Cox-2 inhibitors in Oesophageal Cancer

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4.0 List of Abbreviations in alphabetical order:

AA	Araichdonic Acid
AFC	Abberent Crypt Foci
AhR	Arylhydrocarbon
AOM	Azoxymethane
Ara	arabinofuranosylcytosine
BMI	Body Mass Index
CCBs	Calcium Channel Blockers
CI	Confidence Inrterval
COX	Cycloxygenase
COX-2	Cycloxygenase- 2
Coxibs	Cycloxygenase blockers
EGFR	Epidermal Growth Factor
FFQ	Food Frequency Questionnaire
GEJ	Gastro Oesophageal Junction
GORD	Fgastro oesophageal Reflux Disease
HNU	Human Nutrition Unit
IFR	Institute of Food Research
IGFR	Insulin Like Growth Factor
IL	Interleukin
LOS	Lower Oesophageal Sphinter
MAPK	Mitogen Activated Protein Kinase
MMPs	Matrix Metalloproteinases
NFB	Nuclear Factor B

NOS	Nitric Oxide Synthetase
NSAIDs	Non Steroidal Anti Inflammatorys
OAC	Oesophageal Adenocarcinoma
OC	Oesophageal Cancer
OR	Odds Ratio
PG	Prostaglandins
PPAR	Peroxisome Proliferator Activated Receptor
ROS	Radical Oxide Synthetase
SCC	Squamous Cell Cancer
UK	United Kingdom
US	United State
VEGF	Vascular Endothelial Growth Factor
VGF	Vascular Growth Factor

5.0 Acknowledgements:

I would like to thank a large number of people without whom I would not have been able to finish this massive piece of work.

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6.0 Authors Declaration:

The experimental design of the work presented in this thesis was that of the author and supervisos, Professors Ian Johnson & Aedin Cassidy. The work presented in this thesis was performed by the author, except where the assistance of others is acknowledged.

I declare that this thesis has been composed by myself and is a record of work performed by myself.

Satish Ranka

April 2014

7.0 Thesis Abstract:

Introduction and Aims: The incidence of Oesophageal cancer (OC) has doubled in the last three decades and is increasing. Due to their poor outcome, strategies are being devised to prevent this disease and look into any association between pre-existing risk factors and medications which may aggravate them. Non-steroidal anti-inflammatory drugs (NSAIDs) may be protective by inhibiting Cycloxygenase-2 (COX-2), an enzyme known to induce malignant transformation in cells. Bronchodilators and Calcium channel blockers (CCB's) that relax the Lower oesophageal sphincter (LOS) may increase Gastro-Oesophageal reflux (GORD) and the risk of Oesophageal adenocarcinoma (OAC). Hence I carried out a case control study looking into any association between ingestion of NSAIDs and drugs which may relax the lower oesophageal sphincter and oesophageal cancer.

Case-control study:

Methods: 411 patients in Norfolk, with a primary neoplasm of the oesophagus or cardia were matched with 1644 controls with non-melanotic skin lesions. Data on the use of NSAIDs, bronchodilators and CCB's was collected.

Results: Compared to nonusers, individuals who used NSAIDs had a significantly reduced risk of oesophageal cancer. Use of NSAIDs was associated with approximately 60-70% reduction in OC. The odds ratios (OR) and 95% Confidence Intervals (CI) for different NSAIDs are as follows Aspirin: 0.38, (0.27-0.54); other NSAIDs: 0.29, (0.19-0.41) and COX-2 Blockers (Coxibs) 0.35, (0.16-0.78). LOS relaxing drugs were consumed more frequently in cases of OC as compared to the controls. Other NSAIDs include propionic acid, acetic acid, enolic acid and fenamic acid derivatives. The OR for LOS relaxing drugs are: Inhaled bronchodilators 3.2 (95% CI 2.2 to 4.7), Theophylline 1.9 (95% CI 1.3 to 5.1) and Calcium channel

blockers 2.4 (95% CI 1.2 to 5.0). Data was adjusted for confounding factors like smoking and alcohol consumption. Unadjusted data showed lower negative association with NSAIDs and positive association with drugs which relax the LOS.

Conclusion: NSAIDs were protective against OC development while drugs that relax LOS were associated with increased risk in this case-control study. Furthermore, data unadjusted for smoking and alcohol show reduced effect implying smoking and alcohol consumption may be confounding factors.

However, due to the undesirable side effects of synthetic COX-2 inhibitors, efforts are now directed towards finding natural compounds which have COX-2 inhibiting or antioxidant properties. Hence I conducted another study of validating a food frequency questionnaire with urinary excretion of quercetin, a naturally occurring Cox-2 inhibitor and naringenin, an antioxidant. With this validation, it would be possible to measure the dietary intake of these compounds in an individual's diet and recommend dietary changes by conducting further studies.

Flavonoid study:

Methods: A food frequency questionnaire (FFQ) was used to estimate daily intake of quercetin and naringenin in 49 healthy volunteers. They also provided five 24-hour urine samples over a 2 week period while completing the FFQ. Urinary excretion of quercetin and naringenin was determined by solid phase extraction and high-pressure liquid chromatography.

*Results: T*he estimated mean intake of quercetin and naringenin was 29.4 mg (SD 15.0) and 58.08 mg (SD 62.76) per day respectively. Mean urinary excretion of quercetin was 60.1 μg (SD 33.1) and naringenin was 0.56 mg (SD 0.42) per 24hrs. The correlation between FFQ-estimated intake and levels excreted in the 24hr urines

for quercetin was r = 0.82 (p< 0.0001) and for naringenin was r = 0.251 (p=0.05) respectively.

Conclusion: There was a statistically significant correlation between intake and excretion of quercetin and naringenin. Hence, a FFQ may be used as a tool in epidemiological studies requiring an estimate of naturally occurring COX-2 inhibitors or other chemicals following its validation.

8.0 Introduction: Oesophageal Cancer:

8.1 Epidemiology: Worldwide, OC is a significant and increasing health problem. In 2005, there were 497,700 new cases, and the prevalence is expected to increase by approximately 140% by 2025[1]. It has high mortality, with 416,500 people estimated to have died from oesophageal cancer in 2005. It has two major histological types, squamous cell (SCC) the most common worldwide, and adenocarcinoma. In the United States and many Western countries, OAC has surpassed SCC to become the most prevalent form of oesophageal cancer. Oesophageal adenocarcinoma rates have increased dramatically and has gone from a disease that was not thought to exist over the past few decades, to the fastest increasing cancer in America in the 2000's[2-4]. There were increases in both prostate cancer and melanoma, but these pale in comparison to the six-fold increase in oesophageal adenocarcinoma incidence during that same period[5]. The latest Surveillance Epidemiology and End Results statistics indicate that this alarming increase is continuing in the United States (US), with a more than 460% increase in incidence in white males from 1975 to 2004[4]. The increase has been noted in caucasian females also at a rate of 335% over the same period[4]. The rising incidence is occurring across all disease stages and all age groups, but the greatest increase (> 600%) is in men over 65 years old. Most cases are diagnosed in white males, but Hispanic, Japanese, Chinese, and African-American people also develop oesophageal cancer[1]. In the US in 2008, it was projected that 16,470 new patients will be diagnosed with oesophageal cancer, and more than 50% of cases would be adenocarcinoma[4]. A similar trend has been reported in other Western countries including the Netherlands[5]. OC incidence worldwide was 462126 cases in the year 2002; 315394 cases were diagnosed in males and 146723 cases in females. In

males, the incidence is approximately three times higher than in females. Figures 1 and 2 show that the highest incidence is registered in Ethiopia, China, and Mongolia, where standardized incidence rates are as high as 28.1 per 100.000 in males, and 19.6 per 100.000 in females in the year 2002. In Europe, the highest incidence rates in males are reported in France (11.0), Hungary (9.8), the United Kingdom (UK) (9.6), and Slovakia (9.3 per 100.000). In females, the highest numbers are reported in the United Kingdom (4.4), Netherlands (2.4), and Denmark (2.0 per 100.000).

The incidence of adenocarcinoma of the gastro oesophageal junction (GEJ) is greatest in white males, as seen in oesophageal adenocarcinoma; but unlike oesophageal adenocarcinoma, the incidence does not differ significantly between white and African American females and is similar in African Americans and Asians. Further, the rate of adenocarcinoma of the GEJ is double that of oesophageal adenocarcinoma in these groups[6,7]. The overall incidence of adenocarcinoma of the GEJ (3.1 per 100,000) previously exceeded that of oesophageal adenocarcinoma in the US, but the most recent data indicate that oesophageal adenocarcinoma incidence has surpassed that of adenocarcinoma of the cardia[8,9]. It is the ninth most common carcinoma in adults in the United Kingdom and is the fifth most common cause of death from cancer[10]. Within UK, around 5,000 new cases are diagnosed each year in men and around 2,800 women are diagnosed in women. The overall survival of patients is 5 % in the UK[11]. Only lung and pancreatic cancer have a worse prognosis. It has been estimated that the lifetime risk of developing oesophageal cancer is 1 in 64 for men and 1 in 116 for women in the UK calculated in February 2009 using incidence and mortality data for 2001-2005

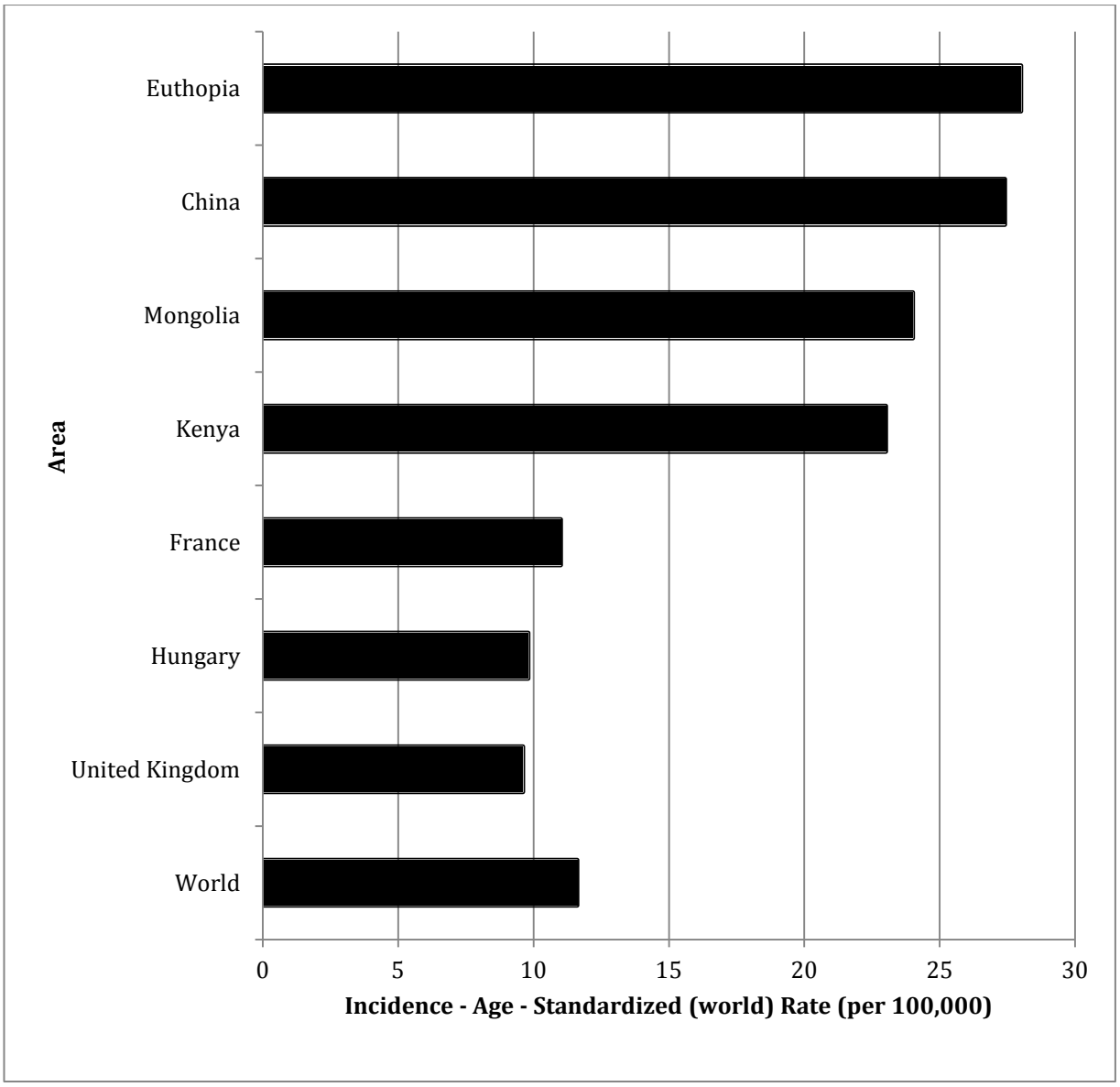


Figure 1: Standardised (world) incidence of oesophageal cancer in selected countries in males in the year 2002. (Courtesy Globocan 2002)

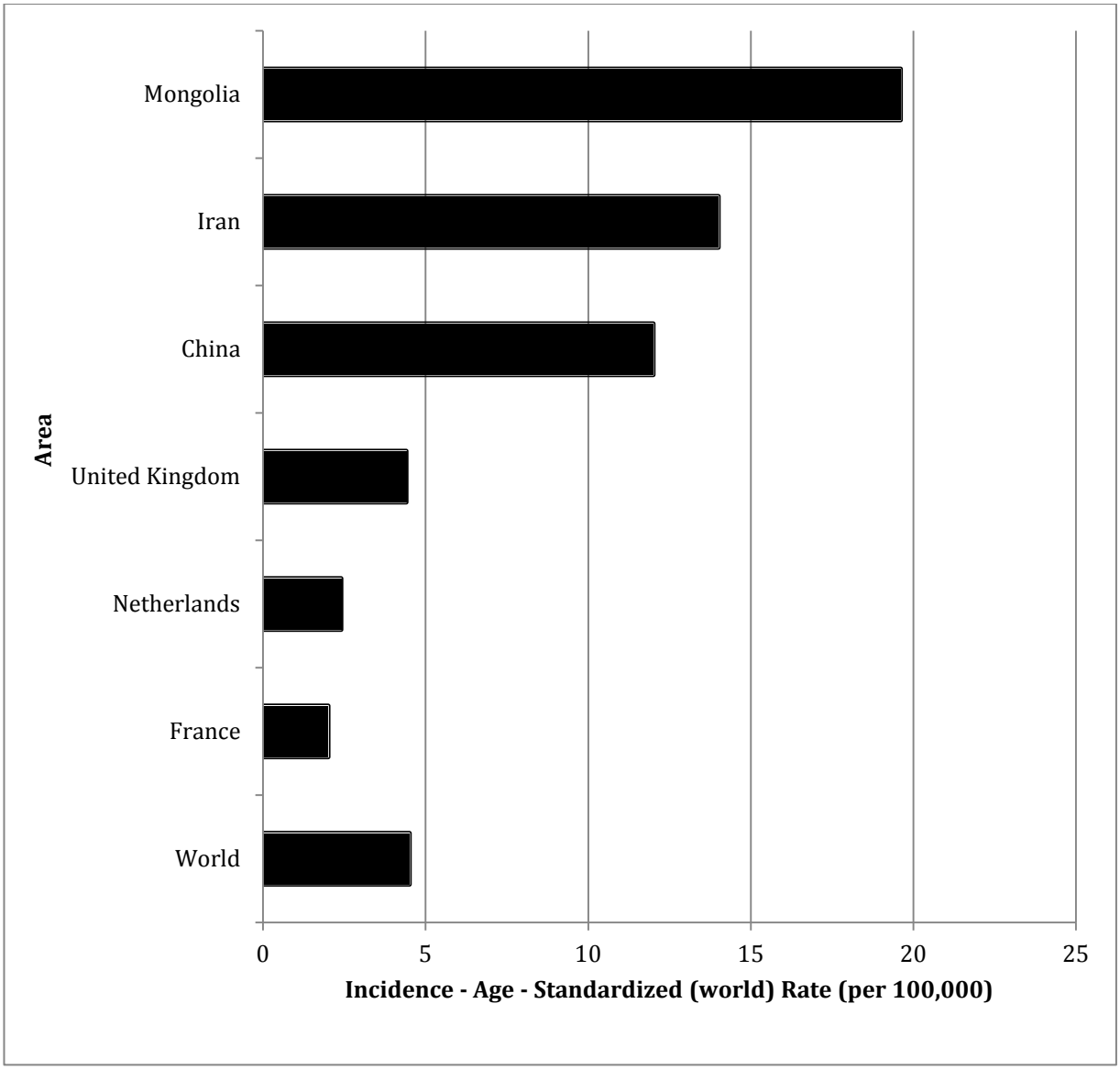


Figure 2: Standardised (world) incidence of oesophageal cancer in selected countries in females in the year 2002. (Courtesy Globocan 2002)

The United Kingdom has the highest incidence of oesophageal adenocarcinoma globally, at 7 cases per 100,000 population[12,13]. By comparison, the average incidence in the US is 2.5 per 100,000, although in some regions the incidence in white males is as high as 5.3 per 100,000[7,9,12]. The incidence of adenocarcinoma of the cardia or gastro-oesophageal junction has also increased significantly since the mid 1970s[14]. However, the average rate stabilized in the US after the late 1980s and is beginning to decline slightly[9].

8.2. Worldwide mortality rates:

Mortality rates represent roughly 90 % of the incidence rates of the disease. Figures 3 and 4 show that the highest number of cases in males is reported in Ethiopia, Kenya, and China, with standardized mortality rates around 27 per 100.000 in the year 2002. In females, the highest numbers are observed in Mongolia, Iran, Kenya and China, where the standardised mortality rates are around 16 per 100.000. Among European countries, the highest mortality rates in males are in Hungary (9.1) and the United Kingdom (9.0 per 100.000). In females, the United Kingdom is in the top position with a standardized mortality rate of 4.1 per 100.000, as well as the Netherlands with the standardized mortality rate 2.2 per 100.000.

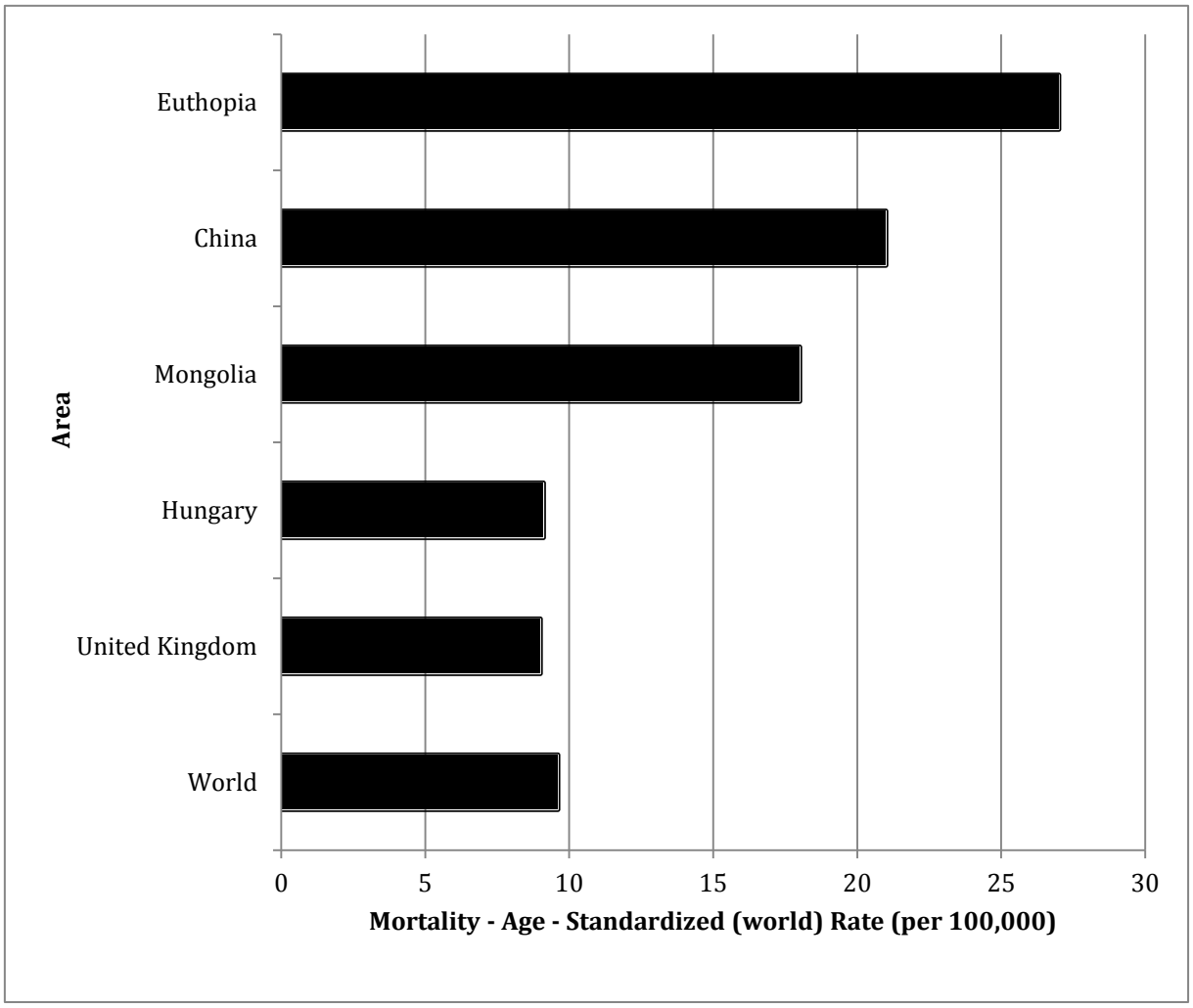


Figure 3: Standardised (world) mortality of oesophageal cancer in males in selected countries in the year 2002. (Courtesy Globocan 2002)

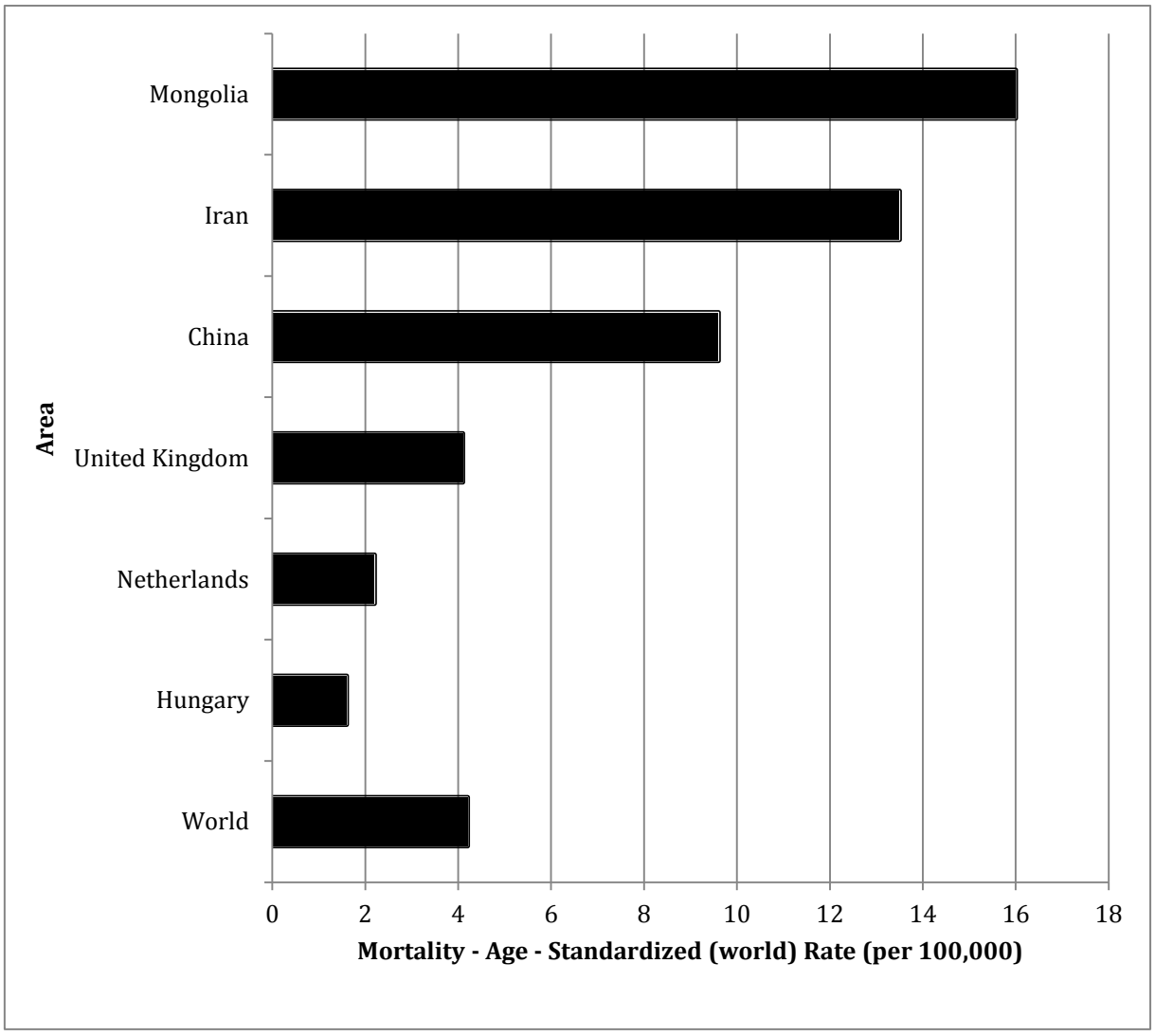


Figure 4: Standardised (world) mortality of oesophageal cancer in females in selected countries in the year 2002. (Courtesy Globocan 2002)

The changing incidence of AC within populations strongly suggests that although there are certain unchangeable risk factors, environmental factors linked to lifestyle changes are also of aetiological importance[15,16].

8.3. Risk factors for OC:

I. Non-modifiable risk factors: These include age, sex and hereditary factors.

The risk of developing the disease increases with age with very few cases diagnosed in people aged under 40 years (Figure 5). Amongst hereditary factors, tylosis characterised by hyperkeratosis of the palms and soles, is a major risk factor and increases the risk of OC by about 70%[17]. Plummer – Vinson syndrome, characterized by sideropenic dysphagia, glossitis, and esophagitis is a risk factor for the development of oesophageal cancer[18]. In persons with a family history, the relative risk of developing OC is around 1.57-2.84 according to several prospective epidemiologic studies[19-21].

II. Modifiable risk factors:

Environmental factors like consumption of alcohol and tobacco have long been recognised to be major risk factors for SCC but their role in OAC is less clear[22,23]. The increased incidence of obesity in Western countries may be an important factor. For example, in both North American and European studies, patients in the highest Body Mass Index (BMI) quartile have a significantly higher risk of developing OAC[24]. The increasing incidence of obesity may therefore be important in explaining the rise in prevalence of OAC in the West, perhaps because obesity leads to increasing intra-abdominal pressure, thereby promoting gastro-oesophageal reflux[25]. However metabolic factors such as a direct effect of insulin, insulin-like growth factors, and adipokines derived from adipose tissue, may also promote proliferation and suppress apoptosis of cells in the lower oesophagus[26].

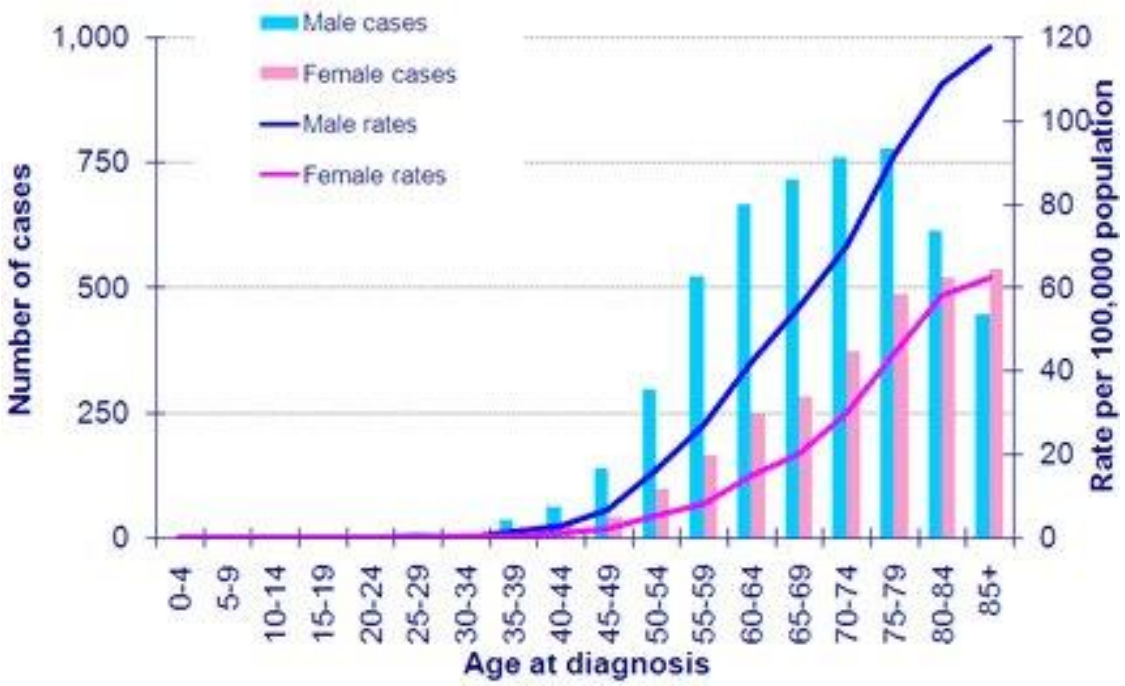
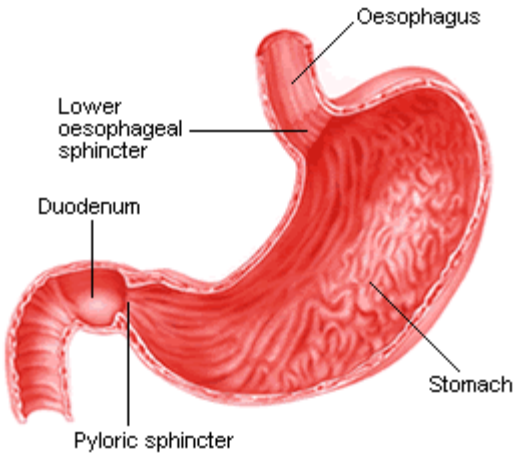


Figure 5: Numbers of new cases and age specific incidence rates, by sex, oesophageal cancer, UK 2006 (Courtesy Cancer research UK)

8.4. Gastro-oesophageal reflux disease (GORD)



GORD is defined as chronic symptoms or mucosal damage produced by the abnormal reflux in the oesophagus[27].

What causes the lower oesophageal sphincter to be faulty?

a. Raised intra-abdominal pressure, e.g. obesity, eating large meals, tight clothing, pregnancy

b. Faulty oesophageal sphincter, e.g. pregnancy - due to hormonal smooth muscle relaxation, smoking, fatty meals, hiatus hernia, after surgery for achalasia

c. Drugs, e.g. tricyclic depressants, anticholinergics which affect the function of the lower oesophageal sphincter

GORD is the strongest identified risk factor for OAC. Recurrent reflux may lead to a marked (almost 8 fold) increase in the incidence of OAC[28]. Since this is a chronic condition, the procarcinogenic effects of exposure to refluxate are likely to act over a long period of time. It is probable, therefore, that ageing is an important factor in the pathogenesis of this disease. Exposure of cultured oesophageal cells to acidified culture media has been shown to stimulate the MAPK pathway, promoting cellular proliferation and inhibition of apoptosis[29,30]. There is also evidence that pulsed acid exposure, as occurs in vivo in GORD, leads to increased cell proliferation in Barrett's metaplasia and over-expression of COX-2 enzyme[31,32]. A number of factors are responsible for GORD, including reduced LOS pressure, delayed gastric emptying, diet and obesity[33-35]. Mechanisms that relax the LOS, may increase the incidence of reflux. Certain medications such as calcium channel blockers, which are widely used in treatment of hypertension, angina and stroke, or bronchodilators such as Salbutamol, Terbutaline, Ipratropium bromide and Theophylline, used in the treatment of asthma, also relax the smooth muscles of the LOS[36-39]. This effect may increase reflux, and contribute to an increased risk of OAC[24].

Potential chemopreventive drugs which block COX-2 enzyme includes Aspirin, Non Steroidal Anti Inflammatory drugs like Ibuprofen, ketoprofen, Indomethacin and specific Cox-2 inhibitors like Celecoxib, Rofecoxib and etoricoxib. Details of doses low and high doses which are clinically prescribed are given in Table 1. Naturally occurring COX-2 inhibitors include flavonoids like quercetin, catechin, epigallocatechin, Luteolin, hesperetin, Isoflavonoids like genistein and daidzein, omega 3 fatty acids like eicosapentaenoic acid, docosahexaenoic acid and resveratrol a naturally occurring phytoalexin. Naturally occurring antioxidants include naringenin, Vitamins A, C, E, carotenoids etc.

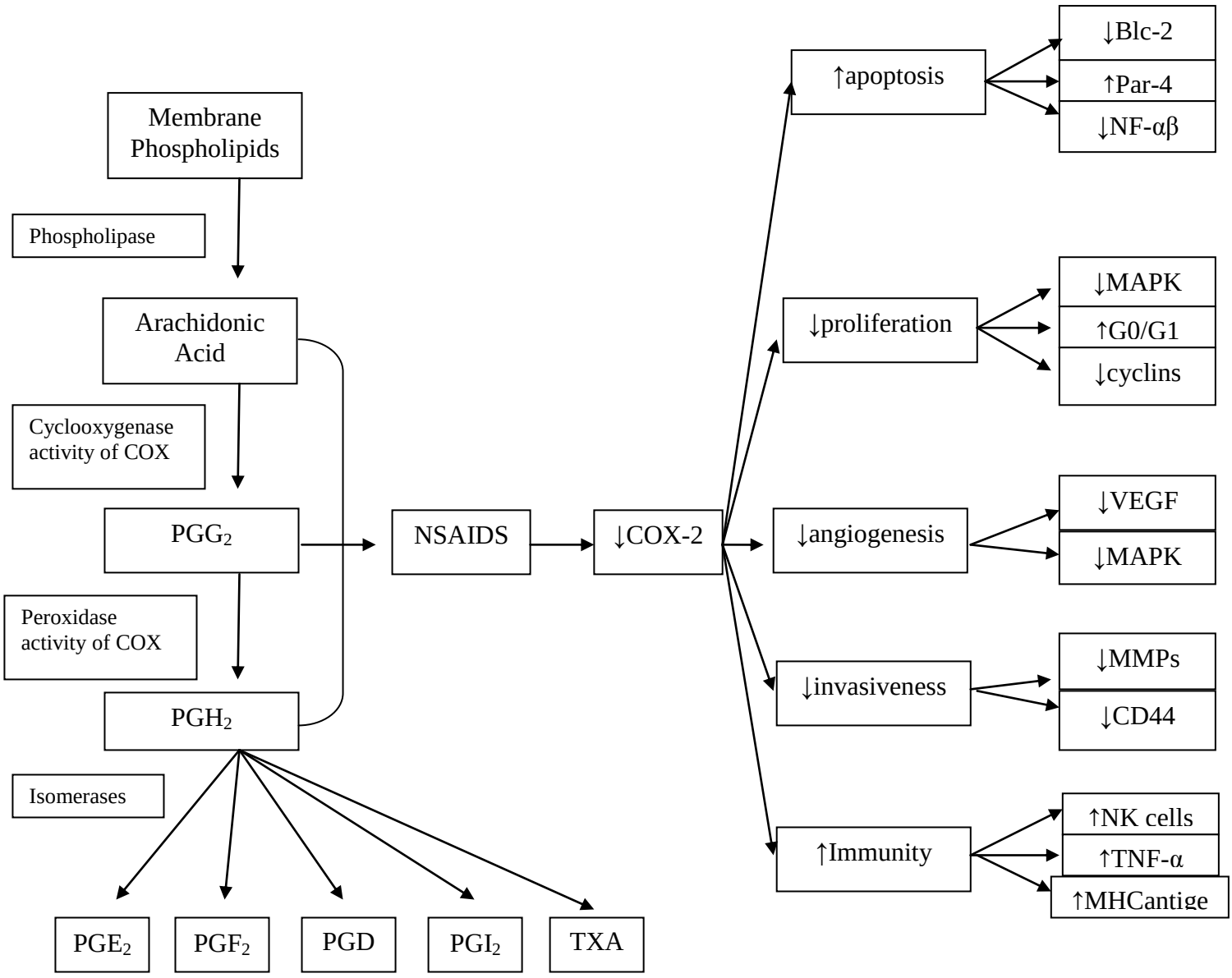
ASPIRIN	Low Dose	High Dose
	75mg	150mg +
OTHER NSAIDS	Low Dose	High Dose
Ibuprofen	200mg	1600mg
Diclofenac	50mg	150mg
Naproxen	500mg	1000mg
Indomethacin	50mg	100mg
Mefenamic acid	500mg	1500mg
Piroxicam	10mg	20mg
Ketoprofen	50mg	200mg
COXIBS		
Celecoxib	100mg	400mg
Rofecoxib	12.5mg	50mg
Etoricoxib	30mg	120mg

Table 1: Details of doses for different Cox-2 inhibitors by types

8.5. Impact of Coxibs on the risk of Oesophageal Cancer:

I. Contribution of COX-2 to Carcinogenesis:

Cycloxygenase (COX) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins (Fig. 6). The first step in prostaglandin synthesis is hydrolysis of phospholipids to produce free arachidonate in a reaction catalysed by phospholipase A2. Next, COX catalyses a reaction in which molecular oxygen is inserted into arachidonic acid. This reaction produces an unstable intermediate, prostaglandin (PG) G2. PGG2 is converted rapidly to PGH2 by the peroxidase activity of COX. Specific isomers then catalyse the reactions of the specific common precursor PGH2 to different prostaglandins and thromboxane, which all have their own range of biological activities[40]. Recently, two Cox isoforms have been cloned (COX-1 and COX-2) which share over 60% identity at amino acid level and have similar enzymatic activities. The most striking difference between the COX enzymes is in the regulation of expression. COX-1 is expressed constitutively in most tissues and seems to mediate production of prostaglandins that control normal physiological functions, such as protection of the stomach, regulation of renal blood flow, and platelet aggregation. COX-2 on the other hand, is usually not detectable in normal tissues, but can be readily induced in response to cell activation by hormones, proinflammatory cytokines, growth factors and tumour promoters. In part, the inducibility of COX-2 is explained by the presence of the nuclear factor responsible for the interleukin-6 expression and the cyclic AMP response element sites in the 5-flanking promoter region of the COX-2 gene . On the other hand, enhanced stability of COX-2 mRNA may also contribute to an increased expression of COX-2 . In the late 1970s it was first noted that neoplastic lesions had elevated



The mechanism of cycloxygenase enzymes (COX1 & COX2) in the biosynthesis of prostaglandins ans he potential targets of inhibitory actions of NSAIDs. PG= prostaglandins, TX= thromboxane, Blc-2= anti-apoptoic protein Blc-2, Pax-4= pro-apoptotic prostate apoptosis response gene, NF αβ: nuclear factor αβ, MAPK= mitogen activated protein kinase pathway, VEGF= vascular endothelial growth factor, MMPs= matrixmetalloproteinases, CD44= adhesion molecule CD44, NK: natural killer cells, TNF-α= tumor necrosis factor α, MHC antigens= major histocompatibility complex antigens

Figure 6

concentrations of prostaglandins, and increased expression of COX-2 was found in many premalignant tissues and malignant tumours (e.g. colorectal, gastric, oesophageal, pancreatic, lung, breast, bladder, cervical and ovarian cancer). Various oncogenes, growth factors and tumour promoters were shown capable of inducing COX-2 expression in malignant cells. Moreover, wild-type but not mutant p53 gene suppresses COX-2 transcription, raising the possibility that p53 is also a determinant of COX-2 expression . There are also data that demonstrate a direct relation between induction of COX-2 expression and carcinogenesis. Knocking out the COX-2 gene in the APC 716 mouse caused a significant reduction in both the number and size of intestinal polyp, whereas overexpression of COX-2 was sufficient to induce tumorigenesis in the mammary gland in transgenic mice[41] . Finally, pharmacological evidence also implies a role for COX-2 in carcinogenesis. Selective COX-2 inhibitors such as celecoxib and rofecoxib reduced the formation of intestinal, breast, skin, lung and bladder tumours in animals, and the non-selective COX inhibitor sulindac causes the reduction of adenomas in familial adenomatous polyposis patients. These data suggest that COX-2 represents a potential molecular target for preventing and treating cancer[40].

II. Mechanisms by Which COX-2 Contributes to Cancer Development:

COX-2 affects many processes that are important in carcinogenesis and is therefore an attractive therapeutic target. These processes include apoptosis, proliferation, angiogenesis, invasiveness, immunosuppression and inflammation[32]. Inhibition of these COX-2 processes by NSAIDs and selective COX-2 inhibitors may be the primary mechanisms of their antineoplastic action described in various studies[42-45] (Table 2).

