the 5 mg group, and only 3 mild cases of gastrointestinal symptoms in the 1 g group that might have been related to resveratrol. The serious adverse events in this trial occurred in the colon group and were unrelated to resveratrol, as evidenced by the fact that they involved control patients.

Resveratrol and its metabolites were detectable in plasma, colon and prostate tissue of humans at both doses. In all tissue types, resveratrol metabolites occurred at higher levels than the parent compound, with resveratrol-3-sulphate being the most abundant metabolite found. The pharmacokinetic profiles were not affected by dose. Plasma $C_{max}$ and AUC values, and average prostate levels were linear to dose.

In colon tissue, luminal concentrations of total [$^{14}$C]-resveratrol equivalents were generally higher than those found in outer tissue. This concentration gradient suggested that in addition to systemic delivery, resveratrol also reached colon tissue topically via the lumen after oral administration. There was no difference in the metabolite profiles of inner and outer tissue, or between benign and malignant samples. Levels of resveratrol and its metabolites were higher in the ascending than the descending colon, which is consistent with the published data (Brown *et al.* 10). Possible reasons for this anatomical pattern include the higher luminal water content, thinner epithelial lining and slower transit time of luminal content in the ascending colon, compared to the descending colon, leading perhaps to better absorption. An unknown metabolite was observed by AMS-HPLC in the colon tissue which was not present in the prostate tissue or the plasma, which may be due to the metabolism of resveratrol by intestinal bacteria.

In conclusion, the pharmacokinetic data from this project showed that the oral administration of dietary and pharmacological doses of resveratrol, in rats and in man, resulted in detectable tissue levels in the organs hypothesised to be the targets of its chemopreventive actions. The clinical significance of the different pharmacokinetic profiles in different tissues after administration of different doses is unclear, as both resveratrol and its metabolites have been postulated to be active chemopreventive agents. The most appropriate pharmacokinetic profile in terms of chemopreventive efficacy is therefore currently unknown.

As the data discussed suggested that resveratrol may be a colon cancer chemopreventive agent, its efficacy at the doses administered in the pharmacokinetic studies was then investigated in *Apc*$^{Min+/-}$ mice, an animal model of colorectal cancer. Resveratrol was not investigated in an animal model of prostate cancer as there was no established colony available in our laboratories. Resveratrol was administered to *Apc*$^{Min+/-}$ mice concurrently with either standard diet, with 16 % of calories derived from fat, or a high fat diet where 60 % of calories were derived from coconut oil. The high fat diet was selected as this has been purported to promote colorectal carcinogenesis, whilst resveratrol has been shown to protect animals from the detrimental metabolic effects induced by high dietary fat intake (Chapter 1.2.4). It was hypothesised that resveratrol may exert its chemopreventive actions by normalising the metabolic insults induced by a high fat diet. Exposure to the study diet began from weaning and continued until the time of culling, at 17 or 14 weeks of age for animals on standard and high fat diets respectively. The different ages at culling were due to the high fat diet decreasing overall survival, with histology suggesting that this was caused by fat-induced atrial thrombosis with associated increases in plasma triglyceride and cholesterol levels. The shortened treatment duration in the high fat diet groups may account for why increased tumour burdens were not seen in these animals, as would be expected if dietary fat was pro-

carcinogenic.

Resveratrol, at 0.7 ppm which was equivalent to 5 mg per day in man, reduced the tumour burden in *Apc*$^{Min+/-}$ mice whilst at 143 ppm, equivalent to 1 g per day in man, resveratrol appeared to be pro-carcinogenic. These effects were not seen in animals on standard diets but only in mice on high fat diets. Animal weights were not altered by resveratrol or dietary fat. High dietary fat intake increased plasma lipid concentrations, but resveratrol did not alter fasting plasma triglyceride, cholesterol, glucose, insulin or IGF1 levels. Histology of major organs as assessed by hematoxylin and eosin staining was within normal limits despite manipulation of dietary fat and resveratrol intake, except for fat-induced atrial thrombosis as discussed. Intestinal IGF1 concentrations were unaffected by resveratrol at 0.7 ppm, but were increased by resveratrol at 143 ppm. It was difficult to conclude if intestinal IGF1 was a valid efficacy biomarker as it was only affected by one resveratrol dose, even though tumour burden was altered by both doses.

Mechanistic data for the actions of resveratrol were further clarified in an *in vitro* study of human HCA7 colon cancer cells, which suggested that different cellular pathways were activated by resveratrol at different concentrations. Although efficacy was demonstrated in mice *in vivo*, mechanisms of action were investigated in human rather than murine cells *in vitro* as it was hoped that the data generated would have greater clinical relevance. Exposure of HCA7 cells to resveratrol for 3 months at 0.01 μM, a typical plasma level achieved in humans after an oral dose of 5 mg, altered the apoptosis, glucose transport and cell adhesion pathways as detected by microarray analysis. Proteomic analysis by immnuodot blot assay, however, could not confirm the involvement of these pathways. This discordance between proteomic and genomic results may not necessarily negate the significance of the microarray

findings as there could be for example, post-translational regulation or alternative splicing of mRNA. Resveratrol at 1.4 μM, a plasma concentration attainable after an oral dose of 1 g, did not significantly alter any pathways. These *in vitro* results were similar to the *in vivo* data, in that the actions of resveratrol differed depending on dose. This correlation was, however, incomplete as high dose resveratrol was active in $Apc^{Min+/-}$ mice, albeit in a detrimental manner but *in vitro*, the equivalent concentration of resveratrol was inactive in altering genomic expression.

Overall, the results from this project suggest that the actions of resveratrol *in vitro* and *in vivo* may differ depending on dose. Resveratrol is not alone in exerting biphasic effects at different doses, as this property has been found for other dietary chemoprevention agents as discussed in Chapter 1.1.2. It is extremely important that the appropriate dose of resveratrol is selected for clinical development, as an incorrect dose may be ineffectual or even detrimental. The data from this project suggest that resveratrol at 5 mg per day, which is an amount that can be consumed from a normal diet, may be a chemopreventive agent for colorectal cancer in man, especially when taken concomitantly with a high fat diet. The clinical relevance of this finding is potentially significant, as resveratrol at dietary dose has not been identified from epidemiological data to result in any known toxicity. Resveratrol could therefore potentially be used in the primary chemoprevention setting for chronic administration to the general population, as well as for high risk individuals. The high dietary fat administered to the $Apc^{Min+/-}$ mice in this project is also clinically relevant, as this is similar to some diets consumed in the Western world. A study in the United States for example, has shown that dietary fat intake accounted for up to 54 % of calories consumed (Ma *et al.* 06).

The information gathered to date, however, is still insufficient for the design of a large clinical efficacy trial of resveratrol. There were wide inter-individual variations in the tumour burden in the *Apc*$^{Min+/-}$ mice and it was not possible from the data in this project to identify a valid *in vivo* biomarker for clinical testing. Without an efficacy biomarker, confirmation of clinical efficacy would be difficult due to the prolonged treatment duration and the large population needed. Mechanistic information may also be useful to pinpoint the most appropriate target population in an efficacy trial, so that only patients likely to benefit from resveratrol would be recruited. There is also a lack of data on other elements of study design, for example the appropriate timing and duration of intervention.

Further work in aiding the clinical development of resveratrol could include a repeat of the *Apc*$^{Min+/-}$ mice experiment in order to ensure that the results seen are of genuine significance. This is already being carried out and preliminary data suggest that the dietary dose of resveratrol is protective against adenoma development, in both male and female *Apc*$^{Min+/-}$ mice. Confirmation of the efficacy of resveratrol in another animal model of colorectal cancer would also strengthen the hypothesis that this may be a chemopreventive agent in humans. *Apc*$^{Min+/-}$ mice are considered as a good model of chemoprevention, as the carcinogenesis pathway is promoted by genetic mutations which can also occur in familial and sporadic human colorectal cancers. Exposure to resveratrol could therefore begin at weaning, before the establishment of macroscopic tumour deposits. Other commonly used animal models involve instead, for example, the implantation of established human cancer cells into nude mice. The administration of resveratrol in this setting would therefore be later in the carcinogenesis sequence than that in *Apc*$^{Min+/-}$ mice. Data gathered from nude mice experiments, however, can also be of value as the use of human rather than murine tissue may mean that the results are of greater clinical relevance.

Further mechanistic actions of resveratrol could be investigated in murine colorectal cancer cells, as the chemopreventive effects were demonstrated in a mouse model. Apc10.1 cells for example, are an established cell line derived from adenomas of *Apc*$^{Min+/-}$ mice (Sale *et al.* 09). Although it can be criticised that the resulting data would have less clinical relevance than information from human cancer cells, it can be difficult to carry out detailed mechanistic studies in *Apc*$^{Min+/-}$ mice or other *in vivo* models due to the limited amount of tissue for analysis. The availability of relevant cell lines such as Apc10.1 cells, however, overcomes this problem and potentially allows an improved understanding of the actions of resveratrol *in vivo*.

Alternatively, expression of specific protein as suggested by the published data could be investigated to corroborate the specific pathways highlighted by the microarray data already gathered. AMPK for example, has been shown to be involved in resveratrol-mediated normalisation of diabetes mellitus in animal models *in vivo*, as discussed in Chapter 1.2.4.2. Investigation of this and other related proteins may confirm the microarray data, and these protein levels could then be measured in *Apc*$^{Min+/-}$ mice tissue for *in vivo* validation of this pathway as an efficacy biomarker of resveratrol.

Finally, it should be noted that the pharmacokinetic data in this project suggested that resveratrol may also be a prostate chemopreventive agent *in vivo* and in man. Preclinical data were not gathered for the efficacy and mechanistic actions of resveratrol in this organ in this project, but these would also be interesting future studies especially in view of the clinical prevalence of prostate cancer.

