
# THE IMPACT OF HIGH PROTEIN-HIGH RED MEAT VS HIGH CARBOHYDRATE WEIGHT LOSS DIETS ON GENOME STABILITY AND BIOMARKERS OF COLORECTAL CANCER RISK IN OVERWEIGHT MEN.

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# ABSTRACT

It has been suggested that high protein diets are associated with an increased risk of colorectal cancer due to the higher content of red meat. However, the study of the overall dietary and lifestyle pattern may prove more important than any individual component when assessing colorectal cancer risk. From this, it is proposed that a dietary pattern used for weight loss that is higher in protein but remains low in fat and high in foods rich in fibre and micronutrients that are required for genome stability may not increase the risk of colorectal cancer, thus providing a safe and effective dietary method of weight loss in overweight subjects.

This thesis describes the development of a novel *in vitro* faecal water genotoxicity test using the cytokinesis-block micronucleus (CBMN) cytome assay in the WIL2-NS cell line. This thesis then investigates faecal water genotoxicity and peripheral blood lymphocyte genome stability in overweight men following a weight loss dietary pattern either high in protein, specifically red meat, or high in carbohydrate.

Results from this thesis indicate that the genotoxic potential of faecal water can be successfully assessed *in vitro* using the CBMN cytome assay. A high protein-high red meat weight loss diet did not increase faecal water genotoxicity or peripheral blood lymphocyte DNA damage, measured with the CBMN cytome assay, differently to a high carbohydrate weight loss diet. Faecal water genotoxicity data suggests weight loss and/or caloric restriction following either a high protein or high carbohydrate diet may beneficially modify the carcinogenic load of the colon in the short term, however this needs to be validated in a study that includes a non-weight loss control group. A lack of relationship was seen between faecal water genotoxicity and genome damage in lymphocytes which may suggest that the assessment of both the genome damage potential of the bowel contents and the assessment of the genome stability profile of peripheral blood lymphocytes may be important in comprehensively assessing the impact on genome damage by different dietary patterns.

# ABBREVIATIONS

| | |
|---|---|
| BN | binucleate |
| CBMN | cytokinesis-block micronucleus |
| Cyto B | cytochalasin B |
| DMSO | dimethyl sulphoxide |
| FBS | foetal bovine serum |
| HBSS | hanks balanced salt solution |
| HC | high carbohydrate |
| HP | high protein |
| MN | micronucleus |
| MNi | micronuclei |
| NDI | nuclear division index |
| NDCI | nuclear division cytotoxicity index |
| NPB | nucleoplasmic bridge |
| NBud | nuclear bud |
| PHA | phytohaemagglutinin |
| RDA | recommended dietary intake |
| SCFA | short chain fatty acid |

# DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

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Bianca J Benassi Date

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# PRESENTATIONS

**2007:** European Nutrition Conference

Poster presentation: High protein-high red meat and high carbohydrate weight loss diets do not differ in their effect on faecal water genotoxicity.

Poster presentation: High protein-high red meat and high carbohydrate weight loss diets do not differ in their effect on lymphocyte DNA damage using the cytokinesis-block micronucleus cytome assay.

Australian Society for Medical Research SA Scientific Meeting

Ross Wishart Memorial Session, Oral Presentation: Impact of a high protein-high red meat vs high carbohydrate diet on biomarkers of colorectal cancer risk

**2006:** International Congress on Obesity

Poster presentation: Short term effect of a high protein-high red meat diet vs. a high carbohydrate diet on biomarkers of colorectal cancer risk

International conference on Nutrigenomics and Gut Health

Oral presentation: Inter- and intra-individual variation in DNA damage potential of faecal water assessed in the WIL2-NS cell line

**2005:** Nutrition Society of Australia national conference

Poster presentation: Benassi B, Clifton P, Fenech M (2005) Inter- and intra-individual variation in DNA damage potential of faecal water assessed in the WIL2-NS cell line, Asia Pac J Clin Nutr, 14 (suppl):S95

# 1. INTRODUCTION

## *1.1 High protein diets*

Over 60% of Australians are overweight and of these, 20% are obese (Figure 1.1) [1]. These people are at higher risk of a number of conditions such as cardiovascular disease, type II diabetes and some cancers including colorectal cancer [2-4]. However, there are currently no agreed guidelines on the most effective weight loss strategy. This indicates that the development of effective and safe strategies for weight loss and weight management is essential in order to reduce the prevalence of obesity and its associated co-morbidities. Since the 1960s, diets that are high in protein and restrictive of carbohydrate intake have been popular among dieters [5]. The replacement of dietary carbohydrate with protein, combined with a low intake of fat, has been shown to induce larger weight loss in overweight subjects when compared with conventional high carbohydrate/low fat diets, suggesting this is a favourable weight loss regime for overweight people [6-9].

NOTE: This figure is included on page 1 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.1 Prevalence of overweight and obesity by BMI in Australian men and women aged 25-64 years from 1980 to 1999/2000.**
Source: [1].

There are several possible mechanisms that may explain this relationship between high dietary protein intake and enhanced weight loss. Baba *et al* (1999) [7] proposed a higher level of weight loss seen with the high protein diet was a result of the thermogenic effect of protein, meaning an increase in energy expenditure after eating
a high protein meal. It has been shown that protein can exert up to three times more

of a thermic effect of feeding compared with isocaloric loads of carbohydrate or fat [10]. High protein intake has also been shown to increase the level of satiety and result in a decrease in total daily energy intake which would also enhance weight loss [11].

There is a strong association between obesity and insulin resistance [12] which is another significant risk factor for cardiovascular disease, type II diabetes [13] and colorectal cancer [14]. The term 'insulin resistance' refers to the reduced sensitivity of the effects of insulin on glucose uptake, metabolism or storage. It has been shown that weight loss significantly improves insulin sensitivity [15]. Results of a study by Baba *et al* (1999) [7] demonstrated that both high carbohydrate and high protein diets reduced fasting insulin levels in hyperinsulinemic obese subjects, however, a diet high in protein induced a more significant reduction that was within the normal range. This suggests that a diet high in protein and low in fat is more effective in achieving both a maximum weight loss and improved insulin sensitivity.

Despite the popularity and effectiveness of high protein diets for weight loss, there have been no long term studies to determine their safety, raising concerns over their long term use in the general public [5, 16, 17]. For the purpose of this thesis, the term 'high protein diet' refers to a moderate protein, moderate carbohydrate, low fat diet (approximately 35% protein, 40% carbohydrate, 25% fat) and high carbohydrate diet refers to high carbohydrate, low protein, low fat diet (15% protein, 60% carbohydrate, 25% fat). This high protein diet is different from severely restricted carbohydrate, moderate protein diets (10-15% carbohydrate) which is typically high in fat. Although controversial, high protein diets have been suggested to have adverse effects on bone, renal function and cancer in some people and the need for more research in this area has been emphasised [16, 18]. In particular, it is suggested that the high red meat content of high protein diets are associated with an increased risk of colorectal cancer and this theory is explored in this thesis.

## *1.2 Colorectal cancer*

Colorectal or bowel cancer is the term used to describe cancer of the colon or rectum. Colorectal cancer has the highest incidence rate of all malignant cancers (excluding skin cancers) (figure 1.2) and is the second most commonly occurring cancer in both men and women in Australia, accounting for 14.6% of all new cancer cases in 2000 [2]. Every 1 in 17 Australian men and 1 in 26 women are likely to develop the disease in their lifetime, with the incidence rates increasing with age (figure 1.3) , and with most cases occurring after the age of 40 [2, 19]. With an aging population in Australia, it can be expected that the number of new cases will increase significantly [19]. Colorectal cancer is the third most common cancer causing death for men and women in Australia, with one in 61 people estimated to die from the disease [2]. By world standards, the incidence of colorectal cancer is higher amongst more developed countries [2]. Incidence rates are lower in Australia than that found in New Zealand but higher than that of the USA, Canada, UK and Northern Europe [2].



**Figure 1.2 Most frequently occurring cancers in both men and women in Australia in 2000.**

Source: [2].

**Figure 1.3 Age-specific incidence and mortality rate of colorectal cancer in men and women in Australia in 2000.**
(a) Age-specific incidence and mortality rate of colorectal cancer in men and (b) Age-specific incidence and mortality rate of colorectal cancer in women.
Source: [2].

## *1.3 Colorectal cancer and the influence of diet*

It has been recognised since the 1970s that the development of colorectal cancer is not entirely genetic in basis and is largely influenced by environmental factors. This is supported with the association of dietary patterns and international differences in the incidence of colorectal cancer [20, 21]. Further, migrants from countries with a low incidence of colorectal cancer who move to a country with a higher incidence tend to acquire the increased risk of that country, suggested to be through a change in diet [20-22]. This variation in diet with cancer rates suggests that diet is an important risk factor for many forms of cancers and supports the possibility that cancer can to some extent, be preventable by dietary changes [23]. For colorectal cancer, it is estimated that up to 90% of cases can be attributed to environmental factors [24]. Although this suggests that the effect of diet on cancer risk is of great public importance, the influence of specific dietary factors on colorectal cancer risk remains largely uncertain [3, 23].

### 1.3.1 Fibre

The 'fibre hypothesis' was first introduced by Burkitt in 1971 [20] who noted an association of high dietary fibre intake and low incidence of bowel cancer in Africa. There are several plausible mechanisms for this apparent protective effect of fibre however the precise mechanism remains unknown. Fibre is proposed to regulate bowel function through increasing stool bulk and decreasing transit time, resulting in dilution of the gut contents and a reduction in exposure time of carcinogens to the colonic mucosa cells [3, 23]. Another possible mechanism is through the fermentation of fibre by colonic bacteria in the large intestine which produces short chain fatty acids such as butyrate, which may potentially protect against colorectal cancer through increasing cell differentiation, inducing apoptosis in damaged cells and inhibiting the production of detrimental metabolites through reducing pH in the lumen [3, 23, 25]. As reviewed by Key *et al* [23] many case-control studies have observed a moderate reduction in risk of colorectal cancer with high consumption of dietary fibre, however results from prospective studies have proven inconsistent. Further, results with high fibre supplements or high fibre diet intervention trials over a 3-4 year period have not been able to show any reduction in colorectal cancer risk. One of the largest prospective studies to investigate dietary fibre intake and colorectal cancer incidence to date involves the European Prospective Investigation into Cancer and Nutrition (EPIC) study, reported by Bingham *et al* which involved 519 978 men and women from 10 European countries [26]. The report concluded that doubling the total fibre intake in those people who consume low intakes of fibre (average of 12 grams per day) could reduce risk of colorectal cancer by 40%. A recent analysis of 13 prospective cohort studies observed a significant inverse association of dietary fibre intake and colorectal cancer risk when adjusted for age only, however this association was not significant after adjustment for other proposed colorectal cancer risk factors [27]. It has been suggested that the conflicting reports may be a result of the difficulty in quantifying dietary fibre or due to differences in the types of fibre consumed [23, 28]. Thus the theory that high fibre intake reduces the risk of colorectal cancer remains largely unestablished.

### 1.3.2 Fat

Wynder first proposed an association between dietary fat and colorectal cancer in 1975 [29]. There are several possible mechanisms for this association. One hypothesis is that dietary fat increases the excretion of bile acids which can be converted to secondary and tertiary bile acids by colonic mucosa, which in association with changes to the gut bacterial flora, are thought to produce tumour promoter substances [30]. Alternatively, it has also been suggested that long chain fatty acids present in red meat act as tumour promoters in the gut, however this is uncertain as the amount of fatty acids that reach the gut intact is not known [31]. Overall, results from case-control and cohort studies generally do not support an independent association of colorectal cancer with fat intake, beyond that associated with total energy intake (reviewed 1.4.1) [23, 31, 32].

### 1.3.3 Red meat

The consumption of meat, specifically red meat, is one of the most studied dietary factors in relation to its risk for colorectal cancer [33]. With an increasing interest in the use of higher protein diets for weight loss and weight management, the risk of meat is of particular importance. It is however, perhaps the most controversial of risk factors associated with colorectal cancer. In 1997 a report by the AICR & WCRF [34] determined that red meat is probably associated with an increase risk of colorectal cancer. This led to the recommendation that the consumption of meat should be no more than 80g per day and that a reduction in red meat intake would decrease the incidence of colorectal cancer and possibly also other cancers. However, this claim was considered unjustified as there was little evidence to support such a recommendation to the general public [35, 36]. A further review by Truswell in 2002 suggested that evidence from studies performed since the AICR & WCRF report indicates that the relationship between meat and colorectal cancer is now even weaker than the 'probable' status it was given in 1997 [37]. Norat *et al* (2005) reports on meat and fish intake and risk of colorectal cancer from the EPIC study, the largest prospective study to date involving 478 040 men and women from 10 European countries [38]. A significant association was found for high red and processed meat intake and colorectal cancer, however in separate analyses processed meat was still statistically significantly associated with colorectal cancer however high red meat intake was not. Norat *et al* (2002) and Chao *et al* (2005) also support that processed

meat may be more strongly associated with colorectal cancer than red meat intake alone [39, 40].

It has been suggested that the inconsistency to define the effect of red meat on colorectal cancer risk may perhaps be due to the lack of ability to identify any underlying specific biological mechanism [41]. There are several putative biological mechanisms that are believed to arise from either the consumption of red meat or through its food processing and cooking techniques, that are suggested to be involved in colorectal carcinogenesis:

***Heterocyclic amines***

A family of compounds called heterocyclic amines (HCAs) which are both mutagenic and carcinogenic are produced through the pyrolysis of amino acids in meat during cooking at high temperatures [42-45]. The quantity of HCAs produced are dependant on the type of meat and the cooking temperature and method [42, 44, 46]. Various HCAs have been shown to induce DNA damage in vitro [43-45, 47] and tumours at various sites including the colon in animal studies [44, 45, 48]. The dosages used in animal studies however can exceed daily human intake by several orders of magnitude and the validity in determination of the genotoxic/cytotoxic potential from the extrapolation of high animal dosages to lower human intake is unclear and as such the contribution of HCAs to colorectal carcinogenesis is suggested to be very small [49, 50]. A recent study using food frequency questionnaires to assess intake of HCAs found a significant association of HCA intake with increased risk of colorectal cancer [51] however a separate study with a different study population found no significant interaction [52].