Apoptosis: The size of a cell population depends on the balance between cell proliferation and programmed cell death (apoptosis). Decreased apoptosis may lead to a prolonged survival of abnormal cells, which favours the accumulation of sequential genetic changes that increase the risk of tumorigenesis, and inhibition of apoptosis will lead to clonal expansion. In vitro studies suggest that carcinoma cells expressing COX-2 have the potential to become resistant to apoptosis. Epithelial cells overexpressing COX-2 have increased amounts of the anti-apoptotic protein Blc-2 and are resistant to butyrate-stimulated apoptosis[46].

	Normal Epithelium	Metaplasia	Low-grade dysplasia	High-grade dysplasia	Adeno-carcinoma
Wilson et al. 1998[42]	0 %	81 %	nd	nd	100 %
Zimmerman et al. 1999[43]	0 %	0 %	nd	nd	78 %
Shirvani et al. 2000[32]	Significant increase in expression of COX-2 (P<0.05)				
Morris et al. 2001[44]	0 %	75 %	83 %	100 %	100 %
Buskens et al. 2002[45]	0 %	50 %	nd	nd	99 %

nd= not determined

COX-2 exprression in normal squamous epithelium of the oesophagus and the metaplasia-dysplasia-adenocarcinoma sequence during malignant degeneration

Table 2

Treatment with NSAIDs reversed this resistance to apoptosis. Recent studies also indicate that both selective and nonselective cyclooxygenase inhibitors induce the expression of the pro-apoptotic gene, prostate apoptosis response, and that accumulation of arachidonic acid caused by the inhibition of cyclooxygenase enzymes activates the production of ceramide, a strong apoptosis inducer. Finally, NSAIDs have been shown to decrease the expression of nuclear factor B (NF-B), a transcriptional factor that prevents apoptosis.

Proliferation: COX-2 is induced by activation of the Ras/Raf/mitogen activated protein kinase (MAPK) pathway, which is one of the key growth-stimulating cascades that causes cellular proliferation and NSAIDs can inhibit this process. It has been demonstrated that selective COX-2 inhibitors have an anti-proliferative effect by preventing epithelial cells from progressing from the quiescent state (G0/G1) into the phase of DNA replication (S-phase). Sulindac decreases the levels of mitotic cyclins, thereby reducing the phosphorylation of the retinoblastoma protein; this process normally allows the cell to enter the S-phase. The decrease in proliferation activity by NSAIDs was also demonstrated on colon carcinoma cells by a significant decrease in the Ki67 antigen, which is a proliferation marker, after treatment with sulindac.

Angiogenesis: The growth of a tumour partly depends on an increase in blood supply. Tumour cells ensure their own growth by secreting growth factors, especially vascular growth factor (VGF), that stimulate angiogenesis[47]. COX-2 has been implicated in this aspect of carcinogenesis as well. Overexpression of COX-2 in cancer cells increases the production of vascular growth factors, and the formation of capillary-like networks in vitro. These effects can be blocked by selective COX-2 inhibitors. It has been demonstrated that selective COX-2 inhibitors also diminish angiogenesis through inhibition of the MAPK pathway in endothelial cells .

Invasiveness: COX-2 is important in modulating the invasive properties of human cancer cells. When COX-2 is overexpressed in cancer cell lines, the production of prostaglandins increases, and the cells become more invasive. This increased invasiveness has been associated with activation of the matrix metalloproteinases (MMPs) 1 and 2. These enzymes digest the collagen matrix of the basement membrane, thus stimulating the invasive and motile phenotype of tumour cells. Additionally, overexpression of COX-2 was associated with increased amounts of CD44, the cell surface receptor for hyaluronate, and specific blockade of CD44 significantly decreased tumour cell invasion. Consistent with these in vitro findings, selective COX-2 inhibitors have been observed to inhibit dissemination in oesophageal adenocarcinoma 33 cells (OE33)[48,49].

Immunosuppression: The growth of tumours is associated with suppression of the immune system. Colony-stimulating factors released by tumour cells activate monocytes and macrophages to syntheize PGE2, which suppresses T-cell and B-cell proliferation, lymphokine production, macrophage activation and the cytotoxic activity of natural killer cells. PGE2 also inhibits the production of tumour necrosis factor while inducing the production of interleukin (IL) 10 , which has immunosuppressive effects[50]. These actions may allow the tumour to escape normal immune surveillance. By inhibiting prostaglandin synthesis, NSAIDs can indirectly enhance immune responses[51,52]. Additionally, they might upregulate expression of major histocompatibility complex antigens, as has been demonstrated in azoxymethane-induced colonic tumours in the rat. Mechanistically, this can depend on cytokine microenvironment, since COX-2 dependent synthesis of prostanoids by lung cancer cells altered release of IL-10 and IL-12 from lymphocytes and macrophages resulting in repression of host immunity.

Inflammation: Chronic inflammation, which is particularly associated with the development of a Barrett's oesophagus, is a recognized risk factor for carcinogenesis. Inflammation induces the synthesis of prostaglandins via a cytokine mediated induction of COX-2. With the data reviewed above, a mechanism can be suggested in which chronic inflammation and an increased expression of COX-2 contribute to the malignant degeneration of a Barrett's oesophagus[53].

NSAID's target the COX enzymes, which convert arachidonic acid (AA) to prostaglandins (PG) and other eicosanoids. COX-2, the inducible isoform, is undetectable in most normal tissues but is activated in response to pro-inflammatory cytokines and growth factors. Many studies have now reported COX-2 overexpression in tumours and its association with increased cell proliferation, metastasis and decreased apoptosis. Prostaglandins are believed to play a major role in these processes because of their effects on cell proliferation, apoptosis, angiogenesis and immune surveillance[54]. COX-2 upregulation has been reported in oesophageal SCC and AC. PGs have also been found to be overexpressed in oesophageal tumours as a consequence of enhanced COX-2 expression and. Moreover, COX-2 expression levels have been found to be elevated in Barrett's oesophagus and have been reported to increase with the progression from metaplasia to dysplasia to adenocarcinoma. A recent report has also indicated that a single nucleotide polymorphism in the COX-2 promoter can elevate COX-2 expression and is associated with a greater risk of developing oesophageal cancer[55]. Thus blocking the PG synthesis by NSAID's or COX-2 inhibitors may also play an important part in reducing the inflammatory process, thus preventing malignant change.

8.6 Impact of Flavonoids on Cancer:

Flavonoids, previously known as “Vitamin P” are water-soluble polyphenolic compounds found in abundance in the human diet. Many flavonoids possess antioxidant, antiplatelet, anti-inflammatory, anti-atherogenic, immunomodulatory and anti-carcinogenic activities in vitro[56-62]. Flavonoids are a large group of plant’s products that have a common C6-C3-C6 structure consisting of two aromatic rings linked through an oxygenated heterocycle (which is fused to one of these aromatic rings). Approximately, 8000 flavonoids have been characterized. Flavonoids containing a hydroxyl group in position C-3 of the C ring are classified as 3-hydroxyflavonoids (flavonols, anthocyanidins, leucoanthocyanidins, and catechins), and those lacking it as 3-desoxyflavonoids (flavanones and flavones). Classification within the 2 families is based on whether and 34how additional hydroxyl or methyl groups have been introduced to the different positions of the molecule. Isoflavonoids differ from the other groups; the B ring is bound to C-3 of ring C instead of C-2. The major classes are flavones (e.g apigenin, luteolin), flavonols (e.g quercetin, kaempferol), flavanones (e.g hesperetin, naringenin), flavanols (e.g epigallocatechin, EGCG), anthocyanins (e.g cyanidnin, delphinidin) and isoflavones (e.g genistein, daidzein). They are present mainly in fruits, vegetables, grains, bark, roots, stems, flowers tea, and wine[63]. Some of the common sources of flavoinoids are depicted in Table 3. Flavonoids possess free radical scavenging properties and have been reported to modulate COX-2 transcription in a number of different cell models. Liang et al. observed that apigenin was the most potent inhibitor of COX-2, inducible nitric oxide synthase (iNOS), and nuclear factor-κB (NF-κB)[64]. On the other hand, Singh and Agarwal noticed that silibinin, a flavonoid isolated from milk thistle, inhibited mitogenic and cell survival signaling such as epidermal growth factor receptor

(EGFR), insulin-like growth factor receptor type I (IGFRI) and NF-κB signaling[62]. The presence of flavonoids in plants depends on several factors including the degree of ripeness, variety, processing and storage. Of the Flavonoids, quercetin, catechin, epigallocatechin, luteolin, hesperetin and hesperidin possess Cox-2 inhibitory properties[59,67-68]. Flavonoids also have antioxidant properties, including preventing the degradation of Vitamin E, scavenging reactive nitrogen species and ROS, chelating metallic ions like Fe^{3+},Cu^{2+} involved in free radical production[69,70]. These properties reduce oxidative stress and a number of other of biological functions which may protect against cancer and other diseases.

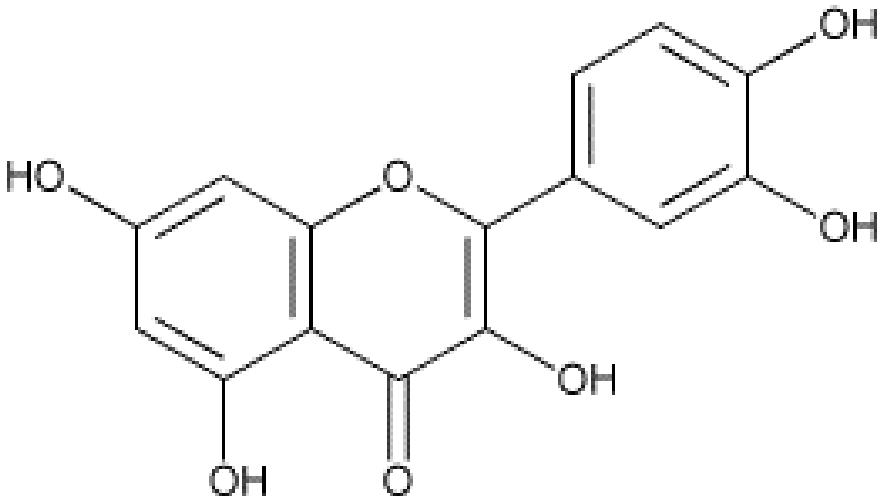
Flavonoids	Major food Sources
Apigenin (Flavonone)	Celery, parsley, thyme, sweet red pepper
Quercetin, kaempferol, myricetin (Flavonol)	Onions, broccoli, tomatoes, kale, berries, apples, grapes, tea
Hesperetin, Naringenin (Flavanone)	Oranges, lemons, prunes
ECGC (Flavanol or catechin)	Apples, plums, green tea
Cyanidnin (Anthocyanin)	Cherries, black grapes
Genistein, diadzein (isoflavone)	Soya beans, chick peas, legumes, rye

Table 3: Some common sources of flavonoids

A number of flavonoids are COX- 2 inhibitors (e.g. apigenin, chrysin, and kaempferol) and some can suppress COX-2 transcription by mechanisms that include activation of the peroxisome proliferator-activated receptor (PPAR) gamma transcription factor, and inhibition of NF-kB expression. COX-2 transcription is inhibited in vitro not just by quercetin aglycone, but also by the metabolites quercetin 3-glucuronide, quercetin 30-sulphate, and 30-methylquercetin 3-glucuronide. These compounds are found in human plasma, and both quercetin and quercetin 30-sulphate also inhibit COX-2 enzyme activity. Quercetin aglycone inhibits COX-2 expression and induces apoptosis in the oesophageal adenocarcinoma cell line OE33 and squamous cell line in vitro[53]. A study by Tanigawa et. al found that quercetin inhibited the proliferation of HepG2 cells through the induction of cell cycle arrest and apoptosis[71]. Also in another study, quercetin suppressed the formation of early preneoplastic lesions in colon carcinogenesis, which occurred in concert with reductions in proliferation and increases in apoptosis[72].

I. Quercetin (2-(3,4-dihydroxyphenyl)- 3,5,7-trihydroxy-4H-chromen-4-one): Quercetin is a bioflavonoid which is claimed to strengthen capillaries and regulate permeability. Frequently quercetin occurs as glycosides (sugar derivatives); e.g., rutin. Quercetin is termed the aglycone, or sugarless form of rutin. Quercetin is the major bioflavonoid in the human diet. The estimated average daily dietary intake of quercetin by an individual in the United States is 25 mg.

Structure of Quercetin: Courtesy Wikipedia.org

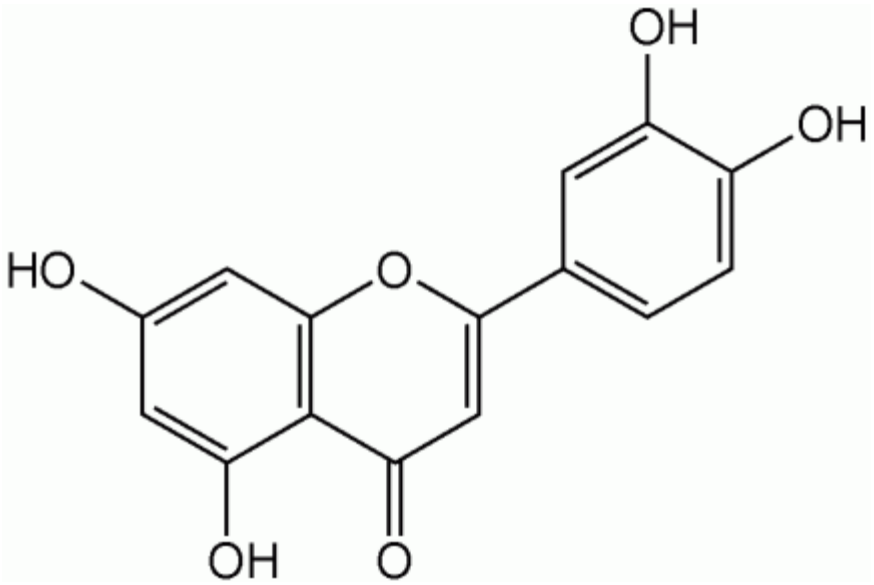


Sources: Foods rich in quercetin include capers (1800 mg/kg), lovage (1700 mg/kg), apples (440 mg/kg), tea (*Camellia sinensis 2000-2500mg/kg*), onion, especially red onion 320mg/kg (higher concentrations of quercetin occur in the outermost rings), broccoli (30mg/kg), bog whortleberry (158 mg/kg, fresh weight), lingonberr1 (cultivated 74 mg/kg, wild 146 mg/kg), cranberry (cultivated 83 mg/kg, wild 121 mg/kg), chokeberry (89 mg/kg), sweet rowan (85 mg/kg), rowanberry (63 mg/kg), sea buckthorn berry (62 mg/kg), crowberry (cultivated 53 mg/kg, wild 56 mg/kg), and the fruit of the prickly pear cactus (50mg/kg). A recent study found that organically grown tomatoes had 79% more quercetin than conventionally grown[73].

II. Naringenin: 5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one:

Naringenin is an flavonone that is considered to have a bioactive effect on human health as antioxidant, free radical scavenger, anti-inflammatory, carbohydrate metabolism promoter, and immune system modulator. It is the predominant flavanone in grapefruit.

Structure of Naringenin: Courtesy supplementscience.org



Sources: Grapefruit, Oranges, and Tomato (skin).

This bioflavonoid is difficult to absorb following oral ingestion[74]. Based on available data, only 15% of ingested naringenin will get absorbed in the human gastrointestinal tract. Ingestion of 8ml/kg of orange juice will supply about enough naringenin to achieve a concentration of about 0.29 mg in urine in 24 hrs[75]. The naringenin-7-glucoside form may be less bioavailable than the aglycol form[76]. Grapefruit juice can provide higher plasma concentrations of naringenin than orange juice[75]. Naringenin can be absorbed from cooked tomato paste[77].

III. Bioavailability of Quercetin and Naringenin:

The bioavailability and metabolism of flavonoids, especially quercetin and (+)-catechin, were investigated by several groups in the 1960s and 1970s[78,79]. In most of these studies, rather high doses of flavonoids were given to laboratory animals and the main focus was on the bioavailability of flavonoid degradation products produced by the gastrointestinal microflora. Today, sensitive methods allowing serum and urine analyses are available for many flavonoids. These methods allow estimations of flavonoid bioavailability in human beings at appropriate dietary intake levels.

It is evident that the bioavailability of flavonoids varies greatly between different subgroups and compounds. This is hardly surprising, considering the differences in their chemical properties (eg, polarity).

Quercetin:

Gugler et al[80] failed to detect quercetin in plasma and urine of subjects receiving 4 g of quercetin aglycone orally, which indicated that quercetin is not absorbed in human beings. Hollman et al[81-84] and de Vries et al[85], however, showed that the compound is bioavailable from onions, tea, apples, red wine, and supplements containing quercetin glycosides. In their studies, bioavailability was examined after ingestion of relatively high amounts of pure compounds or food containing them. Ingestion was either once or over a few days. Relatively few studies have measured plasma quercetin levels after ingestion of quercetin in amounts comparable with those attainable from a normal diet. Recently, 3 studies have been published in regards to this. In these studies, the mean plasma values ranged between 15 and 24 mg/L (50-80 nmol/L) in subjects consuming their habitual diets[86-88] and was 42 mg/L (140 nmol/L) after a diet containing 815 g/d of vegetables, fruits, and berries[88].

Consumption of 100 g/d of berries (lingonberries, bilberries, and black currants) in addition to a normal diet resulted in a mean plasma level of 21 mg/L (70 nmol/L)[87].

Studies with pure compounds have given more precise information about the absorption and kinetics of quercetin. These studies indicate that quercetin aglycone and quercetin glucosides are absorbed from the upper parts of the gastrointestinal tract, probably the duodenum, while quercetin-3-rutinoside is absorbed from the distal parts, probably the colon[89,90]. In one study[89], low doses of quercetin aglycone and quercetin- 3-rutinoside were given to healthy volunteers in a crossover setting. Mean bioavailability of quercetin was similar from the 2 sources, but there was marked interindividual variation in bioavailability from quercetin-3-rutinoside, in particular. A rather interesting finding was that quercetin from quercetin-3-rutinoside was more bioavailable in women compared with men, and plasma levels were highest in women using oral contraceptives. These results suggest interindividual variation, possibly gender specific, in gastrointestinal microflora, or absorption or biotransformation mechanisms[91]. Furthermore, this study also showed that despite previous speculations, quercetin is bioavailable when given as aglycone. This finding was confirmed by Walle et al[92] in a study in which radiolabeled quercetin aglycone was used. The results indicated absolute bioavailability of 36% to 53% for quercetin aglycone and that a substantial portion of quercetin is excreted by the lungs as CO_2. It should be kept in mind that the results are based on recovery of radioactivity, and therefore, the findings may reflect bioavailability of degradation products, which could have been formed, at least partly, before absorption.

The urinary excretion of quercetin has been investigated in several studies reported in a review by Manach and Williamson[93] (Table 4). In these studies, urinary recovery, as a percentage of the ingested dose, ranged between 0.07% and 3%.

Source	Number of Subjects	Dose	Tmax Plasma	Plasma concentration	AUC	Urinary Excretion	Elimination half-life
			h	*µmol/L*	*µmol/L - h/L*	*% of intake*	*h*
Pure quercetin	6	4g		<0.33		<1	
Onions	9 Ileostomizes	89 mg quercetin eq				0.31/13h	
Pure quercetin	10 Ileostomizes	100 mg quercetin eq				0.12/13h	
Fried onions	2	64 mg quercetin eq	2.9	0.65			16.8
Onions	9	68 mg quercetin eq	0.7	0.74	7.7		28
Apples	9	107 mg quercetin eq	2.5	0.3	3.5		
Complete meal	10	87 mg quercetin eq		0.37 at 3h			
Onions	5	186 mg quercetin eq	1.3-1.9	2.18		1.11	
Onions	5	50 mg quercetin eq	2	0.83			
Quercetin 4`-glucoside	9	150 mg	<0.5	3.5	18.8		21.6
Quercetin 3`-glucoside	9	156 mg	0.6	4	19.1	3.6	18.5
Quercetin 4`-glucoside	9	160 mg	0.45	4.5	17.5	3.1	17.7
Pure quercetin	16	8, 20, 50 mg	2, 2.7, 4.9	0.14, 0.22, 0.29	1.74, 2.92, 3.77		17, 17.7, 15
Onions	12	100 mg quercetin eq	0.68	7.6	32.1	6.4	10.9

T_{max} time to C_{max}, AUC: area under the curve. Eq: equivalents

Table 4: Bioavalibility studies with quercetin. (Adapted with permission Manach et al. 2005)

Furthermore, it was lower after ingestion of quercetin-3-rutinoside than after ingestion of onion. Biliary excretion cannot be ruled out and has been shown to be a major route of quercetin elimination in rats[94,95]. In rats fed a diet containing 0.25% quercetin, the concentrations of quercetin and methylated metabolites were approximately 3-fold in bile compared with urine. The high molecular weight of quercetin lucuronides and sulfates and their extensive binding to protein[96-98] could favor their biliary excretion[99].

Naringenin:

Analytical methods allowing the analysis of flavanones in plasma only became available recently[75]. Until then, knowledge about their bioavailability relied on animal studies and human urinary excretion data. The results concerning urinary excretion have varied in different studies (Table 5)[93]. For naringenin, individual urinary recoveries of 5% to 59% (6 subjects)[100], 5% (1 subject)[101], 14% to 15% (2 subjects)[102], and 1% to 6% (6 subjects)[103] are reported after single ingestion of 214 to 700 mg of naringenin as a supplement or in juice. The half-life for naringenin conjugates in urine was estimated as 2.6h[100]. Erlund et al[75] studied the bioavailability and pharmacokinetics of flavanones after single ingestion of 400 to 760 mL of orange juice or grapefruit juice. The resulting plasma concentrations were comparatively high (up to 4 mg/L or 15 nmol/L), which is not surprising, considering that citrus fruits and juices contain quite high concentrations of the compounds (several hundred milligrams per liter). The plasma half-lives of flavanones are relatively short (1-2 h). Furthermore, renal clearance of naringenin appeared to be dependent on dose. Similar plasma levels were reported after consumption of 0.5 or 1 L of orange juice[104]. Bugianesi et al [105] recently made the interesting finding that naringenin is

bioavailable from tomato paste, which is a notable source because of its widespread use, despite its low naringenin content.

Source	Number of Subjects	Dose	Tmax Plasma	Plasma concentration	AUC	Urinary Excretion	Elimination half-life
			h	*µmol/L*	*µmol/L - h/L*	*% of intake*	*h*
Orange Juice	5	22.6 or 45mg naringenin eq	4.6-5	0.06-0.2	0.43-1.29	7.1-7.8	
Orange jiuce	8	23 mg naringenin eq	5.5	0.64	2.6	1.1	1.3
Grapefruit juice	5	199 mg naringenin eq	4.8	5.99	27.7	30.2	2.2
Tomato paste	5	3.8 mg naringenin eq	2	0.12			
Pure compound	6	500 mg naringenin eq				4	
Grapefruit juice	6	7.2mg naringenin eq				8.9	2.6-2.9

T_{max} time to C_{max}, AUC: area under the curve. Eq: equivalents

Table 5: Bioavalibility studies with naringenin. (Adapted with permission Manach et al. 2005)

IV. Inhibition of cancer promotion by flavonoids:

Multiple mechanisms appear to be involved in the inhibition of carcinogenesis by dietary polyphenols (Figure 7). Central to protective effects by dietary flavonoids and other polyphenols at the promotion stage of chemically induced carcinogenesis is the ability to inhibit cell proliferation. The damage that the carcinogens have inflicted on cellular DNA during the initiation stage is being propagated into a new cell population. This machinery, i.e. clonal expansion, is highly complex, geared towards giving the cells immortality by stimulating mitogenesis and/or decreasing cell death by inhibiting apoptosis. Protective effects at this stage are critically important. This has been demonstrated in cell culture with unmethylated flavonoids and other polyphenols, as discussed briefly in Section 1.5 (II), affecting numerous signal transduction pathways. Some of the polymethoxylated flavonoids have also in preliminary studies demonstrated antiproliferative properties[106,107].

Activation of circulating monocytes by hyperglycemia plays a role in inflammatory process. A study by Wu and collegues, examined whether flavonoids (catechin, EGCG, luteolin, quercetin, rutin) - phytochemicals that may possible belong to a new class of advanced glycation end products (AGEs) inhibitors - can attenuate high glucose (15 mmol/L, HG)-induced inflammation in human monocytes[108]. Their results show that all flavonoids significantly inhibited HG-induced expression of proinflammatory genes and proteins, including TNF-α, interleukin-1β (IL-1β), and COX-2, at a concentration of 20 μM. Flavonoids also prevented oxidative stress in activated monocytes, as demonstrated by their inhibitory effects on intracellular reactive oxygen species (ROS) and *N*ε-(carboxymethyl)lysine formation caused by HG. These inhibitory effects may involve inhibition of nuclear factor-κB activation and may be supported by downregulation of the following: i) PKC-dependent NADPH oxidase pathway; ii) phosphorylation of p38 mitogen-activated protein kinase and extracellular signal-regulated protein kinase, and iii) mRNA expression of receptor of

AGEs. In addition, they found for the first time that lower levels of Bcl-2 protein under HG conditions could be countered by the action of flavonoids. Their data suggest that, along with their antioxidant activities, flavonoids possess anti-inflammatory properties as well.

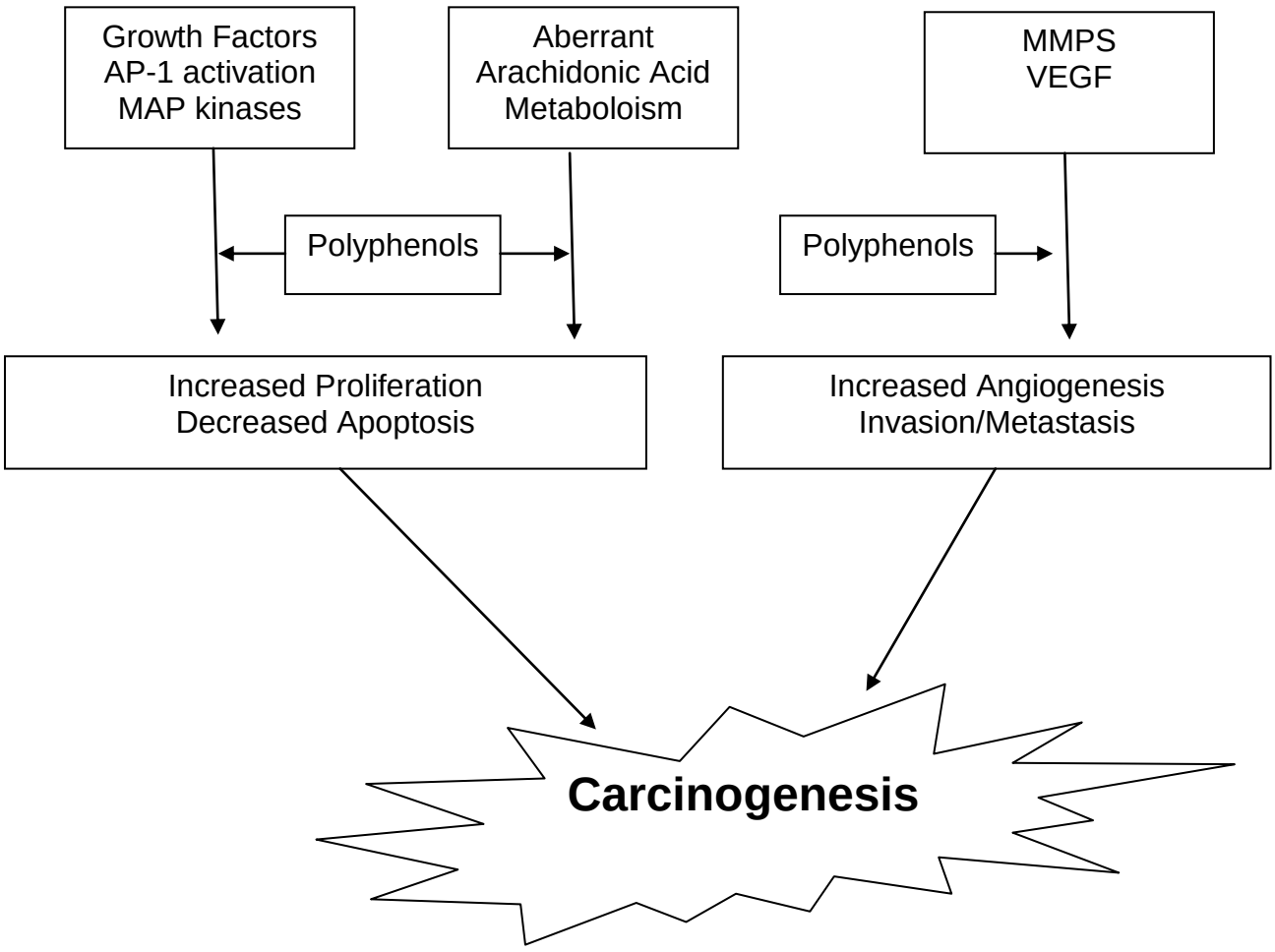


Figure 7: Proposed mechanistic scheme for the prevention of cancer by dietary polyphenols.

The effect of the flavonoid quercetin was investigated on colorectal cancer cells by Dihal and collegues[109]. Male F344 rats (*n* = 42/group) were fed 0, 0.1, 1, or 10 g quercetin/kg diet. Two week after initial administration of experimental diets, rats were given 2 weekly subcutaneous injections with 15 mg/kg body wt azoxymethane (AOM). At wk 38 post-AOM, quercetin dose dependently ($P < 0.05$) decreased the tumor incidence, multiplicity, and size (Table 6). The number of aberrant crypt foci (ACF) in unsectioned colons at wk 8 did not correlate with the tumor incidence at wk 38. Moreover, at wk 8 post-AOM, the number and multiplicity of ACF with or without accumulation of *ß*-catenin were not affected by the 10 g quercetin/kg diet. In contrast, another class of CRC-biomarkers, *ß*-catenin accumulated crypts, contained less *ß*-catenin than in controls ($P < 0.05$). After enzymatic deconjugation, the plasma concentration of 3'-*O*-methyl-quercetin and quercetin at wk 8 was inversely correlated with the tumor incidence at wk 38 ($r = –0.95$, $P \leq 0.05$). Thus quercetin, at a high dose reduced colorectal carcinogenesis in AOM-treated rats, which was not reflected by changes in ACF-parameters.

	Quercetin, g/kg diet			
	0	0.1	1	10
Rats, *n*	22	22	22	20
Tumor bearing rats, *n*	11	9	8	4
Tumor incidence, [1,2] %	50	41	36	20[+]
Tumors, *n*	17	13	11	4
Tumor multiplicity, [3] *n*	1.55 ± 0.93	1.44 ± 1.01	1.38 ± 0.74	1.00 ± 0.0
Tumor size overall, [3] mm	5.94 ± 3.83	5.85 ± 4.22	5.64 ± 3.98	5.00 ± 1.83
Small (<5mm) tumors, % of total	47	46	45	50
Small (≥5mm) tumors, % of total	53	54	55	50
Adenoma, 5 of large tumors	11	--	50	--
Carcinoma, % of large tumors	89	86	33	100

[1] Values are means ± SD or percent. * Different from control, $P < 0.05$.
[2] Quercetin mediated a dose dependent decrease in tumor incidence, $P < 0.05$.
[3] Tumor multiplicity and size were inversely associated with increasing dietary quercetin, both, r = -0.98, $P < 0.05$

Table 6: Tumor characteristics in AOM-treated rats fed control, quercetin diet at wk 38 post-AOM administration. (Adapted with permiss50ion Dihal et al. 2006)

Naringenin, one of the most abundant flavonoids in citrus fruits, has been reported to suppress cytotoxicity and apoptosis in mouse leukemia P388 cells exposed to a typical pro-oxidant, H_2O_2, or an anticancer drug, cytosine arabinoside (1-b -D-arabinofuranosylcytosine; Ara-C) due to its antioxidative properties[110,111]. In order to demonstrate if naringenin has the ability to inhibit tumor growth *in vivo*, a study using ddY mice given a subcutaneous injection of sarcoma S-180 was performed[112]. Naringenin dose-dependently inhibited the growth of sarcoma S-180 when administered at 30, 100 or 300 mg/kg once a day for 5 d, with 99.7%, 72.2% or 57.0% suppression compared with the control (control tumor weight 100%) following intraperitoneal injection, respectively (Fig. 8).

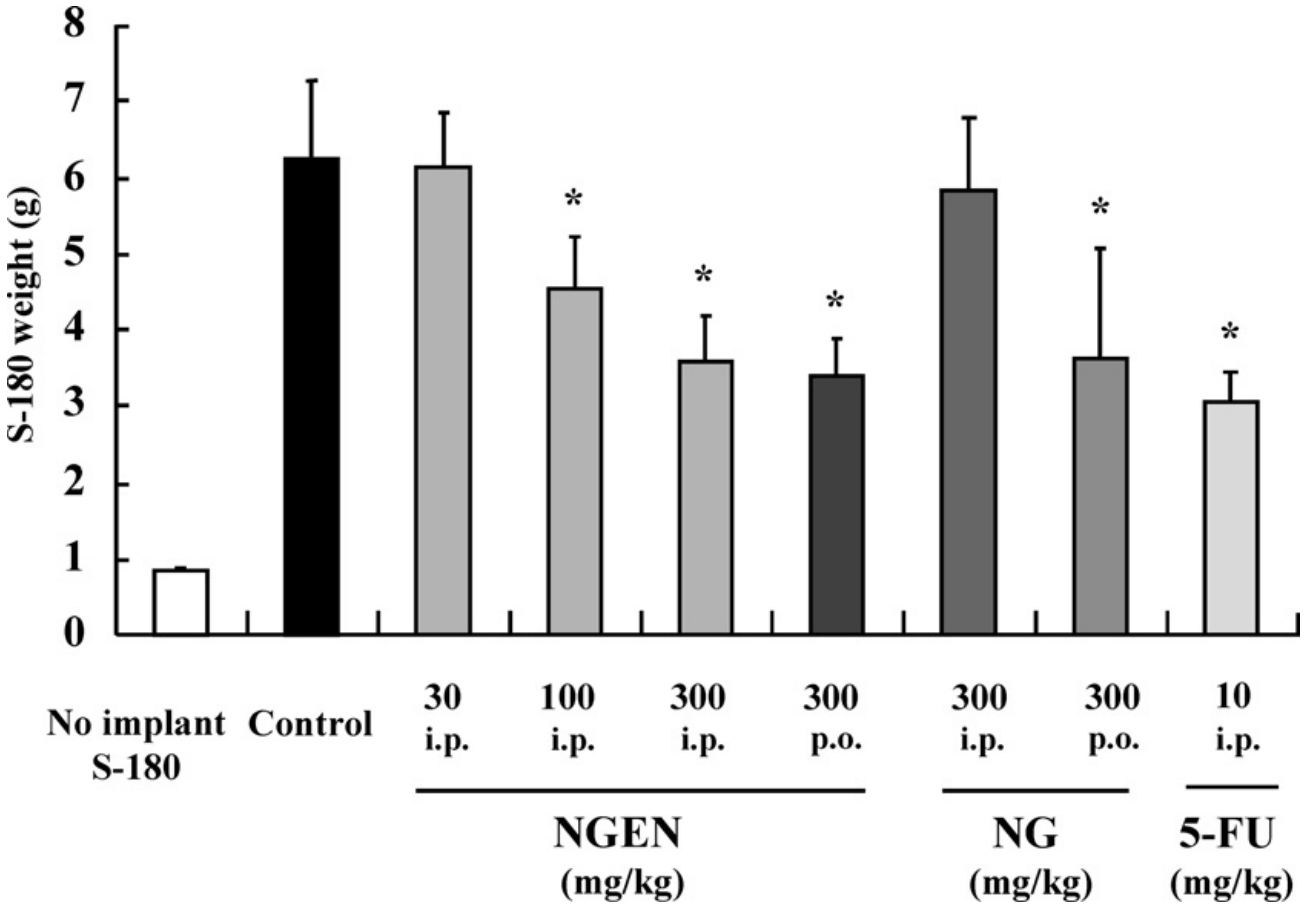


Figure 8: **Inhibitory effects of naringenin on the growth of murine S-180 tumor implanted in ddY mice. (Adapted with permission Kano et al. 2005)**

V. Evidence of Flavonoids in chemoprevention:

Association between intake of flavonoids and the incidences of a variety of cancers have been studied in a number of studies. However, significant associations were observed only for lung cancer and colorectal cancer. Two Finnish cohort studies with relatively low intakes of flavonoids(3-4mg/day), found inverse associations with lung cancer risk (RR: 0.53; 95% CI: 0.29, 0.97; and RR: 0.56; 95% CI: 0.45, 0.69)[113,114]. For catechins, an inverse association was observed for rectal cancer (RR: 0.55; 95% CI: 0.32, 0.9), but not for colon cancer (RR: 0.1; 95% CI: 0.85, 1.44) in postmenopausal women in America[115]. Currently, there is no evidence of an effect of flavonoid intake on the incidence of epithelial cancers such as stomach, urinary tract, prostate or breast[116-119].

Zhang and collegues found that flavones and flavonols exert cytotoxic effects on a human oesophageal adenocarcinoma and squamous cell line by inducing apoptosis[120,121]. Stoner and collegues, evaluated the ability of lyophilized (freeze-dried) black raspberries (Rubus occidentalis), blackberries (Rubus fructicosus), and strawberries (Fragaria ananasia), sources of quercetin to inhibit carcinogen-induced cancer in the rodent esophagus[122]. At 25 wk of the bioassay, all three berry types were found to inhibit the number of oesophageal tumors (papillomas) in NMBA-treated animals by 24-56% relative to NMBA controls. This inhibition correlated with reductions in the formation of the NMBA-induced O6-methylguanine adduct in oesophageal DNA, suggesting that the berries influenced the metabolism of NMBA leading to reduced DNA damage. Furthermore, Naringenin, has anti-estrogen properties and may be responsible for the decreased incidence of breast cancer in woman consuming a large amount of these fruits[123]. In a study from Hawai, where there is a high intake of apples, onions and white grapefruit, rich sources of flavonoids there was a 40-50% reduction in the incidence of lung cancer[124]. Epidemiological

studies suggest ingestion of green tea, a rich source of quercetin, may reduced the risk of oesophageal cancer[125,126].

9.0 Aims of conducting the studies:

It is clear that incidence rates of oesophageal cancer have increased in the UK , and prognosis is poor with only 5 % patient's surviving in the long term. Prevention of oesophageal cancer should be based on early detection and surveillance of precancerous lesions, especially of Barrett's oesophagus, and attention should also focus on chemoprevention, including finding artificial and natural chemical agents which may help reduce its incidence. Carefully designed epidemiological studies, both descriptive and analytical, are required to increase our understanding of the complexity of chemoprevention in oesophageal cancer. Identification of risk factors will allow preventive measures to be developed to reverse the trend of the disease and to decrease its incidence and mortality.

Of the different types of epidemiological studies, a case-control study was conducted by me in Norfolk, United Kingdom, to examine potential associations between exposure to NSAIDs, LOS relaxing drugs and OC. This study method was selected as it was cost effective, quick and easy to conduct. The identification of controls, a major challenge was overcome by selecting patients with non-melanotic skin cancers, as their exposure to NSAIDs would be similar to the general population. Also the results were adjusted for confounding factors like smoking and alcohol consumption.

Epithelial carcinogenesis is a multistep process in which an accumulation of genetic events within a single cell line leads to a progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma. Cancer chemoprevention, as first defined by Sporn in 1976, uses natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression[127]. It is based on the concepts of multifocal field carcinogenesis and multistep carcinogenesis. In field carcinogenesis, diffuse epithelial injury in tissues, such as the aerodigestive tract, results from generalized carcinogen exposure throughout the field and clonal proliferation of mutated cells. Genetic changes exist throughout the field and increase

the likelihood that one or more premalignant and malignant lesions may develop within that field. Multistep carcinogenesis describes a stepwise accumulation of alterations, both genotypic and phenotypic. Arresting one or several of the steps may impede or delay the development of cancer. An example of this is prevention of progression from Barrets oesophagus to OC. Barrets oesophagus is the end result of GORD. Chemoprevention trials are based on the hypothesis that interruption of the biological processes involved in carcinogenesis will inhibit this process and, in turn, reduce cancer incidence[128]. This hypothesis provides a framework for the design and evaluation of chemoprevention trials, including the rationale for the selection of agents that is likely to inhibit biological processes and the development of intermediate markers associated with carcinogenesis.

Cancer chemoprevention may also be achieved by the use of natural, synthetic, or biologic compounds to reverse, suppress, or prevent the development of diseases. Chemoprevention is a potential anti-cancer approach, which has reduced secondary effects in comparison to classical prophylaxis. Natural compounds such as flavonoids reduce oxidative stress, which is the most likely mechanism in the protective effects of these compounds. Even though their exact mechanisms of action are not well understood another mechanism of polyphenolic flavonoids relates to induction of apoptosis[129]. Moreover, flavonoids may modulate protein and lipid kinase signaling pathways. Hence a FFQ was validated by me against the 24hr urinary excretion of quercetin and naringenin, with the aim of potentially using this FFQ to assess the intake of naturally occurring coxibs and in future intervention studies.

To summarise the main aims of conducting the studies were:

- To conduct a case-control study to examine potential associations between exposure to NSAIDs, LOS relaxing drugs and OC

- Validate a FFQ against the 24hr urinary excretion of quercetin and naringenin

10.0 Non-steroidal anti-inflammatory drugs, lower oesophageal sphincter-relaxing drugs and oesophageal cancer: A case-control study.