# 7. References

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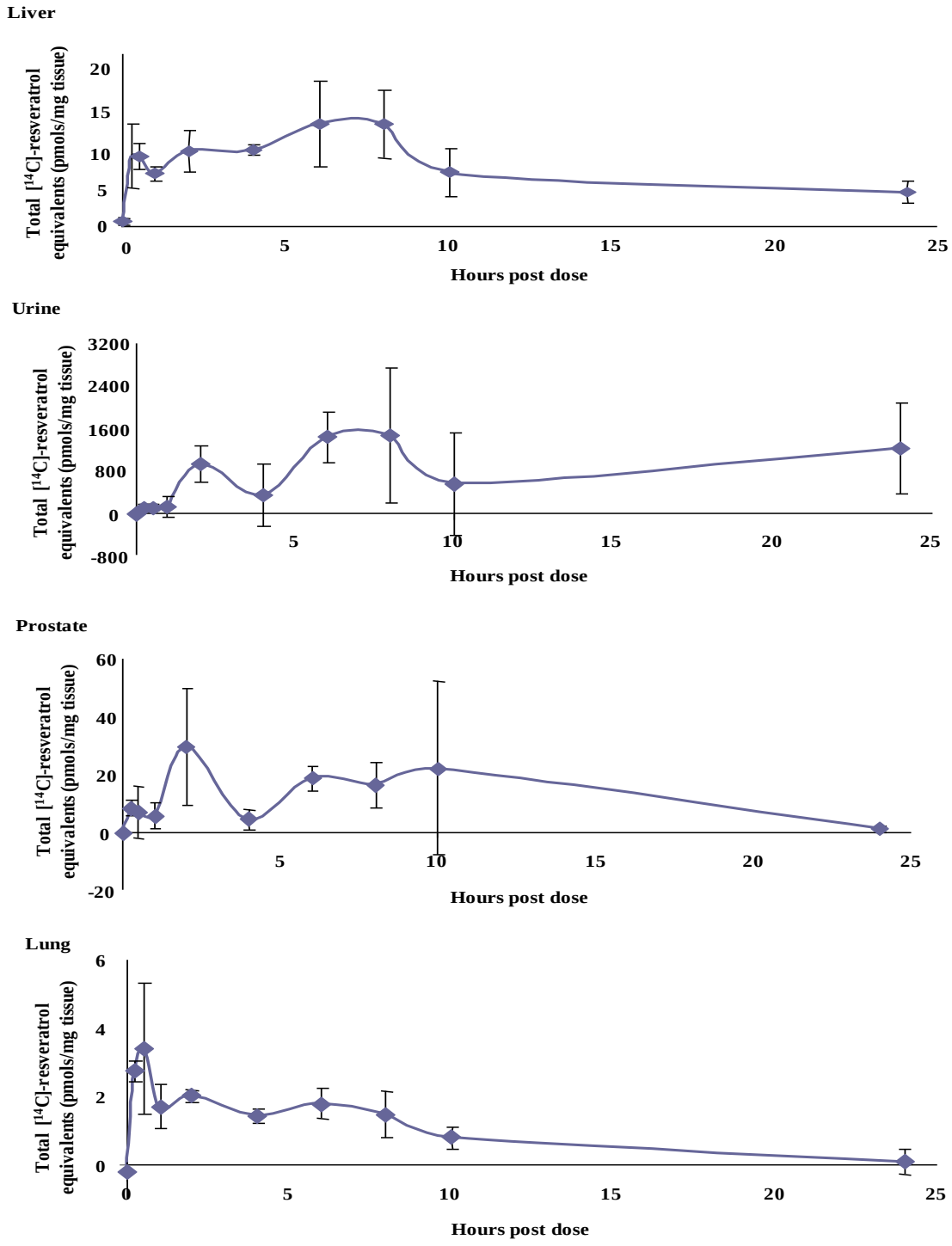
# 8. Appendices

# 8. Appendices

## 8.1 Supplementary data for the pharmacokinetic study of [$^{14}$C]-resveratrol in F344 rats after administration of a single dietary or pharmacological dose by gavage



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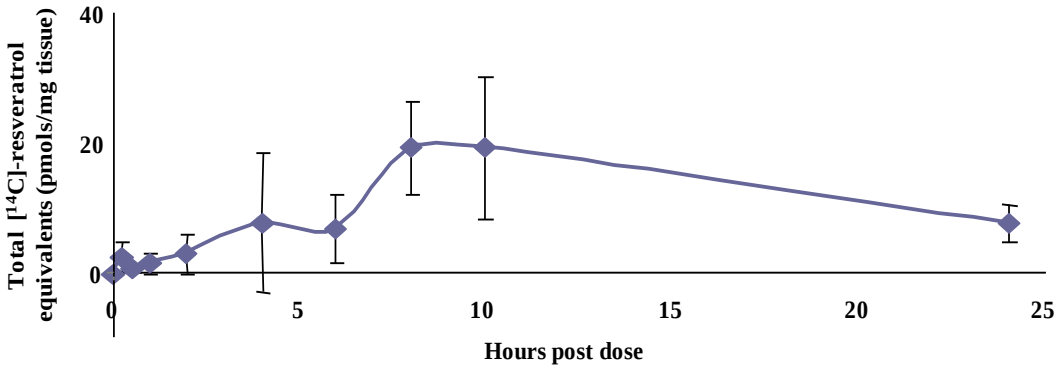
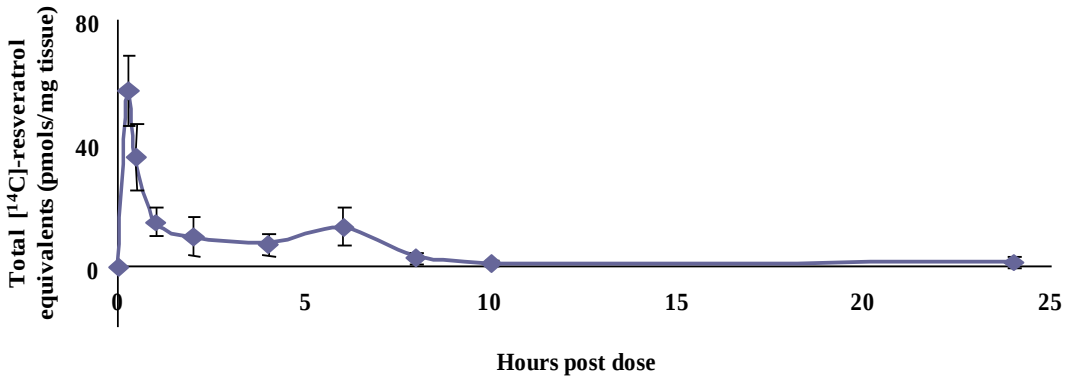
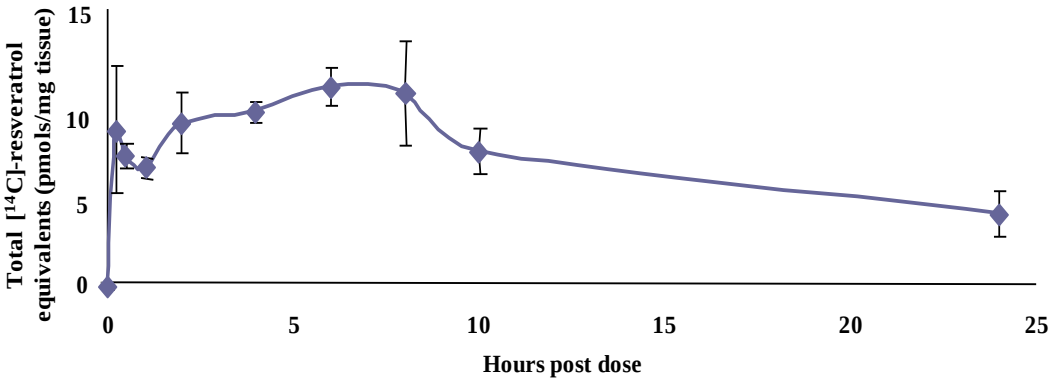