***Polycyclic aromatic hydrocarbons***

Polycyclic aromatic hydrocarbons (PAHs) are formed from the incomplete combustion of organic matter, with the main sources being industrial exposure, for example burning of fossil fuels, cigarette smoking and charcoal-broiled/grilled or smoked meats consumed in the diet [42, 44, 45]. Dietary exposure is thought to be important as blood PAH adducts have been shown to correlate with charcoal-broiled red meat [53] and a recent study has shown intake of PAHs assessed using food frequency questionnaires from meat was associated with increased risk of colorectal

cancer [51, 54], however it is suggested that it cannot be assumed this is a direct association of diet exposure alone [44].

***N-nitroso compounds***

N-nitroso compounds (NOCs) are formed endogenously in the colon from amino acids which are decarboxylised to amines and amides by bacteria on the colon, which then undergo N-nitrosation to form NOCs [55]. Red meat has been shown to increase the level of NOCs that reaches the colon assessed from faecal NOC levels in a dose-dependant manner, but white meat was shown to have no effect, which supports red meat as the likely dietary source [56, 57]. Recently, it has been shown that faecal NOCs, following red meat consumption in humans, increase the formation of DNA adducts in colonic cells [58].

***Haeme iron***

A further hypothesis for the association of red meat with colorectal cancer risk arises from evidence of the ability of dietary haeme to induce faecal water cytotoxicity and hyperproliferation of the colonic mucosa in rats [50, 59]. Dietary haeme has also been shown to dose dependently increase azoxymethane induced aberrant crypt foci in rats [60]. As the haeme content of red meat has been shown to be 10 fold times higher than that in white meat [61], this may also lend support to red but not white meat being associated with increased colorectal cancer.

### 1.3.4 Micronutrients

The relationship between diet and cancer is often explained in terms of carcinogen exposure from dietary factors; however it has been suggested that the deficiency in certain dietary factors high in micronutrients thought to protect against cancer may be as important or even more so [62]. Further, the recommended dietary allowance (RDA) of vitamins and minerals to prevent acute disease, for example scurvy, may not be optimal for reduction of risk of cancer [62]. *In vitro* evidence for the importance of micronutrients in the maintenance of genome stability is described in Chapter 3.4.2.

Micronutrients suggested to be associated with reduced colorectal cancer risk specifically include folate, methionine, Vitamin B6 and B12 which are involved in DNA methylation, synthesis, stability and repair and Vitamins C and E and selenium

due to their antioxidant properties [63, 64]. Vitamin D and calcium are also thought to be protective against colorectal cancer induction with anti-proliferative actions on intestinal cells [65]. Overall however, evidence of a direct protective or anti-carcinogenic effect of micronutrients on colorectal cancer risk from human studies remains largely unclear [34, 66]. The impact of micronutrients is important when assessing the effect of different dietary patterns on colorectal cancer risk as diets high in nutrient-dense protein foods have been shown to be significantly higher in micronutrients required for protection against genome damage [67].

## *1.4 Colorectal cancer risk factors and the influence of high protein diets*

### 1.4.1 Total calories

Considering that the extent to which individual dietary factors are associated with increased risk of colorectal cancer is inconsistent, it has been suggested that the amount of total energy consumption (more so the overconsumption of energy), may be the important factor associated with increased risk rather than any one individual source [68-70]. Steinbach *et al* (1994) demonstrated that caloric restriction reduced the rate of rectal epithelial cell proliferation which is a property of cancer, in rectal biopsies from obese subjects. Furthermore, energy restriction in Zucker obese rats has been shown to retard the appearance of advanced abberant crypt foci, precursors of colorectal carcinogenesis [71] which illustrates that energy restriction exerts an effect on colonic mucosa. This supports results found in a case-control study by Slattery *et al* [70] which determined that, after physical activity was controlled for (energy expenditure), high levels of energy intake were associated with an increase in colorectal cancer risk by approximately 70%, where an increase of 500 calories per day resulted in a 15% increase in colorectal cancer risk in men and 11% increase in risk in women. This increase in risk was not associated with any specific source of energy, suggesting that total energy is more important to risk of colorectal cancer than any one particular source of energy. Therefore, preference of a low calorie diet which is higher in protein over an isocaloric high carbohydrate diet alternative may not increase the risk of colorectal cancer.

### 1.4.2 Physical inactivity and Obesity

There is a consistent association between lack of physical activity and increased risk of colorectal cancer among a number of studies of various study designs, populations and forms of activity [72]. Similarly, energy intake if higher than energy expenditure, results in obesity, characterised by high BMI, which has also been associated with increased risk of colorectal cancer [73-75]. Further, it has been suggested that central adiposity in particular was related to colorectal cancer risk in men [74, 75]. The relationship of obesity and low physical activity with increased risk of colorectal cancer is weaker in women, particularly older women than men [76-78]. The reason for this is unclear at present but it is suggested that colorectal cancer risk is influenced by menopause status, such that the association may even be limited to premenopausal women [76]. Compared with a lower protein diet, a high protein weight loss diet has been shown to increase not only the total amount of weight lost but the amount of abdominal fat lost in women, while the results were less clear in men [79]. Therefore, a high protein diet used for weight loss, in particular if it can reduce central adiposity in men, in combination with regular physical exercise may be effective in reducing the risk of colorectal cancer associated with obesity and physical inactivity.

### 1.4.3 Insulin resistance

It can be suggested from the lack of unifying evidence for the risk of specific dietary and lifestyle factors on risk of colorectal cancer that the interaction among these various components may play a more important role in colorectal carcinogenesis than any individual factor [80]. First proposed by Giovannucci [14] and McKeown-Eyssen [81] was a mechanism whereby the proposed dietary and lifestyle risk factors for colorectal cancer were associated with insulin resistance (IR) and hyperinsulinaemia and that in turn, it is high blood-insulin levels that stimulate the growth of colorectal tumours. This hypothesis was supported primarily by epidemiologic evidence that many risk factors for IR are the same as those suggested for colorectal carcinogenesis, specifically, dietary factors, obesity and physical inactivity [14, 82]. This is supported at a molecular level as insulin has been shown to be a growth factor in colonic mucosa cells and is a mitogen in colonic carcinoma cells in vitro [83, 84]. As reviewed in Chang and Ulrich [85], numerous

epidemiological studies to date which have examined the relationship of diabetes and colorectal cancer risk have generally indicated an increased risk of colorectal cancer to be associated with IR markers and people with diabetes as compared with controls. These studies however, only suggest a role of insulin in colorectal carcinogenesis. Animal studies have shown insulin to promote the growth of abberant crypt foci, precursors of colorectal carcinogenesis [86, 87]. Koohestani *et al* [86] further showed that insulin resistance preceded promotion of colorectal carcinogenesis. These studies provide support for not only a role of insulin in colorectal carcinogenesis but for a causal effect of insulin in the development of colorectal cancer. This suggests that colorectal cancer may be a modifiable disease and it is therefore plausible that dietary intervention which reduces IR may also reduce the risk of colorectal cancer [88]. Weight loss has been shown to improve insulin resistance [15] and diets specifically high in protein have demonstrated a more significant improvement in restoring insulin resistance to normal levels [7]. From this, it is possible to propose that high protein diets, through restoration of insulin resistance, may reduce the risk of colorectal cancer.

## 1.5 Total dietary patterns and colorectal cancer risk

The influence of individual dietary factors on risk of colorectal cancer remains largely undefined. The inter-relation of food components in the diet makes it difficult to define the role of any specific dietary factor on colorectal cancer risk [89]. Association of dietary factors with colorectal cancer risk may also be confounded by lifestyle factors such as BMI and physical activity [23] such that various specific exposures and behaviours may jointly influence colorectal cancer risk. A case control study by Slattery *et al* [90] that assessed broad eating patterns and colorectal cancer risk observed stronger and more consistent associations with general eating patterns than with any individual food source. Therefore, the study of the overall dietary and lifestyle pattern may prove more important than any individual component when assessing the risk of colorectal cancer [39, 89, 90]. Although a high protein-red meat diet may be associated with increasing the risk of colorectal cancer due to the higher content of meat, such a diet is also associated with weight loss and improved insulin sensitivity, risk factors which are also associated with colorectal cancer. From this, it can be suggested that a dietary pattern used for weight loss that is higher in protein but remains low in fat and high in foods rich in fibre and micronutrients that are required for maintenance of DNA, in association with moderate exercise may not

increase the risk or may decrease the net risk of colorectal cancer, thus providing a safe and effective dietary method of weight loss in obese or overweight subjects. This thesis aims to investigate the impact of a dietary pattern that is high in protein, specifically red meat, compared with a high carbohydrate diet on various standard and novel biomarkers of colorectal cancer in overweight men.

# 2. AIMS AND HYPOTHESES

The aims and hypotheses for this thesis, and the corresponding chapters, are described below.

## *AIMS*

*Chapter 5*

To develop and validate a CBMN cytome assay to investigate the genotoxic and cytotoxic potential of human faecal water.

To assess and compare the extent of inter- and intra-individual variation in faecal water genotoxicity and cytotoxicity measured using the CBMN cytome assay.

*Chapter 6*

To investigate whether a high protein weight loss diet, specifically high in red meat, influences the genome damage rate in colonic cells, the genotoxic and/or cytotoxic potential of faecal water and other conventional bowel health biomarkers differently compared to a high carbohydrate weight loss diet.

*Chapter 7*

To investigate whether a high protein weight loss diet, specifically high in red meat, influences the genome stability profile in peripheral blood lymphocytes differently compared to a high carbohydrate weight loss diet.

*Chapter 8*

To investigate whether the genotoxic potential of faecal water is correlated with the genome stability profile of peripheral blood lymphocytes.

## *HYPOTHESES*

*Chapter 5*

Human faecal water induces genotoxic and cytotoxic damage in the WIL2-NS cell line in a dose-dependent manner.

The variation in genotoxic and cytotoxic potential of human faecal water is smaller within one individual than for that observed between individuals.

*Chapter 6*

A high protein-high red meat dietary pattern does not increase the genotoxic/cytotoxic potential of faecal water compared with a high carbohydrate weight loss diet.

A high protein-high red meat dietary pattern does not have a negative impact on conventional bowel health biomarkers compared with a high carbohydrate weight loss diet.

The influence of dietary patterns on the genotoxic/cytotoxic potential of faecal water is correlated with changes seen with conventional bowel biomarkers.

A high protein-high red meat dietary pattern does not increase the micronucleus index in colonic cells compared with a high carbohydrate weight loss diet.

*Chapter 7*

A high protein-high red meat dietary pattern does not increase genome instability in peripheral blood lymphocytes compared with a high carbohydrate weight loss diet.

A high protein-high red meat dietary pattern results in an improved status of micronutrients required for genome stability compared with a high carbohydrate weight loss diet.

*Chapter 8*

The genotoxic potential of faecal water is a risk factor for increased genome damage in peripheral blood lymphocytes.

# 3. GENERAL METHOD: THE CYTOKINESIS BLOCK MICRONUCLEUS CYTOME ASSAY

## *3.1 Introduction*

This chapter is a review of the evolution and use of the cytokinesis block micronucleus (CBMN) 'cytome' assay, utilised in this thesis, as an important tool in the measurement of genomic instability. The development of the assay, current and potential future applications, as well as a detailed description of the scoring criteria used for this thesis will be discussed.

## *3.2 The discovery of micronuclei*

It was independently proposed by both Heddle (1973) and Schmid (1975) that chromosome damage can be detected and assessed through using a method which involves the measurement on micronuclei (MNi) in dividing cell populations, such as bone marrow cells [91, 92]. The assay, termed the micronucleus assay, used in bone marrow, peripheral blood erythrocytes and cultured mammalian cells is now a well established assay in the field of genetic toxicology [93].

MNi arise in dividing cells that contain acentric chromosome fragments (lack a centromere) or whole chromosomes which fail to segregate to the spindle poles during mitosis. During telophase, these lagging fragments are enclosed by a nuclear envelope and assume similar morphology to the main nuclei of the cell, with the exception that they are smaller, hence the name they are given - 'micronucleus' (Figure 3.1) [93]. As MNi are expressed in cells that have undergone nuclear division, they are ideally assessed in once divided cells, recognised as binucleated cells, following cytokinesis block [93].