10.1: Introduction: The evidence for using NSAIDs as a chemopreventive agent in carcinogenesis first came from epidemiological studies. Of the published retrospective observational studies on oesophageal cancer, Thun et al. found that subjects who used aspirin 16 times per month or more often for at least one year have an approximately 40% lower risk of oesophageal cancer (P = 0.054)[130]. Data from the National Health and Nutrition Examination Survey and the National Epidemiological Follow-up Studies show a 90% (95% CI = 0.01–0.76) decreased risk of developing oesophageal cancer in subjects who report occasional aspirin use. Data from an australian study show a reduced risk of AC (OR, 0.48; 95% CI, 0.32-0.72), and squamous cell carcinoma (OR, 0.63; 95% CI, 0.40-0.98) in patients with aspirin use relative to nonusers[131]. Risk reductions for AC among users of aspirin and NSAIDs were greater among those who experienced at least weekly symptoms of reflux (OR, 0.26; 95% CI, 0.12-0.55 and OR, 0.41; 95% CI, 0.21-0.77, respectively) than those who did not experience reflux (OR, 0.96; 95% CI, 0.46-2.00 and OR, 0.78; 95% CI, 0.35-1.72, respectively) . Recently Duan et. al. found regular use of aspirin and non-aspirin NSAIDs was associated with reduced relative risk of AC (OR, 0.60; 95% confidence interval, 0.38-0.95; P(trend) = 0.04)[132]. In addition, in vitro studies and animal studies have been published which support the possible chemopreventive effect of selective COX-2 inhibitors in Barrett's epithelium. Buttar et al. demonstrated in primary cultured endoscopic biopsy specimens from patients with Barrett's oesophagus that selective COX-2 inhibitors significantly decreased COX-2 activity and decreased proliferation of epithelial cells by 55% (95% CI 47.1–63.8)[133]. The same investigators also found in a rat model that two different selective COX-2 inhibitors reduced the relative risk of developing oesophageal cancer by 55% (95% CI

43–66, *P* = 0.008) and by 79% (95% CI 68–87, *P* = 0.001) after oesophagojejunostomy, when compared with controls. However, the prevalence of a Barrett's oesophagus was not significantly different between the groups (*P* = 0.98). Kaur et al. indirectly demonstrated the possible chemopreventive effect of selective COX-2 inhibitors in vivo as well[134]. In this study, biopsy specimens of human Barrett's epithelium were compared with biopsy specimens obtained after 10 days of therapy with rofecoxib 25 mg orally daily. After rofecoxib therapy, the COX-2 expression decreased by 77% (*P* =0.005), the PGE2 content decreased by 59% (*P* =0.005), and the proliferating cell nuclear antigen (PCNA) expression decreased by 62.5% (*P* =0.005). Further evideice of COX-2 inhibitors chemopreventive properties is presented in the following chapter.

10.2. Evidence against chemoprevention:

Evidence demonstrating the overexpression of COX-2 and PG, highlighted the potential of NSAIDs as chemopreventative agents, and may have led to the development of more selective COX-2 inhibitors. However, the mechanisms by which these COX-2 inhibitors exert their antineoplastic effects remain controversial. Apoptotic and/or anti-proliferative effects have been reported in several cell lines including lung , prostate and colon . In oesophageal cancer cells, both growth arrest and apoptosis have been reported to be induced by COX-2 inhibitors. A study by Bardou *et al* failed to demonstrate a lasting effect of chronic ingestion of NSAIDs and cyclooxygenase-2 inhibitors on OC[135]. The COX-2 dependent and independent mechanisms of these inhibitors need to be established in order to achieve safe and effective design of chemo-therapeutic and chemo-preventative protocols. However this case-control study did not differenciate between the histological types of OC. They also could not account for over the counter medication use i.e Aspirin, and NSAIDs. Recent review have assessed the safety of selective COX-2 inhibitors and while gastric toxicity is reduced, some agents have an increased risk of cardiovascular complications[136]. A recent retrospective analysis of the large UK National Barrett's Oesophagus Registry database concluded no difference in risk of development of dysplasia or adenocarcinoma was observed between patients taking aspirin and those not taking aspirin (hazard ratio 1.092, 95% confidence interval 0.358-3.335, P = 0.877)[137]. This study did not examine any relation between NSAIDs use and risk of OC.

Furthermore, a randomized controlled trial by Heath et. al. found administration of 200 mg of celecoxib twice daily for 48 weeks of treatment does not appear to prevent progression of Barrett's dysplasia to cancer[138]. However in this study, few patients were not assigned randomly. They also found treatment with celecoxib for 24 weeks compared with placebo did not change the levels of COX-2 mRNA, regardless of

grade. This could be due to the fact they used only one dose of Celecoxib i.e: 200mg. Increasing dose to 300mg or 400mg amy have given more information.

10.3. Evidence of association of LOS relaxation with OC:

The association of chronic reflux with the development of oesophageal AC suggests that drugs known to decrease the pressure of the lower oesophageal sphincter (and hence predispose to reflux) may be a risk factor for Barrett's esophagus and possibly AC. However, the strength of this association remains uncertain. In a case control study involving 189 patients with newly diagnosed oesophageal carcinoma were compared to 262 patients with adenocarcinoma of the gastric cardia, 167 patients with oesophageal squamous cell carcinoma, and 820 population-based controls[139]. In this study, past use of drugs known to relax the LOS (such as nitroglycerin, anticholinergics, beta adrenergic agonists, aminophylline, and benzodiazepines) was positively associated with the risk of oesophageal adenocarcinoma (Odds ratio 3.8 (95 percent CI, 2.2 to 6.4) for use >five years). Assuming a causal relationship, it was estimated that approximately 10 percent of oesophageal adenocarcinomas occurring in men older than age 60 may be attributable to intake of these drugs. By contrast, in another case control study, an association was detected only between asthma medications (xanthines and beta agonists and not nitrates, calcium channel blockers or benzodiazepines) and Barrett's esophagus among persons younger than age 70[140.] Lui and collegues studied the role of beta-adrenergic signaling in the regulation of growth of an oesophageal squamous-cell carcinoma cell line HKESC-1[141]. They concluded that epinephrine stimulates oesophageal squamous-cell carcinoma cell proliferation via beta-adrenoceptor-dependent transactivation of ERK/COX-2 pathway.

62, 63,10.4. Materials and Methods: Case-Contorl Study

I. Case Identification

The study population lived in Norfolk and presented to one of the three hospitals in the region: The Norfolk and Norwich University Hospital NHS Trust; Norwich, The Queen Elizabeth II Hospital; King's Lynn and the James Paget Hospital; Great Yarmouth which cover the county. The population of Norfolk was approximately 824,200 in mid-2005. Permission was sought from clinicians in each hospital to review patient notes. The International Code of Diseases (ICD), version 10, was used to identify case notes of 411 primary malignant neoplasms of the oesophagus or cardia diagnosed between 1999 and 2004. These codes were: C15.0-C15.5 for cervical oesophagus, thoracic oesophagus, abdominal oesophagus, upper, middle and lower part of oesophagus, C15.8 for overlapping lesions of the oesophagus and C16.0 for lesions of the cardia. These neoplasms were divided into squamous and adenocarcinoma. The study included patients who had been exposed to NSAIDs and LOS relaxing drugs for a minimum period of 4 weeks in the last 10 years. Exposure of these patients to NSAIDs was for analgesia, arthritis, anti-platelet action, and LOS relaxing drugs for asthma, chronic obstructive airway disease, hypertension, ischemic heart disease and cerebro-vascular disease. The case notes were reviewed to confirm the diagnosis.

The study was approved by the Norfolk Research Ethics Committee in June 2004. Protocol of case control study attached as Appendix 1.

II. Control Identification

Patients who had undergone a minor day case procedure for non-melanotic skin tumours, and were under the care of dermatologists and plastic surgeons, formed the control group. They were identified as per the ICD 10 criteria using the code C44. This group of patients was selected because their use of NSAIDs was similar to that in the general population. Any potential control with a past history of oesophageal cancer was excluded. Data were collected on all drugs with respect to their type, dosage and frequency of use. Data on potential confounding factors such as smoking and alcohol consumption were also collected.

III. Data Collection and analysis

Data was collected over a period of 2 years during the period from July 2004 to June 2006. Data was collected by the principal investigator and checked by the Clinical and Non-clinical Supervisors

Demographic and clinical data on drugs, illnesses and surgical management for each proven case were collected on a standard data collection sheet (Appendix 2) and anonymised. The data on drug type, frequency of administration, dosage and any associated illnesses were ascertained from the general practitioner referral letter, medical admission notes and nursing records. Data for controls were obtained from the general practitioner's referral letters and hospital medical and nursing records.

Each case was matched for exact age, sex and year of hospital admission with four controls. A total of 411 cases were matched with 1644 controls. The Odds ratios were calculated using conditional logistic regression with 95% confidence interval. NSAIDs use was divided into the following categories: Aspirin, other NSAIDs and specific Coxibs, LOS relaxing drugs were divided into calcium channel blockers, bronchodilators and Theophylline. The data were adjusted for the confounding effects of smoking and alcohol. STATA SE 8.3 software was used to perform the analysis.

10.5.Results:

Demographic details of cases, control, case tumour characteristics are presented in Tables 7 and 8. Males dominated the study with the gender ratio being 3.2:1 (M:F). The mean age of cases was 71 years and controls 70 years. The most common site of the tumour was the lower third of the oesophagus.

I. Smoking, alcohol consumption and OC

A history of tobacco smoking (past or present) was found in 79% of cases as compared to 28% controls. Seventy one percent of controls never smoked. Data on tobacco smoking was unavailable in 2% of the cases and 1% of the controls. Smoking was 3 times more common in cases with oesophageal cancer as compared to the controls. Alcohol consumption was virtually identical in both the groups (Table 9).

II. NSAIDs and OC

Following adjustment of smoking and alcohol consumption, intake of NSAIDs was lower in cases of OC as compared to the controls, and exposure to any dose of NSAIDs was associated with a signicicant reduction in risk of OC by 60-70%. Amongst the medications, consumption of low dose aspirin (75mg) and other NSAIDs predominated in both cases and controls. The data were therefore sub-divided into low-dose and high-dose NSAIDs, using the minimum daily dose and frequency of consumption of NSAIDs while performing the analysis. Low-dose aspirin appeared to be as effective as high-dose (OR 0.33, CI: 0.23-0.47). There was insufficient data regarding exposure to low-dose Coxibs (n=10), for us to estimate the risk.

III. NSAIDs and OAC

Aspirin and other NSAIDs were more protective than specific Coxibs. As there is an overlap between the CI of OAC and SCC, it was not possible to be certain whether NSAIDs were more beneficial to patients with OAC or SCC.

IV. NSAIDs and SCC

There was a negative association between exposure to aspirin (OR:0.50, CI: 0.25-0.97) and other NSAIDs (OR: 0.36, CI: 0.16-0.81) and SCC of the oesophagus (Table 9). The reduction in risk of SCC was approximately 50% for Aspirin and 65% for other NSAIDs. In this study, no patient with SCC was consuming Coxibs; hence it was not possible for us to evaluate any correlation here.

V. LOS relaxing drugs and OC

Statistically significant positive associations were observed between the risk of OC and exposure to several different drugs that are known to relax the lower oesophageal sphincter. Exposure to these drugs at any level was associated with an odds ratio of between 1.9 and 3.2 (95% CI 1.2 - 5.1) for OC. Exposure to any formulation of CCB, whether short or long acting, was also associated with an odds ratio of 2 to 3 (95% CI: 1.2 - 5.1).

VI. LOS relaxing drugs and OAC

A statistically significant association between the use of drugs which relax the LOS and OAC was observed. Among cases with OAC, 15% had an exposure to a CCB and 22% cases were on anti-asthma medication (Table 9). Cases with OAC were 2-3 times more frequently exposed to LOS relaxing drugs as compared to the controls.

VII. LOS relaxing drugs and SCC

Only weak associations between bronchodilators, CCBs and SCC of oesophagus were observed (Table 9). As only 4 patients with SCC were exposed to Theophylline, no association between Theophylline and SCC could be calculated.

VIII. Data unadjusted for smoking and alcohol

Table 10 & 11 show the data unadjusted for smoking and alcohol. The protective effect of the NSAIDs and the risk with CCBs is reduced when unadjusted for smoking and alcohol.

	Cases	Controls
Patient Numbers	411	1644
Males	285 (70%)	1141 (70%)
Females	126 (30%)	503 (30%)
Mean Age (years)	71 (40-93)	70 (41-94)
Age Range		
41-50 years	15 (3%)	60 (3%)
51-60 years	65 (16%)	260 (16%)
61-70 years	102 (25%)	408 (25%)
71-80 years	148 (36%)	592 (36%)
81-90 years	78 (19%)	312 (19%)
91- 94 years	03 (1%)	12 (1%)

Table 7: Demographics of patients.

Tumour Site	Upper 1/3rd	11 (3%)
	Mid 1/3rd	41 (10%)
	Lower 1/3rd	359 (87%)
Tumour type	Adeno Ca	318 (77%)
	Squamous Ca	93 (23%)
Degree of differentiation		
	Mild	60 (15%)
	Moderate	224 (54%)
	Poor	127 (31%)

Table 8: Oesophageal tumour distribution and histology

	Cases n=411	Controls n=1644	Odds Ratio+	Confidence Interval+
Smoking: Never	79 (19%)	1174 (71%)	-	-
Ever	323 (79%)	398 (24%)	3.18	2.75-3.68
Alcohol : Never	143 (35%)	514 (31%)	-	-
Ever	264 (64%)	1099 (67%)	0.90	0.80-1.01
Oesophageal Cancer				
Aspirin	93 (22%)	524 (32%)	0.38	0.27-0.54
Low Dose Aspirin	56 (14%)	421 (26%)	0.43	0.23-0.47
High Dose Aspirin	37 (8%)	103 (6%)	0.33	0.15-0.98
Other NSAIDs :	79 (19%)	575 (35%)	0.29	0.19-0.41
Low Dose other NSAIDs	23 (6%)	8 (0.5%)	0.44	0.26-0.94
High Dose other NSAIDs	56 (13%)	567 (34%)	0.17	0.10-0.27
Coxibs	55 (12%)	135 (8%)	0.35	0.16-0.78
CCBs:	78 (19%)	112 (7%)	2.41	1.22-5.01
Short Acting CCBs	11 (3%)	26 (2%)	2.08	1.22-5.13
Long Acting CCBs	67 (16%)	86 (5%)	2.90	1.84-4.73
Inhaled Bronchodilators	86 (21%)	108 (7%)	3.21	2.24-4.71
Theophylline	27 (7%)	18 (1%)	1.92	1.36-5.10
Oesophageal Adenocarcinoma				
Aspirin	62 (15%)	345 (21%)	0.35	0.24-0.51
Other NSAIDs	54 (13%)	391 (24%)	0.25	0.16-0.40
Coxibs	55 (13%)	135 (8%)	0.46	0.20-0.94
CCBs	60 (15%)	86 (5%)	2.91	2.12-4.14
Inhaled Bronchodilators	65 (16%)	82 (5%)	3.64	2.67-5.13
Theophylline	23 (6%)	15 (1%)	2.13	1.36-5.12
Oesophageal Squamous Cell Cancer				
Aspirin	31 (7%)	179 (11%)	0.50	0.25-0.97
Other NSAIDs	25 (6%)	184 (11%)	0.36	0.16-0.81
Coxibs	0	0	-	-
CCBs	18 (4.4%)	26 (2%)	1.45	1.22-5.13
Inhaled Bronchodilators	20 (5%)	26 (2%)	1.76	1.53-5.16
Theophylline	4 (1%)	3 (0.2%)	-	-

+ Conditional logistic regression model, adjusting for cigarette smoking status (ever, never) and alcohol consumption (ever, never)

Table 9: Relationship between NSAIDs, Drugs which relax the LOS and Oesophageal Cancer adjusted for Smoking and Alcohol consumption

	Cases n=411	Controls n=1644	Odds Ratio[+]	Confidence Interval[+]
Oesophageal Cancer				
Aspirin	93 (22%)	524 (32%)	0.14	0.11-0.19
Low Dose Aspirin	56 (14%)	421 (26%)	0.10	0.09-0.15
High Dose Aspirin	37 (8%)	103 (6%)	0.29	0.19-0.44
Other NSAIDs :	79 (19%)	575 (35%)	0.11	0.08-0.15
Low Dose other NSAIDs	23 (6%)	8 (0.5%)	0.02	0.01-0.05
High Dose other NSAIDs	56 (13%)	567 (34%)	0.08	0.06-0.11
Coxibs	55 (12%)	135 (8%)	0.33	0.23-0.47
CCBs:	78 (19%)	112 (7%)	5.70	4.50-7.23
Short Acting CCBs	11 (3%)	26 (2%)	5.64	2.88-11.04
Long Acting CCBs	67 (16%)	86 (5%)	6.72	5.13-8.80
Inhaled Bronchodilators	86 (21%)	108 (7%)	6.18	4.90-7.80
Theophylline	27 (7%)	18 (1%)	16.86	9.61-29.60
Oesophageal Adenocarcinoma				
Aspirin	62 (15%)	345 (21%)	0.14	0.11-0.20
Other NSAIDs	54 (13%)	391 (24%)	0.11	0.08-0.15
Coxibs	55 (13%)	135 (8%)	0.33	0.23-0.47
CCBs	60 (15%)	86 (5%)	6.32	4.78-8.36
Inhaled Bronchodilators	65 (16%)	82 (5%)	6.91	5.25-9.11
Theophylline	23 (6%)	15 (1%)	8.30	4.49-15.37
Oesophageal Squamous Cell Cancer				
Aspirin	31 (7%)	179 (11%)	0.14	0.09-0.21
Other NSAIDs	25 (6%)	184 (11%)	0.11	0.07-0.17
Coxibs	0	0	-	-
CCBs	18 (4.4%)	26 (2%)	8.56	4.87-15.05
Inhaled Bronchodilators	20 (5%)	26 (2%)	9.32	5.40-16.10
Theophylline	4 (1%)	3 (0.2%)	18.91	4.30-83.09

Table 10: Relationship between Smoking unadjusted, NSAIDs and Drugs which relax the LOS and Oesophageal Cancer

	Cases n=411	Controls n=1644	Odds Ratio[+]	Confidence Interval[+]
Oesophageal Cancer				
Aspirin	93 (22%)	524 (32%)	0.04	0.03-0.05
Low Dose Aspirin	56 (14%)	421 (26%)	0.03	0.02-0.04
High Dose Aspirin	37 (8%)	103 (6%)	0.08	0.05-0.12
Other NSAIDs :	79 (19%)	575 (35%)	0.03	0.02-0.04
Low Dose other NSAIDs	23 (6%)	8 (0.5%)	0.69	0.30-1.56(p=0.37)
High Dose other NSAIDs	56 (13%)	567 (34%)	0.02	0.01-0.03
Coxibs	55 (12%)	135 (8%)	0.10	0.07-0.14
CCBs:	78 (19%)	112 (7%)	2.47	4.50-7.23
Short Acting CCBs	11 (3%)	26 (2%)	1.73	0.87-3.46(p=0.12)
Long Acting CCBs	67 (16%)	86 (5%)	2.79	2.08-3.75
Inhaled Bronchodilators	86 (21%)	108 (7%)	2.74	2.12-3.55
Theophylline	27 (7%)	18 (1%)	5.76	3.22-10.30
Oesophageal Adenocarcinoma				
Aspirin	62 (15%)	345 (21%)	0.04	0.03-0.06
Other NSAIDs	54 (13%)	391 (24%)	0.03	0.02-0.04
Coxibs	55 (13%)	135 (8%)	0.10	0.07-0.14
CCBs	60 (15%)	86 (5%)	2.55	1.88-3.46
Inhaled Bronchodilators	65 (16%)	82 (5%)	2.84	2.10-3.85
Theophylline	23 (6%)	15 (1%)	5.95	3.15-11.26
Oesophageal Squamous Cell Cancer				
Aspirin	31 (7%)	179 (11%)	0.04	0.02-0.06
Other NSAIDs	25 (6%)	184 (11%)	0.03	0.02-0.05
Coxibs	0	0	-	-
CCBs	18 (4.4%)	26 (2%)	2.76	1.54-4.97
Inhaled Bronchodilators	20 (5%)	26 (2%)	3.04	1.72-5.38
Theophylline	4 (1%)	3 (0.2%)	5.48	1.23-24.35(p=0.2)

Table 11: Relationship between Alcohol unadjusted, NSAIDs and Drugs which relax the LOS and Oesophageal Cancer

10.6. Discussion:

In this case-control study, examining the association between oesophageal cancer and exposure to NSAIDs, Negative associations between both AC and SCC and use of NSAIDs, and observed a positive association with drugs that relax the LOS were observed. In the cases, only 3.5% patients with OC were less then 50 years of age, and, as has been observed by others, male cases considerably outnumbered females (Table 7). The fact that the mean age of our cases with OC was 71 years is also consistent with previous observations suggesting that the development of OC is strongly related to age. An important issue with any study of this type is the choice of hospital-based controls, who may not be truly representative of the general population from which the cases are derived. In the present study, I chose patients with non-melanotic skin tumors as the control group, but cannot entirely exclude the possibility that risk-factors related to non-melanotic skin cancer might lead to unidentified risk-associations.

In previous epidemiological studies it has been established that a combination of smoking and alcohol consumption are strong risk factors for OC. Individually, smoking is a weak risk factor and alcohol intake may not be a risk factor for AC[142,143]. In the present study an association between smoking and OC was observed. The consumption of alcohol was however similar in cases and controls. The associations between medication and OC were observed following adjustment for smoking and alcohol consumption. However, when the data is unadjusted for smoking and alcohol, the protective effect is reduced for both smoking and alcohol. Alcohol unadjusted is weakly associated with the risk of OC in presence of NSAIDs and smokling slightly more. Unadjusted data may also be influenced by other risk factors like obesity, GORD, genetic compositions and dietary factors. I am unable to reproduce any data with respect to other risk factors as they were not part of the study.

These results are also consistent with the prospective data of Vaughan et al. which examined the effects of NSAIDs on the incidence of OAC[144]. They observed a 70 % lower incidence of OAC amongst current users of NSAIDs, a protective effect that was reduced to 30% amongst former users of NSAIDs. My results are also in general agreement with a meta-analysis by Corley et.al[145], but they tend to contradict the suggestion of Bardou et.al[146] that aspirin may not be as beneficial as Coxibs. In the current study, odds-ratios for differences in dosages of Coxibs were not calculated, as exposure was only recorded for a small number of cases. Since no case with SCC was exposed to Coxibs, no association with SCC could be determined.

One potentially important limitation of the study design which needs to be borne in mind relates to the accuracy with which exposure to drugs has been determined. For example there is some risk of differentiation bias, in that the registration of medical details from cancer patients hospitalised due to a malignant disease may have differed from that of controls, who would have undergone mostly day-procedures for a relatively benign form of metaplastic disease. This risk was minimised by verifying that the general practitioner's referral letters contained a list of current and former medications used by the patients that were consistent with nursing records and the inpatient records.

Since this is a case control study, no assessment could be made of any relationship between the time of exposure to the drugs and the disease. However the data were drawn only from patients with a recorded minimum of four weeks exposure to the drugs in the last ten years. Finally it should be noted that the study design did not enable us to account for the effects of any over-the-counter purchase of aspirin or other NSAIDs .

The mechanisms by which NSAIDs and Coxibs protect against oesophageal neoplasias are not clearly established, but plausible possibilities include both suppression of pro-inflammatory mechanisms mediated via the cyclo-oxygenase

pathway, and induction of apoptosis, leading to deletion of precancerous cells[53,147]. Recent studies indicate that in addition to the cyclo-oxygenase pathway, induction of the lipo-oxygenase pathway of arachidonic acid metabolism may be implicated in the development of oesophageal cancer[148,149]. In the present study the greatest negative association of OAC was with other NSAIDs compared to specific Coxibs, implying that leucotrienes formed by lipo-oxygenase activity may play a role in the development of OAC. Negative association between exposure to NSAIDs and the risk of both histological sub-types of oesophageal cancer were observed. Assuming that inhibition of cyclo-oxygenase activity is the main mechanism of chemoprevention, this observation is consistent with the fact that there is over-expression of COX-2 enzyme in both histological sub-types of this disease[150]. However the negative association with NSAIDs was evidently stronger for adenocarcinoma as compared to squamous carcinoma.

11.0: Development of a food frequency questionnaire for the assessment of quercetin and naringenin intake

11.1: Introduction: Over the past few decades conflicting principles of healthy eating have been proposed, generating diverse opinions on what constitutes healthy food choices. Some recommend the exclusion of particular foods, while others profess that the same foods should occupy the central focus of the diet. There is confusion in the general population, including the medical profession, as to which dietary recommendations to follow, and which dietary components may be most important. This confusion has consequences. Clinicians continue to be the most respected source of lifestyle modification information and are exposed to 60 to 70 percent of adult population each year[151,152]. As an example, a study of clinician attitudes highlights these concerns in the delivery of nutrition advice to their patients [150]. Nearly all clinicians were aware of the obesity epidemic and 60 percent of them felt capable of assuming a major role in obesity control, but only 36 percent agreed that they had effective weight-management practices. Clinicians use several strategies, but there are barriers to nutritional counseling which include skepticism about the effectiveness of nutritional interventions, concerns about patient response and compliance, lack of specific knowledge and training a67bout nutrition as it relates to disease, and the perceived unpalatability of nutritional changes[154-157]. Clinicians are able to identify patients at risk, but encounter time constraints, lack of specialty clinics, absence of guidelines, and an inadequate number of dietitians[153]. In addition to appropriate training, physicians need effective nutritional tools and information that can be used in the clinical setting[158].

Dietary choice may reverse or lessen the disease burden of some common risk factors for the main contributors to morbidity and mortality, including coronary heart disease, diabetes, some cancers, and stroke[159]. The dissemination of accurate

dietary information within a medical setting has become an increasing priority to the clinician and patient. Many people believe that a comprehensive plan of complete dietary change is necessary to accomplish goals; however, this is not always the case. Indeed, sometimes simple changes in one area of the diet may make beneficial impact to other areas of the diet[160]. The balance of protective foods is just as important as the avoidance of foods containing excess calories, sugars, saturated or trans fat. Making simple recommendations directed at modifying appropriate risk factors for chronic disease, and providing flexibility within the plan can be more effective over the long term[161]. The goal of nutrition assessment is to identify appropriate and actionable areas of change in the dietary lifestyle.

11.2: Food Frequency Questionnaires

A food frequency questionnaire measures habitual diet over a period of time e.g. over the preceding year. They describe one's usual frequency of food consumption rather than specific meals[162,163]. A comprehensive assessment of diet is necessary which allows a calculation of macro & micro nutrients in individuals. Levels of intake can then be divided into categories such as high, medium and low intake. A number of factors affect the accuracy & compliance of a food frequency questionnaire namely length, number of food items, frequency of intake and portion size. Filling in a lengthy FFQ can lead to fatigue & boredom thus impairing concentration & accuracy. This is true even for a highly motivated cohort as demonstrated by Willett in the United States of America cohort study which studied US nurses[164].

In a FFQ food list should be comprehensive, include all foods which contribute to the nutrient of interest and should also be able to detect between person variations in intake. The foods which contribute most to between person variation, and are therefore the most discriminatory can be calculated statistically by stepwise regression. This process may lead to fewer questions in the FFQ, but which still discriminate between individual's intakes[165]. As intake of food is seasonal, food frequencies are usually described by subjects referring to their diet over the entire previous year. A standard approach is to give subjects a choice of frequency options ranging from never to intake of many times/day e.g. 6 times/day. Defining the options increases clarity and reduces errors compared with open-ended responses where subjects self- report their frequency intake[166].

Controversy exists over the inclusion of portion sizes in FFQ's, which can be achieved by giving descriptive examples or including photographs of different portion sizes[86]. Food items with natural units may be interpreted correctly e.g. a glass of milk, however, portion sizes with-out natural units e.g. a portion of vegetables can be

difficult to describe by subjects[167,168]. Providing ranges of serving sizes e.g. ¼ cup or ½ cup improves clarity as compared to small, medium & large portions[169]. Existing FFQ's can be used to measure individual diets and extra questions added if particular nutrients are required to be studied. Borrud *et al* found that although the frequency distributions of food used by ethnic subgroups differ, a comprehensive FFQ may function well in a diverse population[170].

Also it is widely recognised that self-reporting of individual food items leads to a measurement errors, and FFQs are probably less reliable than other dietary recall methods. For example Michels *et al* used both a FFQ and a 7-day diet diary to correlate fruit and vegetable intake with plasma vitamin C levels[171]. They found similar associations of relative intake with plasma vitamin C, but absolute estimation differed. Day *et al* found FFQ to have a higher regression dilution as compared to 7-day diary[172]. However they examined only absolute rather then energy-adjusted intakes.

11.3. Rationale for using urinary excretion of flavonoids to validate the FFQ:

High intake of fruits and vegetables has been shown to protect against development of many non-communicable diseases like several types of cancers[173]. Accurate estimation of fruit and vegetable intake is critical to further study the association between intake and development of chronic disease. Food diaries, food frequency questionnaires, and dietary recalls represent traditional methods for dietary assessment. All of these methods are associated with large random and systematic errors[174-178]. Blood and urine biomarkers for intake of foods may offer a more objective, universal, and physiologically relevant method for measuring intake. However, a thorough validation of the suggested biomarkers represents a critical and often underrated step in the development and maturation of new biomarkers. Due to large cultural and geographic variation in eating patterns, the validation of biomarkers for food items will have to include several controlled studies testing a large variety of eating patterns.

Several substances found in fruits and vegetables may be potential biomarkers, and serum concentrations of carotenoids and vitamin C have received most attention[179-187]. However, these biomarkers have several limitations; the absorption of carotenoids is subject to high inter-individual variation[188,189] and is affected by factors such as gender, body mass index, physical activity and amount of fat in the diet[190-194]. Moreover, plasma concentration of vitamin C has been shown to be affected by smoking and oxidative status[195]. New candidate biomarkers are the flavonoids that are found ubiquitously in most fruits and vegetables. A recent parallel feeding study has demonstrated a correlation between total urinary excretion of flavonoids and the intake of fruits, berries, and vegetables[196]. Beyond this study, the literature about flavonoids as biomarker of fruit and vegetable intake is scarce. Also a Norwegian

study indicates that urinary excretion of dietary flavonoids may be used to assess changes of mixed fruit and vegetable intake[197].

Another study from Denmark concluded that the habitual intake of fruits and vegetables, determined by 3-day dietary records, correlated significantly with the total excretion of urinary flavonoids, with a coefficient of correlation of 0.35, $P < 0.005$ ($n = 94$)[198]. In addition, highly significant differences in the urinary excretion of different flavonoids were observed in the human intervention study between subjects on diets high or low in fruits, berries, and vegetables. Also, at the individual level a significant positive correlation between changes in fruit and vegetable intake and changes in urinary flavonoid excretion was observed. Furthermore, urinary excretion of quercetin although small, is a constant function of quercetin intake[199].

I. Quercetin and naringenin as a biomarker of intake:

Few studies have attempted to assess the use of plasma or urine quercetin levels as biomarkers of intake. Noroozi et al studied the effect of 2 high-flavonol diets on plasma quercetin concentrations in 10 diabetic subjects. They were supplemented at one of two high flavonols levels (total 77.3 or 110.4 mg/day) provided by supplements of 1500 ml tea daily and 400 g fried white onion in olive oil with and without tomato ketchup and herbs[200]. Fasting plasma flavonols concentrations on habitual diets ranged from 0 to 43.7 ng/ml mean. Regression equations were constricted: total flavonols intake r=0.74, P<0.001 and quercetin intake r=0.744, P<0. 001. From these equations, flavonol intakes from habitual diets were estimated at 17-50, mean 35 mg/day. Of this, 91% was from quercetin.These findings indicate that plasma quercetin concentrations increase with increasing intake.

Radtke et al estimated the intake of several flavonoids from 1- or 7-day dietary records obtained from 48 female students[201]. Intake data were correlated with fasting plasma flavonoid concentrations. For 1-day dietary records (collected on the last day before blood sampling), Spearman correlations were 0.42, 0.64, and 0.47 for

quercetin, hesperetin, and naringenin, respectively. For the 7-day dietary records, the corresponding values were 0.30, 0.32, and 0.35, respectively. These correlations are similar to what has been reported for many other nutrients, the plasma concentrations of which are used as biomarkers of intake.

In another study, middle-aged men consumed either their habitual diets or 100 g/d of berries in addition to their habitual diets. In this study, plasma quercetin was 30% to 50% higher in the subjects consuming berries, compared with the control group[202]. In a strictly controlled dietary intervention study, 77 healthy men and women consumed either 170 or 850 g of fruits, vegetables, and berries daily. Quercetin intake was calculated to be 3 and 24 mg/d on the respective diets. The mean F SD plasma quercetin concentration was 69.2nmol/L during the habitual diet, and it decreased to 50% during the low-vegetable diet and increased to 125% during the high-vegetable diet (changes statistically significant) [203]. In addition to blood samples, 24-h urine samples were also collected from the study and analyzed in Denmark[204]. Urinary quercetin was clearly higher after the high-vegetable diet compared with the low-vegetable diet and decreased during the low-vegetable diet. In another study from Denmark, consumption of rather low amounts of quercetin in 3 increasing doses of fruit juice resulted in a significant increase in urinary quercetin with both dose and time[205]. The results of the 2 studies indicate that the urinary recovery of quercetin in 24-h urine samples also respond to changes in dietary intake.

The use of plasma flavanone levels as biomarkers of intake were investigated in further 2 studies. Bioavailability was studied after both single ingestion and long-term consumption[76,85]. According to the results of the first study, flavanones were clearly bioavailable, but the plasma half-lives were short (1-2 h). Urinary excretion appeared to be dependent on dose[76]. In the second study, 37 Finnish volunteers consumed their habitual diets followed by a diet containing on average 211 g of orange juice, one half orange, and one half mandarin per day for 5 weeks. During the habitual

diets, flavanones were detectable in few samples. After the consumption of citrus, naringenin was detectable in 20% of the fasting plasma samples[85]. The results indicate that fasting plasma flavanone concentrations are problematic as biomarkers of intake. In the controlled dietary intervention study mentioned in the previous paragraph[204], urinary flavanones clearly increased after the high-vegetable diet compared with the low-vegetable diet, which suggests that pooled 24-h urinary samples may be useful as biomarkers of flavanone intake in further studies.

Plasma, serum or urinary quercetin appears to be a good biomarker of intake and can be used for this purpose in epidemiological studies. It should be noted that an accurate assessment of the intake of onion, qualitatively and quantitatively, the most important source of quercetin, is problematic, because onion is a commonly used hidden ingredient of many homemade and processed food. For flavanones, on the other hand, the situation is quite different. The errors in estimating their intake are probably relatively small because they are mainly obtained from citrus fruits and juices. Therefore, in epidemiological studies, a more sensible approach would be to assess their intake from food frequency questionnaires.

II. Interindividual variation in bioavailability of quercetin and naringenin:

Several studies indicate marked individual variation in the bioavailability of flavonoids[75, 89,90] . Such variation is because of both physiological (differences in body weight, body composition, and gastric motility) and molecular factors (differences in the activity or synthesis of transporters or enzymes involved in biotransformation). Individual variation has been reported to occur for secretory transporters, such as P-glycoprotein[206,207] and Multi drug resistence proteins(MRPs)[208], and biotransformation enzymes, such as CYP3A4[209,210], Uridine diphosphate glucuronosyltransferases[211], and sulfotransferases[212]. All of these proteins have been associated with flavonoids; quercetin interacts in vitro with P-glycoprotein[213], MRP[214] and MRP2[215] and is a substrate for uridine diphosphate glucuronosyltransferases[216-218] and

sulfotransferases. In addition, the composition and metabolic activity of the gastrointestinal microflora are likely determinants of the bioavailability of flavonoids absorbed from the distal parts of the gastrointestinal tract.

To date, knowledge about the factors affecting the processes involved in the absorption and gastrointestinal metabolism of flavonoids has been rather fragmentary [219,220]. However, recent advances in molecular methods are expected to result in new information about the influence of environmental and genetic factors on the ctivity and expression of biotranformation enzymes[221,222] and the composition of the microflora. This will almost certainly, in the near future, improve our understanding about the bioavailability of specific compounds such as flavonoids.

In this study, a FFQ was developed to specifically measure flavonoids quercetin and naringenin intake by modifying an existing questionnaire developed at the Institute of Food Research, Norwich[223]. Modifying a FFQ to suit a study does not reducee its potential as documented in literature[224,225]. This existing FFQ measures the intake of basic macro & micro nutrients. The questionnaire was assessed to see if it can be shortened to increase compliance, whilst still be able to differentiate between in take. Secondly it was reviewed to see if portion size adds to the dietary assessment. By modifying a general FFQ, it is possible to assess individual diets with respect to particular issues, such as the intake of particular micronutrients or phytochemicals. Such 'tailoring' of an FFQ can be validated by using it to estimate intake of the target compound, and then determining the correlation between the data obtained and an objective biomarker such as urinary excretion of the compound under investigation, or its metabolites. The FFQ would then be used in future case-control studies to assess if flavonoids protect against gastro-intestinal malignancies.

11.4. Materials and Methods: Flavonoid Study

I. Overview

The study involved the recruitment of apparently healthy subjects via the Human Nutrition Unit (HNU) volunteer databank at the Institute of Food Research, Norwich, advertisement's and email for recruitment of volunteers with in the Norwich Research Park (John Innes Institute, Institute of Food Research and University of East Anglia) and from surgical clinics at the Norfolk & Norwich University Hospital. 64 apparently healthy adult volunteers were recruited. The study involved assessing the dietary intake of the volunteers by a food frequency questionnaire, collection of up to five 24 hour urine samples. Volunteers were not be given any dietary advice as there was no dietary intervention in the study. A flow chart of the overall study design is given in Figure 9 & 10. Flavonoid Study protocol attached as Appendix 2. A slightly modified version of the IFR food frequency questionnaire was used to improve compliance[223]. I as a Principal investigator was involved in the modification of the original FFQ. The FFQ was validated in this research and not the previous version of the FFQ. This is attached as Appendix 3.