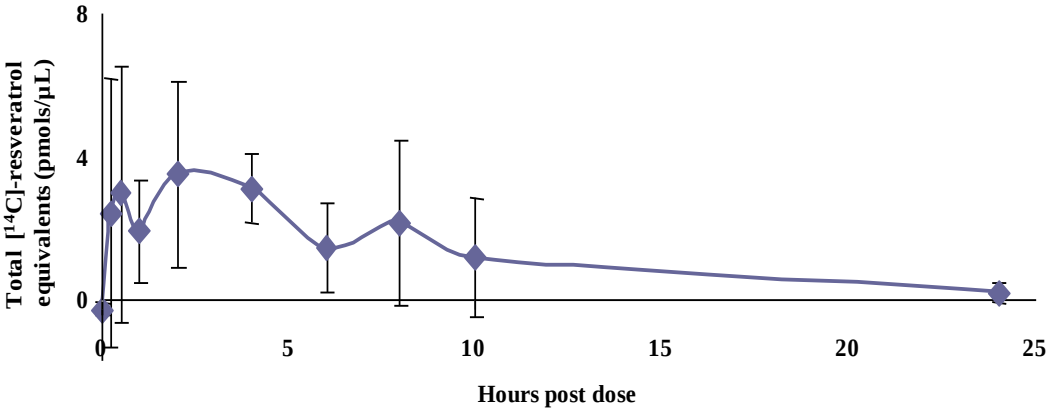

Stomach




Kidney




Blood



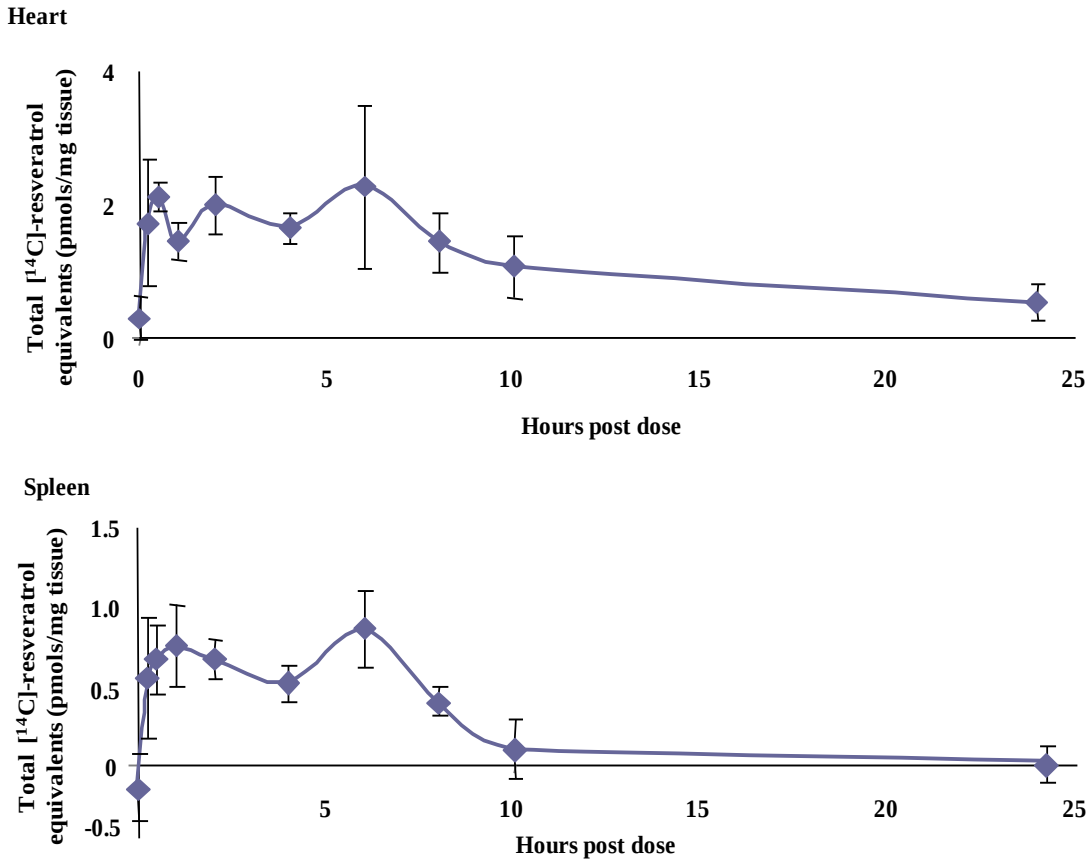
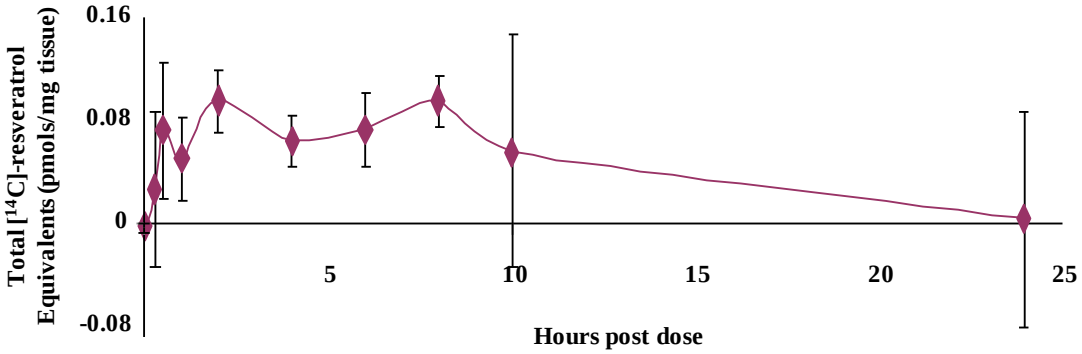
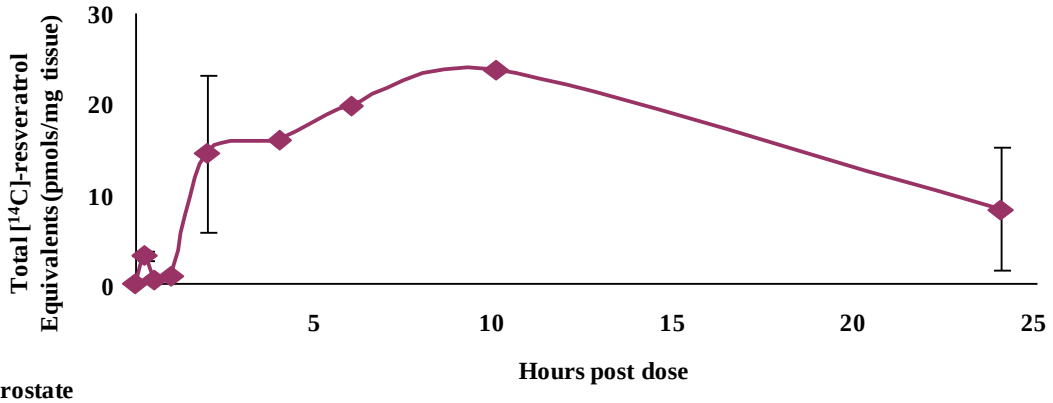



**Figure 8.1 Total [$^{14}$C]-resveratrol equivalent concentrations in organs, blood and urine of F344 rats.** Total [$^{14}$C]-resveratrol equivalent concentrations were measured by liquid scintillation counting after a single dose at 14.3 mg/kg was administered by gavage (see Section 3.2.1, pg 109-115). Data above show mean values n=3 except for urine, where it was only possible to obtain samples from 1-2 animals at each time point. Bars denote standard deviations (SD). Brain tissue was also harvested but data not shown as levels were below the limit of detection, defined as mean of blanks + 2 SD.