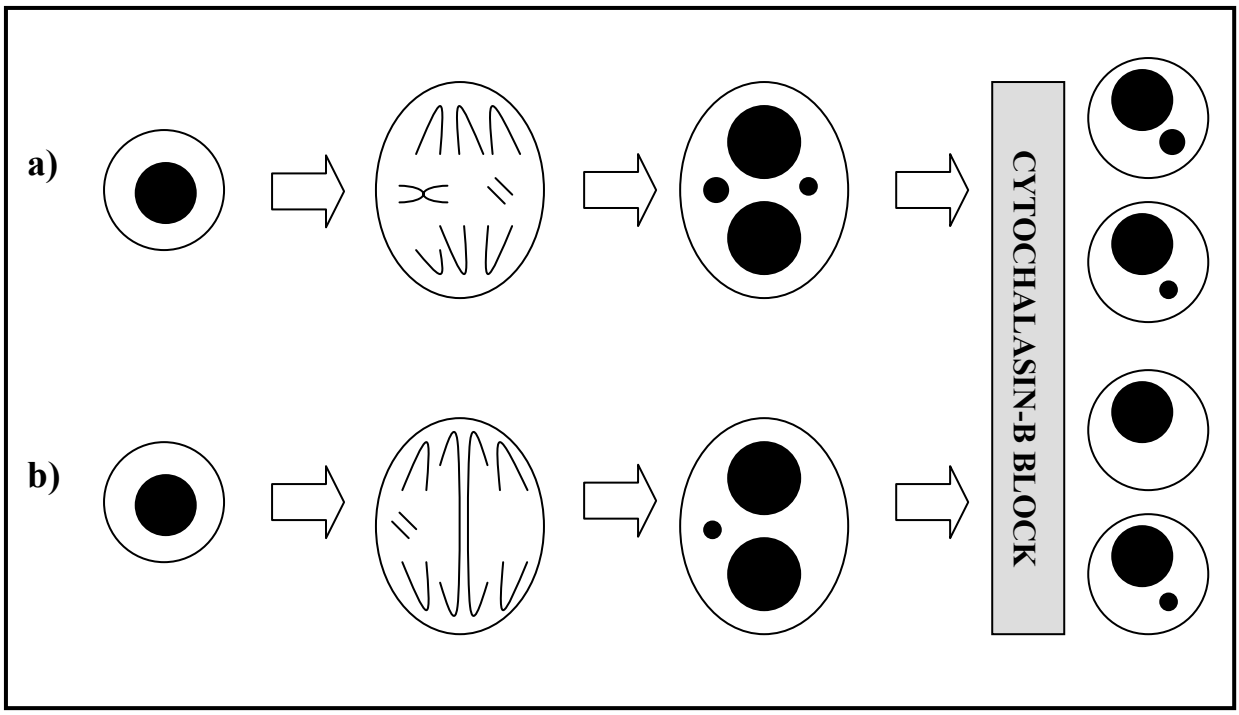


**Figure 3.1. Detection of chromosome damage using the micronucleus assay.**
(a). The origin of micronuclei from lagging acentric chromosome fragments or whole chromosomes during cell division. (b). Micronucleus formed from an acentric chromosome fragment and a nucleoplasmic bridge from a dicentric chromosome where the centromeres have been pulled to opposite spindle poles during cell division. The role of cytochalasin B in blocking dividing cells is indicated in the diagram. Adapted from Fenech 2000 [93].

As this method measures MNi in dividing cells, it cannot be used efficiently or quantitatively in cell populations which have unknown or uncontrolled cell division kinetics. There was therefore a need to be able to distinguish between cells which are dividing and those which are not dividing in a cell population. Several methods were proposed however the most favoured approach, due to its simplicity and precision, was the cytokinesis-block micronucleus (CBMN) assay developed by Fenech and Morley [94, 95]. In the CBMN assay, cells which have completed one nuclear division are blocked from cytokinesis by cytochalasin-B (Cyto-B), which allows for these cells to be readily identified by their binucleated appearance (Figure 3.1). Cyto-B is an inhibitor of actin polymerisation, which is required for the microfilament ring constriction of the cytoplasm between two daughter nuclei during cytokinesis, so that they do not separate and instead appear as a binucleated cell, or a cell with two nuclei [96]. The use of Cyto-B allows for the accumulation of almost all dividing cells at the binucleate stage, regardless of the proportion of dividing cells, and MNi are scored only in these binucleated cells. From this, it is evident that MNi detected in binucleated cells can give an easy and reliable index of chromosome

breakage and loss. This enables a reliable assessment of chromosome damage between cell populations that do not have the same cell division kinetics, which can differ markedly depending on culture conditions, genotype, age and treatment [93].

### *3.3 The evolution of the CBMN assay into a 'cytome' assay*

Apart from MNi, the appearance of nucleoplasmic bridges (NPBs) between the nuclei of a binucleated cell can also be observed with the CBMN assay. These are proposed to arise from dicentric chromosomes, whose centromeres are pulled to opposite spindle poles of the cell during cell division, resulting in a nucleoplasmic connection, covered by a nuclear membrane (Figure 3.1.B, 3.2) [93, 97]. The index of NPBs detected in binucleates gives an additional measurement of chromosome rearrangement and DNA misrepair. It has been suggested that NPBs may also arise from dicentric chromosomes formed as a result of telomere end fusions [98].

More recently, the recognition of the presence of another mechanism of MNi formation known as nuclear budding has emerged. This is believed to occur as a result of the elimination of amplified DNA from the cell [99-101]. Nuclear buds are characterised by having the same morphology as MNi except that they are connected to the main nucleus with a stalk of nucleoplasmic material (Figure 3.2).

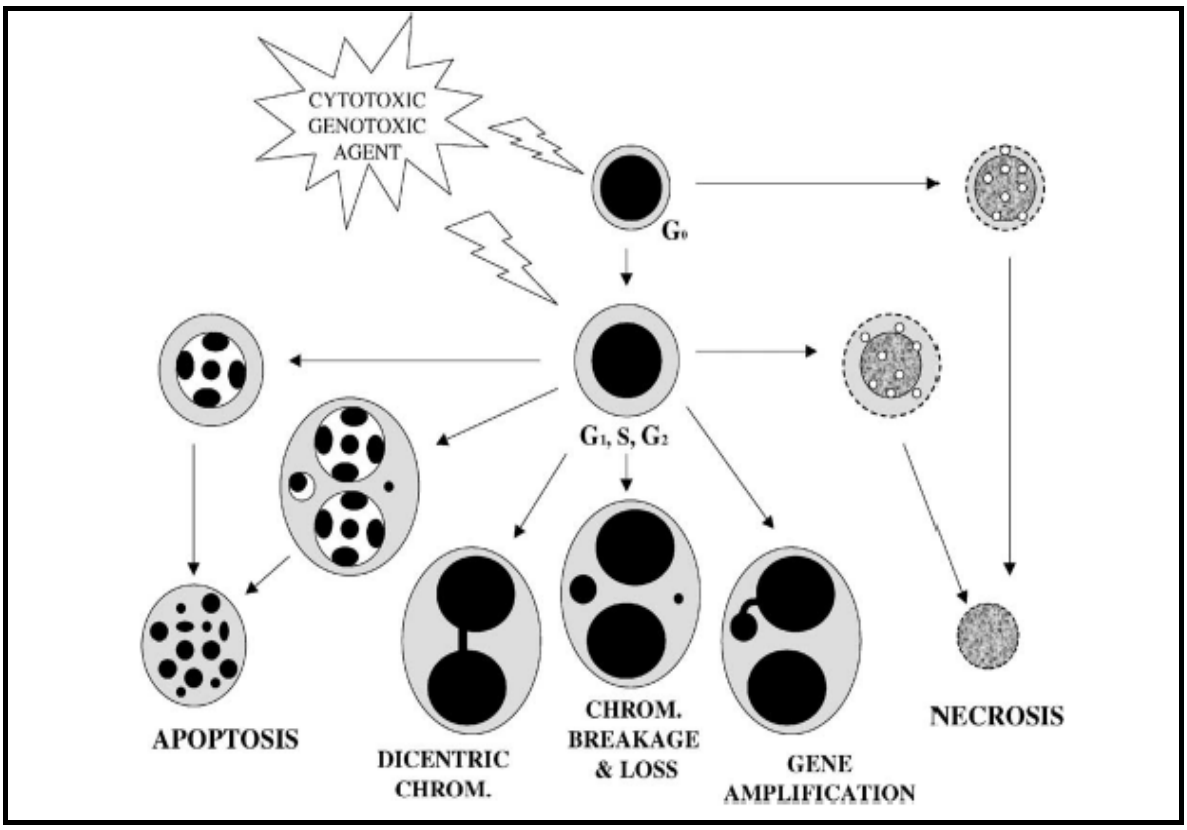


**Figure 3.2. The possible fates of cytokinesis-blocked cells following exposure to a cytotoxic/genotoxic agent.**
Using the CBMN assay, it is possible to detect chromosome breakage and loss (MNi), chromosome rearrangement, such as dicentric chromosomes (NPB) and gene amplification (NBuds). Cell death events (necrosis and apoptosis) can also be assessed. In addition, cytostatic effects can be estimated from the ratio of mono-, bi- and multi-nucleated cells. Adapted from Fenech 2006 [98].

The CBMN technique is an important tool in the measurement of genomic instability, as it provides a comprehensive measure of chromosome damage, as well as cell death (apoptosis and necrosis) rates [98, 102]. Further, cytostatic effects can also be estimated from the ratio of mono-, bi- and multi-nucleated cells. Given that the CBMN assay can be used to provide a comprehensive picture of the genotoxic, cytotoxic and cytostatic effects of a particular cellular insult, this has led to the conception of the CBMN assay as a 'cytome' assay, as every cell in the system is assessed for its viability status (apoptosis/necrosis), mitotic status (mono-, bi-, multi-nucleated) and chromosome instability/DNA damage status (MNi, NPB, NBuds) [98].

## *3.4 Applications of the CBMN cytome assay*

### 3.4.1 The CBMN cytome assay as a biomarker of cancer risk

Considering cancer is likely to be caused by gene and chromosomal mutations and can take years to develop, numerous studies use DNA damage biomarkers for an assessment of cancer risk [102]. The induction of the genomic instability phenotype is considered a crucial early event in carcinogenesis, enabling a cell to evolve into a cancer cell [102]. The ability of the CBMN cytome assay to provide a measure of genomic instability, as the result of DNA damage, therefore indicates its potential as a biomarker of cancer risk [103]. The current methodology whereby lymphocytes from a blood sample are cultured *ex vivo* and once divided cells distinguished by blocking of cytokinesis with cytochalasin-B, allows for a measure of DNA damage that has accumulated while lymphocytes circulate throughout the body, and with a half life of approximately 3-6 months, integrating genotoxic events across body tissues [102, 104].

A causal link between MNi and cancer is supported through a number of observations, as evaluated by Fenech *et al* (1999) [103]:

1. A high frequency of MNi in cancer patients and patients affected by cancer-prone congenital diseases such as Bloom syndrome and ataxia telangiectasia [105-110].
2. Use of MNi in oral mucosa as a surrogate biomarker in clinical chemoprevention trials [111-113].
3. A correlation between genotoxic and carcinogenic agents and increased MN frequency eg ionising radiation, tobacco smoke [114].
4. An inverse association of MN frequency and blood concentration of certain micronutrients associated with increased risk of cancer [115].

The strongest evidence for the association of MN frequency and early events in carcinogenesis in humans comes from a recent large international cohort study, where individuals were tested for MN frequency between 1980 and 2002 and were free of cancer at the time of testing. A significant increase for all cancers were found in subjects in the medium to high MN frequency groups (RR of 1.84 and 1.53 respectively) after adjusting for confounders such as age, gender and smoking status and were strongest for urogenital and gastro-intestinal cancers [108]. Findings from this study support the use of MNi in peripheral blood lymphocytes as a predictive biomarker of cancer risk. A more recent study by Murgia *et al* (2007) showing a

stronger prospective association of MN frequency in peripheral blood lymphocytes with cancer risk also lends further support for the micronucleus assay being one of the best validated cytogenetic test for predicting cancer risk in humans currently available.

### 3.4.2 The CBMN cytome assay as a biomarker of optimal nutritional status

There is a substantial body of evidence that illustrates how a large number of micronutrients (vitamins and minerals) are required either as cofactors of enzymes or as part of the structure of proteins involved in the maintenance of genome stability, for example for DNA synthesis and repair, oxidative damage protection and maintenance of DNA methylation [62, 104, 116, 117]. Importantly, the rate of DNA damage as a result of micronutrient deficiency is of the same sort of magnitude as genomic damage resulting from toxicologically significant doses of environmental genotoxins such as chemical carcinogens and ionizing radiation [104, 117]. Figure 3.3 compares the dose-response effect of x-rays with *in vitro* folic acid deficiency. A reduction in folic acid concentration in cultured lymphocytes from 120 to 12 nmol/l induces a similar MN frequency as acute exposure to 20 rad (0.2 Gy) of low linear energy transfer ionising radiation (e.g. x-rays), an exposure approximately 10 times greater than the annual allowed safety limit of exposure for the general population [104]. The frequency of NBPs and NBuds was also shown to be significantly and negatively correlated with folic acid dose [104, 118, 119]. A strong *in vivo* effect of micronutrient intake on genome damage has also been reported recently [115]. Analysis of micronutrients intake from food frequency questionnaires found retinol, vitamin E, folate, pre-formed nicotinic acid and calcium to be protective against genome damage and conversely riboflavin, pantothenic acid and biotin to be detrimental, as assessed using the CBMN cytome assay [115]. These results suggest that the CBMN cytome assay is a useful a biomarker of genome damage as a result of micronutrient deficiency and its potential use in determining optimal recommended dietary allowances (RDAs) that will not only prevent disease from deficiency, which is rare in the developed world, but also minimise genome damage for the prevention of degenerative diseases such as cardiovascular disease, cancer and Alzheimer's disease [120].