Figure 9. Study Design

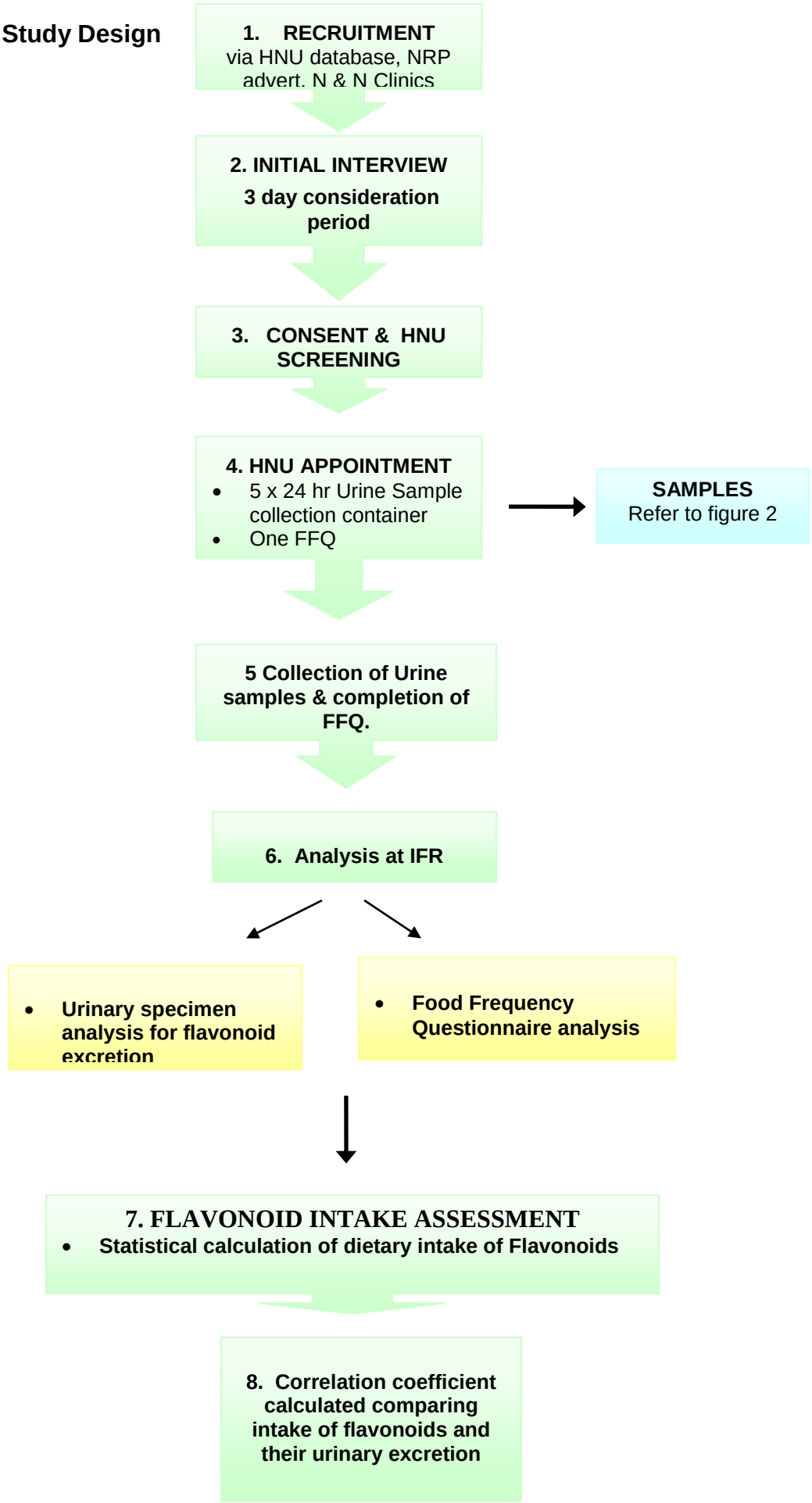
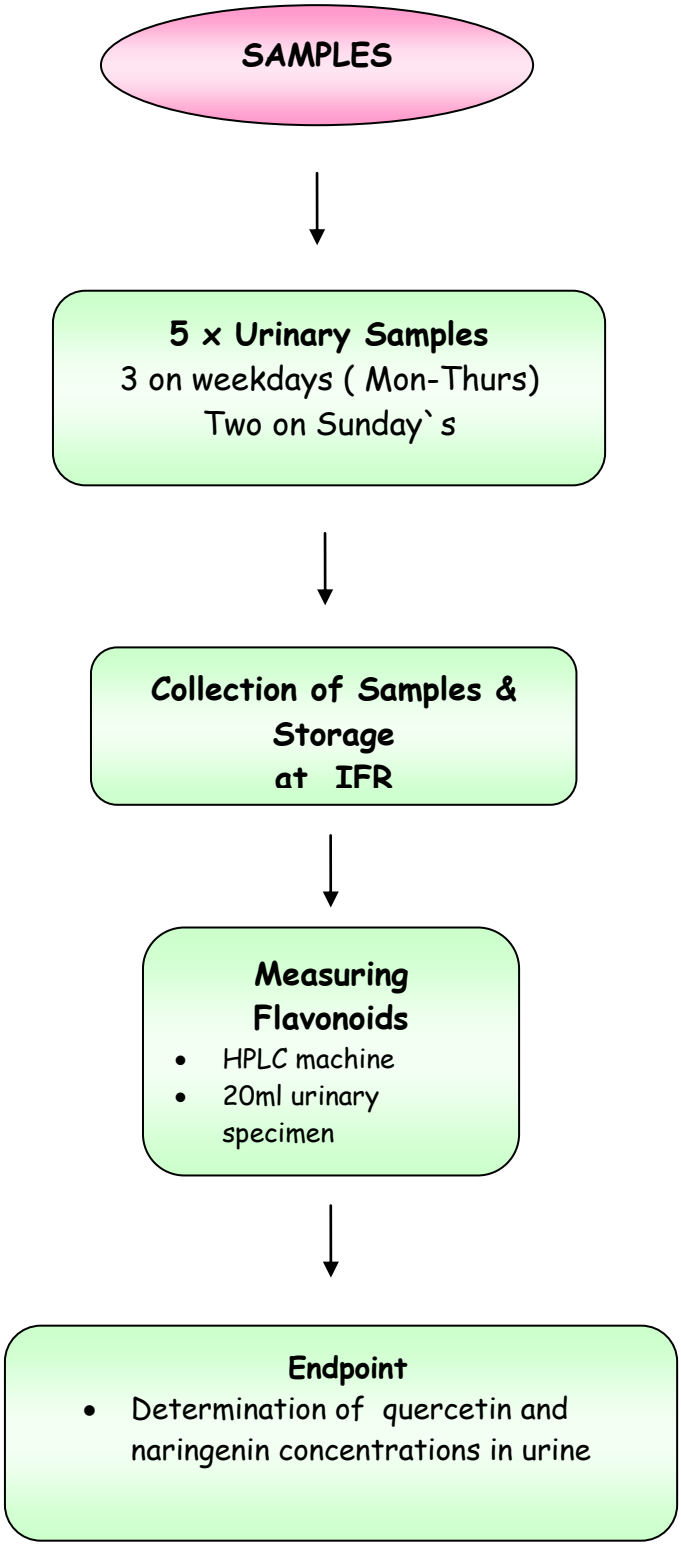


Figure 10: Plan of Urine Sample collection and analysis



II. Volunteer Recruitment

Apparently healthy male/female volunteers were recruited until 64 volunteers, completed the study. Experience has shown that the drop out rate for these types of studies is about 10%. It was envisaged that patients will be recruited onto the study at a rate of 4-5 per week and therefore recruitment continued for approximately 3-4 months. If greater drop out rates occur recruitment would be reviewed.

Advertisements were placed on: the Norwich Research Park***** (University of East Anglia (UEA), John Innes Centre (JIC), Institute of Food Research (IFR)), and in the Human Nutrition Unit (HNU) news letter which is sent to all volunteers on the HNU volunteer database (Annex 1). Patients with minor surgical ailments (e.g. groin hernia`s, cysts etc. that have no impact on dietary habit), their accompanying relatives or friends who attend the Norfolk & Norwich University Hospital surgical clinics, were also be informed of the study, and if interested were sent further information.

Apparently healthy volunteers, who met the basic inclusion criteria, were sent a letter of invitation (Annex 2) to participate in the study. This was supported by the volunteer information sheet (Annex 3). Included was a response slip and pre-paid envelope for returning the slip (Annex 4) if they were interested and wish for further information. The HNU databank contains names and contact details of people who have registered an interest in volunteering for human studies.

Advertisements were placed around the Norwich Research Park (UEA, JIC and IFR) inviting anyone who is interested in receiving information about the study to contact named researchers. NRP staff was also invited to participate in the study by email by seeking adequate permission. Interested responders were sent the volunteer information sheet. This included a response slip and pre-paid envelope in which to return the response slip if they were further interested.

Patients with minor surgical/medical conditions, not affecting their diet or ability to give up to five 24 hour urine samples, if fitting the inclusion criteria, were invited to

participate in the study. Healthy relatives or friends accompanying them were also invited to participate in the study.

Following an expression of interest volunteers were invited to the Human Nutrition Unit for a preliminary interview and given further details of the study. The volunteers were encouraged to ask questions at this point prior to making any commitment. At the end of the interview all volunteers were given a minimum of 72 hours to consider whether they wish to participate in the study. They were also given a small sterile container to take away with them. If they wished to participate, this container was to be used for the urine sample on the morning of the clinical screening visit. If they decided they do not wish to take part in the study they were told to discard the container. During this consideration period the volunteers were not contacted. If, following this period of consideration, the volunteer wished to participate they were asked to contact HNU on telephone number 01603 255305.

All those responding positively following this period of consideration were invited to attend the HNU for a clinical screening. No fasting was required. They were reminded to bring a midstream sample of urine from the first urine of the morning in the container provided at the first interview (This was not be tested until after the consent form has been signed). Volunteers needed to arrive within 2 hours after collection of the urine sample as this was a required specification for the validity of the urine dipstick test.

III. Clinical screening:

On arrival at the HNU the study scientist went through the consent form (Annex 5) with the volunteer and encouraged any questions they may have at this stage, volunteers were then asked to sign a consent form agreeing to participate in the study. A copy of the signed consent form was given to the volunteer to keep. A qualified nurse completed a basic health questionnaire (Annex 6), take and record blood pressure, pulse, height and weight measurements, Body Mass Index (BMI),

perform the urine dipstick test (Combur ^{9} Test®, Roche Diagnostics Ltd).The urine results were known immediately. If any of the results for the urine test were flagged the HNU protocol for abnormal urinalysis results was referred to. If the BMI is <18.5 or >30 the volunteer was excluded from the study.

Volunteers who did not wish to be re-screened or who displayed screening parameters outside the standard reference ranges on both occasions were excluded from the study.

Copies of all clinical results were be sent to the volunteer's GP (Annex 7) and in the event of flagged urine results, the volunteer were informed verbally and advised to speak to their GP to discuss the results. Results were not discussed with the volunteer.

Volunteers who meet the study criteria and whose screening results were satisfactory were included in the study. The GPs of those successfully recruited onto the study were informed of their patient's participation in the study by letter (Annex 8) and were sent copies of all clinical results. The volunteer agreed to this information being sent to the GP by signing the consent form.

Once recruited onto the study, volunteers were assigned a code number with only the named study scientists approved by the Ethics Committee being able to link codes to volunteers. All personal information was kept confidential and known only to the Researcher's, project leader, HNU research nurses, HNU Medical advisor and the volunteer's GP.

The inclusion and exclusion criteria were as follows:

Basic Inclusion criteria

- Aged 40-85 years (Malignancies more common in this age group)
- Male or female
- Non-smoker (Healthy volunteers with out smoking related diseases)

Basic Exclusion criteria

- Pregnant and breastfeeding
- Organ transplant recipients (on immunosuppressant's can effect diet)
- Long term illness requiring active treatment (e.g. Diabetes, cardiovascular disease, anaemia, cancer : may affect diet & participation in study)
- Volunteers currently on antibiotics (ongoing infections can affect the diet)

Screening Exclusion criteria

- BMI < 18.5 or > 30 (exteremes of BMI may be associated with undiagnosed metabolic conditions)
- Abnormal urine analysis results indicative of active illness (Refer to HNU protocol for abnormal Urinalysis results)
- Results of clinical screening which are judged by the HNU Medical Advisor to be indicative of a health problem and could compromise the well-being of the volunteer if they participated, or which would affect the data.
- Volunteers currently on antibiotics (ongoing infections can affect the diet)

Once the volunteer was selected through the screening process, I gave instructions and equipment needed to prepare for the study. The study involved completing a questionnaire on diet and producing up to five 24 hr urine collections over a period of 2 weeks including two on Sundays.

IV. Assessment of Dietary Intake

Diet was assessed by a food frequency questionnaire. The volunteers were asked to complete the questionnaire at home taking into account their diet over the period of the last year. The food frequency questionnaire (annex 9) focuses on habitual diet intake during the previous twelve months and allows an estimate to be made of the habitual intake of flavonoids from food sources. This questionnaire has been modified

from one previously used for studies at the Institute of Food Research, Norwich. The FFQ takes approximately 30 minutes to complete. In addition to recording the habitual intake of a wide range of foods and food groups, the computer software used to interrogate the FFQ data calculated the dietary intake of selected flavonoids e.g. quercetin, naringenin from the information supplied by each volunteer.

The original questionnaire specified 185 foods, divided into 16 major categories (Table 12). This questionnaire was modified as follows: For the purposes of the present study the categories were retained but, in order to minimise the number of questions and at the same time acquire sufficient information on individual fruits and vegetables, some foods with very similar macronutrient content, particularly in the meat section, were grouped together. Also, some previously grouped fruits and vegetables were divided because their flavonoid content was different. Overall, this facilitated an overall reduction in the number of specified foods to 159, which was deemed to have the probable added advantage of improving compliance. Frequency of consumption was based on recall over the past year, and the response divided into six replies: more than once a day, once a day, 4-6 times a week, 1-3 times a week, 1-3 times a month and rarely or never (Table 13). Volunteers were asked to assess portion size (small, medium or large) with the aid of a series of colour photographs of medium portions, on a standard dinner plate with cutlery alongside.

Meats	Biscuits And Puddings
Vegetables	Breakfast Cereals
Pasta And Rice	Bread
Cheese And Cheese Dishes	Chocolate And Sweets
Fish	Alcohol And Other Beverages
Fats	Fruit;
Dairy Products	Nuts
Eggs	Other Foods
Cakes	

Table 12: 16 major categories of the food items retained from the original FFQ.

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
A. Meats Do you eat meat? **YES /NO** (please delete) If 'NO' please go to section B. How often do you usually eat the following?											
1	Salami, Pate or similar meats	see photograph 1c									
2	Meat pies (e.g.pork steak & kidney)	see photograph 2a									
3	Sausage rolls, Cornish pasties	1 small									
4	Ham, corned beef + other cold meats	see photograph 1a									
5	Chicken, turkey etc. including minced & casseroled	see photograph 3b									
6	Lamb chops, cutlets and mince	see photograph 4b									
7	Leg of lamb	see photograph 3b									
8	Leg of pork, pork medallions, steaks and fillets	see photograph 3b									
9	Pork chops	see photograph 3b									
10	Reduced fat pork or beef sausages	1 thick									
11	Sausages, pork or beef	1 thick									
12	Beef steak - rump or sirloin	see photograph 4b									
13	Beef - topside, brisket, forerib, mince	see photograph 3b									
14	Bacon (back, lean, meat and fat), grilled or fried	1 slice									
15	Bacon, streaky, grilled or fried	1 slice									
16	Liver	see photograph 1b									
17	Kidney, pig, stewed and other offal	see photograph 1b									
18	Stew, Shepherds pie, casserole, curry, kebab	see photograp 7b									

Table 13: FFQ used in the study detailing the frequency and portion responses

V. Sample Collection

Upto five 24 hours urinary samples were collected at the HNU, IFR, according to the plan in Figure 9. Ascorbic acid 99% crystalline Sigma Ultra 1gm in 2.5 litres pot was added to the urine collection pots as a preservative and the volunteers informed of its presence. These samples were stored in a cold room at temperatures $+1^0$ C to $+4^0$C for up to a month before being processed.

Urine Samples & Creatnine clearance :

Volunteers were be asked to collect up to five 24 hours urine samples. These samples were stored, processed and analysed at IFR. The procedure for collection of the urinary sample was explained to the volunteers and they had the opportunity to ask questions. Researchers at the Institute of Food Research have many years of experience in collecting urinary samples from volunteers and are able to instruct volunteers to collect samples in a safe and hygienic manner. Creatnine clearance was used as a marker of compliance by determining the amount of creatnine in the 24h sample using a ABX Diagnostic's Creatnine 80 kit on a COBAS Mira Plus analyser. This kit uses the Jaff'e colorimeteric method for determining Creatnine in urine. ABX Diagnostics Human Control N was run as quality control and inter-assay variation was determined.

VI. Sample Analysis

The urinary samples were analysed by the principal investigator for the following flavonoids: quercetin and naringenin. Flavonoids in the urine samples were enzymatically deconjugated, extracted and quantified by HPLC (High Performance Liquid Chomatography) according to the method of Du Pont et.al[226]. Sample handling and analysis was carried by myself as the Principal investigator.

VII. Method of development for extraction of Quercetin: Pilot Study

As a part of method development, in a pilot study, a subject consumed 50gms, 100gms and 150gms of red onions on three different days. The subject collected 24 hr urine samples for each of those days. These samples were then used to extract quercetin in its aglycone form.

VIII. Extraction of flavonoids from Urine, including HPLC analysis:

The process of deconjugation and extraction of flavonoids was based on a previous studies from IFR on flavonoid extraction[226,227].

Chemicals and reagents

HPLC-grade methanol was purchased from Fisher Scientific (Loughborough, UK). Acetonitrile (HPLC grade), trifluoroacetic acid, b-glucuronidase (Helix pomatia type H5), sulfatase (H. promatia type H1), N,O-bis-(trimethylsilyl) trifluoracetamide, rhamnetin, naringenin, hesperetin, perillic acid, ethylbenzoic and propylbenzoic acids, cobalt(II) bromide and 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Pelargonidin-3-glucoside and galangin were obtained from Extrasynthe`se (Genay, France).

Extraction process:

Samples were analysed by HPLC with online UV-diode array and LC-MS detectors. Absorbance at 270 nm was used for quantification, and tandem MS (positive-ion mode) to confirm the identity of the analytes. Acidified urine samples (10·0 ml) were incubated with 5·0 ml phosphate buffer (pH 5·0), 1·0 ml β-glucuronidase (10 000 units) and 1·0 ml sulfatase (1000 units) at 37°C for 2 h. Then 50 µl galangin (0·1 mg/ml) was added as an internal standard before incubation. Flavanones in hydrolysed urine samples were extracted using a solid-phase extraction (SPE) cartridge (Varian Bond Elute C_{18}) conditioned with methanol (5 ml) followed by water

(10 ml). Following application of urine or plasma, the cartridge was washed with water (10 ml) and flavanones eluted directly into vials with 1 % HCl in methanol (1·0 ml for urine) or 1 % HCl in acetonitrile (0·5 ml for plasma). Pelagonidin-3-glucoside (50 µl of 0·1 mg/ml) was added to the SPE eluate as a volume marker immediately before HPLC analysis.

Samples (1 µl) of hydrolysed urine extracts were analysed by HPLC (Agilent HP1100; Agilent Technologies, Waldbronn, Germany) using a Gemini C_{18} column (150 × 2·00 mm, 5 µm particle size; Phenomonex, Macclesfield, Cheshire, UK) eluted at 0·3 ml/min with a gradient of increasing solvent B (0·1 % trifluoroacetic acid in acetonitrile) from solvent A (0·1 % aqueous trifluoroacetic acid) at 30°C over 65 minutes. The eluent was scanned over 200–600 nm by a diode array detector and subsequently an ESI-MS (Agilent Technologies, Waldbronn, Germany). The mass A spectrometer was operated in negative ionisation mode (cone voltage 22 V, source block temperature 120°C, desolvation temperature 300°C) with multiple reaction monitoring. Quantification was based on peak areas at 270 nm. Quantification of the flavonoids was based on standard curves (range 0·1–100 µg/ml) for quercetin and naringenin. Standard curves were linear with regression coefficients >0·99 as per the pilot study.

IX. Identification of Quercetin and its quantitative calculations:

Standards were used to identify (or eliminate) peaks using HPLC retention times. The calculations were based on the area underpeaks from HPLC analysis according to a standard curve (Table 14 & Figure 13). The standard curve was produced by injection of 6 different concentrations (0.5ng, 1ng, 5ng, 10ng, 50ng, 100ng) of authentic quercetin standards over the concentration range 50 to 10,000ng/ml. The slope of this curve was used to calculate the concentration of the post-SPE sample and from the total volume of urine collected over a 24 hour period, the total mass of quercetin excreted.

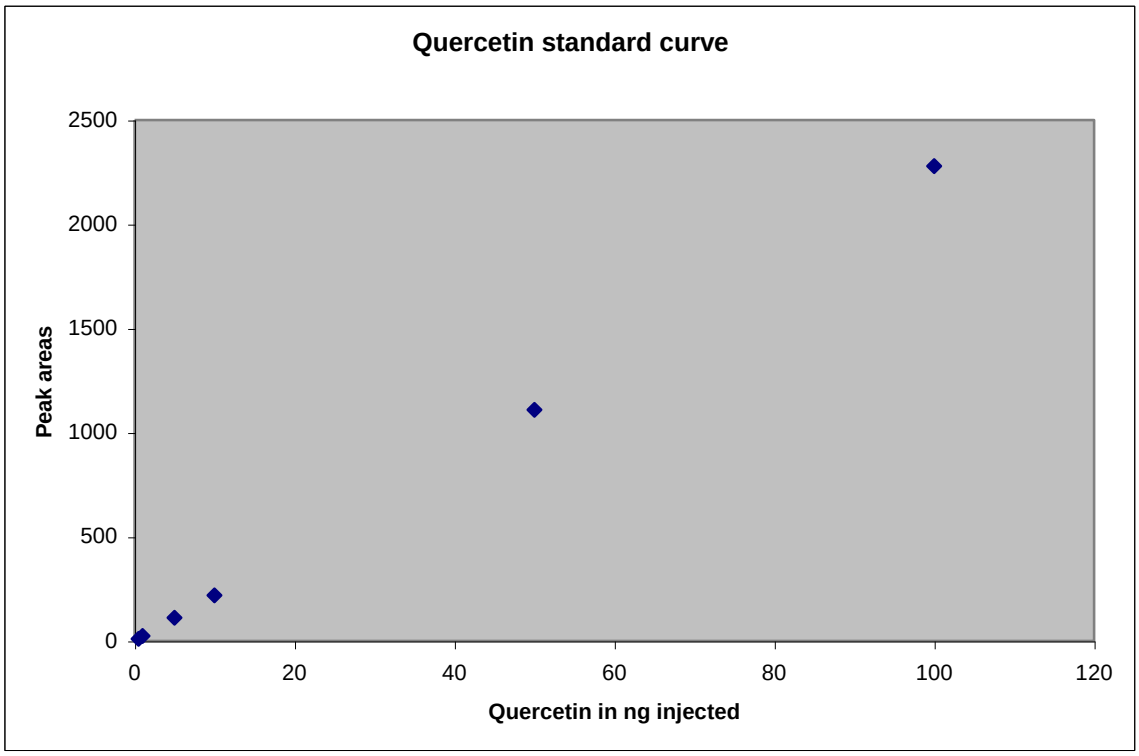
X. Sample Safety

IFR has standard operating procedures for the storage of body fluids. These procedures were adhered to and are integral to IFRs recognition as ISO 9001:2000 compliant and constitute part of process working to the standards of GCP.

Quercetin ng injected	Quercetin area on HPLC
100	2279
50	1109
10	219
5	111
1	24
0.5	10

Relation between quercetin injection and the peak areas on HPLC

Table 14



r=0.999, p<0.0001

Quercetin standard curve for method development

Figure 11

11.5. Statistical Analysis

Total food intake, total energy intake, macro & micronutrients, percentage of energy consumed was calculated using an in-house FFQ which was been re-designed around ACCESS software. The original FFQ from which the new version was derived has been used in previous studies at IFR[223]. The power of the study was been calculated at 80% assuming a group size of 64 to detect a correlation of 0.35 at 5% significance level. All data was analysed using Microsoft excel. The primary analysis was to be using a Pearson's correlation coefficient to measure the strength of the association between dietary assessment of flavonoids and their urinary excretion, for both sexes combined and stratified by sex. If the distribution of the variables was skewed, transformed variables (such as log) was to be used. A secondary analysis would be linear regression, regressing estimated mean dietary flavonoid intake on mean urinary flavonoids, adjusting for covariates such as age, sex and BMI. Both the FFQ and urine results were divided into quartiles of intake/concentrations and compared to check that there are no gross miscalculations between methods.

11.6. Ethical considerations: The project was approved by the Human Research & Governance Committee, Institute of Food Research, Norwich, East Norfolk & Waveney Research Governance Committee, Norwich Local Research Ethics Committee.

11.7. Results:

Sixty-three volunteers were recruited, of whom fourteen were excluded due to various exclusion criteria including body mass index (BMI) < 18.5 or > 30, long term illness requiring active treatment or abnormal urine analysis indicative of active illness. Amongst the 49 volunteers who participated the mean age was 60 yr (Range 40-85). The M:F ratio was 1:1.3 and the average BMI was 26 kg/m^2 (Range 19-36).

I. Results of the pilot study: (Table 15 & Figure 12)

A single volunteer was used as a pilot to determine the accuracy of the method for urinary excretion of quercetin. The process of deconjugation was based from previous IFR studies[226,227]. The pilot study was done to ascertain the process could be duplicated by me, the principal investigator. Raw red onions usually contain 20mg/100gm of quercetin[228]. It was expected that ingestion of 50, 100 and 150 gms of red onions, therefore would yield 10, 20 and 30 mg of quercetin respectively. Urinary excretion of quercetin was between 0.31-0.48% in this volunteer. The correlation coefficient for the intake versus urinary excretion of quercetin was 0.978 ($p<0.0001$).

II. Estimated Nutrient intakes

Details of average energy consumption and intake of major macro-nutrients, fibre, selected minerals and vitamins are given in Table 16.

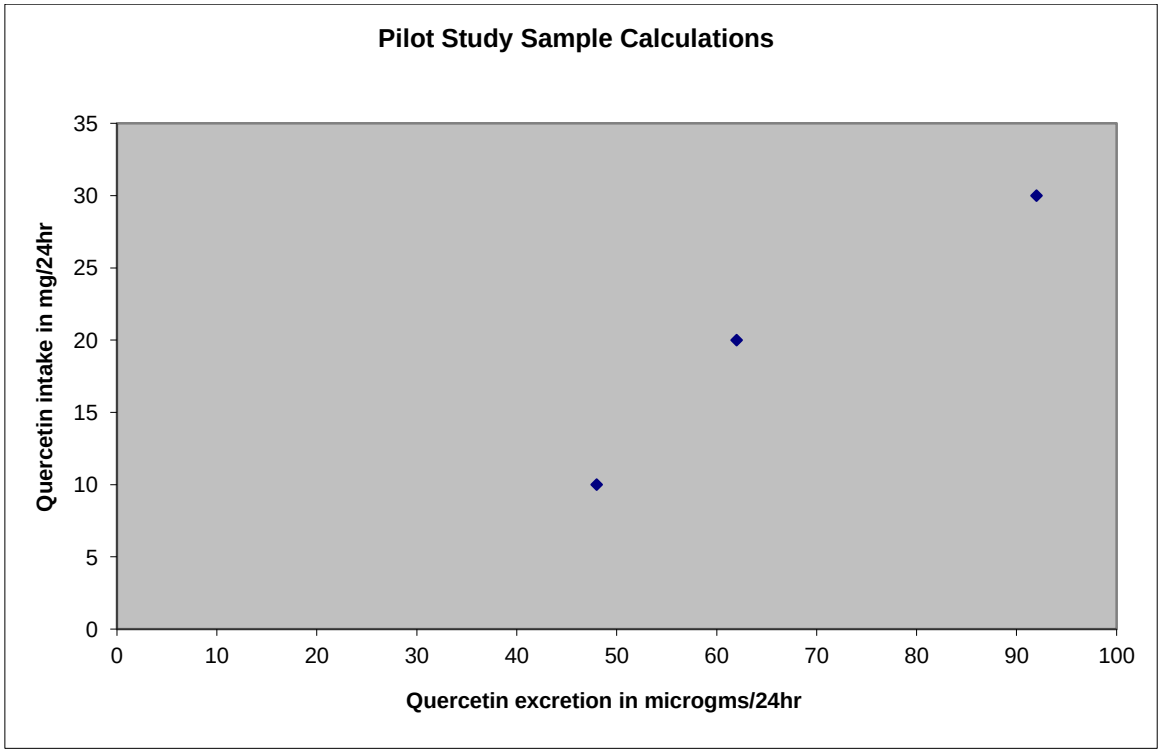
III. Relationship between estimated flavonoid intakes and urinary excretion

The average 24 hr ingestion of quercetin and naringenin estimated from FFQ with inclusion of portion size was 29 mg (SD 15.0) and 56 mg (SD 62.76). The estimated ingestion of quercetin (Fig 13) correlated significantly with urinary excretion ($r = 0.827$; $p < 0.0001$; 95% CI: 0.712 to 0.899) whereas to the relationship for naringenin (Fig 14) was less clear cut ($r = 0.251$; $p<0.05$; 95% CI: -0.032 to 0.497).

When estimates of portion size were not included, the estimated average 24 hr ingestion of quercetin and naringenin from FFQ were 25.9 mg (SD 11.7) and 46.64 (SD 43.38) respectively. Exclusion of portion size reduced the correlations between the estimated ingestion of quercetin ($r = 0.693$, 95% CI: 0.533 to 0.825) and naringenin ($r = 0.157$, 95% CI: 0.54 to 0.26) and their respective urinary excretions (Fig 15 and 16). The correlations between the intakes of quercetin and naringenin estimated from the FFQ, with and without portion size, were 0.856 (95% CI: 0.758 to 0.917) and 0.926 (95% CI: 0.872 to 0.958) respectively. Tests of equality of the two correlation coefficients drawn from the two different samples indicated no statistically significant differences due to inclusion or exclusion of portion size for either quercetin ($p=0.1192$) or Naringenin ($p=0.6378$).

	Day 1	Day 2	Day 3
Raw onions ingested	50 grams	100grams	150 grams
Quercetin from onions	10 mgs	20 mgs	30 mgs
HPLC peaks	115	155	248
Urine Volumes	1900 mls	1850 mls	1700 mls
Quercetin excreted in 24 hr sample	48 μg/L (0.48%)	62 μg/L (0.31%)	92 μg/L (0.34%)

Table 15: Details of pilot study for method development



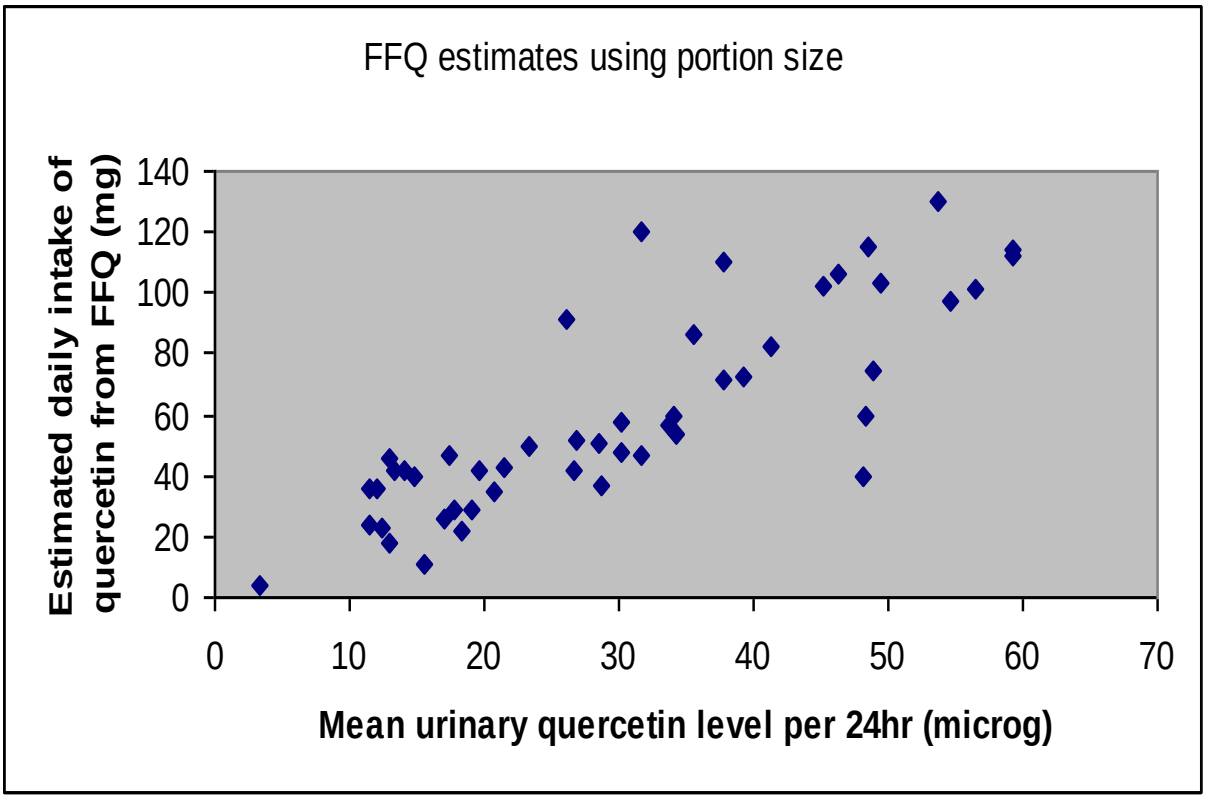
r=0.978, p<0.0001

Correlation between ingestion of quercetin and its urinary excretion in the pilot study

Figure 12

Nutrient	FFQ with portion size	FFQ without portion size
Energy	2749 Kcal	2547 Kcal
Proteins	103 mg	95 mg
Fats	113 mg	105 mg
Carbohydrates	313 mg	291 mg
Fibres	30 mg	28 mg
Iron	18 mg	17mg
Vitamin E	14 mg	14 mg
Vitamin C	284 mg	255 mg
Vitamin B1	2 mg	2 mg
Vitamin B2	2 mg	2 mg
Vitamin B6	3 mg	3 mg
Vitamin B12	12 mg	11 mg

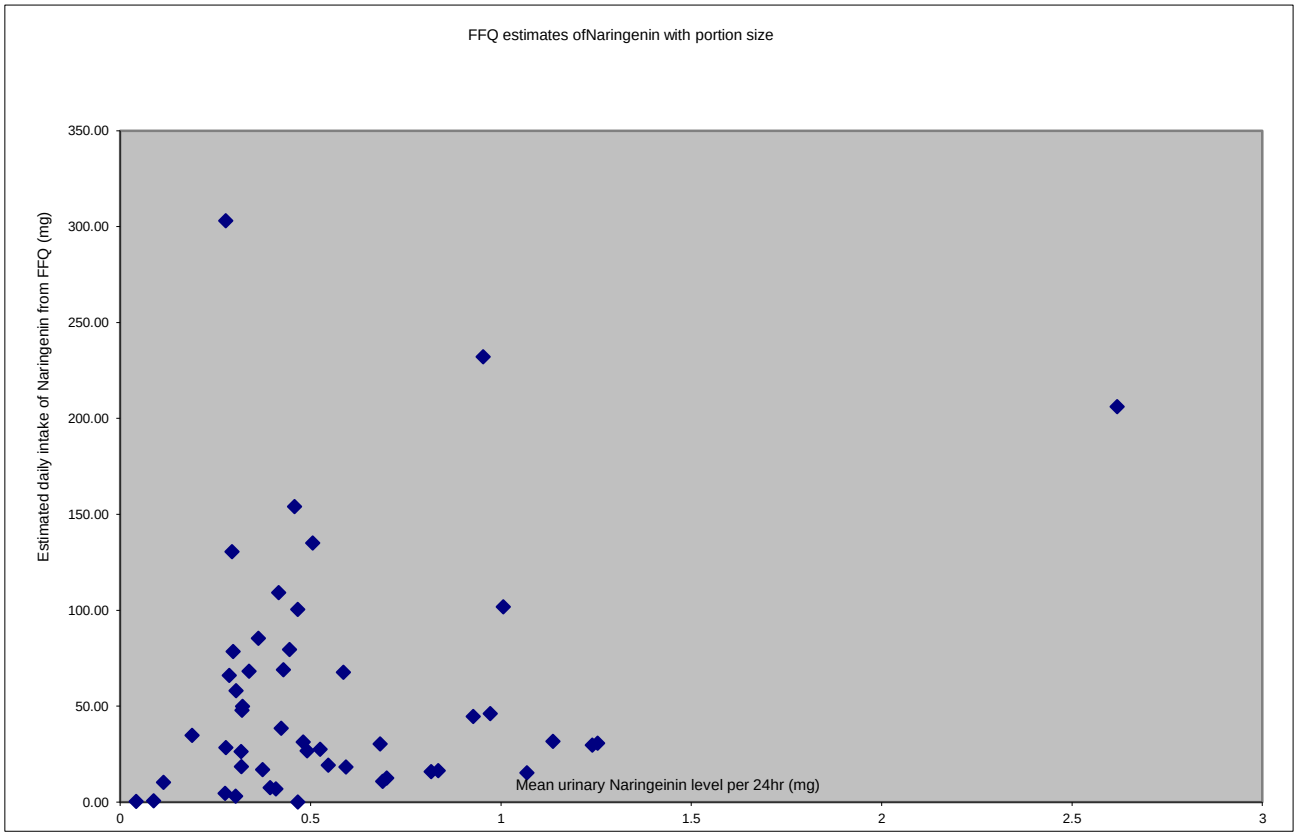
Table: 16: FFQ determination of selected macro and micro nutrients per 24 hrs



r = 0.827, p<0.0001

Correlation between FFQ estimated intake of Quercetin with portion size and its urinary excretion.

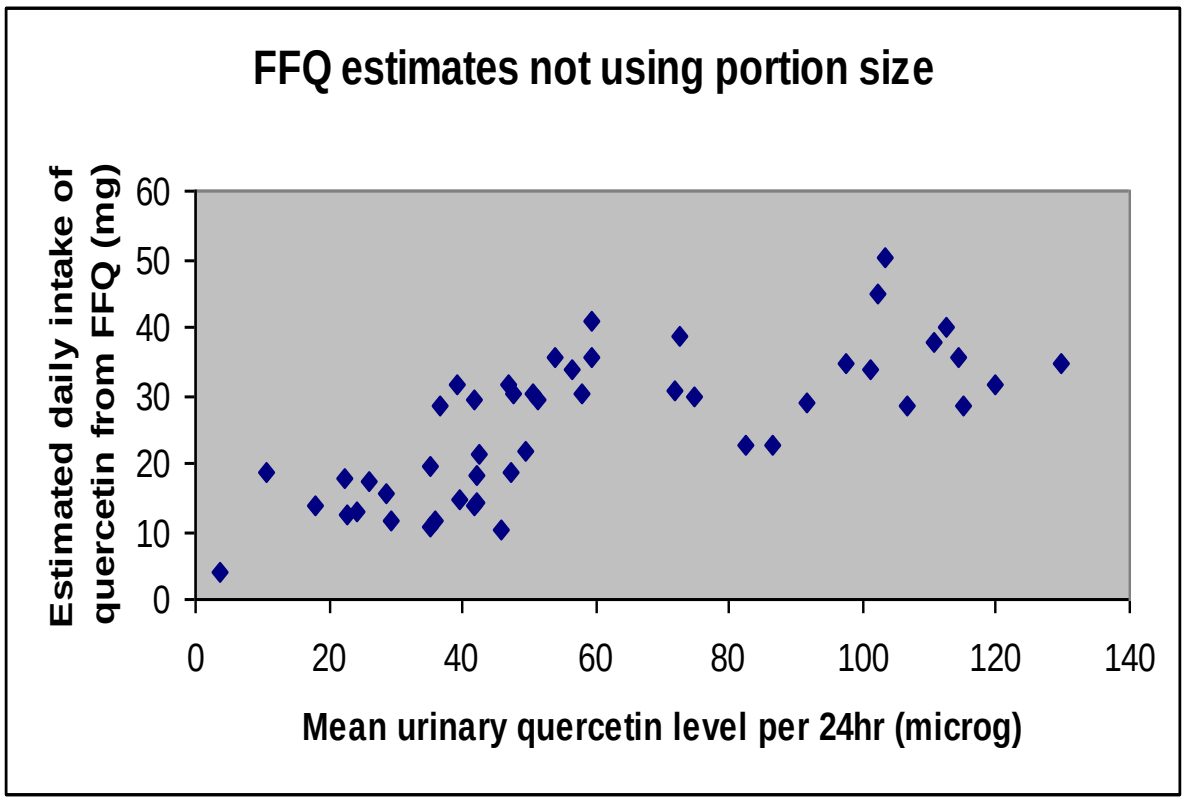
Figure 13



r = 0.2515, p<0.05

Correlation between FFQ estimated intake of Naringenin with portion size and its urinary excretion.

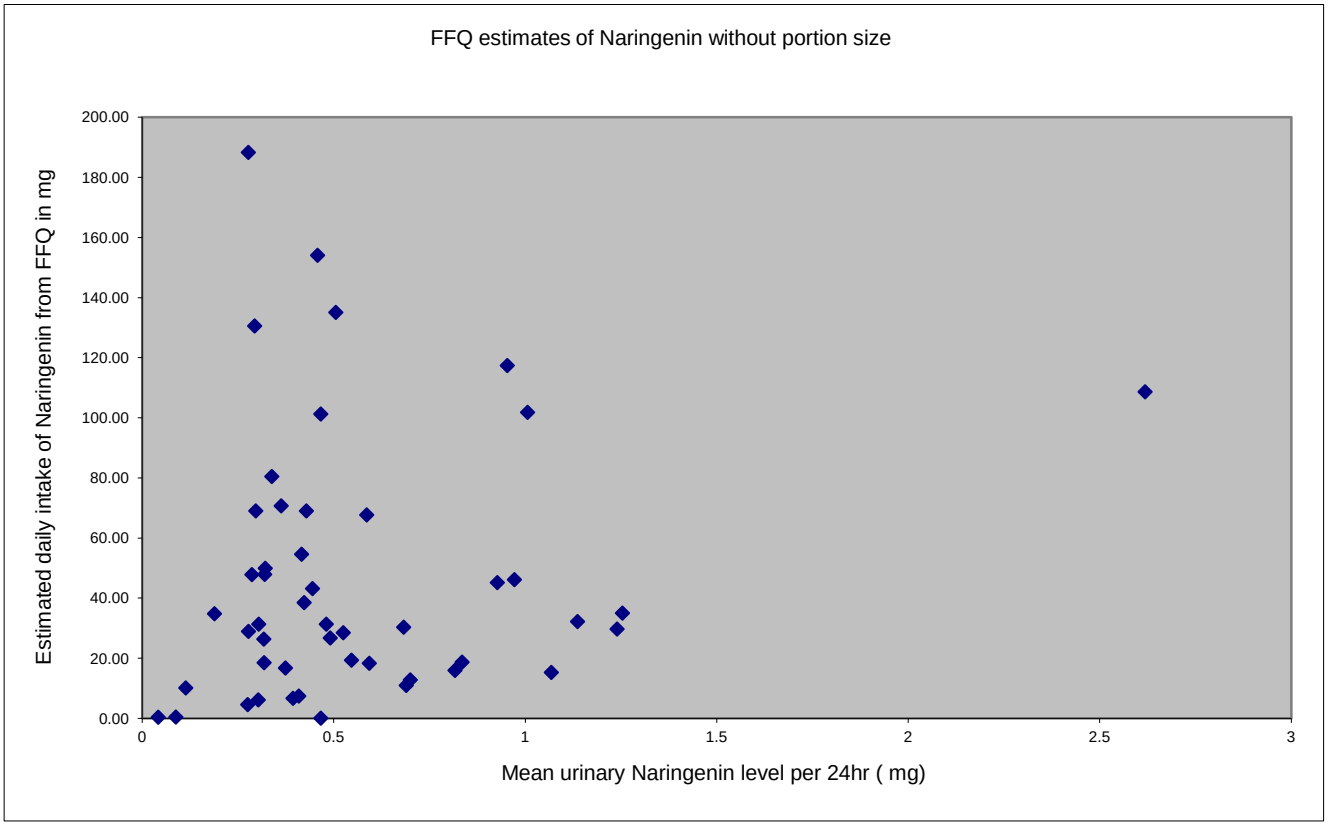
Figure 14



r = 0.693, p<0.0001

Correlation between FFQ estimated intake of Quercetin without portion size and its urinary excretion.