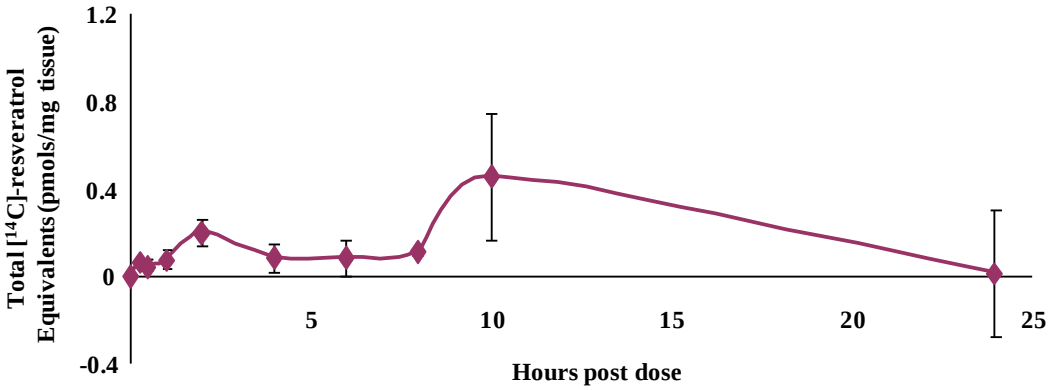
Liver



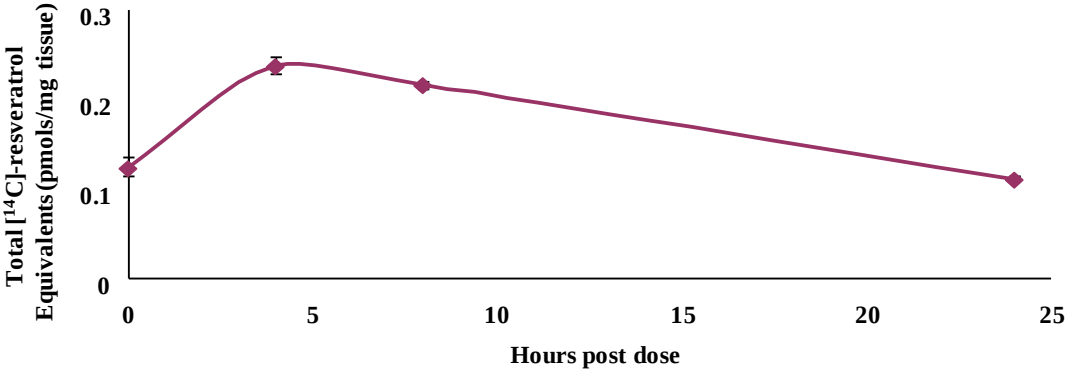

Urine



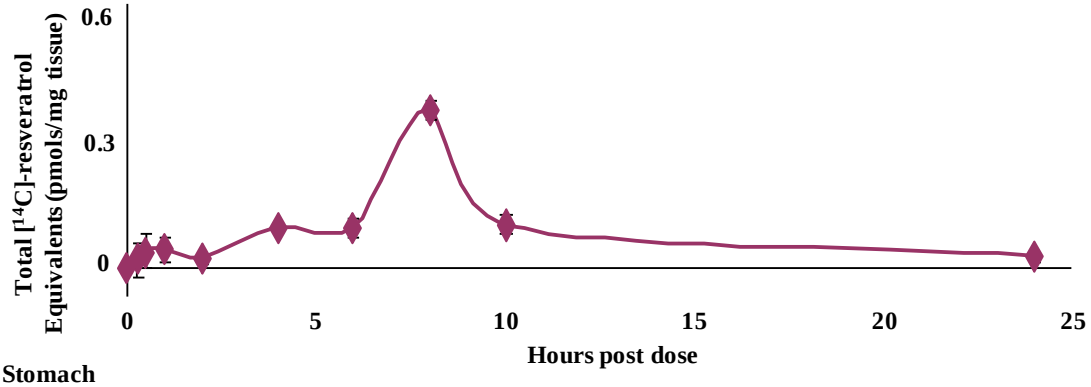

Prostate



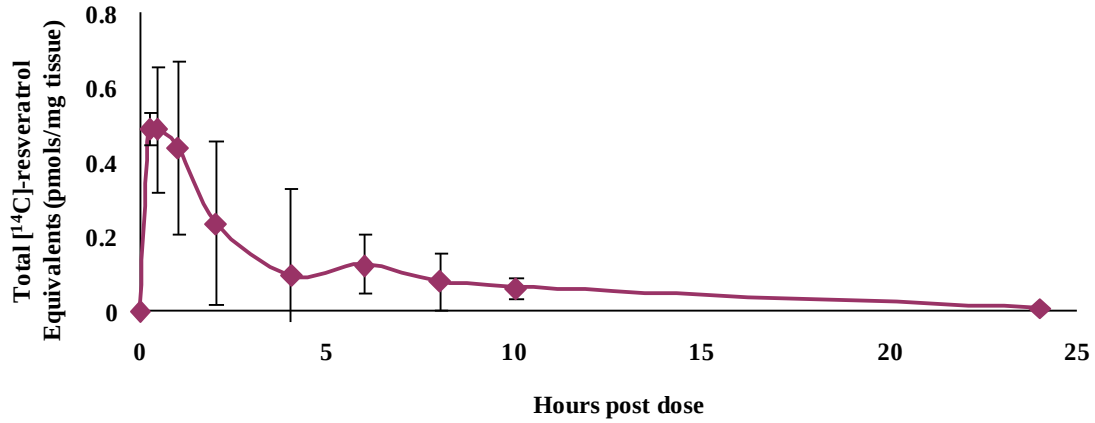

Lung



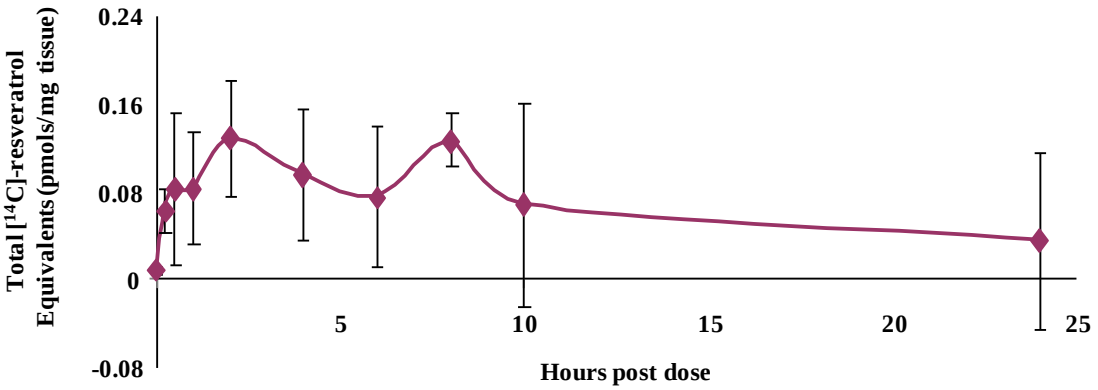
Colon



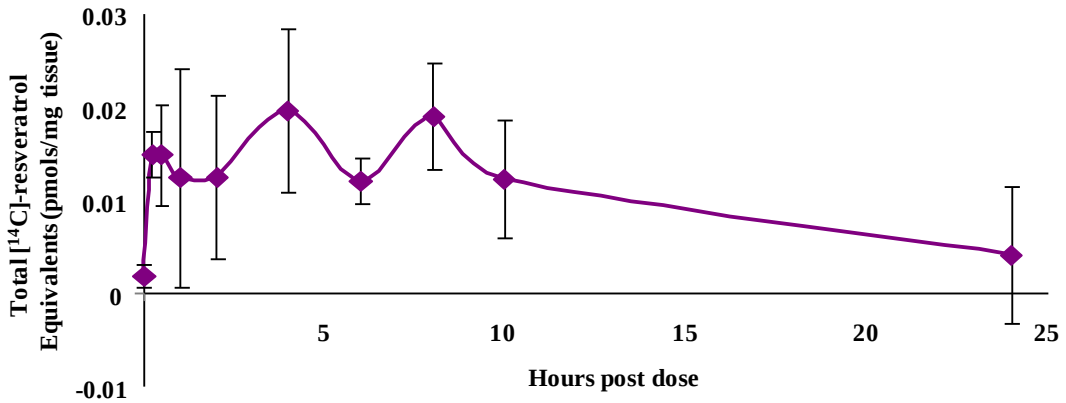

Stomach




Kidney




Blood



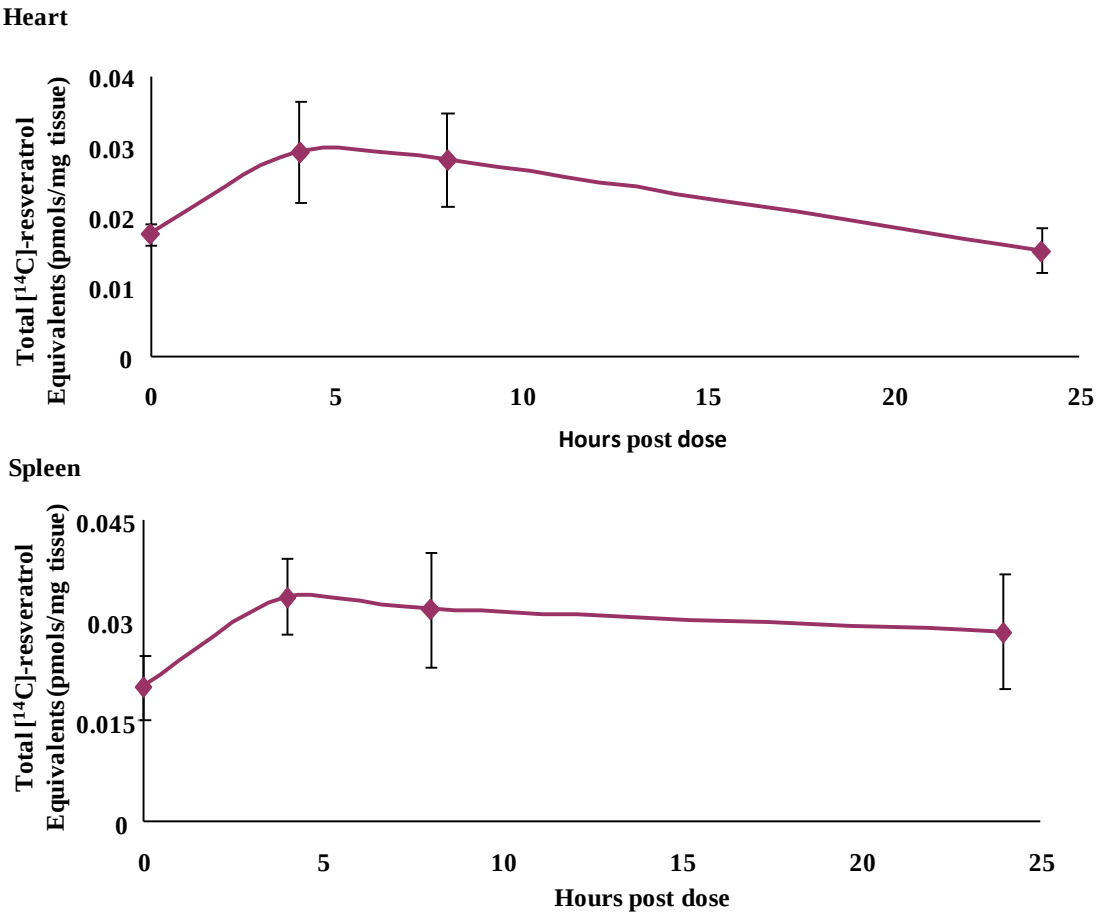



**Figure 8.2 Total [$^{14}$C]-resveratrol equivalent concentrations in organs, blood and urine of F344 rats.** Total [$^{14}$C]-resveratrol equivalent concentrations were measured by liquid scintillation counting after a single dose at 0.07 mg/kg was administered by gavage (see Section 3.2.1, pg 109-115). Data above show mean values n=3 except for urine, where it was only possible to obtain samples from 1-2 animals at each time point. Bars denote standard deviations (SD). Brain tissue was also harvested but data not shown as levels were below the limit of detection, defined as mean of blanks + 2 SD.

## 8.2 Diets to investigate the effect of dose on the pharmacodynamic actions of resveratrol in male C57BL/6J *Apc*$^{Min+/-}$ mice on standard or high fat diet

**Table 8.1 Nutritional content of diets used in *Apc*$^{Min+/-}$ mice experiment**

| | Protein | Fat | Carbohydrate | Fibre | Calcium | Resveratrol |
|---|---|---|---|---|---|---|
| Standard diet (Group 1) 3.89 kcal/g | 17.9 % by weight | 7.1 % by weight | 63.2 % by weight | 5 % by weight | 0.5 % by weight | 0 % by weight |
| | 18.7 % by calorific content | 16.4 % by calorific content | 64.7 % by calorific content | | | |
| Standard diet + low dose resveratrol (Group 2) 3.89 kcal/g | 18.3 % by weight | 7.1 % by weight | 63.2 % by weight | 5 % by weight | 0.51 % by weight | 0.0007% by weight |
| | 18.8 % by calorific content | 16.4 % by calorific content | 64.9 % by calorific content | | | |
| Standard diet + high dose resveratrol (Group 3) 3.89 kcal/g | 18.3 % by weight | 7.1 % by weight | 63.2 % by weight | 5 % by weight | 0.51 % by weight | 0.0143% by weight |
| | 18.8 % by calorific content | 16.4 % by calorific content | 64.9 % by calorific content | | | |
| High fat diet (Group 4) 5.28 kcal/g | 18 % by weight | 35 % by weight | 35.2 % by weight | 5 % by weight | 0.51 % by weight | 0% by weight |
| | 13.7 % by calorific content | 59.7 % by calorific content | 26.7 % by calorific content | | | |
| High fat diet + low dose resveratrol (Group 5) 5.28 kcal/g | 18 % by weight | 35 % by weight | 35.2 % by weight | 5 % by weight | 0.51 % by weight | 0.0007% by weight |
| | 13.6 % by calorific content | 59.7 % by calorific content | 26.7 % by calorific content | | | |
| High fat diet + high dose resveratrol (Group 6) 5.25 kcal/g | 18 % by weight | 35 % by weight | 34.2 % by weight | 5 % by weight | 0.51 % by weight | 0.0143% by weight |
| | 13.7 % by calorific content | 60.1 % by calorific content | 26.1 % by calorific content | | | |