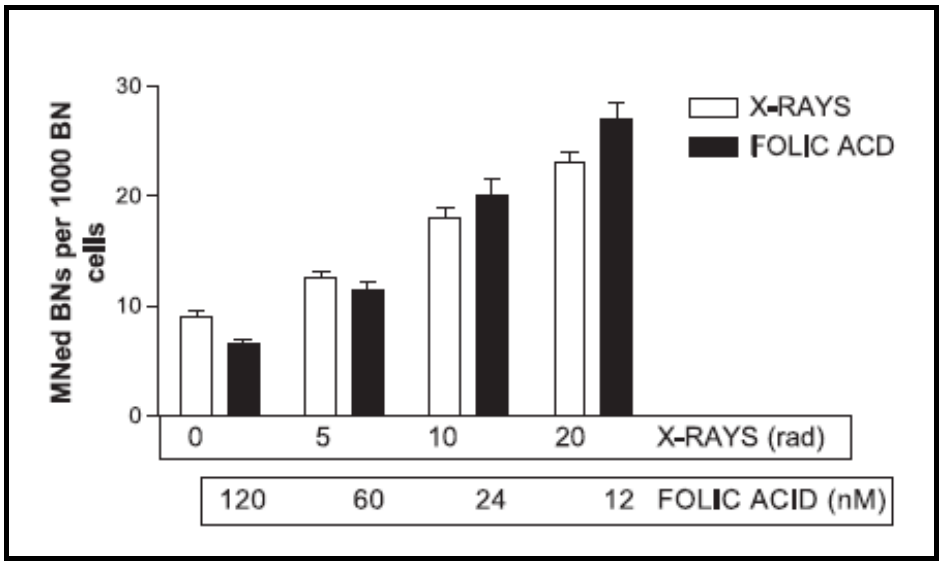


**Figure 3.3 Comparison of the dose-response effect of acute x-ray exposure and folic acid deficiency on MN induction in CBMN cytome assay cultured lymphocytes.**
The dose-response effect of x-rays (n=6) up to 20 rad which is equivalent to 10 times the annual safety exposure limit for the general public and folic acid deficiency (n=20) from 120 and 12 nmol/l, which is considered within the 'normal' physiological range. Results represent mean ± SEM MNed = micronucleated, BN = binucleate. Adapted from Fenech 2005 [104].

### 3.4.3 The CBMN cytome assay as a biomarker of exposure to genotoxic agents

The CBMN cytome assay has become one of the standard cytogenetic measurements used in the genetic toxicology testing of chemicals and radiation *in vitro*. A recent collaborative study by the French branch of the European Environmental Mutagen Society (SFTG) validated the use and effectiveness of the MN assay for detecting genotoxic (clastogens, aneugens or polyploidy inducer) and non-genotoxic agents [121]. Further, the *in vitro* MN assay has been endorsed by the European Commission as a scientifically valid assay for genotoxicity testing. The WIL2-NS cell line used for *in vitro* genotoxicity testing in this thesis, is one cell line that has been validated for use in the in vitro MN assay, due to the low background MN frequency and excellent cellular morphology [122, 123].

### 3.4.4 The genome health clinic concept

*"A paradigm shift in disease prevention based on the diagnosis and nutritional treatment of genome and epigenome damage." Fenech, 2005 [104].*

Considering current research indicates that a) genome damage is a fundamental cause of developmental and degenerative disease, b) micronutrient deficiency induced genome damage is preventable, c) micronutrient deficiency can be assessed using DNA damage biomarkers and d) nutritional status can be diagnosed and optimised through the assessment of genome damage rates, this provides a new initiative in disease prevention [104, 117]. This suggests that it is possible to diagnose and nutritionally prevent genome instability, rather than the current practice of diagnosing and treating the diseases caused by genome damage [104, 117]. Given evidence that dietary requirements may also be influenced by an individuals inherited genes, this has given rise to the concept of 'Genome Health Clinics', where an individual can obtain a diagnosis on the direct effect of their diet and nutritional supplements on their genome [104, 117].

## *3.5 The CBMN cytome assay protocol and detailed scoring criteria*

Preparation of CBMN cytome assay slides for peripheral blood lymphocytes is detailed in Chapter 8 and CBMN cytome assay slide preparation from primary cell cultures (WIL2-NS cell line for faecal water genotoxicity testing) is outlined in Chapter 6. A brief overview of the protocols used is shown below in Table 3.1.

**Table 3.1. Protocols used for CBMN cytome assay in peripheral blood lymphocytes and primary cell cultures.**

| Culture time | Peripheral blood lymphocytes | Primary cell cultures |
|---|---|---|
| **-1** | Set up 0.5ml cultures at $1x10^6$ viable cells/ml | |
| **0** | Add PHA | Set up 0.45ml cultures at $0.3x10^6$ viable cells/ml |
| **20** | | Add test agent |
| **24** | | Add Cyto B |
| **44** | Add Cyto B | |
| **48** | | Harvest cells |
| **72** | Harvest cells | |

All CBMN cytome assay slides were scored by one person (Bianca Benassi) using a Leica DMLB light microscope on 1000X magnification. The following scoring criteria used were based on that described in Fenech *et al* 2003 [124].

### 3.5.1 Data obtained for each slide

1. The proportion of mononucleated, binucleated, multinucleated, necrotic and apoptotic cells for a total of 500 cells (figure 3.4 and 3.6).
2. The frequency of binucleates containing one or more micronuclei in 1000 binucleates.
3. The total number of micronuclei (MNi) found in 1000 binucleates (BNs).
4. The frequency of binucleates containing one or more nucleoplasmic bridges in 1000 binucleates.
5. The frequency of binucleates containing one or more nuclear buds in 1000 binucleates.

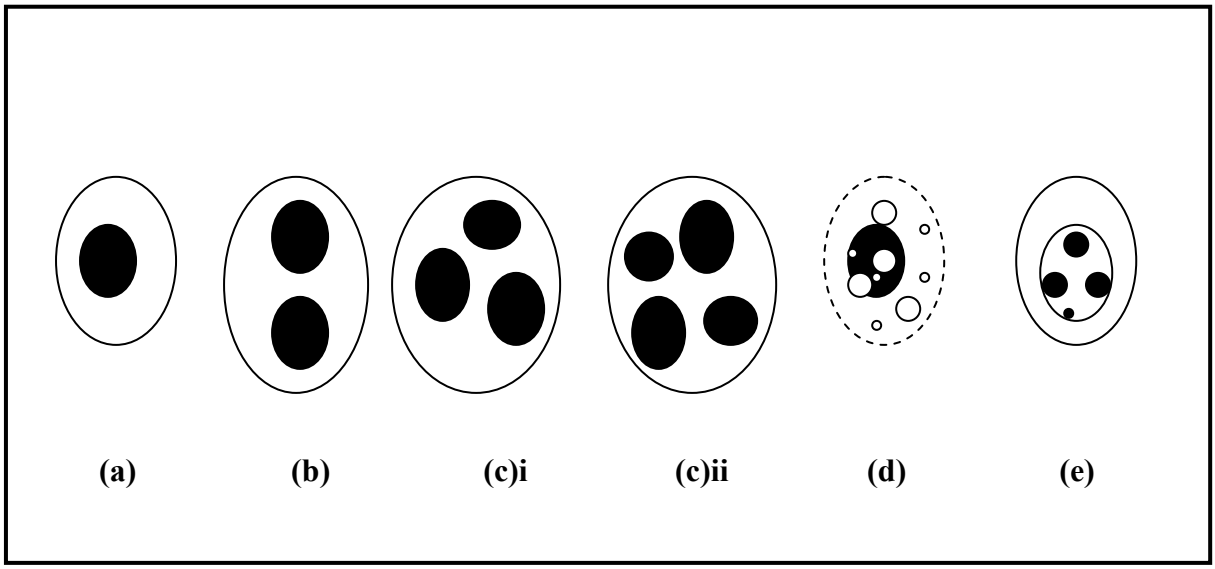


**Figure 3.4. Diagrammatic examples of the various types of cells found in the CBMN cytome assay.**

(a) a typical mononucleated cell, (b) a typical binucleated cell, (c)(i) multinucleated cell with three nuclei and (ii) with four nuclei, (d) necrotic cell, (e) apoptotic cell. Adapted from Fenech *et al* 2003 [124].

### 3.5.2 Criteria for selecting binucleated cells which can be scored for the frequency of micronuclei, nucleoplasmic bridges and nuclear budding

1. The cells should be binucleated.
2. The binucleated cell should have an intact nuclear membrane and be within the same cytoplasmic boundary.
3. The two nuclei should be approximately equal in size, staining pattern and staining intensity.
4. The two main nuclei may touch but cannot overlap each other.
5. The cytoplasmic boundary must be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

### 3.5.3 Criteria for scoring micronuclei

Micronuclei (MNi) have the following characteristics (figure 3.5 and 3.7):

1. The diameter is between 1/16 and 1/3 the size of the mean diameter of the main nuclei.
2. Round or oval in shape.
3. Not linked or connected to the main nuclei.
4. May touch but not overlap the main nuclei such that the micronuclear boundary is distinguishable from the nuclear boundary.
5. Have the same staining characteristics of the main nuclei.
6. There may be more than one MN in a binucleated cell.

### 3.5.4 Criteria for scoring nucleoplasmic bridges

Nucleoplasmic bridges (NPBs) are a nucleoplasmic connection between the main nuclei of a binucleated cell and have the following characteristics (figure 3.5 and 3.7):

1. The width may vary but have a diameter no wider than ¼ the diameter of the largest nucleus.
2. Have the same staining characteristics of the main nuclei.
3. There may be more than one nucleoplasmic connection per binucleated cell, however this is still only scored once, as a BN cell containing one or more NPB.

### 3.5.5 Criteria for scoring nuclear buds

Nuclear Buds (NBuds) are nuclear structures that are morphologically similar to micronuclei attached to one of the main nuclei by a nucleoplasmic connection and have the following characteristics (figure 3.5 and 3.7):

1. Have the same staining characteristics of the main nuclei.
2. There may be more than one nuclear bud per binucleated cell, however this is still only scored once, as a BN cell containing one or more NBuds.

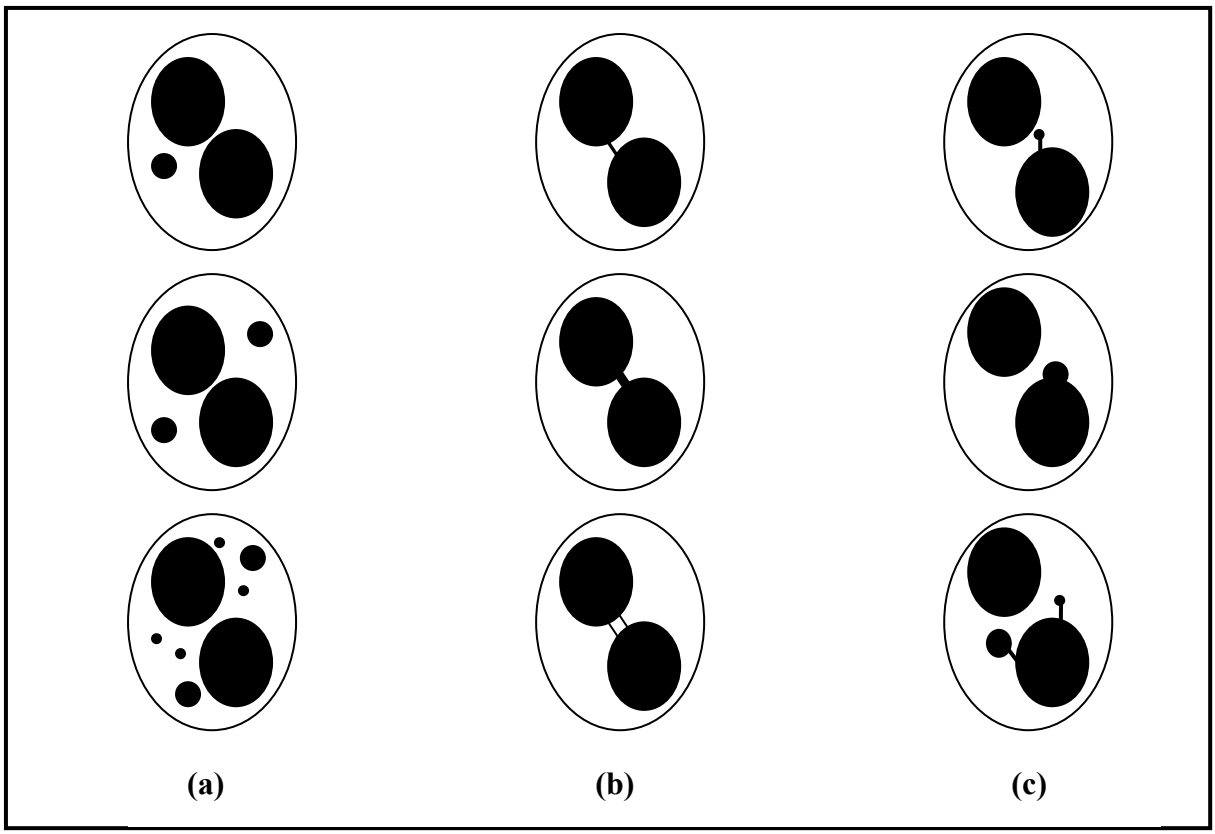


**Figure 3.5. Diagrammatic examples of genome damage events scored in binucleate cells.** (a) binucleated cells with varying size and numbers of micronuclei, (b) binucleated cells with nucleoplasmic bridges of varying widths and number, (c) binucleated cells with nuclear buds of various nucleoplasmic connection, size and number. Adapted from Fenech *et al* 2003 [124].

### 3.5.6 Criteria for scoring apoptotic cells

1. Early apoptotic cells are identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear membranes.
2. Late apoptotic cells are identified by the presence of nuclear fragmentation within an intact cytoplasmic membrane.
3. Staining intensity of the nucleus, nuclear fragments and cytoplasm are generally greater than observed in viable cells. Figure 3.6.