Figure 15



r = 0.1573, p<0.005

Correlation between FFQ estimated intake of Naringenin without portion size and its urinary excretion.

Figure 16

11.8. Discussion:

The primary objective of this study was to assess the ability of the FFQ to estimate dietary flavonoid intake. The relationship between dietary intake of quercetin and naringenin with their urinary excretion was examined to validate a FFQ in 49 volunteers. A pilot study was carried out to asses the accuracy of the method for urinary excretion of quercetin. It confirmed that the process of extraction was accurate with a correlation coefficient r= 0.978 ($p<0.0001$) for the intake versus urinary excretion of quercetin. Similair process was used to extract naringenin.

The results of the flavanoid study suggest that there is a statistically significant correlation between the intake of the flavonoids quercetin and naringenin as estimated by an FFQ and their urinary excretion over a 24h period. Young et al[229] reported that urinary excretion reflects the absorption of quercetin and, therefore is a good marker of its bioavailability. De Vries[230] found urinary flavonoids as biomarkers of dietary consumption, and a Finnish study has shown that urinary flavonoids can be used as a biomarker of dietary fruit and vegetable intake with a correlation co-efficient of 0.35, $p < 0.005$[231]. The present study suggests therefore that FFQ can be used as a practical tool to estimate flavonoid ingestion in epidemiological studies.

Metabolism quercetin and naringenin occurs via a common pathway[232].These polyphenols are absorbed from the small intestine. Quercetin is absorbed in its glycosated form in the stomach and in its aglycone form in the intestine[233]. However, naringenin is absorbed only in its glycosated form in the small intestine. Once absorbed, they are conjugated in the liver and small intestine. They are then excreted via the biliary or urinary route[234]. In our study, the average excretion of quercetin and naringenin was 0.20% and 1% respectively. Our results are in conjunction with previous studies which found, depending upon the dietary source, human characteristics and process of extration, excretion of quercetin[235,236] was in the

range of 0.20% to 0.50% and naringenin in the range of 1-30% of its dietary intake [237-240].

In past, investigators have used 24-hr recalls, prospective food diaries and FFQs as tools to measure fruit and vegetable intake[241-243]. 24-hr recalls are expensive, demand a lot of administrative time and there is a need to obtain multiple recalls. Also there is literacy demand in the estimation of portion size[241]. Food diaries are subject to respondent burden, literacy demands and may be over reported[244]. A cross-sectional study by Brunner *et al* comparing a 7 day diet diary and FFQ with serum cholesteryl ester fatty acid, plasma alpha-tocopherol and beta-carotene found FFQ performed well in comparison with the 7 day diet diary[245].

Over/under reporting of an individuals diet could be one of the other limitations of using a FFQ whilst estimating diet. However, a Brazilian study conducted amongst over weight individuals, concluded FFQ could be used in epidemiological studies to assess the regular food consumption of overweight individuals[246]. They found a higher variability in the reporting of regular food consumption among obese than overweight individuals. Also the exclusion of foods popular to ethnic minority groups that are significant contributors of nutrients could not be taken into consideration. Although historically, FFQ are considered less sensitive to measures of absolute intake for specific nutrient, we modified the FFQ to overcome these limitations. Using a FFQ is a retrospective method that relies upon the respondent's memory. We overcame this constraint by asking volunteers to complete the questionnaire at home taking into account their diet over the period of last year. This was done as we thought they would be more comfortable and undisturbed at home. Also pictures of portion sizes were added to the FFQ.

Thus, FFQs gives the advantage of being cost effective and ability to assess diet over longer period of time[247,248]. It is representative of usual intake, preferable method of measuring intake for nutrients with very high day-to-day variability. Questionnaire

processing is significantly less expensive than food records or diet recalls and can be easy for literate subjects to complete as a self-administered form. It is suitable for very large studies designed to rank individuals according to intake.

12.0. General Discussion:

12.1: Case-control study:

Current evidence from epidemiological studies suggests that long-term non-steroidal anti-inflammatory drugs intake may reduce the risk of developing several types of cancer, including GI malignancies. This evidence is particularly striking in the case of oesophageal cancer. A recent review of nine published epidemiologic investigations found that the reduction in relative risk of oesophageal cancer approaches 73% with daily intake of an NSAID[249]. Similar studies have demonstrated a 40–60% decrease in the relative risk of colon cancer with continuous use of NSAIDs and , and other cancers such as stomach, breast, lung and ovarian also show risk reduction with NSAID intake[250,251]. Two studies from the UK have found significantly lower consumption of NSAIDs in cases of OC as compared to controls[252,253]. A recent meta-analysis showed that the protective effects of aspirin and NSAIDs may be dose-related[254]. It is not clear, however, at what dose aspirin becomes chemo-preventive[255,256]. A study by Bardou *et al* failed to demonstrate a lasting effect of chronic ingestion of NSAIDs and COX-2 inhibitors on OC[257]. NSAIDs have been shown to inhibit tumourigenesis in rodent models of GI cancer. The NSAIDs has been reported to have anti-tumour effects in animal models of oesophageal cancer and in rat models of Barretts oesophagus, selective COX-2 inhibitors inhibited the development of adenocarcinoma[258,259].

Another very important finding of the case-control study is the statistically significant positive association between OAC and exposure to drugs that cause a reduction in lower oesophageal sphincter tone. Previous studies have found only a weak association between β-agonists and OAC and no association with CCBs[260,261]. The same authors also found anti-cholinergics to have the strongest association with OAC. Currently, β-agonists and CCBs are more widely used. Overall, a higher intake

of these drugs was founf in our cases. One possible explanation of our observations that needs to be considered is that of confounding by indication. Drugs that relax the lower oesophageal sphincter are frequently prescribed for asthma, which is also associated with GORD. Thus it is possible that gastro-oesophageal reflux is a cause of both OAC and asthma and that out findings reflect this association[262]. However, treatment of GORD may not always improve asthma control as other factors like food allergy may play a role[263,264].

In view of the increasing incidence of OAC across the western world, it is important to consider potential risk factors like BMI and history of GORD. A recent metanalysis from International BEACON Consortium found that BMI is directly associated with OAC and GEJ adenocarcinoma risk in both men and women and in those with and without GORD symptoms[265]. A prospective cohort study over a 10 year period involving 218,854 participants: 132,288 men and 86,566 women concluded overall obesity was associated with a higher risk of OAC and an increased risk of OAC with increasing abdominal obesity in people with normal BMI[266]. Risk fo GORD and OC have been discussed earlier in section 1.4. It seems that there may be a casual relationship between GORD and OC, however it is Barrets oesophagus which is moresignificantly associated with development of OC[267,268]. Data on BMI and GORD was not collected as this study was primarily done to assess the risk of Cox-2 inhibitors in OC. Hence the possible mechanisms of action by which certain drugs that might exert procarcinogenic effects on the oesophagus[261,269] are considered in this study. One obvious hypothesis is that both CCBs and bronchodilators cause a reduction in lower oesophageal sphincter tone, leading to more frequent reflux and prolonged exposure of the lower oesophageal mucosa to gastric, and possibly duodenal contents[141,261]. As GORD is present in 30-89% of patients with asthma, treatment of this chronic condition may lead to the relaxation of the lower oesophagus due to the presence of β_2 receptors in the LOS[270-272]. Another possibility is that by

blocking the entry of calcium into the cells, CCBs may inhibit apoptosis, thereby reversing one putative protective mechanism that NSAIDs enhance[273]. A possible link with calcium metabolism at cellular level has been suggested by Fitzpatrick et al who found that CCBs increase the incidence of breast carcinoma in postmenopausal women by 2 fold (OR: 2.57, 95%CI, 1.47-4.49)[274]. A positive association between the use of CCBs and cancers in the elderly has been reported by Pahor *et.al*.[275] Furthermore, a Rotterdam study has implicated Verapamil, an L-type Ca^{2+} blocker, with an increased risk of cancer in the elderly[276].

Thus although there is mixed evidence about the effects of coxibs and drugs which relax the LOS with OC, our study potentiates the theory of chemoprevention in oesophageal cancer, especially oesophageal adenocarcinomas. Also as I found an association with the drugs which relax the LOS and OC, however further studies are needed to rule out confounding factors like environmental, genetic and other chemical factors. This leads me to the second part of my thesis about validation of a FFQ against urinary excretion of flavonoids. This study is conceptually linked to the case-control study as following validation of the FFQ, it may be possible to use this FFQ as a dietary tool in further studies to study the impact of intake of naturally occuring compounds with Cox-2 inhibitory and anti oxidant properties on the outcome of various diseases, including cancer.

12.2: Flavonoid Study:

Dietary flavonoids and other polyphenolic food components have over many years been suggested to have preventive properties both at the initiation and the promotion stages of chemically induced carcinogenesis. At the cancer initiation stage, mainly in cell culture studies, polyphenols have clearly been shown to affect many of the carcinogen bioactivating steps necessary for the covalent binding of the carcinogen to cellular DNA, including the major bioactivating CYP1A1 enzyme. Whereas some polyphenols have been shown to act as inducers of CYP1A1 by being agonists of the arylhydrocarbon receptor (AhR), others have been shown to be inhibitors by being AhR antagonists. At the promotion stage, cell culture studies have revealed a wide variety of biochemical mechanisms for the effects of polyphenols on human cancer cells. This most recently includes effects on VEGF and HIF-1 expression via PI3K/AKT pathways or via ARNT, inactivation of EGFR and inhibition of thioredoxin reductase, thymidylate synthase and the MDM2 oncogene as well as effects on cancer cell resistance by targeting the molecular chaperone glucose-regulated protein.

However, *in vivo* cancer chemoprevention studies in animals and especially in humans, using modest, clinically tolerable doses of flavonoids or other polyphenols, have been mostly disappointing. This can be explained by the very poor oral bioavailability of the polyphenols, i.e. their inability to pass intact through the dual intestinal/hepatic barrier into the systemic circulation. In humans, this lack of bioavailability after oral doses has been shown directly for chrysin, quercetin, curcumin and resveratrol. Although the tea flavonoids and the isoflavonoids, such as genistein, have some oral bioavailability, it is still low.

I. Initial approach — Simply asking patients which dietary change(s) would improve their health can reduce time spent in assessment by highlighting areas the patient is willing to consider changing. Patients often can inform the clinician of the areas of weakness in their diet, and which behaviors contribute to body weight or other adverse health risk (such as elevated blood sugars). It is just as important to identify strengths in the diet (fruit, vegetables, other healthy options) in order to build on positive dietary foundations. The clinician may then follow up in subsequent visits to assess patient changes. This follow up is often important for patient motivation.

II. 24-Hour dietary recall — A brief nutritional assessment tool commonly used is the 24-hour dietary recall. The goal of the 24-hour dietary recall is to identify the day-to-day pattern of eating with a minimum of reporting bias. Most people have little variability in dietary habits and are remarkably consistent with caloric intake and food choices.

An abbreviated assessment may be performed by obtaining only the previous evening's intake, which can then be combined with the food frequency assessment. Typically, the evening is when the majority of calories are consumed. Asking what was consumed for the evening meal and any snacks afterward, including beverages may help. For diabetics, timing of intake is also important. Inquire as to portion sizes of meals.

One may also to report the previous day's intake, separating meal occurrences: “What is the first thing you had to eat or drink yesterday?”; "Did you have anything else at the time?"; "What was the next thing you had to eat or drink?" Continuing through to snacks before bedtime and determining the time they went to bed, so as to determine how long after the last meal this occurred may help. This exercise can also elicit behaviors and lifestyle factors that underlie dietary choices.

People frequently will fail to report beverage consumption and most will require regular prompting such as "What did you have to drink with that?" Beverages and snacks often contribute greatly to over or under nutrition[277]. Combined foods such as cream in coffee and butter on bread need to be enquired as well. Mixed dishes such as casseroles may require additional prompting, and a number of other food responses may require follow-up questions to have a clear picture of what was eaten; e.g "Was there a sauce, cheese, or gravy with that?" "What type of beef?", "What percentage of fat in the hamburger?", "What percentage milk?". Be sure to ask about alcohol consumption. As far as assessing trans fatty acids (partially hydrogenated oils), it is important to remember that 40 percent of intake of these detrimental fats comes from cakes, cookies, crackers, pies and breads (processed foods)[278].

Hence there will be difficulties with accurately reporting portion sizes with underreporting of larger portion sizes occurring with almost mathematical predictability[279,280]. Use of comparisons such as a deck of cards (3 ounces), 1 cup of broccoli is about the size of a fist,1 cup of ice cream is about the size of a tennis or racketball may help. Thus using 24 hr dietary assessment may be time consuming, detailed with inter-personal differences and operator differences.

III. Food diary — This is another option for dietary assessment. It entails asking people to keep a three or four-day diary that contains a complete record of foods and beverages consumed over those days. However, this transfers much of the time burden to the person investigated. It quickly highlight foods that need to be changed, in addition to any beneficial choices that may be continued. The diary itself may be an intervention: paeople may alter what they consume because they are required to write it down. A diary can be an excellent tool to help increase awareness of dietary habits and to encourage compliance with recommended dietary changes.

IV. Food frequency questionnaire — This is an assessment tool, which may be the quickest way to identify dietary patterns. Used in combination with the 24-hour recall, this may be one of the best way to identify protective and detrimental components of the patient diet.

The food frequency questionnaire will cover typical intake over a period of time. Focus can be put on one or more key areas that are correlated with health concerns. Asking how often one consumes the food and then probing for greater detail helps. As an example, if the patient has a high LDL cholesterol level, we can explore sources of saturated fat and hydrogenated fats (trans fats), such as meats, cheese, processed foods, snacks, and dairy products including 2 percent milk, whole milk, and ice cream. Also FFQ assessments can reveal whether a person is eating a variety of fruit, vegetables, whole grains, and fish or not. In fact, a simple dietary recommendation of increasing fiber, or lowering saturated fat can also have a beneficial impact on other areas of the diet that are not specifically addressed[281].

However, they may not be able to give absolute nutrient values if they are unstructured, lengthy and do not provide details regarding specific foods[282,283]. A short FFQ may not have an appropriate response rate and good data quality, on the other hand clarity and ease of administration may be compensated in a lengthy questionnaire[284]. Other factors which can affect the subjects compliance in filling a FFQ, and hence the accuracy of a food frequency questionnaire include the number of food items, the method used to estimate frequency of intake and the portion size. Because filling in a lengthy FFQ can lead to fatigue and boredom both concentration and accuracy of response can be impaired. This has been shown to be true, even for a highly motivated cohort, as demonstrated by Willett et al in their study of US nurses[285]. Also, Warneke et al found a 7-item FFQ closely related to mean total fruit and vegetable consumption then a 31-item FFQ[286]. Hence while formulating a FFQ; a

standard approach is to give subjects an adequate length FFW with choice of frequency options ranging from never to intake of many times/day. Defining the options increases clarity and reduces errors compared with open-ended responses where subject's self- report their frequency intake[287]. Another factor to be taken into consideration when interpreting the data. My subjects were a sub-sample of healthy volunteers participating in a prospective study selected based on selfreported dietary intake and therefore may not be representative of the general population with coexistent medical problems which may affect the urinary excretion of flavonoid metabolites. However, this is unlikely to bias the study as my objective was to validate the FFQ rather than estimate levels of flavanoid intake among the population.

Controversy exists over inclusion of portion size in a FFQ. Block et *al* used a reduced dietary questionnaire and compared different portion sizes to a standard portion size. They found estimated values of macronutrients was reduced, however micronutrients were not reduced[288]. Correlations between the same FFQ with and without portion size were 0.9 for the nutrients. A Danish study compared FFQ with and without portion sizes with weighted diet records. Mean correlation coefficients for food groups and nutrients changed slightly[289]. Clapp *et al* found that replacing reported portion size data with standard portion size data may lead to conflicting outcomes for specific nutrients in research concerning the relationship between diet and disease[290]. Recently, a study from Germany concluded that inclusion of portion size may not be important in large epidemiological studies[291]. Portion size, although statistically unimportant in this study, correlates closely to flavonoid excretion when included. Hence I suggest, frequency and portion size may be included in an FFQ, as the correlation grows weaker if portion size is excluded (r = 0.693 & 0.157).

13.0. Conclusion:

In conclusion, the current studies add to the growing evidence that NSAIDs can play a protective role against both squamous and adenocarcinoma of the oesophagus. These data provide interesting evidence that use of CCBs and bronchodilators is associated with increased risk of OAC. One possible explanation for these observations is that the widespread use of these drugs may be partially responsible for the continuing increase in the incidence of OAC. This issue requires further investigation, both to further quantify the level of risk at the population level, and to explore the mechanisms involved.

The Flavonoid study suggests a reasonable reproducibility of the flavonoids ingestion with their urinary validation using data from the FFQ. These findings suggest that FFQ can further be used as a tool to measure the impact of diet on health.

14.0. Future Research:

Many cancers are preventable. Basic lifestyle changes can have a tremendous impact on the rates of cancer. The fact that such changes also protect against other chronic diseases (cardiovascular disease, stroke, and diabetes) makes the case for prevention even more compelling. For several cancers, prophylactic medication can reduce cancer risk for high risk individuals.

The primary effect of the nonsteroidal antiinflammatory drugs is to inhibit cyclooxygenase, thereby impairing the ultimate transformation of arachidonic acid to prostaglandins, prostacyclin, and thromboxanes. Increased expression of COX-2 mRNA and protein has been noted in patients with hypertension, heart failure, and diabetic nephropathy[292]. Differences in a given non-selective NSAID or selective coxibs toxicity may in part be due to the extent to which it inhibits an isoform of cyclooxygenase. A presumed advantage of the coxibs was a reduction in gastrointestinal toxicity compared to other NSAIDs. However, an increased risk of ischemic cardiovascular disease has been described with rofecoxib, celecoxib, and valdecoxib. Not enough data are available to be certain, but a risk may also be present with etoricoxibs and lumiracoxibs. Selective COX-2 inhibition is associated with reduced prostaglandin I2 (PGI2 or prostacyclin) production by vascular endothelium with little or no inhibition of potentially prothrombotic platelet thromboxane A2 production[293]. The relatively selective reduction in prostacyclin activity could predispose to endothelial injury[294]. In addition, selective COX-2 inhibition, as well as non-selective inhibition of COX, elevates blood pressure. These are two of several proposed links between these agents and ischemic cardiovascular events. A similar increase in relative risk of myocardial infarction (RR 2.24, 95% CI 1.24-4.02) or thrombotic cardiovascular events (RR 2.66, 95% CI 1.03-6.86) with rofecoxib therapy was noted in a 2004 meta-analysis and a randomized trial of

rofecoxib versus placebo for secondary prevention of colon cancer that was published after the 2004 meta-analysis[295,296]. The increase in cardiovascular risk may be greater with rofecoxib doses above 25 mg/day [297-299].

In summary, all of the coxibs appear to have potential cardiovascular risk that may be dose-dependent[300]. The magnitude of the risk may differ between agents. As an example, a retrospective study suggested that concurrent therapy with low dose aspirin may have mitigated the risk of myocardial infarction associated with use of low dose rofecoxib but not for doses higher than 25 mg/day[301]. The issue of concomitant aspirin use was also addressed in the TARGET trial but the number of events was too small to have confidence in the results[302].

Furthermore, data from the Cancer Prevention Study II Nutrition Cohort (n = 146,000), with 18,000 cancers diagnosed during a ten year follow-up period, found that the overall cancer incidence in men was lower for those who took ≥325 mg aspirin daily for at least five years compared to no aspirin use; cancer incidence in women was lower, but the difference was not statistically significant[303]. In addition to colorectal cancer, longterm aspirin use was associated with a lower incidence of prostate cancer (RR 0.81, 95% CI 0.7-0.94) and a trend to lower incidence in breast cancer (RR 0.83, 0.63-1.10).

Several theories have been proposed for why aspirin and other NSAIDs are effective in reducing colorectal cancer risk, and possibly effective for other cancers. These medications may cause cell cycle arrest or apoptosis (programmed cell death) of abnormal cells. Reduced risk may also relate to irreversible inhibition of cyclooxygenase-2. Inhibition of this enzyme decreases the synthesis of prostaglandins, which may inhibit tumor growth. Finally, aspirin may influence intracellular signaling through inhibition of phospholipase activity.

Chemoprevention may be helpful in high risk patients but risks and benefits should be weighed carefully. Aspirin and NSAIDs may offer protection against oesophageal cancer, but are may not be recommended for routine use in average risk patients. Thus the adverse effect of Coxibs on cardiovascular disease highlights the challenge of balancing risks and benefits in chemoprevention for cancer. The result of our case control study highlights the significant links between the use of NSAIDs and drugs which relax the LOS. Efforts should be directed at identifying naturally occurring compounds with COX-2 enzyme blocking and anti oxidant properties to help reduce the risks posed by synthetic medications. Deeks and colleagues report the findings of a systematic review and meta-analysis of randomised trials comparing celecoxib with a traditional NSAID or placebo[304]. They identified nine trials including 15172 patients with osteoarthritis and rheumatoid arthritis, in which celecoxib was compared with at least one NSAID (diclofenac, naproxen, or ibuprofen) or with a placebo (five trials). CLASS contributed over half of the patients analysed. They found equivalent efficacy between celecoxib and the comparator NSAIDs, but significantly greater tolerability, in terms of withdrawals from studies as a result of gastrointestinal adverse effects, with celecoxib and a lower incidence of upper gastrointestinal complications, including symptomatic ulcers, perforation and haemorrhage. These results seem also to apply to the subgroup of patients taking low dose aspirin as antithrombotic prophylaxis. However this study was not able to examine longer term sequelae, did not comment on deaths, and did not analyse cardiovascular events. Thus there is evidence about the usefulness of Coxibs and future research should involve minimal risk of harm to the subjects and the amount of benefits must clearly outweighs the amount of risk thus making a point for evaluating a risk/benefit ratio for chemoprevention for individual cases.

Also prospective cohort studies have demonstrated only a weak association between fruit and vegetable intake and cancer risk[305,306]. In a large cohort study (n = 136,089) with 937 incident cases of colon cancer, no association was seen between either total or specific category of fruit and vegetable intake and colon cancer risk[296]. A pooled analysis of fourteen cohort studies (n >750,000), including the previous study, found that eating more than 800 g fruit and vegetables daily, compared to less than 200 g, decreased risk for distal colon cancer (RR 0.74) but not for proximal cancer[307,308].

However with regards to oesophageal cancer, Cheng & Day, in a systematic review of ecologic, case-control, cohort, and intervention studies in 1996 concluded that fruits and vegetables have a protective effect on SCC as compared to OAC[309]. Recently, Freedman and collegues also found a significant inverse association between total fruit and vegetable intake and SCC risk (HR: 0.78, 95% CI: 0.67-0.91), but not OAC risk (0.98, 0.90-1.08)[310]. This may have several further implications. We may need to narrow down research, asking questions about specific fruits and vegetables after identifying the ones with high contents of anti cancer chemicals. Also formulating or modifying a FFQ to study dietary intake of naturally occurring cox 2 inhibitors may provide a better link between natural compounds and cancer.

Hence, there is a scope for further research, especially developing coxibs with less or no cardiovascular side effects. There may be scope to identify more potential naturally occurring coxibs, validating their dietary intake with use of an appropriate dietary tools. This may include modifying existing applications or formation of new ones.

With the evidence available from my studies, it would be possible to develop further studies directed towards identifying characteristics of subgroups that might most benefit from NSAIDs with out adverse effects. Also development of cardioprotection with use of NSAIDs may be another area that needs to be focused on to translate laboratory discoveries into clinical trials and to develop consensus protocols. Also it

would be worth conducting case control studies looking into effects of fruits and vegetables rich in cox 2 inhibitors like quercetin and antioxidants like naringenin on different cancers including oesophageal cancer.

Finally it may also be possible to conduct prospective studies from populations using dietary assessment tools like FFQ may be focussed with estimation of different flavonoid intake, focusing on effect-modifying and confounding factors, such as dietary patterns and lifestyle, until further evidence is available about the role of flavonoids in the chemoprevention of oesophageal and other cancers.

15.0. References.

1. Lambert R, Hainaut P. The multidisciplinary management of gastrointestinal cancer. Epidemiology of oesophagogastric cancer. Baillieres Best Pract Res Clin Gastroenterol 2007;21:921–945.

2. Armstrong RA, Blalock JB, Carrera G. Adenocarcinoma of the middle third of the esophagus arising from ectopic gastric mucosa. J Thoracic Surg 1959; 37:398–403.

3. Hewlett A. The superficial glands of the esophagus. J Exp Med 1900; 5:319–331.

4. Brown LM, Devesa SS, Chow W-H. Incidence of adenocarcinoma of the esophagus among white Americans by sex, stage, and age. J Natl Cancer Inst 2008;100:1184–1187.

5. Crane LM, Schaapveld M, Visser O et.al. Oesophageal cancer in the Netherlands: Increasing incidence and mortality but improving survival. Eur J Cancer 2007;43:1445–1451.

6. Kubo A, Corley DA. Marked regional variation in adenocarcinomas of the esophagus and the gastric cardia in the United States. Cancer 2002;95:2096–2102.

7. Kubo A, Corley DA. Marked multi-ethnic variation of oesophageal and gastric cardia carcinomas within the United States. Am J Gastroenterol 2004; 99:582–588.

8. Pohl H, Welch HG. The role of overdiagnosis and reclassification in the marked increase of oesophageal adenocarcinoma incidence. J Natl Cancer Inst 2005;97:142–146.

9. El-Serag HB, Mason AC, Petersen N, Key CR. Epidemiological differences between adenocarcinoma of the oesophagus and adenocarcinoma of the gastric cardia in the USA. Gut 2002;50:368–372.

10. Cancer Incidence-UK, CRC Factsheet 1.1(1998).

11. Cancer Incidence-UK, CRC Factsheet 1.1 (2002).

12. Bollschweiler E, Wolfgarten E, Gutschow C et al. Demographic variations in the rising incidence of oesophageal adenocarcinoma in white males. Cancer 2001;92:549–555.

13. Botterweck AA, Schouten LJ, Volovics A van den Brandt PA et.al. Trends in incidence of adenocarcinoma of the oesophagus and gastric cardia in ten European countries. Int J Epidemiol 2000; 29:645–654.

14. Cameron AJ. Epidemiology of Barrett's esophagus and adenocarcinoma. Dis of Esoph 2002;15:106–108.

15. Engel LS, Chow WH, Vaughan TL et.al. Population Attributable Risks of Oesophageal and Gastric Cancers. Natl Cancer Inst 2003; 95;1404-1413

16. Nilsson M, Johnsen R, Ye W et.al. Lifestyle related risk factors in the aetiology of gastro-oesophageal reflux. Gut 2004; 53:1730-5.

17. Iwaya, T, Maesawa, C, Ogasawara, S, Tamura, G. Tylosis esophageal cancer locus on chromosome 17q25.1 is commonly deleted in sporadic esophageal cancer. Gastroenterology 1998;114:1206.

18. Ribeiro U Jr, Posner MC, Safatle-Ribeiro AV, Reynolds JC. Risk factors for squamous cell carcinoma of the oesophagus. Br J Surg 1996; 83:1174-85

19. Akbari MR, Malekzadeh R, Nasrollahzadeh D et.al. Int J Cancer.Familial risks of esophageal cancer among the Turkmen population of the Caspian littoral of Iran. 2006 Sep 1;119:1047-51.

20. Gao Y, Hu N, Han X et.al. Family history of cancer and risk for esophageal and gastric cancer in Shanxi, China. BMC Cancer. 2009 Aug 5;9:269.

21. Akbari MR, Malekzadeh R, Nasrollahzadeh D et al. Familial risks of oesophageal cancer among the Turkmen population of the Caspian littoral of Iran. Int J Cancer. 2006; 119:1047–51.

22. Lagergren J, Bergstrom R, Lindgren A, Nyren O. The role of tobacco, snuff and alcohol use in the aetiology of cancer of the oesophagus and gastric cardia. Int J Cancer 2000;85:340-346.

23. Wu AH, Wan P, Bernstein L. A multiethnic population-based study of smoking, alcohol and body size and risk of adenocarcinomas of the stomach and esophagus .Cancer Causes Control 2001;12:721-32.

24. Chow WH, Blot WJ, Vaughan TL. Body mass index and risk of adenocarcinomas of the esophagus and gastric cardia. J Natl Cancer Inst 1998; 90:150-5.

25. Hampel H, Abraham NS, El-Serag HB. Meta-analysis: obesity and the risk for gastroesophageal reflux disease and its complications. Ann Intern Med. 2005;143:199–211.

26 Ogunwobi OO, Beales IL. Adiponectin stimulates proliferation and cytokine secretion in colonic epithelial cells. Regul Pept 2006;134:105-13.

27. DeVault KR, Castell DO. Updated guidelines for the diagnosis and treatment of gastroesophageal reflux disease. The Practice Parameters Committee of the American College of Gastroenterology. Am. J. Gastroenterol 1999;94:1434–42.

28. Lagergren J, Bergstrom R, Lindgren A, Nyren O. Symptomatic gastroesophageal reflux as a risk factor for oesophageal adenocarcinoma. N Engl J Med 1999;340:825-831.

29. Fitzgerald RC, Omary MB, Triadafilopoulos G. Dynamic effects of acid on Barrett's esophagus. An ex vivo proliferation and differentiation model. J Clin Invest 1996;98: 2120-2128.

30. Souza RF, Shewmake K, Terada LS, Spechler SJ. Acid exposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus. Gastroenterology 2002;122:299-307.

31. Leu CM, Wong FH, Chang C, Huang SF, Hu CP. Interleukin-6 acts as an antiapoptotic factor in human oesophageal carcinoma cells through the activation of both STAT3 and mitogen-activated protein kinase pathways. Oncogene 2003;22:7809-7818.

32. Shirvani VN, Ouatu-Lascar R, Kaur BS, Omary MB, Triadafilopoulos G. Cyclooxygenase 2 expression in Barrett's esophagus and adenocarcinoma: Ex vivo induction by bile salts and acid exposure. Gastroenterology 2000;118:487-496.

33. Barham CP, Alderson D: Pathophysiology and investigation of GORD and motility disorders; in Griffin SM, Raimes SA (eds) Upper Gastrointestinal Surgery. London, United Kingdom, 2001:251-282.

34. El-Serag HB, Graham DY, Satia JA, Rabeneck L. Obesity is an independent risk factor for GERD symptoms and erosive esophagitis. Am J Gastroenterol 2005;100:1243-50.

35. El-Serag HB, Satia JA, Rabeneck L. Dietary intake and the risk of gastro-oesophageal reflux disease: a cross sectional study in volunteers. Gut 2005;54:11-17

36. Gelfond M, Rozen P, Gilat T. Isosorbide dinitrate and nifedipine treatment of achalasia: a clinical, manometric and radionuclide evaluation. Gastroenterology 1982;83: 963-969.

37. Stein MR, Towner TG, Weber RW et.al. The effect of Theophylline on the lower oesophageal sphincter pressure. Ann Allergy 1980;45:238-241.

38. Wong RK, Maydonovitch C, Garcia JE, Johnson LF, Castell DO. The effect of terbutaline sulfate, nitroglycerin, and Theophylline on lower oesophageal sphincter pressure and radionuclide oesophageal emptying in patients with achalasia. J Clin Gastroenterol 1987; 9: 386-389.

39. Zfass AM, Prince R, Allen FN, Farrar JT. Inhibitory beta adrenergic receptors in the human distal esophagus. Am J Dig Dis 1970;15:303-310.

40. Oshima M, Dinchuk JE, Kargman SL et.al. Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996; 87: 803-9.

41. Greenhough A, Smartt HJ, Moore AE, Roberts HR, Williams AC, Paraskeva C, Kaidi A. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. Carcinogenesis 2009;30:377-86.

42. Wilson KT, Fu S, Ramanujam KS, Meltzer SJ. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinoma. Cancer Res 1998 58:2929-2934.

43. Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res 1999; 59:198-204.

44. Morris CD, Armstrong GR, Bigley G, Green H, Attwood SEA. Cyclooxygenase-2 expression in the Barrett's metaplasia-dysplasia adenocarcinoma sequence. Am J Gastroenterology 2001;96:990-996.

45. Buskens CJ, van Rees BP, Sivula A, Reitsma JB, Haglund C et.al. Prognostic significance of elevated cyclooxygenase 2 expression in patients with adenocarcinoma of the esophagus. Gastroenterology 2002;122:1800-1807.

46. Zhang X, Miao X, Tan W et.al. Identification of functional genetic variants in cyclooxygenase-2 and their association with risk of oesophageal cancer. Gastroenterology 2005;129:565-76.

47. Su JL, Shih JY, Yen ML, Jeng YM, Chang CC, Hsieh CY, Wei LH, Yang PC & Kuo ML. Cyclooxygenase-2 induces EP1- and HER-2/Neu-dependent vascular endothelial growth factor-C up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. Cancer Research 2004;64:554–564.

48. Rockett JC, Larkin K, Darnton SJ, et al. Five newly established oesophageal carcinoma cell lines: phenotypic and immunological characterization. Br J Cancer. 1997;75:258–263

49. O Jurriaan B. Tuynman, Christianne J. Buskens, Kristel Kemper, Fiebo J. W. ten Kate, G Johan A. Offerhaus, Dirk J. Richel, J Jan B. van Lanschot. Neoadjuvant Selective COX-2 Inhibition Down-Regulates Important Oncogenic Pathways in Patients With Esophageal Adenocarcinoma Ann Surg. 2005 December; 242:840–850.

50. Hung WC, Chang HC, Pan MR, Lee TH, Chuang LY. Induction of p27(KIP1) as a mechanism underlying NS398-induced growth inhibition in human lung cancer cells. Mol. Pharmacol 2000;58:1398–1403.

51. Shaik MS, Chatterjee A, Singh M. Effect of a selective cyclooxygenase-2 inhibitor, nimesulide, on the growth of lung tumors and their expression of cyclooxygenase-2 and peroxisome proliferator- activated receptor-gamma. Clin. Cancer Res 2004;10:1521–1529.

52. Yamazaki R, Kusunoki N, Matsuzaki T, Hashimoto S, Kawai S. Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells. FEBS Lett 2002; 531:278–284.

53. Cheong E, Ivory K, Doleman J, Parker ML, Rhodes M, Johnson IT. Synthetic and naturally occurring Coxibs suppress proliferation in a human oesophageal adenocarcinoma cell line (OE33) by inducing apoptosis and cell cycle arrest. Carcinogenesis 2004;25:1945-52.

54. Conio, G. Lapertosa, S. Blanchi and R. Filiberti, Barrett's esophagus: an 1update, Crit. Rev. Oncol. Hematol 2003;46:187–206.

55. Kristinsson JO, van Westerveld P, te Morsche RH et.al. Cyclooxygenase-2 polymorphisms and the risk of oesophageal adeno- or squamous cell carcinoma. World J Gastroenterol 2009;15:3493-7.

56. Morton LW, Abu-Amsha Caccetta R, Puddey IB, Crpft KD. Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular disease. Clin.Exp.Pharmacol.Physiol 2000;27:152-9.

57. Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Ther 2001; 90:157-77

58. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001;74: 418-25.

59. Duthie GG, Duthie SJ, Kyle JAM. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutr.Res.Rev 2000; 13:79-106.

60. Manthey JA. Biological properties of flavonoids pertaining to inflammation. Microcirculation 2000;7:S29-34.

61. de Whalley CV, Rankin SM Hoult JR, Jessup W, Leake DS. Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. Biochem Pharmacol 1990;39:1743-50.

62. Bors W, Heller W, Michel C. The chemistry of flavonoids. In: Rice-Evans CA, Packer L, eds. Flavonoids in Health and Disease. New York: Marcel Dekker,1998:111-36.

63. Middleton E. Some biological properties of plant flavonoids on. Ann Allergy 1988;61: 53-7.

64. Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 1999;20:1945-52.

65. Singh RP, Dhanalakshmi S, Mohan S, Agarwal C, Agarwal R. Silibinin inhibits UVB- and epidermal growth factor-induced mitogenic and cell survival signaling involving activator protein-1 and nuclear factor-kappaB in mouse epidermal JB6 cells. Mol Cancer Ther. 2006 May;5:1145-53.

66. Morton LW, Abu-Amsha Caccetta R, Puddey IB, Crpft KD. Chemistry and biological effects of dietary phenolic compounds: relevance to cardiovascular disease. Clin.Exp.Pharmacol.Physiol 2000;27:152-9.

67. Birt DF, Hendrich S, Wang W. Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Ther 2001;90:157-77

68. Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. . Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001;74:418-25.

69. Yamanaka, N, Oda O, Nagao S. Green tea catechins such as (-)-epicatechin and (-)-epigallocatechin accelerate Cu2+-induced low density lipoprotein oxidation in propagation phase. FEBS Lett 1997;401:230-4.

70. Scott B C, Butler J, Halliwell B, Aruoma OI. Evaluation of the antioxidant actions of ferulic acid and catechins. Free Radic Res Commu 1993;19:241-53.

71. Tanigawa S, Fujii M, Hou DX. Stabilization of p53 is involved in quercetin-induced cell cycle arrest and apoptosis in HepG2 cells. Biosci Biotechnol Biochem. 2008;72: 797–8041.

72. Warren CA, Paulhill KJ, Davidson LA, Lupton JR, Taddeo SS, Hong MY, Carroll RJ, Chapkin RS, Turner ND. Quercetin may suppress rat aberrant crypt foci formation by suppressing inflammatory mediators that influence proliferation and apoptosis. J Nutr 2009;139:101-5.

73. Mitchell E, Hong YJ, Koh E, Barrett DM, Bryant DE, Denison RF and Kaffka S. Ten-Year Comparison of the Influence of Organic and Conventional Crop Management Practices on the Content of Flavonoids in Tomatoes. Journal of Agricultural and Food Chemistry 2007;55: 6154–9.

74. Ameer B, Weintraub RA, Johnson JV, Yost RA, Rouseff RL. Flavanone absorption after naringin, hesperidin, and citrus administration. Clin. Pharmacol. Ther 1996; 60: 34-40.

75. Erlund I, Meririnne E, Alfthan G, Aro A. Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. J Nutr 2001;131:235- 41.

76. Choudhury R, Chowrimooto G, Srai K, Debnam E and Rice-Evans CA' Interactions of the Flavonoid Naringenin in the Gastrointestinal Tract and the Influence of Glycosylation. Biochemical and Biophysical Research Communications1999; 265; 410-415.

77. Bugianesi R, Catasta G, Spigno P, D'Uva A and Maiani G. Naringenin from Cooked Tomato Paste Is Bioavailable in Men. The American Society for Nutritional Sciences J. Nutr 2002;132:3349-3352.

78. Scheline R. CRC handbook of mammalian metabolism of plant compounds. Boca Raton (FL)7 CRC Press;1991.

79. Griffiths LA, Barrow A. Metabolism of flavonoid compounds in germ-free rats. Biochem J 1972;130:1161-2.

80. Gugler R, Leschik M, Dengler HJ. Disposition of quercetin in man after single oral and intravenous doses. Eur J Clin Pharmacol 1975;9:229- 34.

81. Hollman PC, de Vries JH, van Leeuwen SD, Mengelers MJ, Katan MB. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am J Clin Nutr 199 ;62:1276- 82.

82. Hollman PC, Gaag MV, Mengelers MJ, van Trijp JM, De Vries JM, Katan MB. Absorption and disposition kinetics of the dietary antioxidant quercetin in man. Free Radic Biol Med 1996;21:703-7.

83. Hollman PC, van Trijp JM, Buysman MN, van der Gaag MS, Mengelers MJ, de Vries J. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. FEBS Lett 1997; 418:152-6.

84. Hollman PC, van Het Hof KH, Tijburg LB, Katan MB. Addition of milk does not affect the absorption of flavonols from tea in man. Free Radic Res 2001;34:297-300.

85. de Vries JH, Hollman PC, van Amersfoort I, Olthof MR, Katan MB. Red wine is a poor source of bioavailable flavonols in men. J Nutr 2001;131:745-8.

86. Erlund I, Silaste ML, Alfthan G, Rantala M, Kes7niemi YA, Aro A. Plasma concentrations of the flavonoids hesperetin, naringenin and quercetin in human subjects following their habitual diets, and diets high and low in fruit and vegetables. Eur J Clin Nutr 2002;56:891-8.

87. Erlund I, Marniemi J, Hakala P, Alfthan G, Meririnne E, Aro A. Consumption of black currants, lingonberries and bilberries increases serum quercetin concentrations. Eur J Clin Nutr 2003;57:37- 42.

88. Freese R, Alfthan G, Jauhiainen M et al. High intakes of vegetables, berries, and apples combined with a high intake of linoleic or oleic acid only slightly affect markers of lipid peroxidation and lipoprotein metabolism in healthy subjects. Am J Clin Nutr 2002;76:950- 60.

89. Erlund I, Kosonen T, Alfthan G, et al. Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. Eur J Clin Pharmacol 2000;56:545- 53.