## 8.3 Murine IGF1 ELISA assay validation data

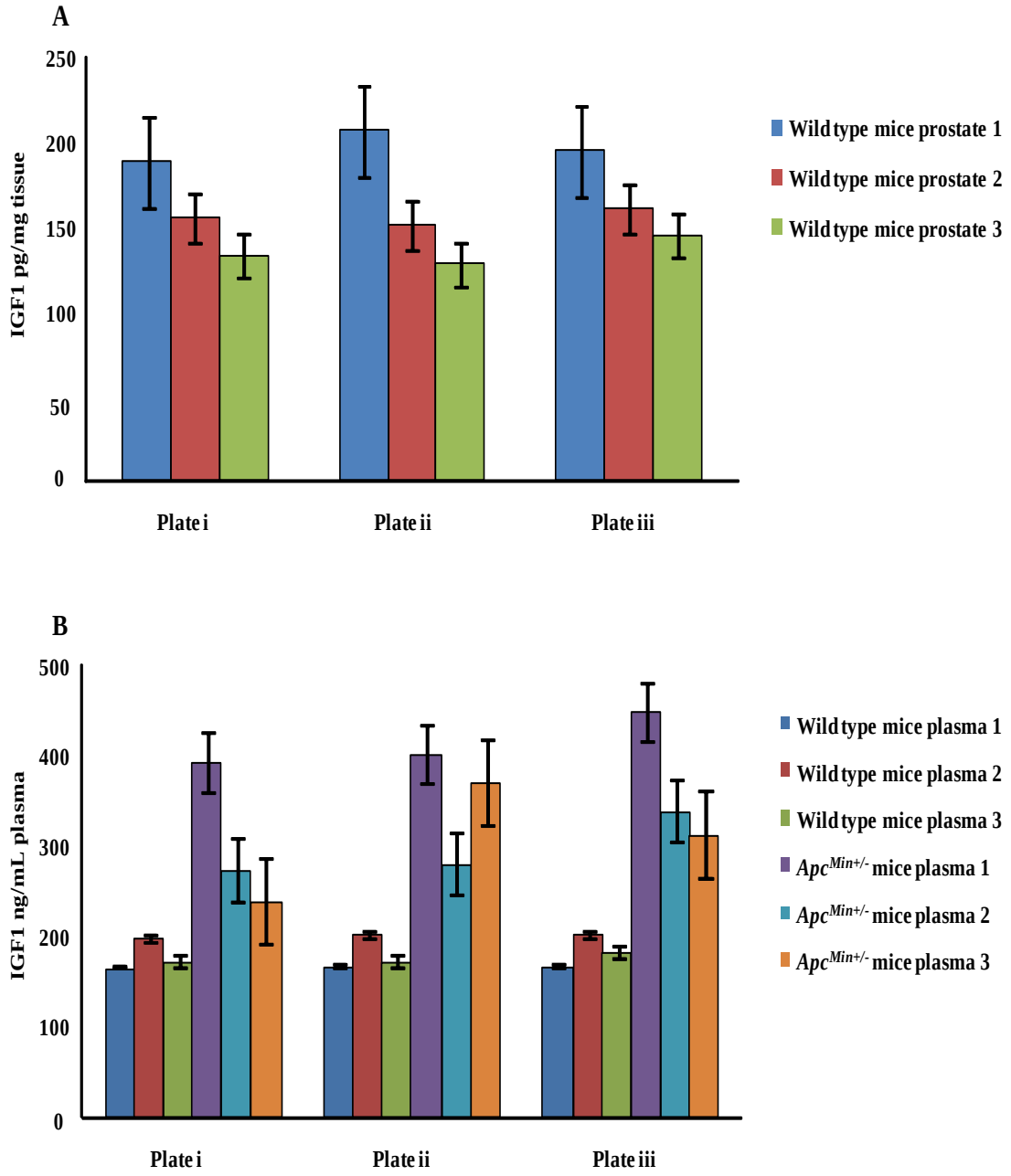



**Figure 8.3 Murine IGF1 ELISA assay validation data.** Prostate tissue and plasma samples were obtained from 3 C57BL/6J wild type and 3 *Apc*$^{Min+/-}$ mice for validation fo the IGF1 assay. Interplate variation between 3 plates was <20 % for both prostate (**A**) and plasma (**B**) samples.

## 8.4 Clinical trial protocol

### i. Participant Selection

**Participating Centre**

The University Hospitals of Leicester NHS trust consist of acute hospitals with adequate facilities and staffing available locally to perform any necessary procedures required for the study. Similar studies have been run locally before without any problems.
The University of Leicester has laboratories based at Leicester Royal Infirmary for sample analysis and storage, as well as facilities at the nearby university site.

**Sources of Recruitment**

**Parts A – Colorectal Patients**

Patients suitable for the study will be identified from pre-assessment clinic. **It will be made clear to all patients invited into the trial that declining to be recruited will not affect their management in any way.**

**Part B – Prostate Patients**

Patients suitable for the study will be identified from multidisciplinary meetings. **It will be made clear to all patients invited into the trial that declining to be recruited will not affect their management in any way.** Patients recruited to this study include:

- Patients with suspected prostate cancer who require biopsy confirmation which will usually be via the transrectal ultrasound guided route (TRUS).
- Patients with benign prostatic hypertrophy who require routine treatment which will usually be by transurethral resection of the prostate (TURP).

**Part C – Human Volunteers**

- flyers
- advertisement in local media

**Method of Participant Numbering**

Each participant is assigned a unique participant identification number (PIN), which will be used on all study forms and specimens. The clinical research fellow's office has a key-locked filing cabinet containing a folder for each study ID which holds the research records. The study identifiers are linkable, but the information linking a number identifier with a participant name is kept in a key-locked storage area that is completely separate from the filing area where the research files are stored. Only the study Clinical Research Fellow or the PI have access to this information.

## ii. Inclusion and exclusion criteria

### Part A Colorectal Patients Inclusion Criteria

- Histological diagnosis of colorectal malignancy
- Surgery is planned as part of the patient's management.
- Willing to abstain from ingesting large quantities of resveratrol-containing foods, drinks and vitamin supplement (diet sheet supplied)
- Premenopausal female patients with intact female reproductive organs must use reliable contraceptive methods and have a negative pregnancy test within 2 weeks of starting resveratrol dosing. Post menopausal status defined as no menses for the last 18 months. All participants must use barrier contraception for the duration of study. Patients or their partners who become pregnant during the duration of study will be referred to the appropriate experts for counselling. Pregnancy tests are not necessary for patients in the control group.

### Part A Colorectal Patients Exclusion Criteria

- Patients receiving neoadjuvant chemotherapy prior to resection
- Unwilling or unable to comply with the protocol
- Excessive alcohol intake (more than 28 units per week for men in UK)
- Undergone chemotherapy or participated in another investigational drug study within 4 weeks of colon tissue sampling.
- Any malabsorption syndrome which will impair oral absorption of resveratrol
- Chronic use of non-steroidal anti-inflammatory drugs, steroids, warfarin or antiepileptic drugs, defined as daily use at time of consent and continued use for the duration of trial, as there is potential drug interaction with resveratrol via cytochrome P450 pathway or interference with biomarker analysis
- Evidence of abnormal liver function tests as defined by:
  1) alanine transaminase (ALT) >2.5 x upper limit normal (ULN) of local laboratory
  2) serum bilirubin >1.5 x ULN
- Evidence of abnormal renal function test defined as creatinine clearance (calculated via Cockroft –Gault formula) < 30ml/min
- Diabetic patients will be excluded as IGF1 and IGFBP3 are potential biomarkers for this study.

### Part B Prostate Patients Inclusion Criteria

- Over 18 years of age
- Able to give written informed consent
- Willing to abstain from ingesting large quantities of resveratrol-containing foods, vitamins and certain drugs for duration of study (list supplied)
- Performance status of WHO 0-2
- Need to undergo biopsy to exclude prostate cancer or routine surgery for treatment of benign prostatic hypertrophy
- Willing to use barrier contraception for the duration of study. Patients with partners who become pregnant during the duration of study will be referred to the appropriate experts for counseling.

**Part B Prostate Patients Exclusion Criteria**

- Unwilling or unable to comply with the protocol
- Excessive alcohol intake (more than 28 units per week for men in UK)
- Undergone chemotherapy or participated in another investigational drug study within 4 weeks of prostate tissue sampling.
- Any malabsorption syndrome which will impair oral absorption of resveratrol
- Chronic use of non-steroidal anti-inflammatory drugs, steroids, warfarin or antiepileptic drugs, defined as daily use at time of consent and continued use for the duration of trial, as there is potential drug interaction with resveratrol via cytochrome P450 pathway or interference with biomarker analysis
- Evidence of abnormal liver function tests as defined by:
  1) alanine transaminase (ALT) >2.5 x upper limit normal (ULN) of local laboratory
  2) serum bilirubin >1.5 x ULN
- Evidence of abnormal renal function test defined as creatinine clearance (calculated via Cockroft –Gault formula) < 30ml/min
- Diabetic patients will be excluded as IGF1 and IGFBP3 are potential biomarkers for this study.

**Part C Healthy Volunteers Inclusion Criteria**

- Men and women aged over 18 years of age
- Able to give written informed consent
- Willing to abstain from ingesting large quantities of resveratrol-containing foods, vitamins and certain drugs for 5 days before study and whilst on study. List supplied to participants.
- Premenopausal women with intact female reproductive organs must use reliable contraceptive methods and have a negative pregnancy test within 2 weeks of resveratrol dosing. Post-menopausal status defined as no menses for the previous 18 months. All volunteers must use barrier contraception for the duration of study. Patients or their partners who become pregnant during the duration of study will be referred to the appropriate experts for counseling.
- Haematological Parameters: white blood cell count, hemoglobin, platelet count within normal limits of local laboratory

  Biochemistry Parameters: Biochemistry - sodium, potassium, urea, creatinine, glucose, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, total protein, albumin, alkaline phosphatase, total, bilirubin, calcium, inorganic phosphate, triglycerides, cholesterol and urinalysis within normal limits of local laboratory
- Performance Status WHO 0-1
- Willing to comply with protocol

**Part C Healthy Volunteers Exclusion Criteria**

- Volunteers who are pregnant or lactating women, or women contemplating pregnancy for the duration of the protocol
- Excessive alcohol intake (more than UK recommended limit - 21 or 14 units per week for men or women respectively)
- Any cancer diagnosis that is currently under treatment, that is clinically detectable, or has been treated within 5 years (basal cell or squamous cell carcinomas are exempt)
- Concurrent participation in any drug studies having finished within the past 6 months
- Any malabsorption syndrome which will impair oral absorption of resveratrol
- Any regular medication except contraceptives

## iii. Agent Information and Administration

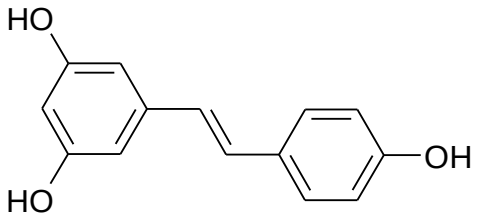
**Name of Agents**
Resveratrol and [$^{14}$C]-resveratrol.