### 3.5.7 Criteria for scoring necrotic cells

1. Early necrotic cells are identified by the presence of a pale cytoplasm and intact nucleus with numerous vacuoles in both the cytoplasm and nucleus.
2. Late necrotic cells are identified by a lack of cytoplasm and damaged nuclear membrane, often with nuclear material leaking from the nucleus.
3. Staining intensity of the nucleus and cytoplasm are generally less than observed in viable cells. Figure 3.6.

### 3.5.8 Calculating Nuclear Division Cytotoxicity Index

The Nuclear Division Cytotoxicity Index (NDCI) gives an estimated measure of the nuclear division status and cell division kinetics in viable cells in relation to the total number of viable and non-viable cells. The proportion of mononucleated, binucleated, multinucleated, necrotic and apoptotic cells scored for a total of 500 cells are used for the equation:

**NDCI = [Apop + Nec + M1 + 2(M2) + 3(M3)] / T**

Where:

Apop = number of apoptotic cells, Nec = number of necrotic cells, M1-M4 = number of viable cells with 1 to 4 nuclei and T = total number of cells (viable and non-viable) scored.

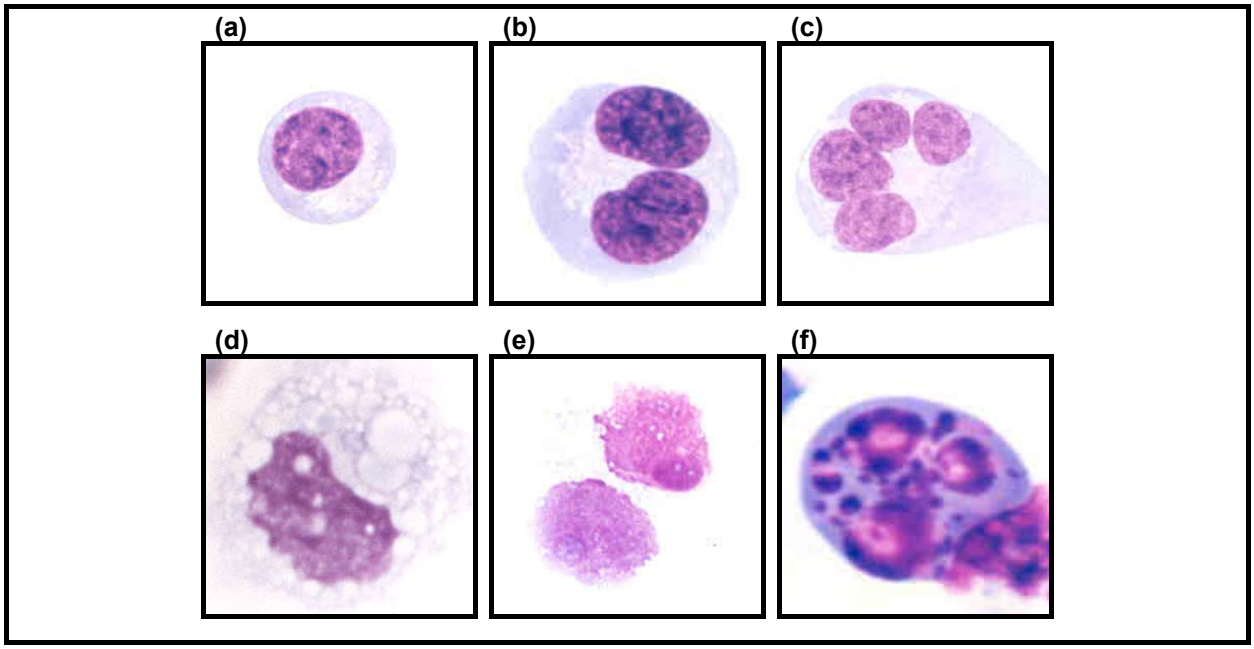


**Figure 3.6. Photograph examples of the various cells found in the CBMN cytome assay.** (a) a typical mononucleated cell, (b) a typical binucleated cell, (c) a typical multinucleated cell, (d) a typical early stage necrotic cell, (e) a typical late stage necrotic cell, (f) a typical apoptotic cell. Adapted from Fenech *et al* 2003 [124].

NOTE: This figure is included on page 28 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 3.7. Photograph examples of genome damage events scored in the CBMN cytome assay.**

(a)-(c) Binucleated cells with one or more micronuclei, (d)-(f) binucleated cells with one nucleoplasmic bridge of varying width, (g)-(i) binucleated cells with both one or more nucleoplasmic bridge and one or more micronuclei, (j)-(l) binucleated cell with one or more nuclear buds. Photographs (a)-(b) show WIL2-NS cells and (c)-(l) lymphocytes, very little difference can be observed between the cell types. Adapted from Fenech *et al* 2003 [124].

# 4. STUDY OVERVIEW

## *4.1 Subjects*

Overweight men were recruited for the study by public advertisement. To be eligible for the study, males had to be aged 20 to 65 years, have a body mass index (BMI) between 27 and 40, have no history of metabolic or coronary disease or type 1 or type 2 diabetes and at least one cardiovascular disease (CVD) risk factor other than obesity. Exclusion criteria included: use of any form of drug therapy or medication or dietary supplements on a regular basis that may interfere with bowel function (e.g. laxatives, antibiotics, anti-diarrhoeals or probiotics), consumption of any over-the-counter medication that could interfere with the study, participation in a bowel health study or in any study of an experimental drug within 30 days of commencement of the study, or a history or presence of gastrointestinal, renal or hepatic disease of any cause. Fifty five men met the selection criteria and were randomly assigned to treatment groups. Subject characteristics were not significantly different at baseline (Table 4.1).

**Table 4.1 Subject characteristics at baseline.**

| | HP | HC |
|---|---|---|
| | *n=24* | *n=31* |
| **Age (years)** | 53.71 ± 1.15 | 50.90 ± 1.53 |
| **Weight (kg)** | 96.68 ± 2.29 | 100.53 ± 2.37 |
| **BMI (kg/m$^2$)** | 31.68 ± 0.67 | 31.78 ± 0.69 |

Values shown at mean ± SEM
HP=high protein group; HC=high carbohydrate group

The protocol and the potential risks and benefits were explained to each subject and written informed consent was obtained prior to the commencement of the study. All experimental procedures were approved by the Human Research Ethics Committees of the CSIRO (Commonwealth Scientific and Industrial Research Organisation) and the University of Adelaide prior to study commencement. All subjects were initially familiarised (2 weeks prior to study commencement) with the dietary requirements/interventions that were carried out during the study.

## *4.2 Dietary protocol and intervention*

The study was a parallel design, comprising of a 12 week intensive weight loss phase with fortnightly visits and follow-up at one year with interim monthly visits. Subjects were assigned to one of two dietary interventions, a high protein–high red meat (HP) diet (35% protein, 40% carbohydrate, 25% fat) or a high carbohydrate-low red meat (HC) diet (17% protein, 58% carbohydrate, 25% fat). The total energy content of both diets was 7000kJ, with some adjustments in kilojoules (while maintaining protein to energy ratios) necessary for individuals to achieve an approximate weight loss of 1 kilo per week. The fibre content and fatty acid profile was the same between diets. Details of the foods prescribed to obtain the planned dietary intakes are described in Table 4.2.

**Table 4.2 Composition of study diets.**

| | HIGH PROTEIN | HIGH CARBOHYDRATE |
|---|---|---|
| **Cereal – high fibre** | 50g | 50g |
| **Dairy - low fat** | 3 serves | 2 serves |
| **Lean protein foods** | Dinner 300g (red meat 4x/week) | Dinner 100g (red meat <1/week) |
| | Lunch 100g chicken/fish | Lunch 30g chicken/fish |
| **Fresh Fruit** | 300g | 450g |
| **Pasta or Rice** | Nil | 70g dry weight |
| **Low starch Vegetables** | at least 2.5 cups | at least 2.5 cups |
| **Oil/spread** | 20g | 20g |
| **Bread (Burgen)** | 105g | 140g |
| **Alcohol** | Up to 1 std drink/day | Up to 1 std drink/day |
| **Total kilojoules** | 6932 | 7027 |
| **Protein g (%)** | 142 (35) | 88 (17) |
| **Fat g (%)** | 53(25) | 51 (25) |
| **Carbohydrate g (%)** | 135 (40) | 198 (58) |
| **Saturated fat g (%)** | 14 (7.6) | 14 (7.5) |

Subjects met individually with a CSIRO dietician once every 2 weeks for the first 12 weeks and once a month for the remainder of the 12 month period. The dieticians provided all dietary counseling such as instruction on the dietary requirements, method for recording food intake and assessment of compliance and were trained on providing consistent information to subjects. Subjects were instructed to keep a checklist of foods consumed each day. Subjects were issued with digital kitchen scales (if needed) to weigh food and advised to consume two and a half cups or more of low energy vegetables per day. A range of additional low-energy foods were

allowed. One standard serve of alcohol was permitted per day. Eating at restaurants was limited to less than once per two weeks. At baseline, subjects were provided with samples of the foods consistent with their allocated diet to encourage compliance. Subjects were advised not to change their physical activity patterns.

Subjects on the HP diet were advised that protein servings at lunch and dinner were compulsory, while other items could be consumed according to appetite but not exceeding amounts specified. HC diet subjects were instructed that the dinner protein serving plus the bread and rice/pasta needed to be consumed and this was isocaloric with the HP protein component.

The composition of diets consumed by subjects and their compliance through the first 12 weeks of the study was assessed by analysis of 3 days of the checklists for each 2 week period. Energy and macronutrient intakes were calculated by using FoodWorks Nutritional Calculation software (Xyris Software, Highgate Hill, Queensland, Australia) based on Australian food composition tables and food manufacturers' data.

At the conclusion of the 12 week diet phase, participants were asked to continue the same diet strategy with a seamless transition from fortnightly to monthly dietician visits, with checklists issued for a 4 week timeframe. Participants who achieved a BMI of less than 25kg/$m^2$ were eligible for the weight maintenance strategy, managed by maintaining the "core diet" and increasing energy intake in 500kJ blocks.

## *4.3 Consort statement*

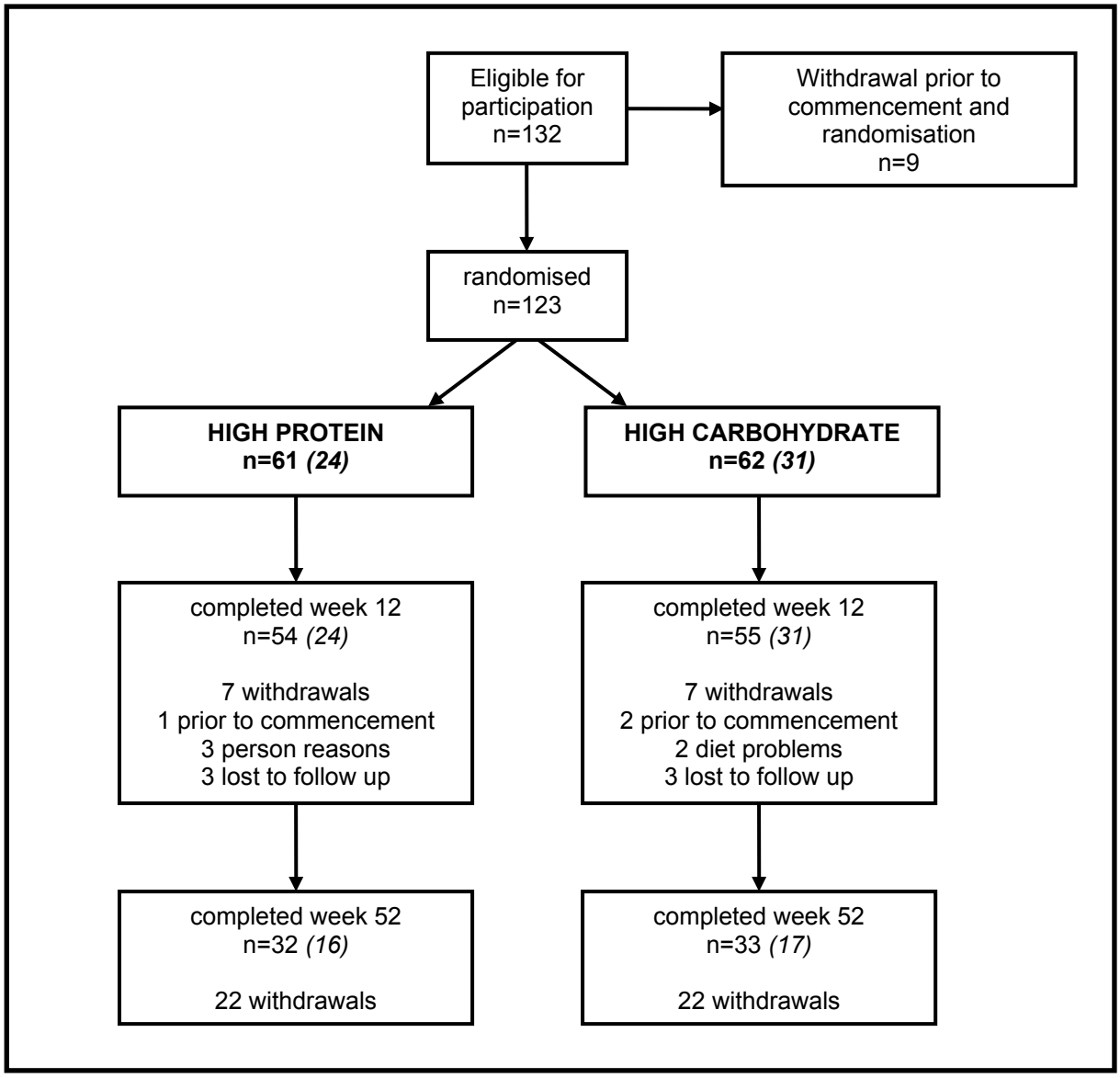


**Figure 4.1. Consort Data.**
Numbers in brackets represent number of subjects in the bowel health arm of the study (data used in this thesis). Other subjects participated in other arms of the trial only.