90. Graefe EU, Wittig J, Mueller S, Riethling AK, Uehleke B, Drewelow B, et al. Pharmacokinetics and bioavailability of quercetin glycosides in humans. J Clin Pharmacol 2001;41:429- 99.

91. Erlund I, Alfthan G, M7enp77 J, Aro A. Tea and coronary heart disease: the flavonoid quercetin is more bioavailable from rutin in women than in men. Arch Intern Med 2001;161:1919- 20.

92. Walle T, Walle UK, Halushka PV. Carbon dioxide is the major metabolite of quercetin in humans. J Nutr 2001;131:2648- 52.

93. Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81: 230S-242S.

94. Manach C, Texier O, Re´ge´rat F, Agullo G, Demigne C, Re´me´sy C. Dietary quercetin is recovered in rat plasma as conjugated derivatives of isorhamnetin and quercetin. Nutr Biochem 1996;7:375- 80.

95. Ueno I, Nakano N, Hirono I. Metabolic fate of [14C]quercetin in the ACI rat. J Exp Med 1983;53:41- 50.

96. Spencer CM, Cai Y, Martin R, Gaffney SH, Goulding PN, Magnolato PN, et al. Polyphenol complexation some thoughts and observations. Phytochemistry 1988;27:2397- 409.

97. Manach C, Morand C, Texier O, Favier ML, Agullo G, Demigne C, et al. Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. J Nutr 1995;125:1911- 22.

98. Boulton DW, Walle UK, Walle T. Extensive binding of the bioflavonoid quercetin to human plasma proteins. J Pharm Pharmacol 1998;50:243-9.

99. Fleck C, Braunlich H. Factors determining the relationship between renal and hepatic excretion of xenobiotics. Arzneimittelforschung 1990;40:942-6.

100. Fuhr U, Kummert AL. The fate of naringin in humans: a key to grapefruit juice–drug interactions? Clin Pharmacol Ther 1995;58:365-73.

101. Ameer B, Weintraub RA, Johnson JV, Yost RA, Rouseff RL. Flavanone absorption after naringin, hesperidin, and citrus administration. Clin Pharmacol Ther 1996;60:34-40.

102. Lee YS, Reidenberg MM. A method for measuring naringenin in biological fluids and its disposition from grapefruit juice by man. Pharmacology 1998;56: 314-7.

103. Ishii K, Furuta T, Kasuya Y. Mass spectrometric identification and high-performance liquid chromatographic determination of a flavonoid glycoside naringin in human urine. J Agric Food Chem 2000; 48: 56-9.

104. Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C, Remesy C. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. Eur J Clin Nutr 2003; 57:235- 42.

105. Bugianesi R, Catasta G, Spigno P, D'Uva A, Maiani G. Naringenin from cooked tomato paste is bioavailable in men. J Nutr 2002;132:3349- 52.

106. Guajardo-Flores D, Serna-Saldívar SO, Gutiérrez-Uribe JA. Evaluation of the antioxidant and antiproliferative activities of extracted saponins and flavonols from

germinated black beans (Phaseolus vulgaris L). Food Chem. 2013 Nov 15;141:1497-503.

107. Murugan K, Iyer VV. Differential growth inhibition of cancer cell lines and antioxidant activity of extracts of red, brown green marine algae. In Vitro Cell Dev Biol Anim. 2013 May;9:324-34.

108. Wu CH, Wu CF, Huang HW, Jao YC, Yen GC. Naturally occurring flavonoids attenuate high glucose-induced expression of proinflammatory cytokines in human monocytic THP-1 cells. Mol Nutr Food Res 2009;53: 984-95.

109. Dihal AA, de Boer VC, van der Woude H et.al. Quercetin, but not its glycosidated conjugate rutin, inhibits azoxymethane-induced colorectal carcinogenesis in F344 rats. J Nutr. 2006;136:2862–7.

110. Kanno S, Shouji A, Asou K, Ishikawa M. Effects of naringin on hydrogen peroxide-induced cytotoxicity and apoptosis in P388 cells. J Pharmacol Sci 2003;92:166-70.

111. Kanno S., Shouji A, Hirata R, Asou K, Ishikawa M. Effects of naringin on cytosine arabinoside (Ara-C)-induced cytotoxicity and apoptosis in P388 cells Life Sci 2004;75: 353-365.

112. Kanno S, Shouji A, Asou K, Ishikawa M. Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. J. Pharmacol. Sci 2003;92:66-70.

113. Knekt P, Jarvinen R, Seppanen R, Hellovaara M, Teppo L, Pukkala E, Aromaa A.Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. Am J Epidemiol 1997;146:223-30.

114. Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P.Flavonol and flavone intake and the risk of cancer in male smokers (Finland). Cancer Causes Control 2001 ;12:789-96.

115. Arts ICW, Jacobs DR Jr, Gross M, Harnack LJ, Folsom AR. Dietary catechins and cancer incidence among postmenopausal women: the Iowa Women's Health Study (United States). Cancer Causes Control 2002;13:373–89.

116. Strom SS, Yamamura Y, Duphorne CM, et al. Phytoestrogen intake and prostate cancer: a case-control study using a new database. Nutr Cancer 1999;33:20–5.

117. Walcott FL, Hauptmann M, Duphorne CM, Pillow PC, Strom SS, Sigurdson AJ. A case-control study of dietary phytoestrogens and testicular cancer risk. Nutr Cancer 2002;44:44–51.

118. McCann SE, Freudenheim JL, Marshall JR, Graham S. Risk of human ovarian cancer is related to dietary intake of selected nutrients, phytochemicals and food groups. J Nutr 2003;133:1937–42.

119. Hertog MGL, Brants HAM, van Poppel G, van den Brandt PA. Intake of flavonoids and cancer risk: a prospective cohort study. In: Armado R, Andersson H, Bardócz S, Serra F, eds. Polyphenols in food. Luxembourg: Office for Official Publications of the European Communities 1998:159–66.

120. Zhang Q, Zhao XH, Wang ZJ. Flavones and flavonols exert cytotoxic effects on a human oesophageal adenocarcinoma cell line (OE33) by causing G2/M arrest and inducing apoptosis. Toxicol In Vitro 2009;23:797-807

121. Zhang Q, Zhao XH, Wang ZJ. Cytotoxicity of flavones and flavonols to a human oesophageal squamous cell carcinoma cell line (KYSE-510) by induction of G2/M arrest and apoptosis. Toxicol In Vitro 2009;23:797-807.

122. Stoner GD, Chen T, Kresty LA, Aziz RM, Reinemann T, Nines R. Protection against oesophageal cancer in rodents with lyophilized berries: potential mechanisms. Nutr Cancer. 2006;54:33-46.

123. Adlercreutz H, Mousavi Y, Clark J. Dietary phytoestrogens and cancer: invitro and invivo studies. J Steroid Biochem 1992;41:331-7.

124. Le Marchand L, Murphy SP, Hankin JH, Wilkens LR, Kolonel LN. Intake of flavonoids and lung cancer. J Natl Cancer Inst 2000;92:154-60.

125. Gao YT, McLaughlin JK, Blot WJ, Ji BT, Dai Q, Fraumeni JF Jr. Reduced risk of oesophageal cancer associated with green tea consumption. J Natl Cancer Inst 1994;86:855-8.

126. Sun CL, Yuan JM, Lee MJ, Yang CS, Gao YT, Ross RK, Yu MC. Urinary tea polyphenols in relation to gastric and oesophageal cancers: a prospective study of men in Shanghai, China. Carcinogenesis 2002;23:1497-503.

127. Sporn MB. Approaches to prevention of epithelial cancer during the preneoplastic period. Cancer Res 1976;36:2699–2702.

128. Lippman SM, Hong WK. Cancer prevention by delay. Commentary re: J. A. O'Shaughnessy et al., Treatment and Prevention of Intraepithelial Neoplasia: An Important Target for Accelerated New Agent Development. Clin. Cancer Res., 8: 314–346, 2002. Clin Cancer Res 2002;8: 305–313.

129. Ramos S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. J Nutr Biochem. 2007;181:427-42.

130. Thun MJ, Blackard B. Pharmacologic effects of NSAIDs and implications for the risks and benefits of long-term prophylactic use of aspirin to prevent cancer. Recent Results Cancer Res 2009;181:215-21.

131. Sadeghi S, Bain CJ, Pandeya N, Webb PM, Green AC, Whiteman DC; Australian Cancer Study.Aspirin, nonsteroidal anti-inflammatory drugs, and the risks of cancers of the esophagus. Cancer Epidemiol Biomarkers Prev 2008;17:1169-78.

132. Duan L, Wu AH, Sullivan-Halley J, Bernstein L. Nonsteroidal anti-inflammatory drugs and risk of oesophageal and gastric adenocarcinomas in Los Angeles County. Cancer Epidemiol Biomarkers Prev 2008;17:126-34

133. Buttar, K.K. Wang, O. Leontovich, J.Y. Westcott, R.J. Pacifico and M.A. Anderson et al., Chemoprevention of oesophageal adenocarcinoma by Coxibs in an animal model of Barrett's esophagus. Gastroenterology 2002;122;1101–1112.

134. Kaur, N. Khamnehei, M. Iravani, S.S. Namburu, O. Lin and G. Triadafilopoulos, Rofecoxib inhibits cyclooxygenase 2 expression and activity and reduces cell proliferation in Barrett's esophagus, Gastroenterology 2002;123:60–67.

135. Bardou M, Barkun AN, Ghosn J, Hudson M, Rahme E. Effect of chronic intake of NSAIDs and cyclooxygenase 2-selective inhibitors on oesophageal cancer incidence. Clin Gastroenterol Hepatol 2004; 2:880-887

136. Farkouh ME, Greenberg BP.An evidence-based review of the cardiovascular risks of nonsteroidal anti-inflammatory drugs. Am J Cardiol 2009;103:1227-37.

137. Gatenby PA, Ramus JR, Caygill CP, Winslet MC, Watson A.Aspirin is not chemoprotective for Barrett's adenocarcinoma of the oesophagus in multicentre cohort. Eur J Cancer Prev 2009;18:381-4.

138. Heath EI; Canto MI; Piantadosi S; Montgomery E; Weinstein WM; Herman JG; Dannenberg AJ; Yang VW; Shar AO; Hawk E; Forastiere AA Secondary chemoprevention of Barrett's esophagus with celecoxib: results of a randomized trial. J Natl Cancer Inst 2007; 99:545-57.

139. Lagergren J; Bergstrom R; Adami HO; Nyren O. Association between medications that relax the lower oesophageal sphincter and risk for oesophageal adenocarcinoma. Ann Intern Med 2000;133:165-75.

140. Corley DA; Levin TR; Habel LA; Buffler PA. Barrett's Esophagus and Medications that Relax the Lower Oesophageal Sphincter. Am J Gastroenterol 2006; 30: 937-44.

141. Liu X, Wu WK, Yu L, Sung JJ, Srivastava G, Zhang ST, Cho CH. Epinephrine stimulates oesophageal squamous-cell carcinoma cell proliferation via beta-adrenoceptor-dependent transactivation of extracellular signal-regulated kinase/cyclooxygenase-2 pathway. J Cell Biochem 2008;105:53-60.

142. Lagergren J, Bergstrom R, Lindgren A, Nyren O. The role of tobacco, snuff and alcohol use in the aetiology of cancer of the oesophagus and gastric cardia. Int J Cancer 2000;85:340-346.

143. Wu AH, Wan P, Bernstein L. A multiethnic population-based study of smoking, alcohol and body size and risk of adenocarcinomas of the stomach and esophagus (United States).Cancer Causes Control 2001;12:721-32.

144. Vaughan TL, Dong LM, Blount PL, Ayub K, Odze RD, Sanchez CA, Rabinovitch PS, Reid BJ. Non-steroidal anti-inflammatory drugs and risk of neoplastic progression in Barrett's oesophagus: a prospective study. Lancet Oncol 2005;6: 945-952.

145. Corley DA, Kerlikowske K, Verma R, Buffler P. Protective association of aspirin/NSAIDs and oesophageal cancer: a systematic review and meta-analysis. Gastroenterology 2003;124:47-56.

146. Bardou M, Barkun AN, Ghosn J, Hudson M, Rahme E. Effect of chronic intake of NSAIDs and cyclooxygenase 2-selective inhibitors on oesophageal cancer incidence. Clin Gastroenterol Hepatol 2004; 2:880-887.

147. Mehta S, Johnson IT, Rhodes M. Systematic review: the chemoprevention of oesophageal adenocarcinoma. Aliment Pharmacol Ther 2005; 22:759-768.

148. Hoque A, Lippman SM, Wu TT, Xu Y, Liang ZD, Swisher S, Zhang H, Cao L, Ajani JA, Xu XC. Increased 5-lipoxygenase expression and induction of apoptosis by its inhibitors in oesophageal cancer: a potential target for prevention. Carcinogenesis 2005; 26:785-791.

149. Zhi H, Zhang J, Hu G, Lu J, Wang X, Zhou C, Wu M, Liu Z. The deregulation of arachidonic acid metabolism-related genes in human oesophageal squamous cell carcinoma. Int J Cancer 2003;106:327-333.

150. Spencer CM, Cai Y, Martin R, Gaffney SH, Goulding PN, Magnolato PN, et al. Polyphenol complexation—some thoughts and observations. Phytochemistry 1988;27:2397- 409.

151. Hiddink GJ; Hautvast JG; van Woerkum CM; Fieren CJ; van 't Hof MA. Consumers' expectations about nutrition guidance: the importance of primary care physicians. Am J Clin Nutr 1997 Jun;65:1974S-1979S.

152. National Ambulatory Medical Care Survey: 2000 Summary. US Department of Health and Human Services. Centers for Disease Control and Prevention, National Center for Health Statistics; www.cdc.gov/nchs

153. Vetter, ML, Herring, SJ, Sood, M, et al. What do resident physicians know about nutrition? AnF evaluation of attitudes, self-perceived proficiency and knowledge. J Am Coll Nutr 2008;27:287.

154. U.S. Preventive Services Task Force. Guide to clinical preventive services, 2nd ed, U.S. Department of Health and Human Services, Office of Disease Prevention and Health Promotion, Washington DC 1996.

155. Truswell, AS. Family physicians and patients: is effective nutrition interaction possible?. Am J Clin Nutr 2000;71: 6.

156. Langner, NR, Hasselback, PD, Dunkley, GC, Corber, SJ. Attitudes and practices of primary care physicians in the management of elevated serum cholesterol levels. CMAJ 1989;141:33.

157. Hyman, DJ, Maibach, EW, Flora, JA, Fortmann, SP. Cholesterol treatment practices of primary care physicians. Public Health Rep 1992;107:441.

158. Al-Ghawi, A, Uauy, R. Study of the knowledge, attitudes and practices of physicians towards obesity management in primary health care in Bahrain. Public Health Nutr 2009; 1.

159. National Center for Health Statistics. Available at the following address www.cdc.gov/nchs/fastats/lcod.htm.

160. Olendzki, BC, Ma, Y, Schneider, KL, et al. A simple dietary message to improve dietary quality: Results from a pilot investigation. Nutrition 2009;25:736.

161. Swain, JF, McCarron, PB, Hamilton, EF, et al. Characteristics of the diet patterns tested in the optimal macronutrient intake trial to prevent heart disease (OmniHeart): options for a heart-healthy diet. J Am Diet Assoc 2008;108:257.

162. Bradburn NM, Rips LJ, Shevell SK. Answering autobiographical questions: the impact of memory and inference on surveys. Science 1987; 236:157-61.

163. Smith AF, Jobe JB, Mingay DJ. Question-induced cognitive biases in reports of dietary intake by college men and women. Health Psychol 1991;10:244-51

164. Willett W C, Stampfer MJ, Colditz GA, Rosner BA, Hennekens CH and Speizer FE. Dietary fat and the risk of breast cancer. N Engl J Med 1987;316:22-8.

165. Heady J A. Diets of bank clerks. Development of a method of classificying the diets of individuals for use in epidemiologic studies. J r Statist Soc 1967;124,336-361.

166. Subar A F, Thompson FE, Smith AF et.al. Improving food frequency questionnaires: a qualitative approach using cognitive interviewing. J Am Diet Assoc 1995;95:781-8.

167. Hankin JH, Rhoads GG, Glober GA. A dietary method for an epidemiologic study of gastrointestinal cancer. Am J Clin Nutr 1975;28:1055-60.

168. Willett WC, Sampson L, Browne ML, Stampfer MJ, Rosner B, Hennekens CH and Speizer FE. The use of a self-administered questionnaire to assess diet four years in the past. Am J Epidemiol 1998;127:188-99

169. Hunter DJ, Sampson L, Stampfer MJ, Coldtiz GA, Rosner B and Willet WC. Variability in portion sizes of commonly consumed foods among a population of women in the United States. Am J Epidemiol 1998;127:1240-1249.

170. Borrud LG, McPhearson RS, Nichalman MZ, Pillow PC, Newell GR. Development of a food frequency instrument: Ethnic differences in food sources. Nutr Cancer 1998;12: 201-211.

171. Michels KB, Bingham SA, Luben R, Welch AA, Day NE. The effect of correlated measurement error in multivariate models of diet.Am J Epidemiol 2004;160:59-67

172. Day N, McKeown N, Wong M, Welch A, Bingham S. Epidemiological assessment of diet: a comparison of a 7-day diary with a food frequency questionnaire using urinary markers of nitrogen, potassium and sodium. Int J Epidemiol 2001;30: 309-17.

173. Steinmetz KA, Potter JD. Vegetables, fruit, and cancer prevention: a review. J Am Diet Assoc 1996;96:1027-39.

174. Cullen KW, Baranowski T, Baranowski J, Hebert D, de Moor C. Pilot study of the validity and reliability of brief fruit, juice and vegetable screeners among inner city African-American boys and 17 to 20 year old adults. J Am Coll Nutr 1999;18:442-50.

175. Goldbohm RA, van't Veer P, van den Brandt PA, et al. Reproducibility of a food frequency questionnaire and stability of dietary habits determined from five annually repeated measurements. Eur J Clin Nutr 1995 49:420-9.

176. Kipnis V, Midthune D, Freedman LS, et al. Empirical evidence of correlated biases in dietary assessment instruments and its implications. Am J Epidemiol 2001;153;394-403.

177. Kipnis V, Schatzkin A, Midthune D, Subar A, Troiano RP. Comparison of a food frequency questionnaire with 24 hour recall for use in epidemiologic studies: results from the observing protein and energy nutrition (OPEN) study. The Fifth International Conference on Dietary Assessment Methods; 2003; Chiang Rai, Thailand.

178. Kipnis V, Carroll RJ, Freedman LS, Li L. Implications of a new dietary measurement error model for estimation of relative risk: application to four calibration studies. Am J Epidemiol 1999;150:642-51.

179. Block G, Norkus E, Hudes M, Mandel S, Helzlsouer K. Which plasma antioxidants are most related to fruit and vegetable consumption? Am J Epidemiol 2001;154:1113-8.

180. Campbell DR, Gross MD, Martini MC, Grandits GA, Slavin JL, Potter JD. Plasma carotenoids as biomarkers of vegetable and fruit intake. Cancer Epidemiol Biomark Prev 1994;3:493-500.

181. McEligot AJ, Rock CL, Flatt SW, Newman V, Faerber S, Pierce JP. Plasma carotenoids are biomarkers of long-term high vegetable intake in women with breast cancer. J Nutr 1999;129:2258-63.

182. van Kappel AL, Steghens JP, Zeleniuch-Jacquotte A, Chajes V, Toniolo P, Riboli E. Serum carotenoids as biomarkers of fruit and vegetable consumption in the New York Women's Health Study. Public Health Nutr 2001;4:829-35.

183. Le Marchand L, Hankin JH, Carter FS, et al. A pilot study on the use of plasma carotenoids and ascorbic acid as markers of compliance to a high fruit and vegetable dietary intervention. Cancer Epidemiol Biomark Prev 1994;3:245-51.

184. Rock CL, Swendseid ME, Jacob RA, McKee RW. Plasma carotenoid levels in human subjects fed a low carotenoid diet. J Nutr 1992;122:96-100.

185. Martini MC, Campbell DR, Gross MD, Grandits GA, Potter JD, Slavin JL. Plasma carotenoids as biomarkers of vegetable intake: the University of Minnesota Cancer Prevention Research Unit Feeding Studies. Cancer Epidemiol Biomark Prev 1995;4:491-6.

186. Benzie IF. Vitamin C: prospective functional markers for defining optimal nutritional status. Proc Nutr Soc 1999; 58:469-76.

187. Drewnowski A, Rock CL, Henderson SA et al. Serum h-carotene and vitamin C as biomarkers of vegetable and fruit intakes in a community-based sample of French adults. Am J Clin Nutr 1997;65:1796-802.

188. Borel P, Grolier P, Mekki N, et al. Low and high responders to pharmacological doses of h-carotene: proportion in the population, mechanisms involved and consequences on h-carotene metabolism. J Lipid Res 1998; 39:2250-60.

189. Parker RS, Swanson JE, You CS, Edwards AJ, Huang T. Bioavailability of carotenoids in human subjects. Proc Nutr Soc 1999; 58:155-62.

190. Roodenburg AJ, Leenen R, het Hof KH, Weststrate JA, Tijburg LB.Amount of fat in the diet affects bioavailability of lutein esters but not of a-carotene, h-carotene, and vitamin E in humans. Am J Clin Nutr 2000; 71: 1187-93.

191. Brady WE, Mares-Perlman JA, Bowen P, Stacewicz-Sapuntzakis M. Human serum carotenoid concentrations are related to physiologic and lifestyle factors. J Nutr 1996;126:129-37.

192. Kitamura Y, Tanaka K, Kiyohara C, et al. Relationship of alcohol use, physical activity and dietary habits with serum carotenoids, retinol and a-tocopherol among male Japanese smokers. Int J Epidemiol 1997;26: 307-14.

193. Olmedilla B, Granado F, Blanco I, Rojas-Hidalgo E. Seasonal and sexrelated variations in six serum carotenoids, retinol, and a-tocopherol. Am J Clin Nutr 1994; 60:106-10.

194. Rock CL, Flatt SW, Wright FA, et al. Responsiveness of carotenoids to a high vegetable diet intervention designed to prevent breast cancer recurrence. Cancer Epidemiol Biomark Prev 1997;6:617-23.

195. Alberg A. The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients. Toxicology 2002;180:121-37.

196. Nielsen SE, Freese R, Kleemola P, Mutanen M. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. Cancer Epidemiol Biomark Prev 2002;11: 459-66.

197. Brevik A, Rasmussen SE, Drevon CA, Andersen LF. Urinary excretion of flavonoids reflects even small changes in the dietary intake of fruits and vegetables. Cancer Epidemiol Biomarkers Prev 2004;13:843-9.

198. Nielsen SE, Freese R, Kleemola P, Mutanen M. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. Cancer Epidemiol Biomarkers Prev. 2002;11:459-66.

199. Young JF, Nielsen SE, Haraldsdóttir J, Daneshvar B, Lauridsen ST, Knuthsen P, Crozier A, Sandström B, Dragsted LO. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Am J Clin Nutr. 1999 Jan;69:87-94.

200. Noroozi M, Burns J, Crozier A, Kelly IE, Lean ME. Prediction of dietary flavonol consumption from fasting plasma concentration or urinary excretion. Eur J Clin Nutr 2000; 54:143-9.

201. Radtke J, Linseisen J, Wolfram G. Fasting plasma concentrations of selected flavonoids as markers of their ordinary dietary intake. Eur J Nutr 2002;41:203-9.

202. Erlund I, Marniemi J, Hakala P, Alfthan G, Meririnne E, Aro A. Consumption of black currants, lingonberries and bilberries increases serum quercetin concentrations. Eur J Clin Nutr 2003; 57:37- 42.

203. Freese R, Alfthan G, Jauhiainen M, Basu S, Erlund I, Salminen I, et al. High intakes of vegetables, berries, and apples combined with a high intake of linoleic or oleic acid only slightly affect markers of lipid peroxidation and lipoprotein metabolism in healthy subjects. Am J Clin Nutr 2002;76:950- 60.

204. Nielsen SE, Freese R, Kleemola P, Mutanen M. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. Cancer Epidemiol Biomark Prev 2002;11:459- 66.

205. Young JF, Nielsen SE, Haraldsdottir J, Daneshvar B, Lauridsen ST, Knuthsen P, et al. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Am J Clin Nutr 1999; 69: 87- 94.

206. Lown KS, Fontana RJ, Schmiedlin-Ren P, Turgeon DK, Watkins PB. Interindividual variation in intestinal mdr1: lack of short term diet effects. Gastroenterology 1995;108: A737.

207. Kerb R, Hoffmeyer S, Brinkmann U. ABC drug transporters: hereditary polymorphisms and pharmacological impact in MDR1, MRP1 and MRP2. Pharmacogenomics 2001;2:51- 64.

208. van der Kolk DM, de Vries EG, van Putten WJ, Verdonck LF, Ossenkoppele GJ, Verhoef GE, et al. P-Glycoprotein and multidrug resistance protein activities in relation to treatment outcome in acute myeloid leukemia. Clin Cancer Res 2000;6:3205- 14.

209. Hall SD, Thummel K, Watkins PB, Lown KS, Benet LZ, Paine MF, et al. Molecular and physical mechanisms of first-pass extraction. Drug Metab Dispos 1999;27:161-6.

210. Dai D, Tang J, Rose S, Hodgson E, Bienstock RJ, Mohrenweiser HW, et al. Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. J Pharmacol Exp Ther 2001;299:825- 31.

211. Fisher MB, VandenBraden M, Findlay K, Burchell B, Thummel K, Hall SD. Tissue distribution and interindividual variation in human UDP-glucuronosyltransferase activity: relationship between UGT1A1 promoter genotype and variability in a liver bank. Pharmacogenetics 2000;10:727- 39.

212. Her C, Szumlanski C, Aksoy IA, Weinshilbaum RM. Human jejunal estrogen sulfotransferase and dehydroepiandrosterone sulfotransferase. Immunochemical characterization of interindividual variation. Drug Metab Dispos 1996;24:1328- 35.

213. Shapiro AB, Ling V. Effect of quercetin on Hoechst 33342 transport by purified and reconstituted P-glycoprotein. Biochem Pharmacol 1997;53:587- 96.

214. Leslie EM, Mao Q, Oleschuk CJ, Deeley RG, Cole SP. Modulation of multidrug resistance protein 1 (MRP1/ABCC1) transport and ATPase activities by interaction with dietary flavonoids. Mol Pharmacol 2001;59:1171- 80.

215. Walgren RA, Lin J-T, Kinne R, Walle T. Cellular uptake of dietary flavonoid quercetin 4V-b-glucoside by sodium-dependent glucose transporter SGLT1. J Pharmacol Exp Ther 2000; 294:837- 43.

216. Cheng Z, Radominska-Pandya A, Tephly TR. Studies on the substrate specificity of human intestinal UDP-glucuronosyltransferases 1A8 and 1A10. Drug Metab Dispos 1999;27:116 -70.

217. Oliveira EJ, Watson DG. In vitro glucuronidation of kaempferol and quercetin by human UGT-1A9 microsomes. FEBS Lett 2000;471:1-6.

218. Boersma MG, van der Woude H, Bogaards J, Boeren S, Vervoort J, Cnubben NH, et al. Regioselectivity of phase II metabolism of luteolin and quercetin by UDP-glucuronosyl transferases. Chem Res Toxicol 2002;15: 662-70.

219. Thummel KE, Kunze KL, Shen DD. Enzyme-catalyzed processes of first-pass hepatic and intestinal drug extraction. Adv Drug Deliv Rev 1997; 27:99- 127.

220. Meibohm B, Beierle I, Derendorf H. How important are gender differences in pharmacokinetics? Clin Pharmacokinet 2002;41:329- 42.

221. Burchell B. Genetic variation of human UDP-glucuronosyltransferase: implications in disease and drug glucuronidation. Am J Pharmacogenomics 2003;3:37- 52.

222. Desai AA, Innocenti F, Ratain MJ. UGT pharmacogenomics: implications for cancer risk and cancer therapeutics. Pharmacogenetics 2003;13:517- 23.

223. Gee JM, Watson M, Matthew JA, Rhodes M, Speakman CJM, Stebbings WSL and Johnson IT. Consumption of fish oil leads to prompt incorporation of eicosapentaenoic acid into colonic mucosa of patients prior to surgery for colorectal cancer, but has no detectable effect on epithelial cytokinetics. J. Nutr 1999;129:1862-1865.

224. Vandenlangenberg GM, Mares-Perlman JA, Brady WE, Klein BE, Klein R, Palta M, Block G. Incorporating fat-modified foods into a food frequency questionnaire improves classification of fat intake. J Am Diet Assoc.1997;97:860-6.

225. Greeley S, Storbakken L, Magel R. Use of a modified food frequency questionnaire during pregnancy. J Am Coll Nutr. 1992;11:728-34.

226. DuPont MS, Day AJ, Bennett RN, Mellon FA, Kroon PA. Absorption of kaempferol from endive, a source of kaempferol-3-glucuronide, in humans. Eur J of Clin Nutr 2004; 58:947-954.

227. DuPont MS, Bennett RN, Mellon FA, Williamson G. Polyphenols from alcoholic apple cider are absorbed, metabolized and excreted by humans. J Nutr. 2002 Feb;132:172-5.

228. USDA Database for the Flavonoid Content of Selected Food. (http://www.nal.usda.gov/fnic/foodcomp/Data/Flav/flav.pdf.)

229. Young JF, Nielsen SE, Haraldsdottir J et. al. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Am J Clin Nutr 1999; 69: 87-94.

230. de Vries JH, Hollman PC, Meyboom S, Buysman MN, Zock PL, van Staveren WA, Katan MB. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. Am J Clin Nutr. 1998;68:60-5.

231. Nielsen SE, Freese R, Kleemola P, Mutanen M. Flavonoids in human urine as biomarkers for intake of fruits and vegetables. Cancer Epidemiol Biomarkers 2002; 11: 459-66.

232. Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. J Nutr 2000;130:2073S-85S.

233. Crespy V, Morand C, Besson C, Manach C, Demigne C, Remesy C. Quercetin, but not its glycosides, is absorbed from the rat stomach. J Agric Food Chem 2002; 50: 618-21.

234. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727-47.

235. Hollman PC, van Trijp JM, Buysman MN, van der Gaag MS, Mengelers MJ, de Vries JH, Katan MB. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. FEBS Lett 1997;418:152-6.

236. Young JF, Nielsen SE, Haraldsdottir J, Daneshvar B, Lauridsen ST, Knuthsen P, Crozier A, Sandstrom B, Dragsted LO. Effect of fruit juice intake on urinary quercetin excretion and biomarkers of antioxidative status. Am J Clin Nutr 1999;69:87-94.

237. Ishii K, Furuta T, Kasuya Y. Mass spectrometric identification and high-performance liquid chromatographic determination of a flavonoid glycoside naringin in human urine. J Agric Food Chem 2000;48 56-9.

238. Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C, Rémésy. Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. Eur J Clin Nutr 2003;57:235-42.

239. Erlund I, Meririnne E, Alfthan G, Aro A.Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. J Nutr 2001;131:235-41.

240. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Bioavailability and bioefficacy of polyphenols in humans. Review of 97 bioavailability studies. Am J Clin Nutr 2004;79:727-47

241. Block, G.Human dietary assessment: methods and issues. Prev Med 1989;18: 653-60.

242. Thompson, F. E and Byers T. Dietary assessment resource manual. J Nutr 1994; 124:2245-2317.

243. Crawford PB, Obarzanek E, Morrison J, Sabry ZI. Comparative advantage of 3-day food records over 24-hour recall and 5-day food frequency validated by observation of 9- and 10-year-old girls. J Am Diet Assoc 1994;94: 626-30.

244. Willett WC. Nutritional epidemiology. 2nd ed. New York: Oxford University Press, 1998.1234

245. Brunner E, Stallone D, Juneja M, Bingham S, Marmot M. Dietary assessment in Whitehall II: comparison of 7 d diet diary and food-frequency questionnaire and validity against biomarkers. Br J Nutr 2001;86:405-14.

246. Salvo VL, Gimeno SG. Reproducibility and validity of a food frequency questionnaire. Rev Saude Publica. 2002 Aug;36:505-12.

247. Resnicow K, Odom E, Wang T, Dudley WN, Mitchell D, Vaughan R, Jackson A, Baranowski T.Validation of three food frequency questionnaires and 24-hour recalls with serum carotenoid levels in a sample of African-American adults. Am J Epidemiol 2000; 152: 1072-80.

247. Newby PK, Hu FB, Rimm EB, Smith-Warner SA, Feskanich D, Sampson L, Willett WC. Reproducibility and validity of the Diet Quality Index Revised as assessed by use of a food-frequency questionnaire. Am J Clin Nutr 2003;78:941-9.

249. Abnet CC, Freedman ND, Kamangar F, Leitzmann MF, Hollenbeck AR, Schatzkin A. Non-steroidal anti-inflammatory drugs and risk of gastric and oesophageal adenocarcinomas: results from a cohort study and a meta-analysis. Br J Cancer 2009; 100:551-7

250. Harris RE, Beebe-Donk J, Doss H, Burr Doss D. Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade. Oncol Rep 2005;13:559-83.

251. Epplein M, Nomura AM, Wilkens LR, Henderson BE, Kolonel LN. Nonsteroidal antiinflammatory drugs and risk of gastric adenocarcinoma: the multiethnic cohort study. Am J Epidemiol 2009;170:507-14

252. Cheng KK, Sharp L, McKinney PA, Logan RF, Chilvers CE, Cook-Mozaffari P, Ahmed A, Day NE. A case-control study of oesophageal adenocarcinoma in women: a preventable disease. Br J Cancer 2000;83:127-132.

253. Sharp L, Chilvers CE, Cheng KK, McKinney PA, Logan RF, Cook-Mozaffari P, Ahmed A, Day NE. Risk factors for squamous cell carcinoma of the oesophagus in women: a case-control study. Br J Cancer 2001;85:1667-1670.

254. Corley DA, Kerlikowske K, Verma R, Buffler P. Protective association of aspirin/NSAIDs and oesophageal cancer: a systematic review and meta-analysis. Gastroenterology 2003;124:47-56.

255. Patrono C, Coller B, Dalen JE, FitzGerald GA, Fuster V, Gent M, Hirsh J, Roth G. Platelet-active drugs: the relationships among dose, effectiveness, and side effects. Chest 2001;119:39-63.

256. Friis S, Sorensen HT, McLaughlin JK, Johnsen SP, Blot WJ, Olsen JH. A population-based cohort study of the risk of colorectal and other cancers among users of low-dose aspirin. Br J Cancer 2003;88:684-688.

257. Bardou M, Barkun AN, Ghosn J, Hudson M, Rahme E. Effect of chronic intake of NSAIDs and cyclooxygenase 2-selective inhibitors on oesophageal cancer incidence. Clin Gastroenterol Hepatol 2004;2:880-887.

258. Hao J, Liu B, Yang CS, Chen X. Gastroesophageal reflux leads to oesophageal cancer in a surgical model with mice. Gastroenterol 2009;9:59.

259. Oyama K, Fujimura T, Ninomiya I, Miyashita T, Kinami S, Fushida S, Ohta T, Koichi M. A COX-2 inhibitor prevents the oesophageal inflammation-metaplasia-adenocarcinoma sequence in rats. Carcinogenesis 2005;26:565-70.

260. Lagergren J, Bergstrom R, Adami HO, Nyren O. Association between medications that relax the lower esophageal sphincter and risk for esophageal adenocarcinoma. Ann Intern Med 2000;133:165-175.

261. Vaughan TL, Farrow DC, Hansten PD et.al. Risk of oesophageal and gastric adenocarcinomas in relation to use of calcium channel blockers, asthma drugs, and other Medications that promote gastroesophageal reflux. Cancer Epidemiol Biomarkers Prev 1998; 7:749-756.

262. Corley DA, Levin TR, Habel LA, Buffler PA. Barrett's esophagus and medications that relax the lower esophageal sphincter. Am J Gastroenterol. 2006 May;101:937-44.

263. Astarita C, Gargano D, Cutajar M, Napolitano A, Manguso F, Abbate GF. Gastroesophageal reflux disease and asthma: an intriguing dilemma.Allergy 2000;55: 52-5.

264. Leggett JJ, Johnston BT, Mills M, Gamble J, Heaney LG. Prevalence of gastroesophageal reflux in difficult asthma: relationship to asthma outcome. Chest 2005; 127:1227-31.

265. Hoyo C, Cook MB, Kamangar F, Freedman ND et.al. Body mass index in relation to oesophageal and oesophagogastric junction adenocarcinomas: a pooled analysis from the International BEACON Consortium. Int J Epidemiol. 2012 Dec; 41:1706-18.

266. O'Doherty MG, Freedman ND, Hollenbeck AR, Schatzkin A and Abnet CC . A prospective cohort study of obesity and risk of esophageal and gastric adenocarcinoma in the NIH-AARP Diet and Health study.Gut. 2012 September; 61:1261–1268.

267. Lagergren J.Oesophageal cancer and gastro-oesophageal reflux: what is the relationship. Gut. 2004 Aug; 53:1064-5.262.

268. Solaymani-Dodaran M, Logan RF, West J, Card T, Coupland C. Risk of oesophageal cancer in Barrett's oesophagus and gastro-oesophageal reflux. Gut. 2004 Aug; 53:1070-4.

269. Newnham A, Quinn MJ, Babb P, Kang JY, Majeed A. Trends in oesophageal and gastric cancer incidence, mortality and survival in England and Wales 1971-1998/1999. Aliment Pharmacol Ther 2003;17:655-664.

270. Harding SM, Richter JE. Gastroesophageal reflux disease and asthma. Semin Gastrointestinal Dis 1992;3:139-150.

271. Sontag SJ, Schnell TG, Miller TQ, Khandelwal S, O'Connell S, Chejfec G, Greenlee H, Seidel UJ, Brand L. Prevalence of oesophagitis in asthmatics. Gut 1992;33: 872-876.

272. DiMarino AJ, Jr., Cohen S. Effect of an oral beta2-adrenergic agonist on lower oesophageal sphincter pressure in normals and in patients with achalasia. Dig Dis Sci 1982; 27: 1063-1066.

273. Wyllie AH. Apoptosis (the 1992 Frank Rose Memorial Lecture). Br J Cancer 1993; 67:205-208.

274. Fitzpatrick AL, Daling JR, Furberg CD, Kronmal RA, Weissfeld JL. Use of calcium channel blockers and breast carcinoma risk in postmenopausal women. Cancer 1997; 80:1438-1447.

275. Pahor M, Guralnik JM, Ferrucci L, Corti MC, Salive ME, Cerhan JR, Wallace RB, Havlik RJ. Calcium-channel blockade and incidence of cancer in aged populations. Lancet 1996;348:493-497.

276. Beiderbeck-Noll AB, Sturkenboom MC, van der Linden PD, Herings RM, Hofman A, et.al. Verapamil is associated with an increased risk of cancer in the elderly: the Rotterdam study. Eur J Cancer 2003;39:98-105.

277. Stookey, JD, Constant, F, Gardner, CD, Popkin, BM. Replacing sweetened caloric beverages with drinking water is associated with lower energy intake. Obesity 2007;15:3013.

278. Katcher, HI, Hill, AM, Lanford, JL, et al. Lifestyle approaches and dietary strategies to lower LDL-cholesterol and triglycerides and raise HDL-cholesterol. Endocrinol Metab Clin North Am 2009;38:45.

279. Olendzki, et al. Underreporting of energy intake and associated facotrs in a Latino population at risk of developing type 2 diabetes. J Am Diet Assoc 2008;108:1003.

280. Wansink, B, Van Ittersum, K. Portion Size Me: Downsizing our consumption norms. J Am Diet Assoc 2007;107:1103.

281. Olendzki, BC, Ma, Y, Schneider, KL, et al. A simple dietary message to improve dietary quality: Results from a pilot investigation. Nutrition 2009; 25:736.

282. Larkin FA, Metzner HL, Thompson FE, Flegal KM, Guire KE.. Comparison of estimated nutrient intakes by food frequency and dietary records in adults. J Am Diet Assoc 1989;89:215-23.

283. Briefel RR, Flegal KM, Winn DM, Loria CM, Johnson CL, Sempos CT. Assessing the nation's diet: limitations of the food frequency questionnaire.J Am Diet Assoc 1992; 92:959-62.

284. Subar AF, Ziegler RG, Thompson FE et.al. Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial Investigators. Is shorter always better? Relative importance of questionnaire length and cognitive ease on response rates and data quality for two dietary questionnaires. Am J Epidemiol 2001;153:404-9.

285. Willett W C, Stampfer MJ, Colditz GA, Rosner BA, Hennekens CH and Speizer FE. Dietary fat and the risk of breast cancer.N Engl J Med 1987;31:22-8

286. Warneke CL, Davis M, De Moor C, Baranowski T. A 7-item versus 31-item food frequency questionnaire for measuring fruit, juice, and vegetable intake among a predominantly African-American population. J Am Diet Assoc 2001;101:774-9

287. Subar A F, Thompson FE, Smith AF, Jobe JB, Ziegler RG, Potischman N, Schatzkin A, Hartman A, Swanson C, Kruse L, Hayes RB, Lewis DR and Harlan IC. Improving food frequency questionnaires: a qualitative approach using cognitive interviewing. J Am Diet Assoc 1995; 95:781-8.