**Dose Groups and Duration of Exposure**

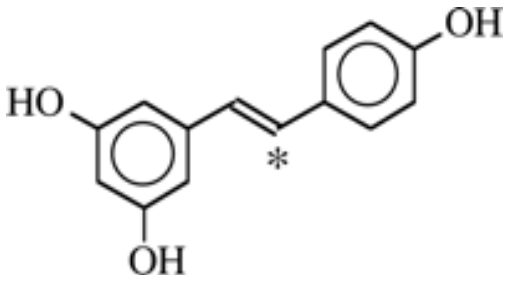
| | Part A Colorectal patients | Part B Prostate patients | Part C Healthy Volunteers |
|---|---|---|---|
| Dietary dose arm | 6 x 5mg unlabelled doses + 1 x 5mg [$^{14}$C]-resveratrol dose | ≤13 x 5mg unlabelled doses + 1 x 5mg [$^{14}$C]-resveratrol dose | 1 x 5mg [$^{14}$C]-resveratrol dose |
| Pharmacological dose arm | 6 x 1g unlabelled doses + 1 x 1.005g [$^{14}$C]-resveratrol dose (latter = 4x250 mg non-radiolabelled capsules + 1x5 mg radiolabelled capsule) | ≤13 x 1g unlabelled doses + 1 x 1.005g [$^{14}$C]-resveratrol dose (latter = 4x250 mg non-radiolabelled capsules + 1x5 mg radiolabelled capsule) | 1 x 1.005g [$^{14}$C]-resveratrol dose (= 2x500 mg non-radiolabelled capsules + 1x5 mg radiolabelled capsule) |
| Control arm | No capsules | No capsules | Not applicable |
| Effective radiation exposure/participant | 0.962 µSv | 0.962 µSv | 0.962 µSv |
| Total number of participants recruited | 10 in each arm | 10 in each arm | 10 in each arm |

Doses will not be changed once the initial dose has been prescribed.
5mg dose = 1x5mg capsule
1g dose = 2x500 mg or 4x250mg capsules
1.005g dose = (2x500mg or 4x250 mg unlabelled capsules) + (1 x 5mg radiolabelled capsule)

**Formulation and drug description**
Structural of unlabelled resveratrol:




Structure of $^{14}$C-resveratrol:




* = [$^{14}$C] label

| | |
|---|---|
| Molecular Formula: | $C_{14}H_{12}O_3$ |
| Molecular Weight: | 228.247 |
| Appearance: | white solid |
| Melting Point: | 253-255 °C |
| Solubility: | readily soluble in water |
| Chemical Name: | *trans*-3,4',5-trihydroxystilbene |
| International non-proprietary name: | resveratrol |
| CAS Number: | 501-36-0 |
| Formulation: | capsules containing 5 mg, 250 mg or 500 mg |
| Administration: | oral |
| Storage: | room temperature |
| Administered radiation dose | 1.2 µCi or 44.5 kBq per trial participant |
| Estimated effective radiation dose | 0.962 µSv per trial participant |

**Manufacturer and Supplier**
Royalmount Pharma (Montreal, Canada) will facilitate the supply of resveratrol powder from the manufacturer (Orchid Chemicals and Pharmaceuticals Ltd, Bombay, India). Non-radiolabelled resveratrol will be encapsulated by Nova Laboratories (Leicester, UK). [$^{14}$C] labeled resveratrol poweder will be produced by BioDynamics Research Ltd (Northamptonshire, UK) and encapsulated by Pharmaceuticals Profiles Ltd (Nottingham, UK).

**Packaging and Labels**

For colorectal and prostate patients in Part A and Part B of this study respectively, bottles will be used for dispensing non-radiolabelled resveratrol for the patients to take home. The [$^{14}$C]-labelled resveratrol dose will be supplied separately to the research team for in-patient administration to avoid confusion. 2 extra non-radiolabelled doses are supplied to each patient in case there is any delay in surgery.

For volunteers in Part C of this study, a single [$^{14}$C]-labelled resveratrol dose will be supplied to the research team for administration.

Non-radiolabelled resveratrol is provided in 5mg, 250 mg and 500 mg capsules and is dispensed by the University Hospitals of Leicester pharmacy. All drug bottles are labeled with the participant identification number, study number, number of capsules, dose and schedule to take plus study contact number. Radiolabelled resveratrol is provided in 5 mg capsules and will be dispensed to the research team by radiopharmacy for patient administration, rather than to the patient directly.

**Dose Modification**

If the adverse event is graded as 2 and is prolonged (over 48 h period), or grade 3 or above and thought to be possibly, probably or definitely drug related, the individual should be withdrawn from further dosing. The participant will be followed up until resolution of their adverse events. No doses will be reduced due to toxicity.

**Adherence/Compliance**

For colorectal and prostate patients in Part A and Part B:

● *Adherence to non-radiolabelled resveratrol, low resveratrol diet and concomitant medication restriction*

This will be assessed through the Food Diary and dosing calendar. Participant will be instructed to return all unused capsules to the investigator. Patients who have been non-compliant with the low resveratrol diet, non-radiolabelled resveratrol dosing or avoidance of concomitant medications will not be replaced.

● *Adherence to [$^{14}$C]-resveratrol capsule*

Patients who have taken their [$^{14}$C]-resveratrol capsule earlier than planned due to unexpected surgical delay will still have colorectal or prostate tissue samples taken for analysis, but this time lapsed will be noted and they will be replaced. Patients who have not taken their [$^{14}$C]-resveratrol capsule for whatever reason before biopsy / surgery will be replaced.

● *Delay in biopsy*

At the University Hospital of Leicester it is very unlikely that the surgery date will be delayed. In the event of this occurrence:

For patients who have already begun the dosing schedule but have not taken the [$^{14}$C]-resveratrol dose, they will be asked to continue on non-radiolabelled resveratrol capsules for up to 2 extra doses, delaying their radiolabelled dose until the day before surgery.

For patients who have not yet commenced their resveratrol capsules, the start date will be delayed so that it is 7 or 14 days before surgery date for colorectal and prostate patients respectively.

For patients who have already taken their [$^{14}$C]-resveratrol capsule, colorectal or prostate

tissue samples will still be taken for analysis but the time lapsed between [$^{14}$C]-resveratrol dose and surgery will be noted and these patients will be replaced.

For healthy volunteers in Part C, compliance to resveratrol is not an issue as patients will receive only a single dose. Patients who are non-compliant with the low resveratrol diet or the pharmacokinetic sampling may be replaced at the Principal Investigator's discretion, depending on the degree of non-compliance.

**Drug accountability**
The hospital pharmacist will maintain an adequate record of the receipt and distribution of all study supplies of resevratrol using a drug accountability form. These forms must be available for inspection at any time.

**iv. Study procedures**
**Part A - Recruitment of colorectal patients**

Patient information sheets will be posted together with details of the preassessment clinic appointment to ensure that only patients who are aware of the diagnosis of cancer and the need for an operation will be recruited. Patient information sheets are posted before the pre-assessment clinic to allow patients adequate time to read the information.
● *Informed Consent*
● *Medical History*
Includes past medical history, drug history, social history of alcohol or tobacco use. Participation in other studies noted including enrollment and ending dates as well as the nature of the study. Method of contraception documented. Age, gender and ethnicity noted.
● *Physical Examination*
Vital signs of temperature, blood pressure, pulse and respiratory rate noted. Height and weight noted.
● *Urinalysis*
- Any protein, blood, glucose and ketones noted.
- Baseline levels of [$^{14}$C], resveratrol and its metabolites measured.
● *Pregnancy test valid for 14 days prior to dosing*
Post menopausal status defined as no menses for 18 months prior to study. All other female patients will need a negative urine pregnancy test. Pregnancy tests not necessary for patients in the control arm.
● *Food and Drug Avoidance Information Sheets, Food Diary*
Avoid resveratrol containing vitamins, foods, drink and certain drugs for duration of study.
● *Blood tests taken as routine care but used for trial inclusion/exclusion criteria*
- Full blood count including haemoglobin, differential white blood cell count and platelet
- Biochemistry including sodium, potassium, blood urea, creatinine, alanine aminotransferase, albumin, alkaline phosphatase, bilirubin
- Baseline levels of [$^{14}$C], resveratrol and its metabolites measured
● *Resveratrol Dosing*
Participants will be supplied with 8 doses of 5 mg or 9 doses of 1 g non-radiolabelled resveratrol capsules in a bottle with a calendar to take each dose at the same time. The patient will record time and date of dose, number of capsules remaining in box and initial on a daily basis. Patients will be asked to take ≤9 doses of non-radiolabelled resveratrol and only the last dose will contain [$^{14}$C]. The resveratrol bottle will be returned with the remaining resveratrol capsules and the dosing calendar. The last radiolabelled [$^{14}$C]-resveratrol dose will not be given to the patient, but will be dispensed separately by pharmacy

to be taken the evening before the operation as an inpatient.

**Presurgery Day for colorectal patients**

On the evening before or the morning of surgery, the patient will be met on the ward. The resveratrol bottle, dosing calendar and Food Diaries collected. Patients will receive their radiolabelled resveratrol dose from the research team.

**Day of operation for colorectal patients**

Patients who are non-compliant with a low resveratrol diet or avoidance of certain concomitant drugs will not be replaced but this will be noted for result analysis. Blood and urine samples will be collected on the day of the operation, but before general anaesthetic as anaesthetic drugs can interfere with biomarker analysis. Samples collected for measurement of [$^{14}$C], biomarkers, resveratrol and its metabolites are as follows:

● *Blood Specimens*

40 ml venous blood and 10-20ml portal venous blood will be obtained at operation for drug assay. 50 % will be used for biomarker analysis and 50 % for levels of resveratrol, its metabolites and [$^{14}$C].

● *Tissue Specimens*

All patients will have non-malignant and malignant tissue samples taken at operation by the surgeon and prepared in the pathology department. This tissue will be in the form of a wedge excision biopsy or multiple shavings. The tissue samples will only come from the resected specimen, at the discretion of the pathologist responsible for the patient's diagnosis. The harvest method has been approved by the Leicester Pathology Department and deemed not to interfere with the staging of the tumor specimens, with the same method currently being used in other clinical trials. All harvest sites will be clearly marked with pins and the specimens also clearly labeled to the effect that additional samples have been taken.

Colorectal specimens will be pinned out on corkboards and the bowel opened longitudinally up to the tumour site but not including it, as stipulated by the UK Royal College of Pathologists Database Guidelines.

Samples will then be taken from the tumour intraluminally. No tumor samples will be taken from within 30 mm of the tumor resection margin. The maximum diameter of tumour encroachment intraluminally will be left untouched. No lymph nodes, peritoneum, or samples near the dentate line/internal anal sphincter will be taken.

All specimens will be stored in a suitable freezer. The interval between the last dose of resveratrol and the sampling of tissue will be recorded for every patient and maintained as constant between patients as possible.

● *Completion of Study*

Once all specimens and information relating to drug compliance has been collected, the participant will be deemed off-study and no further contact will be required.

**Part B – Recruitment of prostate patients**

Patients will be approached either at urology outpatients clinic or an information sheet will be posted to them together with their biopsy or operation date. By posting the information sheet

together with the date of biopsy or surgery, we will ensure that only patients who are aware of the need of an operation will be approached. This latter method of recruitment will also allow patients adequate time to read the information sheet before their surgery or biopsy.