## *4.4 Weight loss*

Fasted (overnight) volunteers were weighed by a CSIRO dietician (model AMZ14, Mercury Digital Scales, Japan) wearing light clothing and no shoes at week 0, 12 and 52. Figure 4.2 and Table 4.3 shows volunteers weight at week 0 and week 12. Two-way ANOVA shows there was a significant (P<0.001) weight loss from week 0 to 12, with no significant difference between the diets. Average weight lost was 8.4 ± 0.5kg. Figure 4.3 and Table 4.4 show weight at week 0, 12 and 52 for volunteers who completed the study. Bonferroni multiple comparison test indicates that average weight lost from week 0 to week 12 in volunteers who completed the study was 9.3 ± 0.7kg and this weight was maintained at week 52 with no significant difference between dietary patterns.

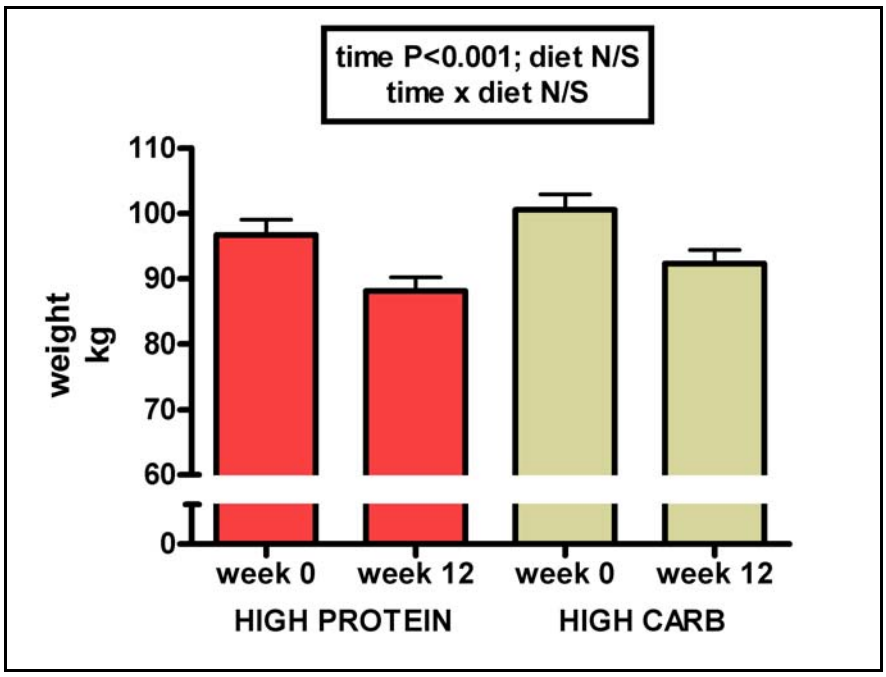


**Figure 4.2 Effect of 12 weeks following a high protein-high red meat or a high carbohydrate dietary pattern on body weight.**

**Table 4.3 Weight loss after 12 weeks following a high protein-high red meat or a high carbohydrate dietary pattern.**

| | | | Week 0 | Week 12 | *t*-test^ |
|---|---|---|---|---|---|
| **weight** | *HP* | *n=24* | 96.68 ± 2.29 | 88.10 ± 2.08 | P<0.001 |
| | *HC* | *n=31* | 100.53 ± 2.37 | 92.30 ± 2.04 | P<0.001 |
| | ***t*-test^^** | | P=0.26 | P=0.16 | |

Data represented as mean ± SEM
^ paired t-test, comparing effect of time for each diet group
^^independent t-test, comparing the diets at each time point

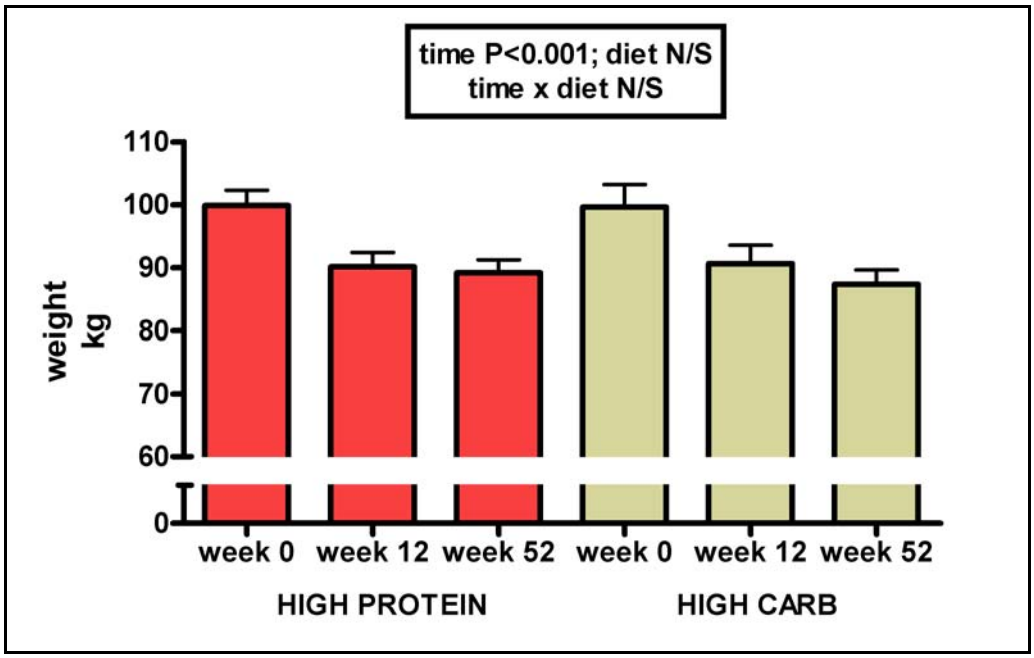


**Figure 4.3 Effect of 12 and 52 weeks following a high protein-high red meat or a high carbohydrate dietary pattern on body weight.**

**Table 4.4 Weight loss after 12 and 52 weeks following a high protein-high red meat or a high carbohydrate dietary pattern.**

| | | | Week 0 | Week 12 | Week 52 | One-way ANOVA^ |
|---|---|---|---|---|---|---|
| **weight** | *HP* | *n=16* | 99.84 ± 2.45[a] | 90.14 ± 2.27[b] | 89.15 ± 2.10[b] | P<0.001 |
| | *HC* | *n=17* | 99.58 ± 3.62[a] | 90.61 ± 2.96[b] | 87.34 ± 2.30[b] | P<0.001 |
| | ***t*-test^^** | | P=0.95 | P=0.90 | P=0.57 | |

Data represented as mean ± SEM
Values in one row not sharing a letter are significantly different P<0.05
^ One-way ANOVA, comparing effect of time for each diet group
^^independent t-test, comparing the diets at each time point

## *4.5 Methods for sample collection and storage*

### 4.5.1 Blood collection

At week 0, 12 and 52, approximately 9ml of venous blood was collected from fasted (overnight) volunteers in one Vacuette®-Heparin tube by a CSIRO nurse for isolation of lymphocytes and kept at room temperature. Approximately 4ml of venous blood was collected in one Vacuette® tube containing no additives and transported at room temperature to the Institute of Medical and Veterinary Science (IMVS) within 2 hours for quantification of folate and vitamin B12 in plasma. Approximately 2ml of venous blood was collected in one Vacuette®-EDTA tube, placed on ice and transported to the IMVS within 2 hours for quantification of homocysteine in plasma.

#### *Isolation and storage of lymphocytes*

Normally, 9 ml of blood yields 5-10 x $10^6$ lymphocytes, however this depends on the donor and the competency of cell isolation and storage. All blood samples were processed within 4 hours of collection. All following steps in the protocol were carried out in a biohazard fume hood.

1. The tubes were inverted several times to ensure thorough mixing of the anticoagulant.
2. Blood was transferred to sterile 50ml pots (Sarstedt, Adelaide, Australia) and diluted 1:1 with Hanks Balanced Salt Solution (HBSS; Thermo Electron, Melbourne, Australia) at room temperature and mixed well by gently pipetting the solution (approximately 18ml total).
3. The diluted blood was then gently layered onto Ficoll-Paque (Amershan Biosciences, Uppsala, Sweden) in sterile 50ml Falcon conical tubes (Becton Dickinson Labware, NJ, USA) at a ratio of 1:3 (ie 6ml Ficoll-Paque to 18ml diluted blood), with care taken not to disturb the interface.
4. Tubes were then centrifuged at 400g for 30 minutes at 18°C.
5. After centrifugation, the solution is divided into 3 distinctive phases. Red blood cells penetrate the Ficoll-Paque and reside at the bottom of the tube. The middle layer consists of the Ficoll-Paque and the upper layer contains the HBSS and plasma. The lymphocytes form a cloudy layer between the Ficoll-Paque and HBSS/plasma interface.
6. Lymphocytes were removed using a sterile glass pasteur pipette (plugged with cotton wool and fitted with a rubber teat) and transferred to a new sterile

Falcon conical tube. Typically 5ml of the lymphocyte solution is removed, with care taken not to disturb the red blood cell layer.

7. The lymphocyte solution was diluted 1:3 in HBSS (ie 15ml HBSS for 5ml lymphocyte solution) and centrifuged at 180g for 10 minutes at 21-23°C.
8. The supernatant was discarded and the pellet resuspended in 2x the volume of removed HBSS (ie 10ml HBSS) and centrifuged at 100g for 10 minutes at 21-23 °C.
9. The supernatant was discarded and the pellet resuspended in 1.8ml foetal bovine serum (FBS; Thermo Electron, Melbourne, Australia).
10. Lymphocyte-FBS solution was divided into two (2x 900ul) and transferred to 1.8ml cryovials (Nunc, Roskilde, Denmark) and placed on ice.
11. 100ul of dimethyl sulphoxide (DMSO; Sigma, Sydney, Australia) was added to the solution, mixed well, and returned to ice.
12. Within 15 minutes of adding DMSO, cryovials were transferred to a cold (4°C) Stratacooler® cryo preservation module (Stratagene, CA, USA) and placed at -80°C for 24 hours.
13. After 24 hours, cryovials were quickly transferred from -80°C to storage in liquid nitrogen until required.

The candidate was responsible for the collection and freezing of blood samples from the clinic and performing all genotoxicity assays described throughout the thesis, collection of data end points related to the described hypotheses and the analysis and interpretation of the data.

### 4.5.2 Faecal collection and storage

At week 0, 12 and 52, bowel actions from a 24 hour period (typically from midnight Saturday to midnight Sunday) were collected by volunteers and stored in an esky with frozen ice bricks until they were brought to the clinic the following morning and then stored at -20°C.

#### *Isolation and storage of faecal water*

1. Faecal samples were thawed at room temperature for 3 hours.
2. Samples from each volunteer were pooled, mixed and loaded into 38ml Oakridge capacity thick walled polycarbonate tubes and sealed with aluminium caps.

3. Tubes were then centrifuged at 145 510 g for 50 minutes at 4°C.
4. The supernatant was collected and aliquoted in 500ul fractions and stored at -20°C.

Isolation of faecal water was performed by Ms Sylvia Usher, a Research Project Officer at CSIRO Human Nutrition.

The candidate was responsible for the collection of isolated faecal water from Ms Usher and storage of the samples, performing all genotoxicity assays described throughout the thesis, collection of all data end points related to the hypotheses and the analysis and interpretation of the data.

# 5. DNA DAMAGE POTENTIAL OF HUMAN FAECAL WATER MEASURED USING THE CYTOKINESIS-BLOCK MICRONUCLEUS CYTOME ASSAY AND THE WIL2-NS CELL LINE

## *AIM*

To develop and validate a CBMN cytome assay to investigate the genotoxic and cytotoxic potential of human faecal water.

To assess and compare the extent of inter- and intra-individual variation in faecal water genotoxicity and cytotoxicity measured using the CBMN cytome assay.

## *HYPOTHESES*

Human faecal water induces genotoxic and cytotoxic damage in the WIL2-NS cell line in a dose-dependent manner.

The variation in genotoxic and cytotoxic potential of human faecal water is smaller within one individual than for that observed between individuals.

## *5.1 Introduction*

Colorectal cancer is the third most commonly diagnosed cancer in the world (after lung and breast cancers), accounting for 9.4% of the world total, and is the only cancer that occurs at about the same frequency in both men and women (ratio 1.2:1.0) [125]. There is a 25-fold variation in occurrence of colorectal cancer worldwide. Incidence rates appear to be high in countries such as North America, Australia, New Zealand and Western Europe and low in Africa and Asia [125]. It has been recognised since the 1970s that the development of colorectal cancer is not entirely genetic in basis and is largely influenced by environmental factors. This is supported by the association with different dietary patterns and international differences in the incidence of colorectal cancer [20, 21]. This variation in cancer rates with diet suggests that diet is an important risk factor for many forms of cancers and supports the possibility that cancer can to some extent, be preventable by dietary changes [23]. For colorectal cancer, it is estimated that up to 90% of cases can be attributed to environmental and dietary factors [24].