288. Block G, Hartman AM. Issues in reproducibility and validity of dietary studies. Am J Clin Nutr 1989;50:1133-8.

289. Tjonneland A, Haraldsdottir J, Overvad K, Stripp C, Ewertz M, Jensen OM. Influence of individually estimated portion size data on the validity of a semiquantitative food frequency questionnaire. Int J Epidemiol 1992;21:770-7.

290. Clapp JA, McPherson RS, Reed DB, Hsi BP. Comparison of a food frequency questionnaire using reported vs standard portion sizes for classifying individuals according to nutrient intake. J Am Diet Assoc 1991;91:316-20.

291. Noethlings U, Hoffmann K, Bergmann MM. Boeing H. Portion Size Adds Limited Information on Variance in Food Intake of Participants in the EPIC-Potsdam Study. J Nutr 2003;133:510-5.

292. Khan, KN, Stanfield, KM, Harris, RK, Baron, DA. Expression of cyclooxygenase-2 in the macula densa of human kidney in hypertension, congestive heart failure, and diabetic nephropathy. Ren Fail 2001;23:321.

293. Caughey, GE, Cleland, LG, Penglis, PS, et al. Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2. J Immunol 2001;167:2831.

294. Cheng, Y, Austin, SC, Rocca, B, et al. Role of prostacyclin in the cardiovascular response to thromboxane A2. Science 2002;296:539.

295. Juni, P, Nartey, L, Reichenbach, S, et al. Risk of cardiovascular events and rofecoxib: cumulative meta-analysis. Lancet 2004;364:2021.

296. Kerr, DJ, Dunn, JA, Langman, MJ et al. Rofecoxib and cardiovascular adverse events in adjuvant treatment of colorectal cancer. N Engl J Med 2007;357:360.

297. Ray, W, Stein, C, Daugherty, J,= et al. COX-2 selective non-steroidal anti-inflammatory drugs and risk of serious coronary heart disease. Lancet 2002;360:1071.

298. Solomon, DH, Schneeweiss, S, Glynn, RJ, et al. Relationship between selective cyclooxygenase-2 inhibitors and acute myocardial infarction in older adults. Circulation 2004;109:2068.

299. Graham, DJ, Campen, D, Hui, R, et al. Risk of acute myocardial infarction and sudden cardiac death in patients treated with cyclo-oxygenase 2 selective and non-selective non-steroidal anti-inflammatory drugs: nested case-control study. Lancet 2005; 365:475.

300. Andersohn, F, Suissa, S, Garbe, E. Use of first- and second-generation cyclooxygenase-2-selective nonsteroidal antiinflammatory drugs and risk of acute myocardial infarction. Circulation 2006;113:1950.

301. Levesque, LE, Brophy, JM, Zhang, B. The risk for myocardial infarction with cyclooxygenase-2 inhibitors: a population study of elderly adults. Ann Intern Med 2005; 142:481.

302. Farkouh, ME, Kirshner, H, Harrington, RA, et al. Comparison of lumiracoxib with naproxen and ibuprofen in the Therapeutic Arthritis Research and Gastrointestinal Event Trial (TARGET), cardiovascular outcomes: randomised controlled trial. Lancet 2004; 364:675.

303. Jacobs EJ; Thun MJ; Bain EB; Rodriguez C; Henley SJ; Calle EE A large cohort study of long-term daily use of adult-strength aspirin and cancer incidence. J Natl Cancer Inst 2007;99 608-15.

304. Deeks JJ, Smith LA, Bradley MD. Efficacy, tolerability, and upper gastrointestinal safety of celecoxib for treatment of osteoarthritis and rheumatoid arthritis: systematic review of randomised controlled trials. BMJ 2002;325:619-623

305. Feskanich D; Ziegler RG; Michaud DS; Giovannucci EL; Speizer FE; Willett WC; Colditz GA. Prospective study of fruit and vegetable consumption and risk of lung cancer among men and women. J Natl Cancer Inst 2000;92:1812-23.

306. Smith-Warner SA; Spiegelman D; Yaun SS et.al. Intake of fruits and vegetables and risk of breast cancer: a pooled analysis of cohort studies. JAMA 2001;285:769-76.

307. Michels KB; Edward Giovannucci; Joshipura KJ et.al. Prospective study of fruit and vegetable consumption and incidence of colon and rectal cancers. J Natl Cancer Inst 2001;92:1740-52.

308. Koushik A; Hunter DJ; Spiegelman D et.al. Fruits, vegetables, and colon cancer risk in a pooled analysis of 14 cohort studies. J Natl Cancer Inst. 2007;99:1471-83.

309. Cheng KK, Day NE. Nutrition and esophageal cancer. Cancer Causes Control. 1996;7:33-40.

310. Freedman ND, Park Y, Subar AF, Hollenbeck AR, Leitzmann MF, Schatzkin A, Abnet CC. Fruit and vegetable intake and esophageal cancer in a large prospective cohort study. Int J Cancer. 2007 Dec 15;121:2753-60.

16.0. Appendices:

Appendix 1: Case Control Study Protocol

SUMMARY:

The incidence of oesophageal cancer is increasing and is associated with a poor survival rate of less than 5 % at 5 years. This has lead to increasing efforts to identify potential chemo-preventive agents. There is limited evidence that aspirin and non-steroidal anti-inflammatory drugs are protective in a dose dependent manner. However, there are no studies of adequate power to compare the potential protective affects of aspirin and cyclo-oxygenase inhibitors, specifically cyclo-oxygenase-2 drugs in a UK population using multiple methods for patient identification. This investigation will measure the incidence of oesophageal cancer in Norfolk and the data acquired will be used to plan future aetiological investigations. It will facilitate the calculation of the population size and monitoring period required for subsequent aetiological case-control studies. Patient's records from individuals who have been diagnosed with oesophageal cancer will be reviewed to determine both the frequency and the use of aspirin and cyclo-oxygenase inhibitor drugs, including selective COX-2 blockers. These data will be compared with the use of the same drugs in a control group of patients selected from dermatology clinics, to determine if there is a protective effect. Demonstration of such an effect would suggest a preventive strategy for oesophageal cancer in both the general population and in high risk groups.

AIMS :

1. To measure incidence of oesophageal cancer in Norfolk between 1st April 1995 and December 31st 2004.

2. To determine if aspirin and cyclo-oxygenase-2 inhibitor drugs protect against the development of oesophageal cancer in a case-control study.

INTRODUCTION :

Great Britain has one of the highest incidences of oesophageal cancer in Europe, with approximately 7,000 new cases been diagnosed annually [(1)]. The overall incidence is rising with a two fold increase in Scotland in the last 8 years rising from 8/100,000 cases/year to 15/100,000 cases/year[(2),]**.** The disease is commoner in men than women and incidence increases with age. Furthermore, the highest incidence of oesophageal cancer in the world in women is in United Kingdom[(3)]. The prognosis for patients with oesophageal cancer is poor with an overall 5 year survival of less then 5%, primarily because most patients present with advanced disease.

Clinical and epidemiological studies have reported a change in the two main histological types of oesophageal cancer i.e. squamous cell carcinoma and adenocarcinoma**[(4)]**. Thirty years ago squamous cell cancer was commoner then adenocarcinoma but now this trend is reversed[(5)]. Also a greater number of gastric tumours are being diagnosed at the cardia and gastro-oesophageal junction compared to the distal stomach**[(6)]**. The reasons for these shifts in anatomical and histological pattern are currently unknown.

The well demonstrated risk factors for squamous cell cancer are tobacco and alcohol, each increasing the risk by 2-3 fold[(7)]. In the case of adenocarcinoma noted factors are obesity, a history of gastro oesophageal reflux disease and dietary deficiencies such as lack of fruits and vegetables are potential risk factors. Barrett's oesophagus is a recognised pre-malignant condition for adenocarcinoma, increasing the risk by 50 times[(8,9)].

Potential protective factors for oesophageal cancer are both aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) with two studies from the UK showing a protective effect of 40 %[(7, 11)]. However, these studies had several limitations including being restricted only to women, small patient numbers and lack of differentiation between aspirin and selective cyclo-oxygenase-2 and all COX

inhibitors. NSAIDs are currently classed as either COX enzyme inhibitors which block both the COX I & 2 subtypes of enzymes or COX 2 inhibitors which are specific for this form of the enzyme. Furthermore, the potential effects of selective cyclo-oxygenase-2 inhibitors were not investigated in the two studies cited above [7,10].

The potential protective effects of NSAIDs are supported by plausible biological mechanisms. The cyclo-oxygenase 2 enzyme is over expressed in oesophageal cancer tissue and Barrett's oesophagus, leading to an excess production of prostaglandins[9]. This over expression firstly prevents apoptosis, the process of programmed cell death and secondly induces angiogenesis, both of which promote neoplastic changes. COX-2 inhibitors have been shown to reduce cellular proliferation in Barrett's cell lines[11] and can reduce the risk of oesophageal adenocarcinoma induced by reflux in a rat model[10]. Furthermore, NSAIDs decrease the risk of development of Barrett's oesophagus by reducing the inflammatory response associated with intestinal metaplasia[12]. Finally cyclo-oxygenase-2 selective inhibitors suppress mitogen or growth factor induced prostaglandin production and inflammation thus reducing cellular proliferation and oxidative damage from free radicals [13]. The potential protective effect of NSAIDs are thus mediated by the promotion of apoptosis via a reduction in PGE2 production and elevation of arachdonic acid levels[14] .

The incidence and case-control studies proposed here will have several advantages over previous work. Firstly multiple databases will be used to identify patients with oesophageal cancer, including pathology records, discharge data and endoscopy databases. The case control study has been designed to achieve adequate power, include both men and women and to also specifically investigate selective cyclo-oxygenase-2 inhibitors. Demonstrating a positive association between the use of NSAIDs and a reduced rate of oesophageal cancer would suggest a preventive strategy for managing the progression of Barrett's oesophagus to cancer and help to evaluate the mechanism of carcinogenesis. The incidence study will

generate invaluable data for planning future case-control studies to investigate the effect of diet in the aetiology of oesophageal cancer.

METHODS:

A) **An incidence study of oesophageal cancer in Norfolk and a cross - sectional analysis of aspirin and NSAID use in patients.**

i. ***Disease definition*:** All primary malignant neoplasm's of the oesophagus and cardia diagnosed between 1st April 1995 and 31st December 2004 will be included using the International Classification of Diseases -10 codes. These codes are :

- C15.0 : Cervical part of oesophagus
- C15.1 : Thoracic part of oesophagus
- C15.2 : Abdominal part of oesophagus
- C15.3 : Upper part of oesophagus
- C15.4 : Middle part of oesophagus
- C15.5 : Lower part of oesophagus
- C15.8 : Overlapping lesion of oesophagus
- C16.0 : Cardia :
    - Cardiac orifice
    - Cardio-oesophageal junction
    - Gastro-oesophageal junction
    - Oesophagus and stomach

These neoplasm's will be divided into squamous cell carcinoma and adenocarcinoma and include patients who have received either surgical or palliative treatment.

ii. ***Patient identification*****:** The data will be collected by a retrospective review of hospital notes of patients identified from pathology databases, endoscopy databases and hospital discharge records. The use of these multiple sources will improve the inclusion rate of appropriate patients. Information will be collected regarding the site of tumour, histology, treatment, outcomes and use of medication.

iii. ***Study population*****:** The study population will comprise both men and women in the postal region of Norfolk. Details of the structure of this population approximately 500,000 people will be obtained from the 2001 census. Patients living in this region may present to one of the three hospitals: The Norfolk & Norwich University Hospital NHS Trust; Norwich; The Queen Elizabeth II Hospital, King's Lynn and The James Paget Hospital, Great Yarmouth. Clinicians in each hospital will be approached for permission to review patient notes.

iv. ***Data collection:*** Demographic and clinical data on drugs, illnesses and surgical management for each proven case will be collected on a standard data collection form (Appendix - 1) and anonymised. The data on drug type, frequency of administration and dosage and the illnesses will be ascertained from the general practitioner referral letter, medical admission notes and nursing records. Drugs use will be subdivided into regular (daily) use and non-regular (less than daily) use. Data on the consumption of alcohol, smoking, past history of Barrett`s will be collected.

The incidence study will also allow us to describe the surgical management of the patients. These data will include the clinical presentation of the patient, type of surgical management, the ASA anaesthetic grading of the patient's fitness for surgery and outcome. The aim of collecting these data will be to identify what is current practice in the

surgical management of oesophageal cancer, including the outcome of the different types of surgical procedures. The study does not aim to examine or judge the management of individual cases but to identify trends in clinical management and patient outcomes. All patient and hospital information will be anonymised and kept strictly confidential.

v. ***Analysis:*** The mean crude incidence per 100 000 per annum in Norfolk will be calculated with 95% confidence intervals. A gender adjusted rate will be computed correcting for differences in age structures between men and women. By using population data from the 2001 census, age and sex specific case fatality rates will be calculated as well as the total number of expected cases in the UK based on the incidence rate in Norfolk.

vi. ***Approach*** : Permission will be requested from all Consultants who have treated patients in the study, Hospital Research & Development Committee and relevant Ethics Committee to review the medical notes.

B) **A Case control study to determine if aspirin and cyclo-oxygenase inhibitors including specific COX-2 inhibitors protect against the development of oesophageal cancer.**

i. ***Subject recruitment:*** Cases will be identified and confirmed by the methods described in the incidence study above. Cases in which there is either no histological confirmation of cancer or in which a diagnosis is suspected but there are no confirmatory investigations will be excluded. The number of such patients should be small. Dermatology patients will form the control group with 4 controls matched to each case for gender, age (+/-1year) and year of diagnosis. Data will be collected on controls by reviewing the notes of the dermatology patients, under the care of dermatologists and plastic surgeons, who have undergone a minor day case procedure for non-melanotic skin tumours. Patients with non-melanotic skin tumours will be identified as per the ICD 10 criteria. This control group has been selected as their use of NSAIDs should be similar to that in the general population. Any control who has a past history of oesophageal cancer will be excluded. Data will be collected on the dosage & frequency of use of NSAIDs and potential confounding factors including smoking and alcohol (Appendix - 2). This information will be obtained from the general practitioner's referral letters and hospital medical and nursing records.

ii. ***Analysis:*** Crude odds ratios will be calculated using conditional logistic regression and adjusted for the effects of smoking and alcohol. Power calculations show that 411 cases are required matched with 4 controls per case to show a protective effect of NSAIDs of 0.5 with 80 % power at a 5 % significance level.

Data Collection Sheet A: (Case Group) **Appendix-1**

Patient Label

Dermographic Data :

Code :

1. Name
2. Date of Birth
3. Age on admission
4. Hospital No.
5. Study Identifier
6. Postcode (e.g. NR=1)
7. Hospital
   (Norwich=1; Gr Yarmouth=2; Kings Lynn=3)
8. Gender (Male=1; Female=2)
9. Ethnic Group
   (Caucasian=1; Asian=2; Afro-Caribbean=3;
   Other=4)

Risk Factors

1. Smoking
   (No=0; Ex smoker=1; Current smoker=2)
2. Number of cigarettes/day
3. Alcohol
   (No=0; Ex alcohol=1; Current alcohol=2)
4. Amount of alcohol (units/week)
5. Known Barrett's
   (No=0; Yes=1)

6. Length of Barrett's
   (0-3cms=1; >3cms=2)

7. Actual length in cms

8. Previous endoscopic histology
   (Normal=0; Mild Dysplasia=1; Moderate Dysplasia=2; Severe Dysplasia=3)

9. Time between diagnosis of dysplasia and diagnosis of cancer

10. Time between first diagnosis of Barrett's and cancer diagnosis in years.

Previous Investigations:

1. Have had previous Endoscopy
   (No=0; Yes=1)

2. If Yes, Date : dd/mm/yyyy

3. Macroscopic findings of last endoscopy
   (Normal=0;Barrett`s=1;Achalasia=2)

4. Time between last gastroscopy and diagnosis of cancer

Current Investigations:

1. Tumour Site (From mouth in cms)

2. Present histology
   (Adenoca=1,Squamous=2)

3. Degree of differentiation
   (Mild=1; moderate=2; severe=3)

4. Radiological Investigation
   (CT=1; Barium=2; EUS=3)

5. Disease code (ICD)
   (C15.0=1;15.1=2;15.2=3;15.3=4;15.4=5;
   15.5=6, C16.0=7)

6. Preoperative Staging
   T_1= 1, T_2= 2,T_3= 3, T_4= 4
   N_1= 1, N_2= 2, N_3= 3, N_4= 4
   M_0= 1, M_1= 1

Treatment

1. ASA Grading
   (I=1, II= 2, III= 3, IV= 4)
2. Treatment
   (Surgery=1; Palliation=2)

3. Surgery
   (Ivor-Levis=1; Trans-hiatial=2; Lt Thoraco-abd; Other=3)

4. Histological Staging
   T_1= 1, T_2= 2, T_3= 3, T_4= 4
   N_1= 1, N_2= 2, N_3= 3, N_4= 4
   M_0= 1, M_1= 1

Prognosis

1. 30 Day Mortality
   (Alive= 1, Dead= 2)

2. Cause of Death (30 day period)
   (Anastomotic leak= 1, DVT/PE= 2, MI= 3, Stroke= 4, Resp infection= 5, Aspiration=6, Other= 7)

3. Length of hospital stay in days

4. Five year survival post diagnosis
   (Not relevant= 0, No= 1, Yes= 2)

5. Five year survival in years

6. Patient Died
   (Due to Ca=1; Not due to Ca=2.; Survived=3)

7. Cause of Death
   (Recurrence= 1, Other=2)

Drug History

Drugs on Admission (No=1; Yes=2)	Dose	Frequency
i. Aspirin		
ii. Steroids		
iii. All NSAIDs (Excluding aspirin)		
iv. COX-2 specific inhibitors (No=1, Yes=2)		
v. Ca^{++} channel blockers (Any= 1, Short acting= 2, Long acting=3)		
vi. Salbutamol		
vii. Theophylline		

Other illnesses on admission

(No=1; Yes=2)

i. Rheumatological

ii. Orthopaedic

iii. Other malignancy

iv. Gastrointestinal

v. Cardiac

vi. Respiratory

vii. Endocrine

viii. Renal

ix. Neurological

x. Psychiatric

xi. Renal

Data Collection Sheet B: (Control Group) Appendix-2

Patient Label

Dermographic Data :

Code:

1. Name
2. Date of Birth
3. Age on admission
4. Hospital No.
5. Study Identifier
6. Postcode (e.g. NR=1)
7. Hospital
   (Norwich=1; Gr Yarmouth=2; King's Lynn=3)
8. Gender (Male=1; Female=2)
9. Ethnic Group
   (Caucasian=1; Asian=2; Afro-Caribbean=3;
   Other=4)

Risk Factors

1. Smoking
   (No=0; Ex smoker=1; Current smoker=2)
2. Number of cigarettes/day
3. Alcohol
   (No=0; Ex alcohol=1; Current alcohol=2)
4. Amount of alcohol (units/week)
5. Known Barrett's

(No=0; Yes=1)

6. Length of Barrett's
   (0-3cms=1; >3cms=2)

7. Actual length in cms

8. Endoscopic histology
   (Normal=0; Mild Dysplasia=1; Moderate Dysplasia=2; Severe Dysplasia=3)

Previous Investigations:

1. Have had previous Endoscopy
   (No=0; Yes=1)

2. If Yes, Date : dd/mm/yyyy

3. Macroscopic findings of endoscopy
   (Normal=0;Barrett`s=1;Achalasia=2)

4. Time between endoscopy & diagnosis of skin tumours.

Current Investigations:

1. Diagnosis
   (BCC=1; SCC=2)

2. Disease code (ICD)
   (C44.0=1;44.1=2; C44.2=3; C44.3=4;
   C44.4=5; C44.5=6; C 44.6=7; C44.7=8
   C44.8=9;C44.9=10)

Drug History

Drugs on Admission Y/N Dose Frequency
(No=1; Yes=2)

i. Aspirin

ii. Steroids

iii. All NSAIDs (Excluding aspirin)

iv. COX-2 specific inhibitors (Rofecoxib=1, Celecoxib=2)

v. Ca^{++} channel blockers (Any= 1, Short acting= 2, Long acting=3)

vi. Salbutamol

vii. Theophylline

Other illnesses on admission

(No=1; Yes=2)

i. Rheumatological

ii. Orthopaedic

iii. Other malignancy

iv. Gastrointestinal

v. Cardiac

vi. Respiratory

vii. Endocrine

viii. Renal

ix. Neurological

x. Psychiatric

xi. Renal

Appendix 2: Flavonoid Study Protocol:

SUMMARY:

Dietary flavonoids may protect against the development of cancers of the gastro-intestinal tract. There is epidemiological evidence that antioxidants have a protective effect of up to 40-50 % against oesophageal cancer. Flavonoids are found ubiquitously in the diet and have potential anticarcinogenic properties including antioxidant, cyclo-oxygenase & lipo-oxygenase enzyme inhibiting action. They are present mainly in citrus fruits, vegetables, tea, and wine. Quercetin is the most commonly studied flavonoid and is easily detectable in the urine up to 24 hrs after ingestion. Food frequency questionnaires (FFQ) are used to measure habitual diet over a specified time period. However, currently there are limited studies, which have correlated a FFQ on fruits and vegetable intake against urinary flavonoids. A suitable FFQ for total dietary flavonoid intake as measured against urinary markers in a United Kingdom population is required. In this study, subjects will complete one FFQ`s and provide up to five 24 hour urinary samples to measure the flavonoid excretion for which a correlation coefficient will be calculated for dietary intake against urinary excretion. This exercise will be repeated at an interval of 3-4 months to check its reproducibility. This FFQ could then be used in case-control studies investigating if dietary flavonoids protect against cancer such as oesophageal cancer.

AIM:

To correlate a food frequency questionnaire measuring flavonoid intake against the urinary excretion of these compounds. As previous work from animal models and cellular studies has shown that flavonoids may protect against development of oesophageal cancer, this particular tumour will be discussed in detail. The aim would be to use such a FFQ in a case-control study investigating if flavonoids protect against the development of oesophageal cancer.

INTRODUCTION:

OESOPHAGEAL CANCER:

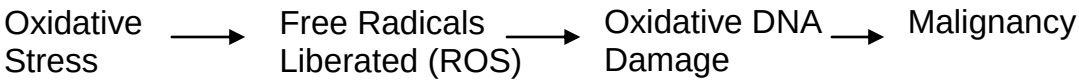
i) Epidemiology: Oesophageal cancer is the 9th commonest cancer in adults in the United Kingdom and the 5th most common cause of death from cancer. The UK has the highest incidence of oesophageal cancer in women in the world and it is rising. The neoplasm is commonest above the age of 50 years with a Male: Female ratio of 4:3. Furthermore the incidence of oesophageal cancer is higher in whites than in blacks with a ratio of approximately 5:1 ratio.

ii) Clinical Aspect: There are two histological types of oesophageal cancers, namely adenocarcinoma and squamous cell carcinoma. During the last century there were changes over time in the histological type & tumour site. Before the mid 70's adenocarcinoma accounted for less than 5 % of oesophageal cancer, but by the 1990's it has increased to greater than 25 % in several countries including the USA, Australia and Sweden. In the 1990's adenocarcinoma changed from being a rare neoplasm to becoming more common than squamous carcinoma of the oesophagus in the USA. The overall survival of patients with oesophageal cancer is only 5 % in the UK, with only a worse prognosis in those with lung and pancreatic cancer.

iii) Risk Factors: Major risk factors for squamous cell carcinoma of the oesophageal cancer are tobacco & alcohol, others include exposure to hot beverages & dietary deficiencies. For adenocarcinoma, obesity, smoking, diet lacking in fruits & vegetables have been identified. The risk of adenocarcinoma of the oesophagus and gastric cardia persists, however for nearly 30 years after cessation of smoking. A Barrett's segment of greater than 3 cms, increases the risk of oesophageal cancer in the normal population by 30-125 times. Also the presence of a high grade dysplasia, large hiatus hernia and Barrett's ulcer are recognised precursors for the development of oesophageal cancer.

iv) Mechanism of Carcinogenesis: All the above risk factors lead to metaplastic changes in the oesophageal mucosa by a synergistic action of acid, pepsin and development of gastro-oesophageal reflux. Although the oesophagus-metaplasia-dysplasia-carcinoma sequence is clear, molecular mechanisms leading to the genetic changes & adenocarcinoma are poorly defined. In oesophageal cancer & Barrett's oesophagus, oxidative stress may induce malignant transformation of cells.

Figure 1



Oxidative Stress (OS) is a harmful condition that occurs when there is excess of free radicals, a decrease in antioxidant levels, or both. Oxidative stress may possibly be because of excess free radicals which are increased in reflux disease. Oxidative stress has been suggested as being the driving force for the development of adenocarcinoma. Reactive oxygen species (ROS), such as free radicals and peroxides, are a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. There are many different sources by which the reactive oxygen species are generated. Most reactive oxygen species come from endogenous sources as by-products of normal metabolic reactions[28]. These include energy generation from mitochondria or the detoxification reactions involving the liver cytochrome P-450 enzyme system. Exogenous production of ROS may follow exposure to cigarette smoke, environmental pollutants such as emission from automobiles and industries, alcohol excess, asbestos, ionizing radiation, and bacterial, fungal or viral infections.

MECHANISM OF ROS INDUCED DAMAGE

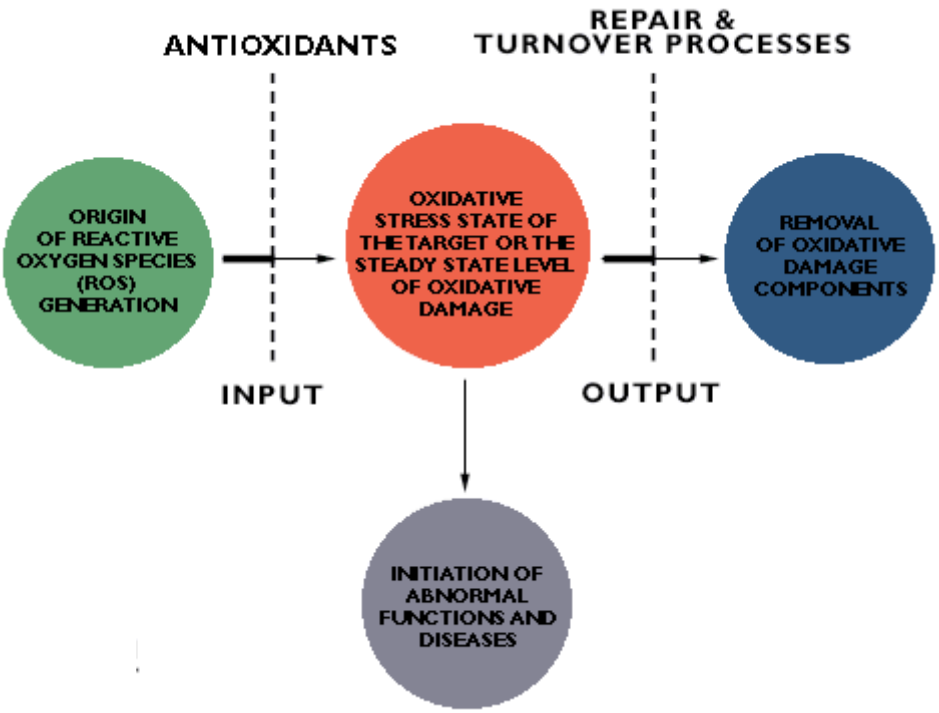


FIGURE 2:Production of ROS

Free radicals such as superoxide and hydroxyl radicals can cause injury to cells by damaging proteins, cell membranes or DNA. Deficiency in antioxidant defence mechanisms further amplifies oxidative stress and tissue injury. Oxidative stress induces the formation of high levels of DNA adducts which have been discovered in Barrett's epithelium. These DNA adducts can interfere with DNA replication and therefore initiate mutagenic & carcinogenic processes by producing mispaired DNA sequences.

Recently anti-oxidants have been shown to be inversely associated with risk factors for oesophageal cancer. Examples of antioxidants are ascorbic acid, beta-carotene & alpha-tocopherol. In rats, reflux-related oesophageal mucosal damage and lipid peroxidation may be reduced by antioxidants. A Swedish case-control study found an epidemiological association of higher intake of antioxidants and decreased risk for oesophageal cancer. They studied intakes of Vitamin C, beta-carotene and alpha-tocopherol in a nationwide population- based, case-control study and found that subjects with high intake of these antioxidants showed a 40-50 % decreased risk of both histological types of oesophageal cancer as compared to subjects with a low intake. Mechanism of action of flavonoids, which have antioxidant properties, will be discussed in the following section.

FLAVONOIDS:

Introduction : Flavonoids, previously known as “Vitamin P”, are water soluble plant pigments. They are polyphenolic compounds found in abundance in the human diet. There are more than 4000 naturally occurring flavonoid compounds with 3 major subclasses namely, Flavonols, Flavones & Flavanols. Major flavonols are quercetin, kaempferol, myricetin and isorhamnetin and are found for example in onions, broccoli and lettuce. The most abundant flavones are luteolin and apigenin and are found in celery, sweet pepper and citrus fruit. The principle flavanols are catechin, epicatechin, gallocatechin, epigallocatechin & gallic acid esters found in citrus fruits, chocolates, tea and wine. The presence of flavonoids in plants depends on several factors including the degree of ripeness, variety, processing and storage.

Previous case-control studies have shown that a high intake of fruits and vegetables protect against several cancers including oesophageal cancer, which may be due to their high flavonoid content.

Mechanism of Action: There are several plausible biological mechanisms for the protective effects of flavonoids as they posses antioxidant, antiplatelet, anti-inflammatory, anti-artherogenic, immunomodulator and anti-carcinogenic activities. There is some evidence that they also exhibit cycloxygenase & lipoxygenase enzyme inhibitor properties. Cyclo-oxyxygenase enzyme is found in high concentration in neoplastic tissue and recent evidence suggests that cycloxygenase inhibitor drugs, namely NSAID’s may reduce the incidence of oesophageal cancer by 40%. Flavonoids have antioxidant properties, including preventing the degradation of Vitamin E, scavenging reactive nitrogen species and ROS, chelating metallic ions like Fe^{3+},Cu^{2+} involved in free radical production. These properties reduce oxidative stress and a number of other of biological functions which may protect against cancer and other diseases.

Epidemiological Studies: Previous epidemiological studies have found that people with a high intake of food containing quercetin and other flavonoids tend to have a lower incidence of lung cancer. Preliminary studies from the Mayo clinic suggest that they may help prevent or treat prostate cancer by blocking male hormones. In vitro studies have found that flavonoids inhibit breast cancer cell proliferation, delay mammary tumorigenesis and have anti proliferative properties towards human colon cancer cells. However no epidemiological studies have specifically assessed whether dietary flavonoids reduce the risk of oesophageal cancer.

Metabolism: Flavonol glycosides can be absorbed as intact molecules and absorption varies according to food source. High performance liquid chromatography can achieve a detection limit of about 50ng/ml plasma. In humans only, data on the bioavailability of flavonols are available. Quercetin reaches a peak in the plasma 20 minutes after absorption (range 0.5-9 hours). Urinary elimination of quercetin is slow and may take up to 24hrs. However flavanols are rapidly excreted, with elimination half lives of only 1-6hrs.

A Finnish study has shown that urinary flavonoids can be used as a biomarker of dietary fruit and vegetable intake with a correlation co-efficient of 0.35, $p < 0.005$. However, this questionnaire could not be used to estimate the total dietary intake of flavonoid. Research is therefore needed to correlate a food frequency questionnaire which measures dietary intake of flavonoids against a urinary biomarker.

FOOD FREQUENCY QUESTIONNAIRES:

A food frequency questionnaire (FFQ's) measures habitual diet over a period of time e.g. over the preceding year. They describe one's usual frequency of food consumption rather than specific meals. A comprehensive assessment of diet is necessary which allows a calculation of macro & micro nutrients in individuals. Levels of intake can then be divided into categories such as high, medium and low intake. A number of factors affect the accuracy & compliance of a food frequency questionnaire namely length, number of food items, frequency of intake and portion size. Filling in a lengthy FFQ can lead to fatigue & boredom thus impairing concentration & accuracy. This is true even for a highly motivated cohort as demonstrated by Willett in the United States of America cohort study which studied US nurses.

In a FFQ food list should be comprehensive, include all foods which contribute to the nutrient of interest and should also be able to detect between person variations in intake. The foods which contribute most to between person variation, and are therefore the most discriminatory can be calculated statistically by stepwise regression. This process may lead to fewer questions in the FFQ, but which still discriminate between individual's intakes. As intake of food is seasonal, food frequencies are usually described by subjects referring to their diet over the entire previous year. A standard approach is to give subjects a choice of frequency options ranging from never to intake of many times/day e.g. 6 times/day. Defining the options increases clarity and reduces errors compared with open-ended responses where subjects self- report their frequency intake.

Controversy exists over the inclusion of portion sizes in FFQ's, which can be achieved by giving descriptive examples or including photographs of different portion sizes[63]. Food items with natural units may be interpreted correctly e.g. a glass of milk, however, portion sizes with-out natural units e.g. a portion of vegetables can be difficult to describe by subjects. Providing ranges of serving sizes e.g. ¼ cup or ½

cup improves clarity as compared to small, medium & large portions[63]. Existing FFQ's can be used to measure individual diets and extra questions added if particular nutrients are required to be studied. Borrud found that although the frequency distributions of food used by ethnic subgroups differ, a comprehensive FFQ may function well in a diverse population.

In this study, we aim to develop a FFQ to specifically measure flavonoid intake by modifying an existing questionnaire developed at the Institute of Food Research, Norwich. This existing FFQ measures the intake of basic macro & micro nutrients. The questionnaire will be assessed to see if it can be shortened to increase compliance, whilst still be able to differentiate between in take. Secondly it will be reviewed to see if portion size adds to the dietary assessment. This modified FFQ will then be correlated against urinary flavonoids, which are a biomarker of dietary intake. The FFQ will then be used in future case-control studies to assess if flavonoids protect against gastro-intestinal malignancies.

Objectives

To asses whether the dietary intake of flavonoids correlates with their urinary excretion.

Hypothesis

Dietary intake of flavonoids may prevent against malignancies such as oesophageal cancer. Once a food frequency questionnaire is correlated against the urinary biomarkers, it will then be used to study whether a higher intake of flavonoids, as recorded by FFQ, protects against oesophageal cancer.

STUDY DESIGN

Overview

The study involves the recruitment of apparently healthy subjects via the HNU volunteer databank at the Institute of Food Research, Norwich, advertisement's and email for recruitment of volunteers with in the Norwich Research Park (John Innes Institute, Institute of Food Research and University of East Anglia) and from surgical clinics at the Norfolk & Norwich University Hospital. 64 apparently healthy adult volunteers will be recruited. The study involves assessing the dietary intake of the volunteers by a food frequency questionnaire, collection of up to five 24 hour urine samples and a repeat of the whole exercise 3-4 months later to check the reproducibility of the results Volunteers will not be given any dietary advice as there is no dietary intervention in the study. A flow chart of the overall study design is given in Figure 1 & 2

Figure 1. Study Design

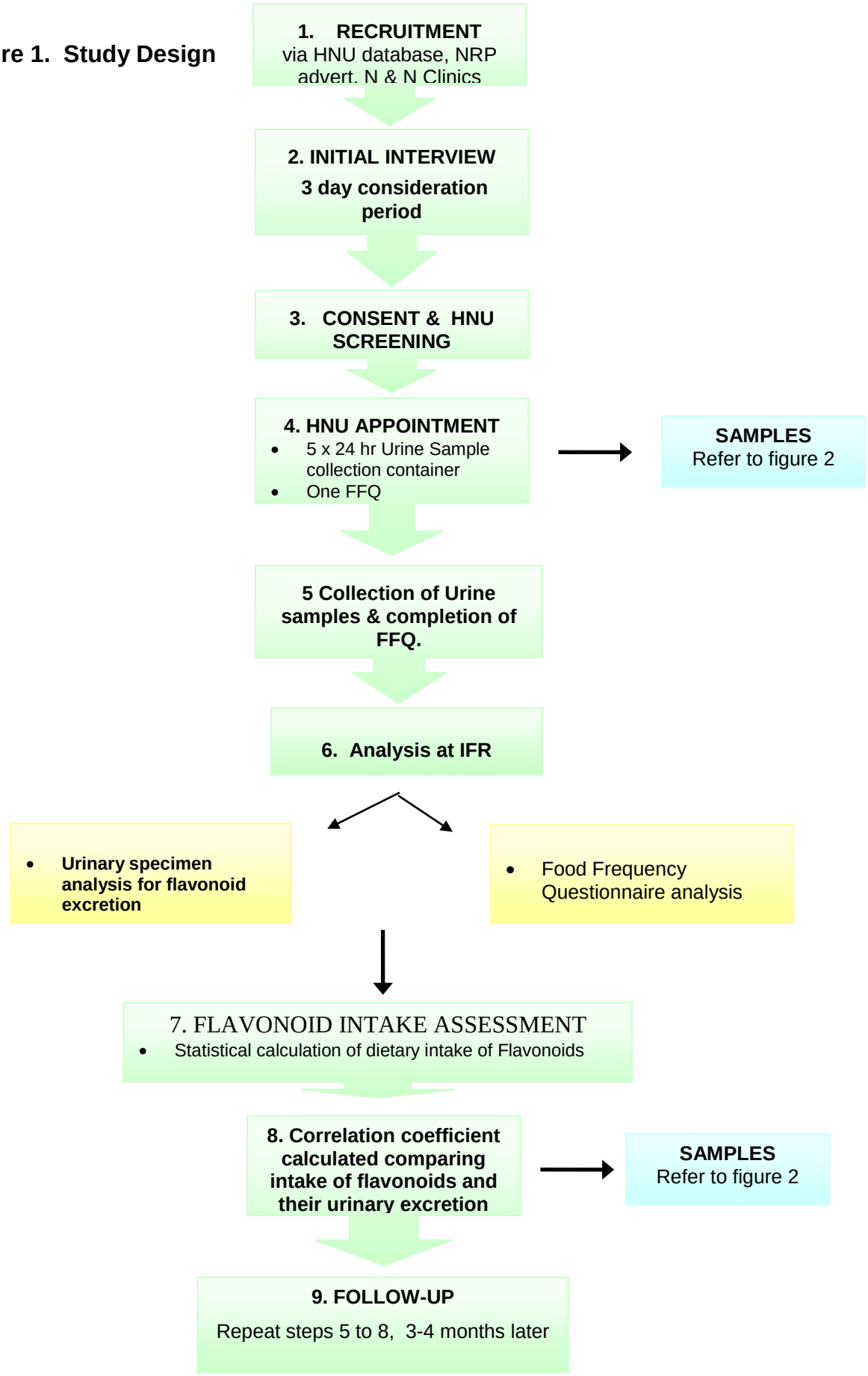
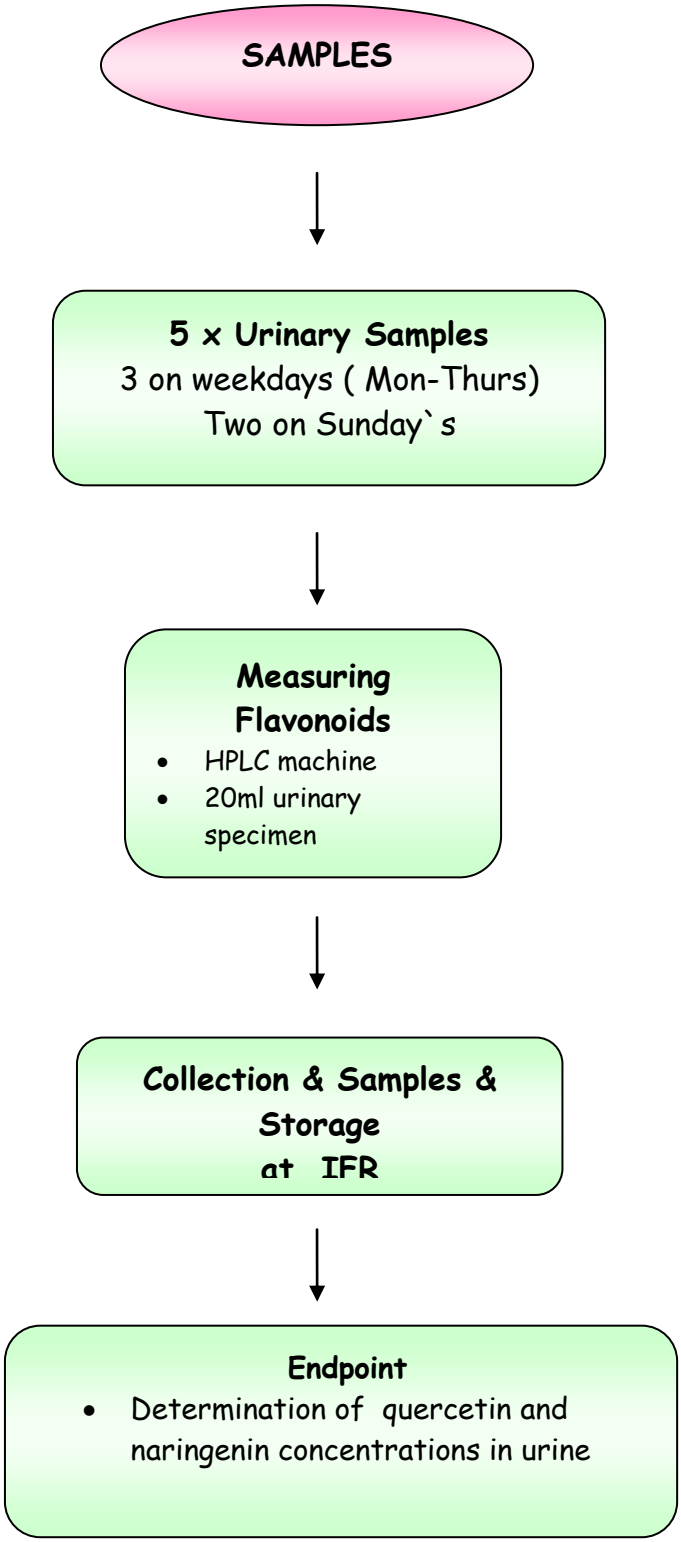


Figure 2: Plan of Urine Sample collection and analysis



Volunteer Recruitment

Apparently healthy male/female volunteers will be recruited until 64 volunteers, have completed the study. Experience has shown that the drop out rate for these types of studies is about 10%. It is envisaged that patients will be recruited onto the study at a rate of 4-5 per week and therefore recruitment may continue for approximately 3-4 months. If greater drop out rates occur recruitment will be reviewed.