**Allocation of prostate patients to treatment arms**

Patients approached in the urology clinic in person are contacted via telephone after they have had at least 24 hours to read the information sheets. Patients who have received an information sheet in the post will be approached on the day of their surgery or biopsy for possible recruitment to the control arm of the trial.
Interested biopsy or surgical patients will be allocated to one of the trial arms:
- control arm
- low dietary dose resveratrol arm
- higher pharmacological dose resveratrol arm

Patients in the control arm will receive no further intervention until the day of their biopsy or surgery. Patients in the two resveratrol arms will return to hospital for a dosing visit.

**Dosing visit for prostate patients allocated to receive resveratrol**

The dosing visit may coincide with the pre-assessment clinic or may be an extra hospital visit.
- *Informed Consent*
- *Medical History*

Includes past medical history, drug history; social history of alcohol or tobacco use. Participation in other studies noted including enrollment and ending dates as well as the nature of the study. Performance status, age, ethnicity and method of contraception noted.
- *Physical Examination*

Weight, height, temperature, blood pressure, pulse and respiratory rate noted.
- *Food and Drug Avoidance Information Sheets, Food Diary*

Avoid resveratrol containing foods, drink and certain drugs for duration of study.
- *Resveratrol dosing*

Participants will be supplied with 15 doses of 5 mg or 16 doses of 1 g non-radiolabelled resveratrol capsules in a bottle with a calendar to take each dose at the same time. Treatment will commence ≤14 days before biopsy / surgery. 2 extra non-radiolabelled doses are supplied in case there is delay in biopsy. The patient will record time and date, number of capsules remaining in the box and initial on a daily basis. Patients will be asked to take ≤16 doses of non-radiolabelled resveratrol and only the last dose will contain [$^{14}$C]. The resveratrol bottle should be returned with any remaining resveratrol capsules and the dosing calendar. The last and radiolabelled resveratrol dose will be taken morning of or evening before biopsy / surgery, depending on time of procedure. This will be dispensed separately from the unlabelled resveratrol doses.

- *Blood tests*

Baseline bloods taken for levels of [$^{14}$C], resveratrol, its metabolites and biomarkers.

The research team will liaise with the urology department so that participants will be telephoned 14 days before their biopsy / surgical date to remind them to commence resveratrol. If there is a delay in the biopsy / surgical date then patients will be asked to take their resveratrol doses as per section 5.7.

**Day of prostate biopsy / surgery**

For patients allocated to the control arm:
Biopsy patients are asked routinely to arrive an hour early to receive their pre-biopsy antibiotics. Surgical patients will arrive early the morning of their operation in preparation for their procedure. All patients will be met by the research team to sign the informed consent form for additional tissue, blood and urine samples. Patient examined and observations taken. Blood and urine samples taken for levels of [$^{14}$C], resveratrol, its metabolites and biomarkers for control.
2 extra prostate tissue samples will be taken in addition to the usual 10 samples as routine treatment for biopsy patients. For surgical patients, 2 tissue samples will be taken from the routinely resected prostate for trial analysis. The patient is deemed off study after the biopsy specimens have been taken.

For patients allocated to receive resveratrol:
The pre-biopsy antibiotic, ciprofloxacin, can interfere with resveratrol levels via the cyctochrome P450 enzyme pathway. For biopsy patients, blood and urine samples are therefore taken before antibiotic administration for levels of $^{14}$C, resveratrol and its metabolites and biomarkers. Surgical patients will receive a different antibiotic (gentamicin) which will not interact with resveratrol.
Compliance assessed for adherence to diet, concomitant drugs and resveratrol dosing. Patients who are non-compliant with any of these factors will not be replaced but this will be noted for results analysis. Patients who have taken their [$^{14}$C]-labelled resveratrol dose earlier than planned due to unexpected surgical delay will still have tissue samples taken for analysis, but the time lapsed noted.
2 extra prostate tissue samples will be taken in addition to the usual 10 samples that are taken as standard for biopsy patients. For surgical patients, 2 tissue samples will be taken from the routinely resected prostate for trial analysis. These tissue samples will be analyzed for levels of [$^{14}$C], resveratrol and its metabolites as well as biomarkers.

● *Completion of Study*
Once all specimens and information relating to drug compliance has been collected, the participant will be deemed off-study and no further contact will be required.

**Part C – Recruitment of Healthy Volunteers**
Interested participants will be instructed to contact the research team who will conduct a telephone enquiry to ensure fulfilment of inclusion and exclusion criteria as much as possible, to save unnecessary screening visits for unsuitable volunteers. Food habits noted. Suitable volunteers will then be sent a patient information sheet. After the volunteer has had at least 24 hours to consider the patient information sheet, a screening visit will be arranged over the telephone.

**Screening Visit for Healthy Volunteers**

● *Informed consent*
● *Medical History*
Includes past medical history, drug history; social history of alcohol or tobacco use. Participation in other studies noted including enrollment and ending dates as well as the nature of the study. Performance status and method of contraception will be documented. Age, gender and ethnicity noted.
● *Physical Examination*

Temperature, blood pressure, pulse, respiratory rate, height and weight noted.

● *Blood Tests valid for 2 weeks prior to dosing*

Haematology - haemoglobin, haematocrit, platelet count, differential white blood cell count

Biochemistry - sodium, potassium, urea, creatinine, glucose, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, total protein, albumin, alkaline phosphatase, total, conjugated and unconjugated bilirubin, calcium, inorganic phosphate, triglycerides and cholesterol.

● *Urine dipstick*

Any protein, blood, glucose and ketones noted.

● *Pregnancy test valid for 2 weeks prior to dosing*

Post menopausal status defined as no menses for 18 months prior to study. All other female patients will need a negative urine pregnancy test.

● *Food and Drug Avoidance Information Sheets, Food Diary*

Avoid resveratrol containing vitamins, food and drink for 5 days before and the duration of study, compliance checked by Food Diary. Avoid certain drugs whilst on study as potential interaction.

**Confirmation of Healthy Volunteer participation**

Participants are contacted via telephone with their blood test results to confirm eligibility. A convenient date, at least 5 days after the volunteer has been on a low resveratrol diet and within 14 days of when the screening blood tests were taken, will be arranged for the volunteer to undergo pharmacokinetic sampling.

**Day of [$^{14}$C]-resveratrol administration to Healthy Volunteers**

Participants must fast from midnight prior to the morning of [$^{14}$C]-resveratrol administration and can eat 3-4 hours after their [$^{14}$C]-resveratrol dose. Compliance to low resveratrol diet checked, and blood and urine samples taken prior to drug administration as control.

A single dose of [$^{14}$C]-resveratrol at either 5 mg or 1.005 g will be given by the research team and this time is noted. Repeat blood samples will be taken at 0.25 h, 1 h, 1.5 h, 3 h, 5 h, 10 h, 24 h and 48 h post dose. A new needle must be used each time for blood taking to prevent [$^{14}$C] contamination between samples. Urine samples will be taken at 12 hourly intervals for 48 h post dose. Volunteers will not need to stay at the research centre overnight but must return at the previously stated times for repeat blood and urine sampling. The Food Diary will be collected for formal assessment of diet compliance.

● *Completion of Study*

Once all specimens and information relating to drug compliance has been collected, the participant will be deemed off-study and no further contact will be required.

**Contact Information**

In event of problems related to the trial, participants will be advised of telephone numbers for contact.

**Information for General Practitioner**

An information letter, containing brief information concerning the trial and contact details, will be sent to the patient's General Practitioner (primary physician).

## v. Specimen Preparation

### Plasma

As soon as possible post collection specimens will be centrifuged at 4˚C at 5000*g* for 15 min, the plasma layer and leucocytes removed, split and stored in separate, equal volumes in labeled polypropylene tubes at -20°C or -80°C until assay.

### Tissue

Colorectal and prostate tissues obtained will be stored in a suitable freezer or processed into paraffin blocks until analyses. Biopsy sample weight will be approximately 10 mg, containing approximately 800 µg protein.

- *Control Samples*
- *Post-Intervention Samples*

Samples will be stored for analyses of biomarkers, resveratrol or its metabolites, or sent to the Lawrence Livermore National Laboratory, Livermore, California, USA for AMS analysis. Samples will be exposed to as little light as practically feasible.

### Sample Extraction for HPLC Analysis

Resveratrol and its metabolites will be extracted from tissue, plasma and urine samples by solid or liquid phase extraction.

### HPLC Analysis Conditions

The HPLC systems will consist of a system designated for AMS samples only. Chromatographic separation is typically accomplished by injecting the sample onto a Waters Atlantis 4.6x150 mm, 3-µm C18 T3 column (Waters, Elstree, UK). Fractions may be collected for AMS analysis.

### Biomarker Analysis

Biomarkers analysed from blood, urine and tissue samples may include markers of lipid peroxidation and oxidation (e.g. *N7*-hydroxyethylguanine DNA adduct and $M_1$dG DNA adduct), markers of apoptosis (e.g. caspase 3), DNA damage (e.g. comet assay) and indicators of proliferation (e.g. Ki67).

## vi. Adverse Events

### Definition

An adverse event (AE) is any untoward medical occurrence in a patient which appears or worsens after the participant has enrolled in an investigational study. It does not necessarily have a causal relationship with the treatment. An adverse event can be any unfavorable or unintended sign including an abnormal laboratory finding, symptom or disease. All adverse events will be noted on the Adverse Event Case Report Form (CRF), whether or not related to study drug.

An adverse drug reaction (ADR) is any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject.

An unexpected adverse reaction is an adverse reaction, the nature and severity of which is not

consistent with the information about the medicinal product in question set out in the investigator's brochure.

A serious adverse event (SAE), serious adverse drug reaction or suspected unexpected serious adverse reaction (SUSAR) is defined as those events, occurring at any dose, which meet any of the following criteria:

- Results in death
- Immediately life threatening
- Requires in-patient hospitalization or prolongation of existing hospitalization
- Results in persistent or significant disability/incapacity
- Is a congenital anomaly/birth defect
- In addition, events that may not meet these criteria, but which the investigator finds very unusual and/or potentially serious as may jeopardize the patient or require intervention to prevent one of the other outcomes listed in the definitions above, will also be reported in the same manner.

Progression or deterioration of the underlying colorectal cancer including metastasis and death due to disease progression will not be reported as an SAE. An SAE occurring during hospitalization or treatment of colorectal cancer during the study period will be reported.