Considering the role diet plays on risk of colorectal cancer, it can be suggested that the genotoxicity of the contents of the bowel is an important factor in the process of transformation of cells lining the bowel to cancerous cells [126]. Colorectal cancer is thought to arise from direct-acting carcinogens in food, or by interactions between the colonic flora and epithelium which may generate genotoxic species within the colon. As human faeces represent the outcome of the digestion process as well as the metabolic products of colonic bacteria, the assessment of the faecal contents provides a non-invasive procedure for studying the environment in the colon and its contribution to risk of colorectal cancer [45, 127]. Of particular importance is the faecal water or aqueous phase of the faeces as it has been suggested that this faecal fraction is more likely to exert an effect on the colonic cells than compounds which are bound to food residues and bacterial mass [128]. This suggests that the genotoxic potential of faecal water may be a suitable biomarker in studying the effect of diet on risk of colorectal cancer in humans.

A variety of methods have been used to measure the genotoxic effects of faecal or caecal water. These methods include single cell gel electrophoresis or Comet assay or measurement of DNA adducts [129-131]. The consumption of a diet rich in fat, meat and sugar but poor in vegetables and wholemeal products has previously been shown to increase the genotoxic potential of faecal water as measured by the comet assay [132]. The comet assay has also been used to demonstrate the genotoxic activity of bile acids which are thought to be promoters of colorectal cancer [133]. However, the comet assay is limited by confounding of DNA strand breaks in early apoptotic and necrotic cells, the lack of detection of mis-repair of DNA strand breaks and the fact that chromosome malsegregation events or telomere end fusions are not detectable by this method all of which are important events in the colorectal carcinogenic process [131, 134, 135].

The cytokinesis-block micronucleus cytome (CBMN Cyt) assay is an alternative comprehensive approach to measure the genotoxicity and cytotoxicity of caecal or faecal water [98, 136-138]. This assay is a well established test for genotoxicity measurement at the chromosomal level using three distinct biomarkers namely: Micronuclei (MN), a biomarker of chromosome breakage and/or loss ; Nucleoplasmic bridges (NPBs), a biomarker of DNA strand break mis-repair and/or telomere end fusion; Nuclear buds (NBUDs), a biomarker of gene amplification and/or elimination of DNA repair complexes. Furthermore in the same assay one can measure frequency of apoptotic and necrotic cells (biomarkers of cell death) and Nuclear Division Index (NDI), a measure of cytostatic effects [98, 136-138]. Genomic instability, induced by exposure to genotoxins or as a result of acquired or inherited defects in genome maintenance, is a fundamental property of cancer cells and therefore biomarkers of genomic instability are often used as reliable predictors of cancer risk [102]. The CBMN Cyt assay is considered an important tool for the measurement of genomic instability at a chromosome level because the technique provides a comprehensive measure of chromosome breakage or loss (MN), assymetrical chromosome rearrangement (NPB), gene amplification (NBuds), as well as cell death and cytostasis [102].

The ability of faecal water to induce DNA damage as measured by the CBMN cytome assay, has not been assessed previously, such that the extent to which individuals vary in the ability of their faecal water to induce DNA damage using the cytokinesis-block micronucleus assay is unknown. In this study, the sensitivity in the

measurement of genotoxic potential of faecal water was assessed using the CBMN cytome assay in the WIL2-NS cell line as well as the extent of inter- and intra-individual variation. The WIL2-NS cell line is a human B lymphoblastoid cell line originally derived from the spleen of a Caucasian male who was free from malignancy [139, 140]. A mutation in the p53 gene allows WIL2-NS cells which would have normally undergone apoptosis following a major genome damage event to continue through the cell cycle and express chromosome damage, rather than die via apoptosis, making it easier to assess the genotoxic load [141, 142]. This cell line also has a high Nuclear Division Index (NDI) and excellent cellular morphology which makes it ideal for scoring the genome damage markers in cytokinesis-blocked binucleated cells [123]. This study aimed to validate the use of the CBMN cytome assay system as a biomarker for the assessment of faecal water genotoxicity by determining the appropriate concentration for the assay, the extent of increase in genome damage that may be detected, as well as the extent of variation that may be expected within and between faecal samples collected from the same or different individuals.

## *5.2 Methods*

### 5.2.1 Subjects and faecal collections

Faecal samples were collected from six subjects on one occasion, when convenient within the week. One of these subjects also provided one faecal sample per week for six weeks. The volunteer was required to collect the faecal sample directly into a plastic bag which was carefully sealed and immediately stored at -20ºC. All samples were stored frozen at -20ºC until required.

### 5.2.2 Isolation of faecal water

Faecal samples were thawed at room temperature for 3 hours. Samples were homogenised and loaded into 20ml oakridge tubes with seal adaptors and centrifuged at 40 000 rpm at 4ºC for 50 minutes. The supernatant was collected and aliquoted in 500ul fractions and stored at -20ºC until required for CBMN cytome assay.

One faecal water fraction per subject sample was thawed at room temperature on the day of the CBMN cytome assay. Faecal water was diluted to 10%, 1%, 0.1% and 0.01% in RPMI 1640 medium and sterile filtered to determine the concentration which produced a sufficiently large genotoxic effect to be measured without excessive cell death and cytostasis. Faecal water from all six individuals was tested at the various concentrations. Only 1% faecal water was tested for the six separate samples from the one individual as this was found to be the optimal concentration for performing the CBMN cytome assay.

### 5.2.3 Cell culture and cytokinesis-block micronucleus cytome assay

WIL2-NS cells were obtained from the American Type Culture Collection, USA. Cells were cultured and maintained in RPMI 1640 medium (Thermo Electron, Melbourne, Australia) containing 5% foetal bovine serum (FBS; Thermo Electron, Melbourne, Australia), 1% penicillin-streptomycin solution (Thermo Electron, Melbourne, Australia) and 1% L-glutamine (Sigma, Sydney, Australia) at 37ºC in a humidified atmosphere with 5% $CO_2$ ($CO_2$ incubator; Quantum Scientific, Brisbane, Australia). Cell count and percentage viability tests were performed to determine the number of viable cells. 60ul of the cell suspension was transferred into 20ml vials containing 15ml Isoton-II solution (Beckman Coulter, Miami, USA) and cell number determined using a Coulter Counter (Beckman Coulter, Miami, USA) according to the

manufacturer's instructions. Cell viability was determined by mixing 50ul of cell suspension to 50ul trypan blue (Sigma, Sydney, Australia) which was loaded onto a haemocytometer (ProSciTech, Kirwan, Australia) and a minimum of 100 cells were manually counted using a Leica DMLB light microscope on 40X magnification. Viable (live) cells remained unstained whilst non-viable (dead) cells stained dark blue. Viable cell growth was calculated by multiplying cell number by percentage viability. Duplicate 0.45ml cultures per sample were set up in 24-well plates at a concentration of $0.3x10^6$ viable cells/ml and placed in a humidified atmosphere with 5% $CO_2$ ($CO_2$ incubator). (Figure 1).

20 hours later, sterile faecal water was added to the cultures (50ul of diluted faecal water added to 450ul cell cultures) and returned to the $CO_2$ incubator.

4 hours after exposure to faecal water, 38ul of cytochalasin B (final concentration of 4.5 ug/ml in cultures; Sigma, Sydney, Australia) was added to each culture and returned to the $CO_2$ incubator.

24 hours later cells were harvested by cytocentrifugation using a Shandon Cytospin Cytocentrifuge (Shandon Scientific, Chesire, England). Cells were thoroughly resuspended by pipette and 120 µl of each sample were transferred to cytocentrifuge chambers (consisting of a slide, filter card and cytocentrifuge sampling cup) and spun in the cytocentrifuge at 600 rpm for 5 minutes, to give a spot of cells on the slide. Slides were then rotated and a second 120 µl sample transferred, using the same conditions, to generate a second spot of cells. Slides were removed from the cytocentrifuge chamber, laid flat and left to airdry for 10 minutes.

Slides were stained using the commercial kit 'Diff Quik' (Lab Aids, Narrabeen, Australia). This involved fixing the cells by immersing the slides in fixative for 10 minutes. Slides were then removed and immediately transferred first in the Diff-Quik solution 1 (red stain) for approximately 10 seconds and then in Diff-Quik solution 2 (blue stain) for a further 6 seconds. Staining time was adjusted slightly depending on the age of the stain. Slides were then washed thoroughly under running water to remove excess stain. Slides were then left to airdry completely for at least 30 minutes. Slides were then coverslipped using Depex (BDH Laboratory Supplies, Poole, England) and left to set overnight. Slides were then stored in a slide box until required.

CBMN cytome assay slides were scored according to criteria described in Chapter 3.5.

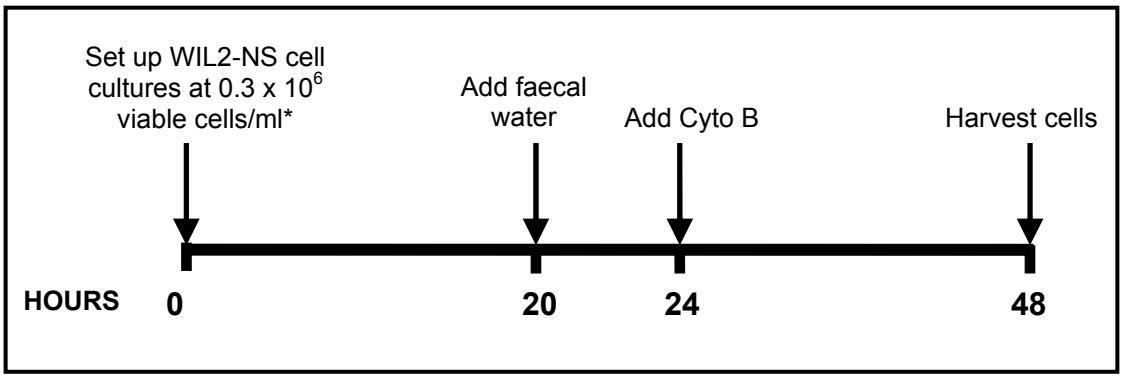


**Figure 5.1. Schematic protocol of faecal water CBMN cytome assay experiment.**
* WIL2-NS cell cultures were set up from a bulk culture of cells originally obtained from the American Type Culture Collection.

### 5.2.4 Statistical Analysis

Data is shown as mean ± standard error of the mean (SEM). Dose response effects were analysed by repeated measures ANOVA and Bonferroni multiple comparison test was used to determine significance of difference between treatments. Differences at $P<0.05$ were considered significant. All calculations were performed using GraphPad Prism version 4.00 software.

## *5.3 Results*

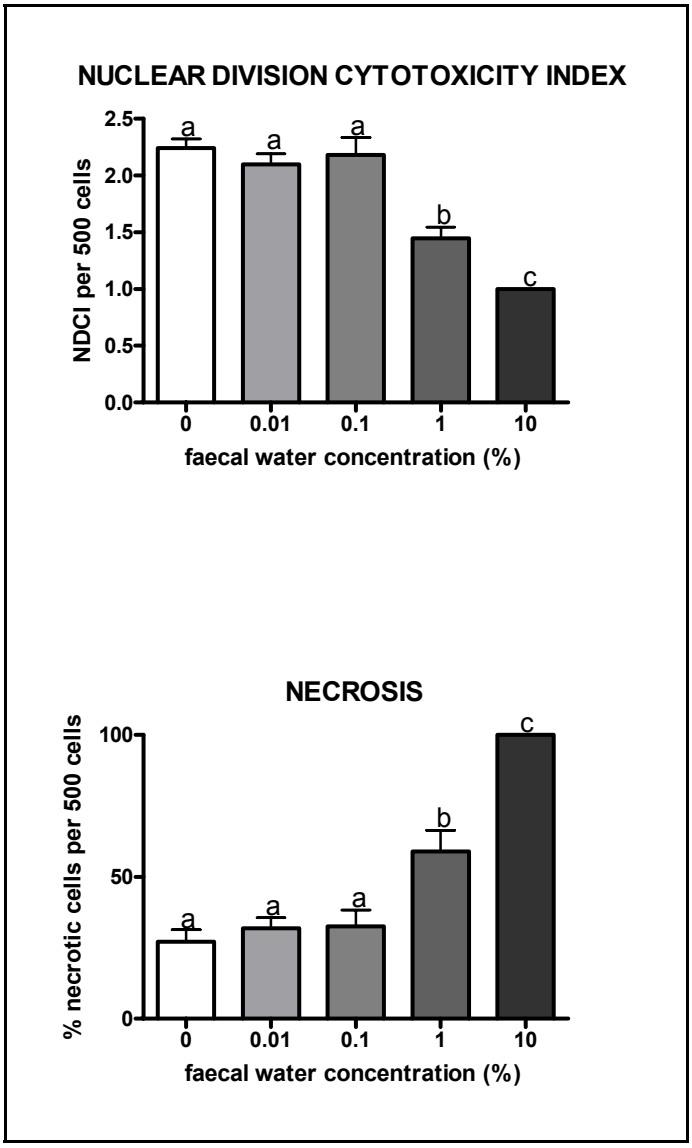


**Figure 5.2. Dose response effect of faecal water concentration on NDCI and necrosis assessed in the WIL2-NS cell line.**

WIL2-NS cells ($0.3\times10^6$ viable cells/ml) were exposed to faecal water from six subjects at various concentrations (0, 0.01, 0.1, 1 and 10%) using the CBMN cytome assay. Results represent mean ± SEM for the six faecal water samples (the result for each sample was derived from 2 replicate cultures). Values not sharing the same letter are significantly different from each other ($P<0.05$).