Advertisements will be placed on: the Norwich Research Park***** (University of East Anglia (UEA), John Innes Centre (JIC), IFR), *and in the HNU news letter which is sent to all volunteers on the HNU volunteer database* (Annex 1). Patients with minor surgical ailments (e.g. groin hernia`s, cysts etc. that have no impact on dietary habit), their accompanying relatives or friends who attend the Norfolk & Norwich University Hospital surgical clinics, will also be informed of the study, and if interested will be sent further information. If insufficient numbers of volunteers are recruited from these areas advertisements will be placed in the local press.

Apparently healthy volunteers, who meet the basic inclusion criteria, will be sent a letter of invitation (Annex 2) to participate in the study. This will be supported by the volunteer information sheet (Annex 3). Included will be a response slip and pre-paid envelope for returning the slip (Annex 4) if they are interested and wish for further information. The HNU databank contains names and contact details of people who have registered an interest in volunteering for human studies.

Advertisements will be placed around the Norwich Research Park (UEA, JIC and IFR) inviting anyone who is interested in receiving information about the study to contact named researchers. NRP staff will also be invited to participate in the study by email by seeking adequate permission. The researchers will send interested responders the volunteer information sheet. This will include a response slip and pre-paid envelope in which to return the response slip if they are further interested.

Patients with minor surgical/medical conditions, not affecting their diet or ability to give up to five 24 hour urine samples, if fitting the inclusion criteria, will be invited to participate in the study. Healthy relatives or friends accompanying them will also be invited to participate in the study. If they show an interest to participate in the study, further details in the form of patient information sheet will be sent to them.

Following an expression of interest volunteers will be invited to the Human Nutrition Unit (HNU) for a preliminary interview and given further details of the study. The volunteers will be encouraged to ask questions at this point prior to making any commitment. At the end of the interview all volunteers will be given a minimum of 72 hours to consider whether they wish to participate in the study. They will also be given a small sterile container to take away with them. If they wish to participate, this container will be used for the urine sample on the morning of the clinical screening visit. If they decide they do not wish to take part in the study they will be told to discard the container. During this consideration period the volunteers will not be contacted. If, following this period of consideration, the volunteer still wishes to participate they will be asked to contact HNU on telephone number 01603 255305.

All those responding positively following this period of consideration will be invited to attend the HNU for a clinical screening. No fasting will be required. They will be reminded to bring a midstream sample of urine from the first urine of the morning in the container provided at the first interview (This will not be tested until after the consent form has been signed). Volunteers need to arrive within 2 hours after collection of the urine sample as this is a required specification for the validity of the urine dipstick test.

Clinical screening:

On arrival at the HNU the study scientist will go through the consent form (Annex 5) with the volunteer and encourage any questions they may have at this stage, volunteers will then be asked to sign a consent form agreeing to participate in the study. A copy of the signed consent form will be given to the volunteer to keep. A qualified nurse will then complete a basic health questionnaire (Annex 6), take and record blood pressure, pulse, height and weight measurements, Body Mass Index (BMI), perform the urine dipstick test (Combur ^{9} Test®, Roche Diagnostics Ltd).The urine results will be known immediately. If any of the results for the urine test are flagged the HNU protocol for abnormal urinalysis results will be referred to. If the BMI is <18.5 or >30 the volunteer will be excluded from the study.

Volunteers who do not wish to be re-screened or who display screening parameters outside the standard reference ranges on both occasions will be excluded from the study.

Copies of all clinical results will be sent to the volunteer's GP (Annex 7) and in the event of flagged urine results, the volunteer will be informed verbally and advised to speak to their GP to discuss the results. Results will not be discussed with the volunteer.

Volunteers who meet the study criteria and whose screening results are satisfactory will be included in the study. The GPs of those successfully recruited onto the study will be informed of their patient's participation in the study by letter (Annex 8) and will be sent copies of all clinical results. The volunteer will agree to this information being sent to the GP by signing the consent form.

Once recruited onto the study, volunteers will be assigned a code number with only the named study scientists approved by the Ethics Committee being able to link codes to volunteers. All personal information will be kept confidential and known only

to the Researcher's, project leader, HNU research nurses, HNU Medical advisor and the volunteer's GP.

The inclusion and exclusion criteria are as follows:

Basic Inclusion criteria

- Aged 40-85 years
- Male or female
- Non-smoker

Basic Exclusion criteria

- Pregnant and breastfeeding
- Organ transplant recipients (on immunosuppressant's can effect diet)
- Long term illness requiring active treatment (e.g. Diabetes, cardiovascular disease, anaemia, cancer : may affect diet & participation in study)
- Volunteers currently on antibiotics (ongoing infections can affect the diet)

Screening Exclusion criteria

- BMI < 18.5 or > 30
- Abnormal urine analysis results indicative of active illness (Refer to HNU protocol for abnormal Urinalysis results)
- Results of clinical screening which are judged by the HNU Medical Advisor to be indicative of a health problem and could compromise the well-being of the volunteer if they participated, or which would affect the data.
- Volunteers currently on antibiotics (ongoing infections can affect the diet)

Once the volunteer is selected through the screening process, the scientist will give instructions and equipment needed to prepare for the study. The study involves you completing a questionnaire on diet and producing up to five 24 hr urine collections over a period of 2 weeks including two on Sundays.

The first phase of the study will be the collection of up to five 24 hour urinary sample's. They will be asked to deposit the sample at the Human Nutrition Unit, IFR. The second phase of the study will be filling the food frequency questionnaire. In the final phase the volunteers will be asked to repeat the whole exercise 3-4 months later.

Assessment of Dietary Intake

Diet will be assessed by a food frequency questionnaire. The volunteers will be asked to complete the questionnaire at home taking into account their diet over the period of the last year. The food frequency questionnaire (annex 9) focuses on habitual diet intake during the previous twelve months and allows an estimate to be made of the habitual intake of flavonoids from food sources. This questionnaire has been modified from one previously used for studies at the Institute of Food Research, Norwich. The FFQ takes approximately 30 minutes to complete. In addition to recording the habitual intake of a wide range of foods and food groups, the computer software used to interrogate the FFQ data will calculate the dietary intake of selected flavonoids e.g. quercetin, naringenin and catechins from the information supplied by each volunteer.

Sample Collection

Up to five 24 hours urinary samples will be collected at the HNU, IFR, according to the plan in Figure 2. Ascorbic acid 99% crystalline Sigma Ultra 1gm in 2.5 litres pot will be added to the urine collection pots as a preservative and the volunteers informed of its presence. These samples will be stored in a cold room at temperatures $+1^0$ C to $+4^0$C for up to a month before being processed.

Urine Samples & Creatnine clearance :

Volunteers will be asked to collect up to five 24 hours urine samples. These samples will be stored, processed and analysed at IFR. The procedure for collection of the urinary sample will be explained to the volunteers and they will have the opportunity to ask questions. Researchers at the Institute of Food Research have many years of experience in collecting urinary samples from volunteers and are able to instruct volunteers to collect samples in a safe and hygienic manner. Creatnine clearance will be used as a marker of compliance by determining the amount of creatnine in the 24h sample using a ABX Diagnostics` Creatnine 80 kit on a COBAS Mira Plus analyser. This kit uses the Jaff'e colorimeteric method for determining Creatnine in urine. ABX Diagnostics Human Control N will be run as quality control and inter-assay variation will be determined.

SAMPLE ANALYSIS

The urinary samples will be analysed for the following flavonoids: quercetin, naringenin and catechins. Flavonoids in the urine samples will be enzymatically deconjugated, extracted and quantified by HPLC (High Performance Liquid Chomatography) according to the method of Du Pont et.al[68]. Briefly, urine samples (20ml) will be incubated with phosphate buffer (3ml, 0.1M, pH 6.2), internal standard (100µg), β- glucuronidase (200U) and sulphatase (20U) for 3 h at 37^0C.Menthol (2ml, containing 1mM ascorbic acid), acetic acid (200µl, 50%) and acetonitrile (to a final

volume of 40 ml) will be added to precipitate proteins and extract flavonoids. The samples will be vortex-mixed for 30s every 2min over a 10 min period, before centrifugation (13 600g, 10min, 4^0C). The supernatant will be evaporated. Evaporated samples will be taken up in menthol/water (300µ1; 1/1, v/v) and filtered for HPLC analysis. A new extraction procedure based on SPE (Solid Phase Extraction) is being devised at IFR to replace the existing procedure[68], and if proved to be more effective will be incorporated into the analysis protocol.

Sample Safety

IFR has standard operating procedures for the storage of body fluids. These procedures will be adhered to and are integral to IFRs recognition as ISO 9001:2000 compliant and constitute part of our process working to the standards of GCP.

STATISTICAL ANALYSIS

Total food intake, total energy intake, macro & micronutrients, percentage of energy consumed will be calculated using an in-house FFQ which has been re-designed around ACCESS software. The original FFQ from which the new version has been derived has been used in previous studies at IFR[67, 69]. The power of the study has been calculated at 80% assuming a group size of 64 to detect a correlation of 0.35 at 5% significance level. All data will be analysed using STATA 8.3SE. The primary analysis will be using a Pearson's correlation coefficient to measure the strength of the association between dietary assessment of flavonoids and their urinary excretion, for both sexes combined and stratified by sex. If the distribution of the variables is skewed, transformed variables (such as log) will be used. A secondary analysis would be linear regression, regressing estimated mean dietary flavonoid intake on mean urinary flavonoids, adjusting for covariates such as age, sex and BMI.

Both the FFQ and urine results will be divided into quartiles of intake/concentrations and compared to check that there are no gross miscalculations between methods.

All data will be analysed using STATA 8.3SE

ETHICAL CONSIDERATIONS: The project is being submitted to the Human Research & Governance Committee, Institute of Food Research, Norwich, East Norfolk & Waveney Research Governance Committee, Norwich Local Research Ethics Committee.

Appendix 3: Modified FFQ.

“Correlation of dietary flavonoid intake by Food Frequency Questionnaire with measurement of urinary excretion of flavonoid metabolites”

FOOD FREQUENCY QUESTIONNAIRE

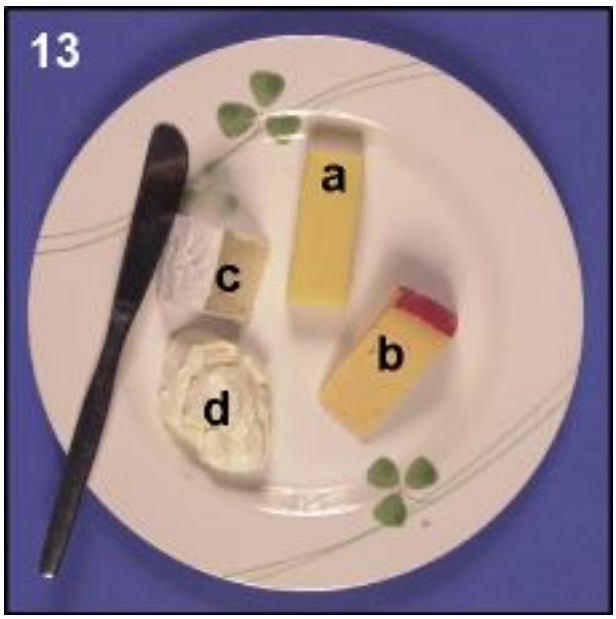
Please

1. Answer every question.
2. Fill in the portion sizes for foods you eat & please note that the **photos** are of medium portion size.
3. Tick only one box for portion size.
4. Tick only one box for how often you eat a particular food.

The research scientist will show you how to complete the questionnaire using the following example:

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
D. Cheese and cheese dishes How often do you usually eat the following?											
70	Reduced fat cheddar cheese	see photograph 13a		√					√		

The colour pictures at the back of the questionnaire show **medium portions** of various foods. In this example photograph 13a is used to decide on the size of the portion that is normally eaten.



This example indicates that the portion of cheese eaten is of medium size in as shown in the photograph, and that cheese is eaten between one and three times a week.

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
A. Meats Do you eat meat? **YES /NO** (please delete) If 'NO' please go to section B. How often do you usually eat the following?											
1	Salami, Pate or similar meats	see photograph 1c									
2	Meat pies (e.g.pork steak & kidney)	see photograph 2a									
3	Sausage rolls, Cornish pasties	1 small									
4	Ham, corned beef + other cold meats	see photograph 1a									
5	Chicken, turkey etc. including minced & casseroled	see photograph 3b									
6	Lamb chops, cutlets and mince	see photograph 4b									
7	Leg of lamb	see photograph 3b									
8	Leg of pork, pork medallions, steaks and fillets	see photograph 3b									
9	Pork chops	see photograph 3b									
10	Reduced fat pork or beef sausages	1 thick									
11	Sausages, pork or beef	1 thick									
12	Beef steak - rump or sirloin	see photograph 4b									
13	Beef - topside, brisket, forerib, mince	see photograph 3b									
14	Bacon (back, lean, meat and fat), grilled or fried	1 slice									
15	Bacon, streaky, grilled or fried	1 slice									
16	Liver	see photograph 1b									
17	Kidney, pig, stewed and other offal	see photograph 1b									
18	Stew, Shepherds pie, casserole, curry, kebab	see photograp 7b									
18	Moussaka or Lasagne	see photograph 6a									
20	Bolognese, Chili con carne	see photograph 7b									
21	Burgers, plain	1									
22	Regular hamburger with bun	1									

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
23	Venison, rabbit , hare or other game	see photograph 3b									
B. Vegetables How often do you usually eat the following?											
24	Potatoes roast, fried, regular, chips	see photograph 4a									
25	Potatoes mashed, boiled or baked	see photograph 2b									
26	French fries	see photograph 10a									
27	Potato crisps	1 small bag									
28	Onions, leeks, spring onions	see photograph 2c									
29	Mushrooms, fried	see photograph 2c									
30	Tomatoes : fresh/tinned	1 tomato									
31	Vegetable curry or casserole	see photograph 9b									
32	Runner, french or broad beans	see photograph 4c									
33	Red kidney or butter beans	see photograph 4c									
34	Baked beans, canned in tomato sauce	see photograph 12b									
35	Sweetcorn, on the cob, boiled, or tinned	1 cob									
36	Coleslaw	see photograph 14b									
37	Potato salad	see photograph 14b									
38	Lentils, chic peas etc	see photograph 7b									
39	Brussel sprouts or cabbage	9 sprouts or photograph 8c									
40	Cauliflower or broccoli	see photograph 8b									
41	Peas	see photograph 4c									
42	Spinach	see photograph 8c									
43	Swede	see photograph 9a									
44	Turnips, parsnips or carrots	see photograph 9a									
45	Vegeburger	see photograph 8a									
46	Lettuce	see photograph 4c									

	Food category	Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
47	Watercress	see photograph 4c									
48	Beetroot	see photograph 4c									
49	Peppers	see photograph 4c									
50	Cucumber &	see photograph 4c									
51	celery	see photograph 4c									
C. Pasta and Rice											
How often do you usually eat the following?											
52	Longgrain rice, boiled	see photograph 5b									
53	Fried rice	see photograph 5b									
54	Spaghetti, canned in tomato sauce	see photograph 11a									
55	Other pasta (e.g. spaghetti, macaroni, pasta shells)	see photograph 7a									
D. Cheese and cheese dishes											
How often do you usually eat the following?											
56	Reduced fat hard cheese e.g low fat cheddar, edam	see photograph 13a									
57	Hard cheese e.g. Cheddar, cheshire	see photograph 13a									
58	Soft cheese e.g. brie, camembert , cream cheese	see photograph 13c									
59	Veined cheese e.g. Danish blue, silton	see photograph 13a									
60	Cottage cheese, cheese spreads e.g. philadelphia	see photograph 13d									
61	Fromage frais, crème fraiche	see photograph 13d									
62	Cheese topped pizza	see photograph 11b									
63	Quiche lorraine or cheese flan	see photograph 10b									
64	Cheese sauce	¼ pint (125ml)									
E. Fish											
How often do you usually eat the following?											
65	Haddock, plaice, cod or trout steamed	see photograph 12a									
66	Haddock, plaice or cod, fried	see photograph 12a									
67	Herring, mackerel or tuna	see photograph									

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
		14c									
68	Tuna, canned in oil	see photograph 14c									
69	Fish cakes, fried	1									
70	Fish fingers, fried	1									
71	Shellfish	1 small jar or 20 prawns									
F. Fats How often do you usually eat the following?											
72	Butter (or other spread) on bread, toast or sandwiches	see photographs 16c and 15									
73	Butter (or other spread) on jacket potatoes or other vegetables	see photograph 16c									
74	Butter (or other spread) on crackers or crispbread	see photograph 16b									
G. Dairy Products How often do you usually eat the following?											
75	Milk on breakfast cereals	see photograph 17c									
76	Glass of milk	see photograph 17b									
77	Dried milk powder (e.g.Coffeemate)	1 teaspoon									
78	Cream in drinks or soups	1 tablespoon									
79	Cream on puddings or fruit	see photograph 23b									
80	Cream in other recipes	1 tablespoon									
81	Whole yoghurt	1 small pot 25g									
82	Yoghurt, low fat	1 small pot 25g									
H. Eggs How often do you usually eat the following?											
83	Egg, boiled, poached or raw (e.g. in baking)	1 size 3									
84	Fried or scrambled egg	1 size 3									
85	Egg, omelette	2 eggs size 3									

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
I. Cakes, Biscuits and Puddings How often do you usually eat the following?											
86	Chocolate biscuit bars e.g. KIT-KAT, PENGUIN	1									
87	Chocolate biscuit	1									
88	Plain or sweet biscuits	1									
89	Cream crackers, Ritz type biscuits, CRISPBREADS	1									
90	Biscuits shortbread	1									
91	Chocolate/plain sponge with icing, cream cakes, cheese cake	see photograph 18a									
92	Fruitcake	see photograph 18b									
93	Fruit pie or apple crumble	see photograph 19b									
94	Rice pudding or other milk pudding	see photograph 20									
95	Trifle, dairy deserts, moose etc	see photograph 20									
96	Ice-cream	see photograph 21									
97	Scones or teacakes or currant buns	1									
98	Danish pastries	1									
99	Steamed pudding	see photograph 22a									
100	Other cakes	1 slice									
J. Breakfast cereals How often do you usually eat the following?											
101	Muesli	see photograph 24									
102	All Bran or similar cereal	see photograph 25									
103	Shredded wheat, Weetabix or similar cereal	1 biscuit									
104	Rice Krispies, Special K or similar cereal	see photograph 25									
105	Cornflakes, Sugar Puffs or similar cereal	see photograph 25									

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
106	Porridge	see photograph 20									
107	Oatmeal, raw	see photograph 24									
K. Bread How often do you usually eat the following?											
108	Bread, wholemeal, wholewheat, granary or soft grain	1 medium slice									
109	Bread, white or malted	1 medium slice or one roll/bap									
110	Bread, white, fried	1 medium slice									
111	Naan, chapattis or pitta	½ naan or 1 chapati/pitta									
112	Other breads (papudum etc.)	1									
L. Chocolate and sweets How often do you usually eat the following?											
113	Chocolate e.g. Mars bar	1									
114	Chocolate e.g. Bounty bar	1									
115	Chocolate, plain	1									
116	Chocolate, milk	1									
117	Other sweets or toffees	1									
M. Alcohol and other beverages How often do you usually drink the following?											
118	Beer, lager, cider (normal strength)	1 pint									
119	Strong beer or lager	1 pint									
120	Wine : Red	1 measure									
121	Wine : White/rose	1 measure									
122	Sherry, liqueurs and spirits	1 measure									
123	Fizzy drinks (not low calorie)	1 can or see photograph 17b									
124	Fruit squashes	1 can or see photograph 17b									
125	Pure fruit juices : Citrus	1 can or see									

Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
		photograph 17b									
126	Pure fruit juices : Apple, pear etc	1 can or see photograph 17b									
127	Fruit Juice : Tomato	1 can or see photograph 17b									
128	Tea	see photograph 17a									
129	Coffee	see photograph 17a									
130	Chocolate drinks	see photograph 17a									
N. Fruit How often do you usually eat the following?											
131	Bananas	1									
132	Avocado pears	½									
133	Olives	9									
134	Apples, pears or apricots	1									
135	Oranges, tangerines, mandrins, clementines : raw	1									
136	Grapefruit or pineapple	1 or 1 slice									
137	Nectarine, peach, plum, kiwi fruit, grapes or melon	1 piece, 30 grapes or 1 slice melon									
138	Strawberries or raspberries etc	9 berries									
139	Black/blueberries etc	9 berries									
140	Tinned fruit	see photograph 23a									
141	Dried fruit	1 tablespoon									
O. Nuts How often do you usually eat the following?											
142	Brazil or cashew nuts	1 small bag									
143	Peanuts and other nuts	1 small bag									
P. Other food How often do you usually eat the following?											
144	Peanut butter, smooth	see photograph 16a									
145	Reduced calorie mayonnaise or	1 tablespoon									

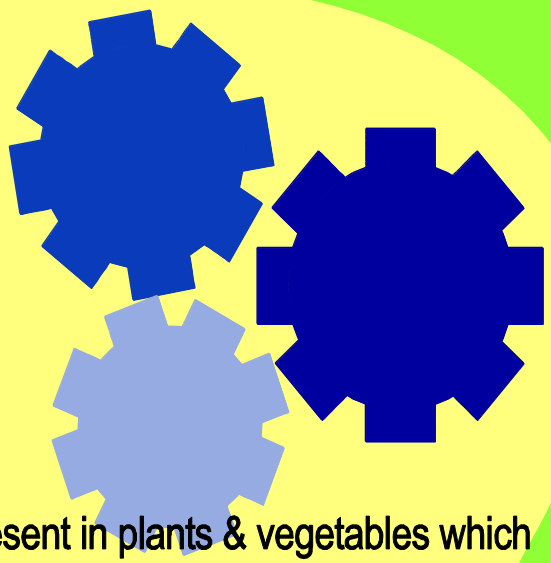
Food category		Size of medium portion	Your portion size			How often					
			S	M	L	3 or more per day	1 - 2 per day	4-6 times per week	1-3 times per week	1-3 times per month	Rarely or never
	salad cream										
146	Mayonnaise or salad cream	1 tablespoon									
147	Custard	see photograph 22b									
148	Gravy	see photograph 2d									
149	Sugar,white	1 teaspoon									
150	Preserves (jam. marmalade or honey)	see photograph 16a									
151	Soups	1 small tin									
152	Pickles or sauces (except tomato or soy)	1 teaspoon									
153	Tomato sauce	see photograph 17a									
154	Soy sauce	see photograph 17a									
155	Hummus	see photograph 14b									
156	Taramasalata	see photograph 14b									
157	Cod liver oil	1 capsule									
158	Bran	1 tablespoon									
159	Wheatgerm	1 tablespoon									

Are there other foods which you eat more than once a week ? Yes ☐ ☐ **No**

Food Item	**Usual serving size**	**Number of times eaten each week**

Institute of Food Research

The Flavonoid Study



Flavonoids are chemicals present in plants & vegetables which may help prevent several cancers.

Male & female volunteers between 40-85 yrs are required to take part in this study.

You can take part if you are a non-smoker, without long standing medical illness, non-pregnant or breastfeeding and currently not on antibiotics.

Volunteers will be required to fill in a dietary Food Frequency Questionnaire and give upto five 24hr urine samples & repeat the whole exercise 3-4 months later.

For further information please contact:
Human Nutrition Unit,
Institute of Food Research
Tel: 01603 255305 **email:** satish.ranka@bbsrc.ac.uk

"An expression of interest does not commit you to taking part"

Volunteers Required

www.ifr.ac.uk

Annex1

Annex 2

DATE

Dear

Thank you for your interest in studies at the Institute of Food Research.

I have sent you the details of one of the studies in progress at present, as your details on the volunteer database fit the criteria for this study. If you are further interested, please contact the Human Nutrition Unit, Institute of food Research, Norwich.

If, however, any of your details have changed or change in the future or you would prefer to no longer remain on the database please could you inform the Human Nutrition Unit on; 01603 255305.

Thank you

Yours sincerely

Norwich Research Park, Colney, Norwich NR4 7UA, UK
www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

Annex 3

VOLUNTEER INFORMATION SHEET

For

"THE FLAVONOID STUDY"

"Correlation of dietary flavonoid intake by Food Frequency Questionnaire with measurement of urinary excretion of flavonoid metabolites"

Norwich Research Park, Colney, Norwich NR4 7UA, UK
www.ifr.ac.uk Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

THE FLAVONOID STUDY

Information for Volunteers: "*The Flavonoid Study"*

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

Thank you for reading this.

What is the study called ?

*"*Correlation of dietary flavonoid intake by Food Frequency Questionnaire with measurement of urinary excretion of flavonoid metabolites"

or

SHORT TITLE *"The Flavonoid Study "*.

What is it about ?

Studies have shown that chemicals present in fruits and vegetables called flavonoids may help prevent several cancers. It is known that flavonoids prevent the formation of chemicals dangerous to the body, which may lead to cancer. However, we currently do not have an accurate questionnaire which estimates the consumption of flavonoids from what you eat. The aim of this study is to produce a suitable Food Frequency questionnaire (FFQ). Further studies at a later date can then be carried out to find out whether supplementing the diet with these chemicals helps prevent cancer.

Do I have to take part ?

It is up to you to decide whether or not to take part. If you take part you are free to withdraw at anytime, without giving a reason. A decision to withdraw, or a decision not to take part, will not affect your participation in future studies. An expression of interest at this stage does not commit you to take part in the study.

Why have I been chosen ?

You have received this information about the study because you have responded to an advertisement about the study or your name has been given to us from the volunteer database held at the Institute of Food Research (IFR) Human Nutrition Unit ((I) or you have shown interest to participate in the study whilst attending a

Norfolk & Norwich University Hospital surgical clinic.

We are aiming to recruit a total of 64 people aged 40-85 years who are apparently in good health.

You will not be able to volunteer if :

- You are a smoker
- You are taking part in other studies
- You have a diagnosed long term medical illness
- You are *pregnant or have been pregnant in the last 12 months* or are breastfeeding
- You are currently taking antibiotics

If you are in any doubt about whether or not you are a suitable volunteer please do not hesitate to contact us.

I have a medical condition but it does not affect what I do, so why can't I be included?

We know you can live a normal life with many medical conditions and recognise that you are not ill. However, these conditions may affect the data required for the study research or prevent us performing some study procedures necessary to collect the data; therefore we may have to exclude you from this particular study.

How do I say No?

Please feel free to say no by not responding to the letter we have sent you. Do not worry, no one will contact you and try to persuade you to join the study. The decision to participate is yours and we are grateful for your time.

If you are on the volunteer database, saying no will not affect the likelihood of you being contacted for other studies.

How do I say Yes?

Anyone interested in finding out more about participating in the study should contact HNU on 01603 255305 or

Email : satish.ranka@bbsrc.ac.uk

Alternatively, you can complete the attached response form and return it using the pre-paid envelope enclosed.

What will happen when I have said yes?

After you have contacted us to tell us you are interested in participating, Dr Satish Ranka will make an appointment for you to visit the Human Nutrition Unit (HNU) at IFR, when it is convenient for you, to discuss the study further. Please ask any questions you may have at this or any stage during the study. You are free to withdraw from the study at any time without giving a reason.

At the end of this visit you will be given a small container to take away with you. If you do decide to participate you will use

this container to bring a urine sample to the next visit to the HNU (screening visit).

At least 72 hours must elapse between your first and second visit to allow you to decide whether or not you wish to participate in the study and during this time we will not contact you.

If you decide not to participate, dispose of the urine bottle and take no further action (no one will check up on you).

If you decide you do wish to participate, please contact HNU, IFR to arrange an appointment for you, at your convenience, to visit the HNU for screening.

The health screening visit will be in the morning and you will be asked to bring a mid-stream urine sample collected when you get up that day, in the container we gave you. The urine sample has to arrive within two hours of collection to make the test valid. You do not need to avoid eating or drinking at any time.

All volunteers will be asked to sign a consent form agreeing to participate in the study. You will be given a copy of this to keep.

A qualified research nurse will then complete a brief health questionnaire with you. You will have your height, weight and body mass index (BMI) measured and your blood pressure and pulse rate recorded. A dip stick test will be carried out on your urine.

We are quite flexible and if you work, we try, where possible, to make this appointment at a time that suits you best.

If you require transport, this can be provided to and from the HNU.

What are you going to do with my screening urine sample?

The urine dipstick test is a quick way to check that there is nothing unusual in your urine that might affect the study data. If you have any questions about the test please ask one of the research nurses.

If the result of your urine is outside the standard reference ranges we will tell you. We cannot tell you what your results may mean as we are not medically qualified to do so. You should not worry if this happens as it may be a one-off result or it may be perfectly normal for you.

Please remember these tests are performed to determine if you are suitable for the study.

Are there any risks or side affects from participating in this study?

We do not anticipate any adverse effects of taking part in the study.

Will my GP be informed?

Yes, it is routine practice for us to inform your GP that you are participating in a study at IFR and to forward details of your clinical results. This is done whether the results are inside or outside the standard reference ranges to keep your GP informed.

This is one of the things you are agreeing to when you sign the consent form for taking part in the study. If any of your results are outside the standard reference ranges, we will recommend that you speak to your GP about the results.

Results outside the reference ranges are referred to the HNU Medical Advisor. Advice will be given by the Medical Advisor as to whether we may offer you the opportunity of a second screening appointment. If the results from the second test are within range you may still be able to participate. If not you will probably be excluded from the study.

My screening results are ok – what happens next?

Once you are selected through the screening process, the scientist will give you the instructions and equipment you need to participate in the study. The study involves you completing a questionnaire on your diet and producing five urine samples, each collected over a period of 24 hours. Your involvement in the study will last for 3–4 months. If you have questions at any stage then you are free to contact the study scientists for further information at any time.

Completing a Questionnaire on my diet.

One part of the study involves us finding out about your diet. This will involve filling in a questionnaire. The questionnaire concentrates on the food that you eat. It asks you how often you eat certain foods, and what portion sizes you eat.. There will be coloured photographs of medium portion sizes to help you decide. This questionnaire takes about 30 minutes to complete.

How do I collect the Urinary samples ?

You will be given one container at IFR, after the screening process. The scientist will give you instructions for collecting 24 hour urine samples. Over a 2 week period, you will be asked to collect up to three 24 hour urine samples during weekdays (Mon-Thurs) and one 24 hour urine sample on two consecutive Sundays. You will be asked to fill in the dietary questionnaire when you have time, between these collections. Once you have collected the sample, you contact one of the members of the study team at the HNU to deposit it or we can arrange to pick the sample up. We will then supply the second container. Once you have supplied the samples and completed the questionnaire, you will be asked to repeat the whole procedure 3-4 months later. The study does not involve supplying any blood samples

What happens to the questionnaire and the urine samples ?

Questionnaires: The questionnaires will be analysed using a computer to measure the intake of flavonoids from your diet.

Urine specimen : These will be tested in a special machine at IFR to determine the amount of flavonoid excreted in 24 hours.

If you turn to page _9_ there is a simple flow chart outlining what the study involves

After the analysis of the questionnaire and the urinary sample, we will be able to check how accurate the questionnaire is in measuring flavonoid intake.

Any sample of urine remaining after the information has been collected will be disposed off into the institute sewer system and the containers disinfected and thoroughly cleaned.

What are the possible benefits of taking part ?

The study will allow us to develop a questionnaire which will accurately measure flavonoids intake. We hope that this will be used in future work to see if alteration in diet may help prevent cancer.

Do I get paid for doing this?

Participating in these studies is done on a voluntary basis, however we do recognise that being involved in the study can cause you some inconvenience and that there are travel costs associated with you visiting the HNU.

Therefore you will receive small inconvenience payments for your urine samples and filling in the questionnaire.

Travelling expenses will be reimbursed on production of a receipt for buses or trains and 30.5 pence/mile for travel by private car. If you require transport to and from the HNU please let us know and we will order a taxi and pay for it.

Payments are liable to tax and in the case of IFR staff will be taxed at source.

Is what you find out about me going to be kept safe?

Any information relating to you will be held in strictest confidence. Once you are recruited you will be issued with a volunteer code number. This number will be used on all your samples so that no one else will know, or be able to work out that they are yours.

Access to your records is restricted to the approved scientists who are running the study, the HNU research nurses, HNU Medical Advisor and your GP.

Will I be told my results?

No, as a volunteer you are valuable to us but we are unable to tell you any of your results.

Some results will be published but no reference is made to individuals. At the end of the study we will try to provide feedback of what we have found as a result of your help and what it may mean for the future research.

Is there anything I should tell you?

We do need you to tell us some things for your safety and for the success of the study. Please tell us if you have **ANY** episodes of illness even if it is just a headache, or if you become injured in any way, become pregnant. Some medication may affect the information we are collecting so you need to tell us if you take **ANY** medication, this includes anything you may have bought over the counter from the chemist e.g. paracetamol.

What if something happens to me while I am on the study?

The Institute of Food Research accepts responsibility for carrying out trials, and as such will give sympathetic consideration to claims from participants for harm suffered by them as a direct result of participating in the trial, with the exception of those claims arising out of negligence by the participant. Like other publicly funded bodies, the Institute is unable to insure and thus cannot offer advance indemnity cover for participants. Please note that the Institute will not fund any legal costs arising from any action unless awarded by a court.

Who is funding this study?

The research is taking place at IFR, Norwich. The research is being funded by money received by hospital Consultants to support medical research in Norwich. It is hoped that some local cancer charities will also offer financial support.

Who has reviewed this study ?

This study has been reviewed by the IFR Human Research Governance Committee (HRGC), East Norfolk & Waveney Research Governance Committee, Norwich and Norwich Local Research Ethics Committee (NLREC).

What should I do now ?

If you are interested in taking part in the study then please complete the reply slip overleaf and return it in the FREEPOST envelope provided (no stamp required). If you are not interested in taking part then you need do nothing, no one will contact you about the study.

Contact for further information

If you would like further information about the study then you can contact the study team:
HNU on 01603 255305 or
Email : satish.ranka@bbsrc.ac.uk

Taking part in the research is entirely voluntary!

You will be given a copy of this information sheet and your signed consent form to keep.

YOU ARE FREE NOT TO PARTICIPATE AND MAY WITHDRAW FROM THE STUDY AT ANY TIME.

Institute of Food Research
Norwich Research Park, Colney, Norwich NR4 7UA, UK Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

"THE FLAVONOID STUDY FLOWCHART"

1. RECRUITMENT
into "The Flavonoid Study"

↓

2. INITIAL INTERVIEW

3 day consideration period

↓

3. CONSENT & SCREENING

↓

4. Entry into study

- 5 x 24 hr Urine Sample collection container
- One FFQ

↓

5 Collection of Urine samples & completion of FFQ.

↓

- Food Frequency Questionnaire analysis

- Urinary specimen analysis for flavonoid excretion

↓

6. FLAVONOID INTAKE ASSESSMENT

- Statistical calculation of dietary intake of Flavonoids

↓

7. . Calculations to check whether the FFQ correlates with the urinary excretion of flavonoids

Annex 4

"THE FLAVONOID STUDY"

I am interested in finding out more information about the *FLAVONOID STUDY.*

(Please complete the personal details below)

Name: ……..

Address: ……..

……..

……..

Date of Birth: ……..

Daytime telephone no: ……..

Evening telephone no: ……..

Email address: ……..

Please return this form in the FREEPOST envelope provided to :

Dr Satish Ranka
Research Scientist
W312 Institute of Food Research 1
FREEPOST
Norwich Research Park
Colney
NORWICH
NR4 7BR

Expressing an interest in the study does not commit to you taking part!

Institute of Food Research
Norwich Research Park, Colney, Norwich NR4 7UA, UK Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

Annex 5

INFORMED CONSENT FORM FOR RESEARCH STUDY

Full Study Title: Correlation of dietary flavonoid intake by Food Frequency Questionnaire with measurement of urinary excretion of flavonoid metabolites

Have you read the Volunteer Information Sheet; Version No: 5. 0:01.2005
Date: ……..……YES/NO

Do you agree that you do not fall within the basic exclusion criteria listed for this research study?.....YES/NO*
*If you have answered NO to this question we are unable to accept you on this study.

Have you had an opportunity to discuss this study and ask questions; including the exclusion criteria
and your responsibilities as a volunteer?.....YES/NO

With whom have you discussed the information for this research study with?.....

Have you received sufficient information about the study?..... ..YES/NO

Have you received satisfactory answers to all your questions?
……YES/NO

Do you understand that you are free to withdraw from the study:

- at any time
- without having to give a reason for withdrawing
- without withdrawal affecting future participation in other research studies……YES/NO

Are you aware that your personal information will be held confidentially?.....YES/NO

Do you agree to us informing your General Practitioner of your participation in this study and
of all clinical results?.....
.....YES/NO*
* If you have said NO to this question then we are unable to accept you on this study.

Institute of Food Research
Norwich Research Park, Colney, Norwich NR4 7UA, UK Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

Name and address of your General Practitioner:

……

……
……..

Do you understand that all research is subject to Inspection and Audit*……YES/NO
*Although your records may be accessed for this purpose your personal information remains confidential

Do you agree to take part in this study?.....YES/NO

Signed: …… Date:……

(Name in BLOCK Letters:)……..

Date of Birth:……

Scientist (I confirm that the volunteer above has been given a full verbal and written explanation of the study)

Signed:…… Date:……

Name in BLOCK Letters: ……

A copy of the signed consent form will be given to the volunteer to keep.

NDH/Version 7/ AB/Rev. July 04

Institute of Food Research
Norwich Research Park, Colney, Norwich NR4 7UA, UK Tel: +44(0) 1603 255000 GTN 6626 5000 Fax: +44 (0)1603 507723

Annex 6 **CONFIDENTIAL**

Volunteer Health Declaration Form

Volunteer code number:…… Sex:……..

D.O.B.:……. Age:……

Height:……. Weight:……

B.P.:…… Pulse:……..

Urine: …….. Body Mass Index (BMI)……

Have you ever had any of the following:
If yes give details below each relevant section.

Angina/Heart disease: Y N
……
……

Thrombosis: Y N
……
……

High blood pressure: Y N
……
……

High Cholesterol: Y N
……..
……..

Chest problems: Y N
……
……

Diabetes: Y N
……..
……..

Depression or anxiety: Y N
……
……

Digestive problems: Y N
……
……

Skin Conditions: Y N
……..
……

Other:
……
……

Are you currently on any:

Prescribed medication:…….

Dietary supplements:..... Herbal remedies:……

If yes give details:……

Have you had a major physical injury/operation:……

If yes give details:……..
……

Volunteer No:…….

Are you currently suffering from any illness/injuries: Y N
If yes give details:…….
……..

Are you/could you be pregnant: Y N

Do you/ or have you ever smoked: Y N

When did you stop smoking:……

If yes how many per day:……..

Do you drink alcohol: Y N

How many units per week:……

Have you any known allergies: Y N
Food:…….
Other::……
……

Special dietary requirements: Y N

Do you agree to us, informing your General Practitioner of your participation in this study or of any results found: Y N

If you have answered NO to this question then we are unable to accept you on this study.

What is the name and address of your General Practitioner?
…….
……
……
Tel. No…….

Form completed by (print):……Signature…….

Date:……

Annex 7

HNU/letter

Date!!

Dear Doctor ……..

Your patient, ……, has volunteered to take part in a human nutrition study at the Institute of Food Research entitled " The Flavonoid Study

Following consent it is our standard practice to screen the volunteers to exclude any health factors which may affect the study data or indicate an issue which may require further investigation. We are looking for healthy people who have no chronic illness and are not taking any prescribed medication which may affect the study data.

Some of your patient's results fell outside the standard reference range on this occasion.

Please find results enclosed.

These results **will/will not** affect the study data.

Your patient **will/will not** be able to participate in the study.

We have informed your patient that one or more of the results from their screening were outside these reference ranges. We have not discussed these results with them. However, we have asked them to speak to you as soon as convenient to discuss these results, in order that you may take further action/investigation if necessary.

Yours sincerely

Annex 8

Date

Dear Doctor ……..

This is to inform you that your patient …….. has consented to participate in a nutrition study at the Institute of Food Research.

The study titled “The Flavonoid Study” as been approved by the Norwich Local Research Ethics Committee and the study co-ordinator Dr Satish Ranka can be contacted on …….. if you require further information.

It is our policy to forward copies of all results (normal or abnormal) obtained during the study to the Volunteer’s GP.

We anticipate completion of the study by ……..

Yours sincerely