**Severity**

All adverse events, whether drug related or not, will be graded by a numerical score according to National Cancer Institute Common Toxicity Criteria (NCI CTC), version 3.0 published 12 December, 2003. Adverse Events not included in the defined NCI CTC will be scored according to their impact on the participant's ability to perform daily activities as follows:

**Mild:** Causing no limitation of usual activities. (Grade 1)
**Moderate**: Causing some limitation of usual activities. (Grade 2)
**Severe**: Causing inability to carry out usual activities. (Grade 3)

**Laboratory Test Abnormalities**

Laboratory test results will be recorded on the laboratory results page of the CRF. Laboratory test abnormalities should not be reported on the AE page of the CRF as adverse event unless they are treatment emergent and they satisfy one or more of the following conditions for clinical significance:

- Accompanied by clinical symptoms.
- Leading to permanent discontinuation of resveratrol.
- Requiring a change in concomitant medication (e.g. the addition of or interruption or change of concomitant medication).

**Project Specific Adverse Events Definition**

Progression or deterioration of the underlying colorectal cancer disease including metastasis and death due to disease progression are not reported as AEs or SAE.

Signs and symptoms of the underlying colorectal cancer should only be reported if:

- Newly emergent (not at baseline) and the association of the underlying malignancy and new metastatic lesions is unclear.
- The investigator attributed deterioration of the colorectal cancer and symptoms directly to the study drug.

Any uncertainty about the attribution of the colorectal cancer to the AE, it should be reported as an AE, SAE or SUSAR accordingly.

**Relationship of Toxicity to Drug**

For AEs of severity Grade 2 or higher, investigators will discriminate any drug related (possible, probably or definite) AEs from non drug-induced AEs. If such a differentiation is not possible then the adverse effects will be considered drug-induced.

**Action after an Adverse Event**

All adverse events will be reported to a medically trained Investigator immediately and as soon as possible to the site Principal Investigator (PI). The Sponsor shall keep detailed records of all adverse events reported to him by the investigators for submission to the MHRA should they be requested.
Actions will be as follows:
● *Drug Related (definitely, probably, possibly or unlikely) Grade 1 AE*
The adverse event should be monitored carefully, with additional safety bloods and examinations as clinically appropriate. All data will be collected in a CRF.

● *Drug Related (definitely, probably, possibly or unlikely) Grade 2 or above AE*
If the adverse event is graded as 2 and is prolonged (over 48 h period) or grade 3 or above and thought to be drug related, the individual should be withdrawn from further dosing. All data will be collected in a CRF.

● *Non Drug Related*
The individual should be monitored throughout this period with clinically appropriate examinations and investigations. Investigators may withdraw volunteer at any time. All data will be collected in a CRF.

**Actions after Serious Adverse Event**

The investigators shall report all serious adverse events as defined in section 8.1 within 24 h to the Sponsor which may be made orally or in writing, followed by detailed written reports which will be submitted within 48 h of the event as per clinical trials directive 2001/20/EC (April 2001). The initial and follow-up reports shall identify subjects by unique code numbers. An example of the Serious Adverse Event Form is attached as Appendix A. For reported deaths of a subject, the investigators will supply the Sponsor and Ethics Committee with any additional information required.
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**Actions after Suspected Unexpected Serious Adverse Reaction**

The Sponsor shall report all SUSAR which occur during the clinical trial as per clinical trials directive 2001/20/EC (April 2001). Any SUSAR which is fatal or life-threatening will be reported as soon as possible to the MHRA and the relevant Ethics Committee not later than seven days after the Sponsor was first aware of the reaction. Any additional relevant information should be sent within eight days of the initial report.
Any SUSAR which is not fatal or life-threatening should be reported as soon as possible to the MHRA and not alter than 15 days after the Sponsor was first aware of the reaction.
The data elements included in a SUSAR report will be consistent with Annex 3 of Detailed

guidance on the collection, verification and presentation of adverse reaction reports arising from clinical trials on medicinal products for human use – April 2006. The Sponsor will provide follow up information for an adequate analysis of causality when they are available should the information be incomplete at the time of initial reporting.

**Follow-up**

All AEs, including laboratory abnormalities that in the opinion of the Investigator are clinically significant, if drug related or not, will be followed up according to Good Clinical Practice and documented as such.

In the event of unexplained abnormal laboratory values, the tests should be repeated immediately and followed up until they have returned to normal range/baseline values or an adequate explanation of the abnormality is found. If a clear explanation is found it needs to be recorded on the CRF.

**Pregnancy**

Any female patient that becomes pregnant during the study should have the resveratrol stopped immediately. A female patient must be instructed to inform the investigator if a pregnancy has been confirmed or suspected. Pregnancy occurring in a partner of a patient participating in the study should also be reported to the investigator and the sponsor. These actions are not applicable to patients in the control arms.

**Annual and Biannual Safety Reports**

The Sponsor will submit a safety report to the MHRA and the Ethics Committee once a year from the date of the CTA approval throughout the clinical trial, or on request. The aim of the Annual Safety Report is to describe concisely all new relevant safety information and to assess the safety of subjects included in these studies.

The Sponsor will submit a report of all SUSARs every 6 months to the MHRA and the relevant Ethics Committee. These reports will only include SUSARs reported within the period covered by the report.

### vii. Off Study Criteria

**Study Completion**

A participant will have completed the study when the events as defined in Section 4 and as per schema pg 4-9 are completed. Participants will be deemed off study once all specimens and dose related information have been collected and no further contact will be made. Once completed the End of Study CRF will be completed and the reason for off-study be recorded.

**Premature Removal of a Participant / Replacement**

● *Volunteer Withdrawal*

A participant may withdraw voluntarily from the study at any time without giving a reason.

● *Adverse Events / SAE*

If a participant develops adverse effects, investigators will follow guidance in section 7.0 regarding possible termination of that participant on the study. They will be medically treated, as clinically necessary, and will be followed up until such events resolve.

● *PI or Sub -investigator Instigated*

The Principal Investigator or Sub-investigator can decide to end a participant's participation in the study at any time. This decision could be based on factors such as unacceptable adverse

events or for other safety concerns, or non compliance with protocol procedures.
● Compliance to resveratrol doses, concomitant medication, low resveratrol diet
See section 5.7.
● Death of a Participant

### viii. Study Administration

**Research Ethics**

Ethics Committee approval will be obtained prior to initiating the study. During the study, the following will be submitted to the Ethics Committee for their review:
● Changes to the Investigator's Brochure.
● Reports of adverse events that are serious, unexpected and associated with the investigational drug.
● All protocol amendments and revised patient information sheets (if any).
● At least once a year, the Ethics Committee will be asked to review and re-approve the clinical study.
● At the end of the study, the investigator will notify the Ethics Committee about the study completion.

**Indemnity**
The University Hospitals of Leicester NHS Trust will be the sponsor of this study. NHS indemnity scheme and professional indemnity will apply to cases where harm arises to participants in the conduct of research. No arrangements have been made for compensation in the event of harm to the research participants or investigators where no legal liability arises.

**Publications**

The Clinical Research Fellow, Dr EN Scott, will take responsibility for publication of the results of this study.

**Guidelines for informed consent**

Prior to enrolment, written informed consent must be obtained from each patient or his legally authorised representative. The informed consent must contain all of the elements prescribed by the relevant regulatory authorities, and must be appropriately signed and witnessed. The basic elements of informed consent are presented below, and should be incorporated as appropriate when preparing the informed consent document.
● A statement that the study involves research, an explanation of the purposes of the research, the expected duration of the subject's participation, a description of procedures to be followed, and identification of any procedures which are experimental.
● A description of any reasonably foreseeable risks or discomforts to the subject.
● A description of any benefits to the subject, which reasonably may be expected as a result of the research.
● A disclosure of appropriate alternative procedures or courses of treatment, if any, which may be advantageous to the subject. A statement describing the extent to which the confidentiality of records identifying the subject will be maintained, and noting the possibility that the regulatory agencies may inspect the records.
● If the research involves more than minimal risk, a statement describing what compensation (if any) that will be paid, and an explanation as to whether any medical treatment is available

if an adverse reaction occurs, what the treatment consists of, and/or where further information can be obtained.

● A list of people to contact in the event of a study-related adverse experience and to answer pertinent questions about the research, including the subject's rights.

● A statement that the participation in the study is voluntary, refusal to take part in the research will not result in a penalty or loss of benefits to which the subject is otherwise entitled, and the subject may withdraw from the study at any time without penalty or loss of benefits to which he or she is otherwise entitled.

● A statement that the particular treatment or procedure may involve currently unforeseeable risks to the subject (or embryo or fetus, if the subject is or may become pregnant).

● Circumstances under which the subject's involvement may be terminated by the investigator without regard to the patient's consent.

● Additional costs to the subject, if any, which may result from participation in the research.

● The consequences that may result from the subject's decision to withdraw from the research, and the procedures for an orderly termination of the patient's participation.

● A statement that significant new findings will be provided to the subject during the course of the study that may relate to his or her willingness to continue in the program.

## 8.5 Submitted and published papers and abstracts

- EN Scott, H Cai, P Greaves *et al.* Opposing effects of resveratrol at dietary and clinically relevant doses on adenoma burden in *Apc*$^{Min+/-}$ mice fed a high fat diet. Shortlisted for BACR Young Investigator Prize, National Cancer Research Institute Annual Meeting, Liverpool 2010.
- EN Scott, MA Khan, D Hemingway *et al.* Comparison of [$^{14}$C]-resveratrol pharmacokinetics and target tissue distribution in humans following a pharmacological and dietary dose using accelerator mass spectrometry (AMS). Shortlisted for BACR Young Investigator Prize, National Cancer Research Institute Annual Meeting, Liverpool 2010.
- EN Scott, AJ Gescher, WP Steward, K Brown. Development of dietary phytochemical chemopreventive agents: biomarkers and choice of dose for early clinical trials. Cancer Prev Res. 2009;2:525-30.
- EN Scott, WP Steward, AJ Gescher *et al.* Gene and protein changes in HCA-7 colon cancer cells after exposure for three months to resveratrol at therapeutic and dietary relevant concentrations. Proceedings of the 100th Annual Meeting of the American Association for Cancer Research; 2009 Apr 18-22; Denver, Colorado, abstract no. 951.
- EN Scott, LM Howells, WP Steward *et al.* Pharmacokinetics in rats of a dietary and a clinically feasible dose of the putative cancer chemopreventive agent resveratrol. Poster presentation at the National Cancer Research Institute Annual Meeting, Birmingham 2009.