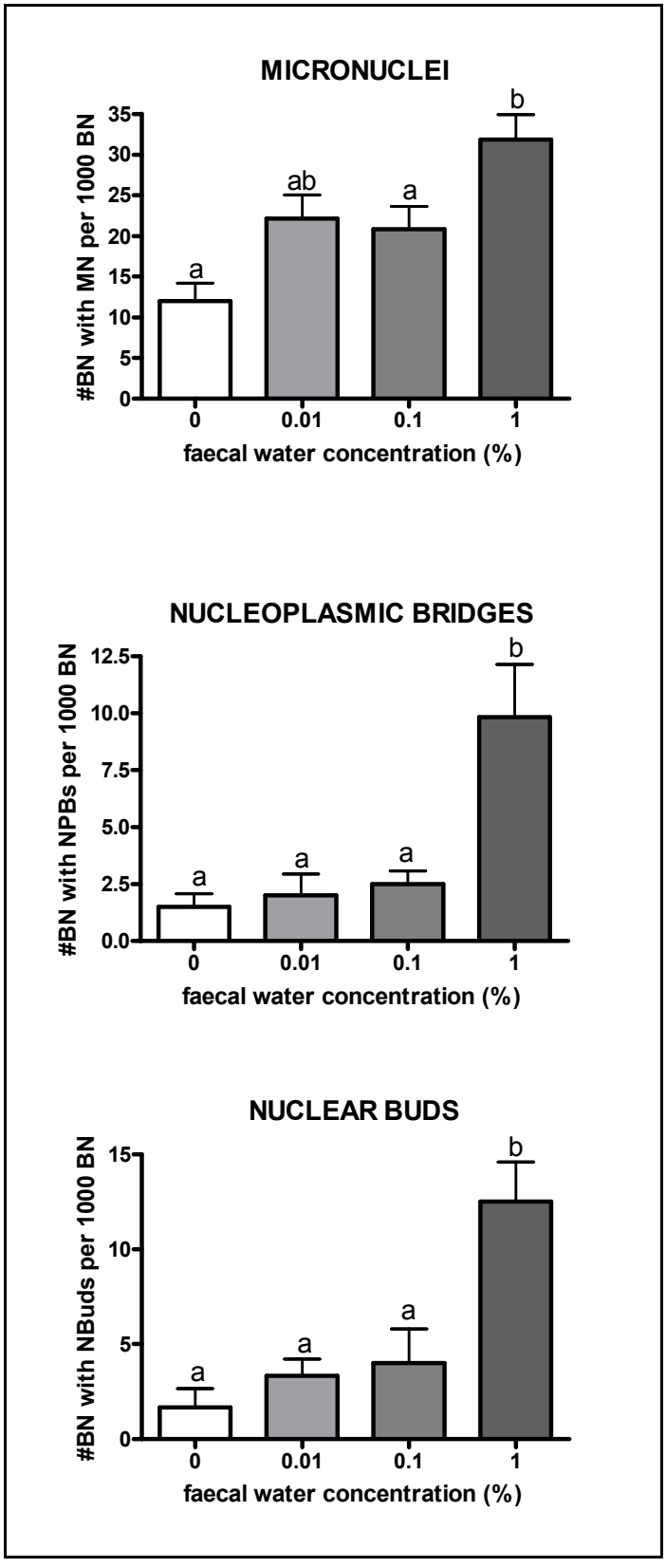


**Figure 5.3. Dose response of faecal water on CBMN cytome assay genome damage biomarkers (MN, NPB, NBuds) assessed in the WIL2-NS cell line.**

WIL2-NS cells ($0.3\times10^6$ viable cells/ml) were exposed to faecal water from six subjects at various concentrations (0, 0.01, 0.1 and 1%. 10% not shown as all cells were necrotic) using the CBMN cytome assay. Results represent the mean ± SEM. for the six faecal water samples (the result for each sample was derived from 2 replicate cultures). Values not sharing the same letter are significantly different from each other ($P<0.05$). BN=binucleated cells.

A first set of experiments was designed to determine the dose response of faecal water on DNA damage biomarkers as measured by the CBMN cytome assay in the WIL2-NS cell line of faecal water. For this, faecal water collected from six different subjects was tested at 4 different concentrations (0.01, 0.1, 1 and 10%) in duplicate and compared with a control which contained no faecal water. There was a dose related increase in necrosis and decrease in NDCI such that for 10% faecal water all cells were non-viable (necrotic) and that only 1% faecal water concentration or lower provided sufficient BN cells to score DNA damage biomarkers reliably (Figure 5.2.). MN, NPB and NBuds at 1% faecal water were all significantly increased relative to the control (Figure 5.3.). Exposure to 0.01 and 0.1% faecal water treatment only marginally increased genome damage rate relative to the control but these changes did not achieve statistical significance. From this it was determined that using 1% faecal water was optimal to measure DNA damage biomarkers in the CBMN cytome assay using the WIL2-NS cell line.

Table 5.1 shows means ± SEM of CBMN cytome assay DNA damage and cytotoxicity biomarkers for 1% faecal water in the WIL2-NS cell line for the six different individuals. All biomarkers were significantly increased ($P<0.05$) in the presence of 1% faecal water relative to the control; the fold increases (relative to the control) in order of magnitude were 7.5 (NBuds), 6.5 (NPB), 2.6 (MN) and 2.2 (necrosis).

**Table 5.1. CBMN cytome assay DNA damage and cytotoxicity markers for 1% faecal water in the WIL2-NS cell line for six different individuals.**

| BIOMARKER | CONTROL | 1% FAECAL WATER | FOLD INCREASE |
|---|---|---|---|
| **micronuclei** ^ | 12.00 ± 2.18 | 31.83 ± 3.10* | 2.65 |
| **nucleoplasmic bridges** ^ | 1.50 ± 0.56 | 9.83 ± 2.31* | 6.55 |
| **nuclear buds** ^ | 1.66 ± 0.99 | 12.50 ± 2.11* | 7.53 |
| **necrosis** ^^ | 27.17 ± 4.25 | 59.00 ± 7.40* | 2.17 |
| **NDCI** ^^^ | 2.24 ± 0.08 | 1.44 ± 0.10* | 0.64 |

The results shown are the mean values from faecal water samples from 6 different individuals, each tested in duplicate.
^ Results per 1000 binucleate cells
^^ Results are % of 500 total cells
^^^ Results calculated from cell ratios per 500 total cells
* Significantly different from control, $P<0.05$

The next step of validation was to test the variability of faecal water genotoxicity and cytotoxicity using the CBMN cytome assay. Table 5.2 shows the CBMN cytome assay DNA damage and cytotoxicity biomarkers for 1% faecal water in the WIL2-NS cell line for faecal water samples from one individual. All DNA damage biomarkers and necrosis significantly increased ($P<0.05$) in the presence on 1% faecal water relative to the control. The fold increases (relative to the control), in order of magnitude, were 28.7 (NBuds), 5.9 (NPB), 4.3 (MN) and 2.4 (necrosis), which followed a similar trend observed for the 6 different individuals.

**Table 5.2. CBMN cytome assay DNA damage and cytotoxicity markers for 1% faecal water in the WIL2-NS cell line for one individual (six times).**

| BIOMARKER | CONTROL | 1% FAECAL WATER | FOLD INCREASE |
|---|---|---|---|
| **micronuclei** ^ | 8.00 ± 0.00 | 34.67 ± 1.09* | 4.33 |
| **nucleoplasmic bridges** ^ | 2.50 ± 0.22 | 14.67 ± 1.15* | 5.87 |
| **nuclear buds** ^ | 0.50 ± 0.22 | 14.33 ± 2.31* | 28.66 |
| **necrosis** ^^ | 28.00 ± 2.24 | 67.00 ± 3.99* | 2.38 |
| **NDCI** ^^^ | 2.31 ± 0.01 | 1.41 ± 0.07* | 0.61 |

The results shown are the mean values from faecal water samples collected on 6 different occasions from one individual, each tested in duplicate.
^ Results per 1000 binucleate cells
^^ Results are % of 500 total cells
^^^ Results calculated from cell ratios per 500 total cells
* Significantly different from control, $P<0.05$

The coefficient of variation (%CV) for results from the inter- and intra-individual experiments for the CBMN cytome assay DNA damage and cytotoxicity biomarkers is compared in Table 5.3. %CV of all CBMN cytome assay biomarkers for the intra-individual experiment was smaller than that found in the inter-individual experiment, particularly for MN (7.67 vs 23.79%) and NPB (19.12 vs 57.68%), while the %CV did not differ substantially for NBuds. The fold increase/CV ratios for the various biomarkers was then compared to identify those biomarkers that gave the highest response relative to observed variation (Table 5.4). From this analysis, it is evident that the genome damage markers (MN, NPB and NBuds) are more sensitive endpoints than the cytotoxicity markers (i.e. necrosis and NDCI).

**Table 5.3. Inter- and intra-individual coefficient of variation (CV) of CBMN cytome assay DNA damage and cytotoxicity markers for 1% faecal water in the WIL2-NS cell line.**

| BIOMARKER | INTER-INDIVIDUAL CV(%) | INTRA-INDIVIDUAL CV(%) |
|---|---|---|
| **micronuclei** | 23.79 | 7.67 |
| **nucleoplasmic bridges** | 57.68 | 19.12 |
| **nuclear buds** | 41.34 | 39.63 |
| **necrosis** | 30.73 | 14.59 |
| **NDCI** | 16.81 | 12.17 |

**Table 5.4. Inter- and intra-individual 'fold increase to CV ratio' for CBMN cytome assay DNA damage and cytotoxicity markers for 1% faecal water in the WIL2-NS cell line.**

| | FOLD INCREASE TO %CV RATIO | |
|---|---|---|
| **BIOMARKER** | **INTER-INDIVIDUAL** | **INTRA-INDIVIDUAL** |
| **micronuclei** | 0.11 | 0.57 |
| **nucleoplasmic bridges** | 0.11 | 0.31 |
| **nuclear buds** | 0.18 | 0.72 |
| **necrosis** | 0.07 | 0.16 |
| **NDCI** | 0.04 | 0.05 |

## *5.4 Discussion*

Diet is an important risk factor in the development of colorectal cancer and the study of the genotoxicity of the contents of the bowel has been suggested as a non-invasive biomarker for risk assessment [45, 127]. Faecal water is thought to be the component of faeces most likely to exert an effect on the colonic cells as it is unbound to the food and bacterial mass [128]. The comet assay (or single cell gel assay) has been used to determine faecal water genotoxicity; however, is limited by confounding of DNA strand breaks in early apoptotic and necrotic cells, the lack of detection of mis-repair of DNA strand breaks and the fact that chromosome malsegregation events or telomere end fusions are not detectable by this method all of which are important events in the colorectal carcinogenic process [131, 134, 135]. Here, we have assessed DNA damage potential of faecal water between individuals and also within an individual using the cytokinesis-block micronucleus (CBMN) cytome assay [98] in the WIL2-NS cell line. This assay allows for a more comprehensive analysis of DNA damage, and is an important tool for measuring genomic instability as it assesses not only chromosome breakage and loss (evident as micronuclei) but also a number of other biomarkers such as asymmetrical chromosome rearrangement (nucleoplasmic bridges) and gene amplification (nuclear buds) [98, 102]. In addition it allows apoptotic and necrotic cells to be clearly distinguished from viable cells and in addition allows cytostatic effects to be measured, making it a truly comprehensive cytome assay [98]. Importantly, the micronucleus assay in lymphocytes is predictive of cancer risk in human cohorts followed over a 15-20 year period [108].

To our knowledge, this is the first study to use the CBMN cytome assay for evaluation of DNA damage potential of human faecal water and as such, we first had to determine the optimal dilution of faecal water to be used. Previous studies have shown that undiluted faecal water was cytotoxic to HT-29 cells [143]. It seems reasonable to assume that colonic cells *in vivo* would not be exposed to the same level of faecal water as in the lumen, due to a concentration gradient (decreasing from lumen to cells) which is the result of the excretion or exocytosis of compounds such as bile acids from the colonic cells into the enterohepatic circulation and also the mucin layer that coats the epithelial cells, which makes the diffusion of compounds from the lumen to the cells more difficult [143]. The study further illustrated that the degree of dilution of faecal water required for use in a cell line *in*

*vitro* with sufficient viability was 1% and that a 10% dilution resulted in complete cytotoxicity. This suggests that the effective concentration of faecal water solutes reaching the colonic cells in vivo is likely to be of an order of approximately 1%, however this has yet to be tested directly *in vivo* or *in vitro* with colonic epithelial cells.

As this is the first study to use the CBMN cytome assay to assess DNA damage potential of faecal water, the sensitivity and extent of variation of the biomarkers within the assay were previously unknown. From the present study, it can be surmised that the CBMN cytome assay biomarkers provide a good measure of variation of faecal water both between and also within individuals. The data show that the CBMN cytome assay using the WIL2-NS cell line to measure the genotoxicity of faecal water is a reliable and reproducible method, in that the CV was found to be smaller for repeated measures of separate samples from one individual as compared to CV of samples from different individuals. The results also suggest that the genotoxicity of faecal water from an individual who maintains a relatively unchanged dietary pattern is not significantly different from one week to the next, in that a one off faecal sample is largely indicative of the genotoxic potential of the individuals dietary pattern. A more detailed dose-response curve may have allowed for a more precise determination of the dilution to use in further faecal water challenge experiments. Considering that previous research, as well as the present study, have shown that 10% or greater faecal water is completely cytotoxic to cells, and the present study also shows no significant response to faecal water below 1%, perhaps testing from 2% to 8% faecal water may have provided a more optimal concentration for detection of a genotoxic response in a larger proportion of faecal water samples from the wider population than faecal water diluted to 1% allowed.

It is noted that a good 'biomarker' or surrogate end point of disease (in this case colorectal cancer) and the exposure (faecal water) must prove to reliably indicate whether there is an association between the exposure and the disease [144]. Considering the CBMN cytome assay is a tool for measuring genomic instability, which in turn is considered a crucial early event in carcinogenesis [102], and faecal water is considered the fraction most likely to exert an effect on colonic cells in vivo [128], using the CBMN cytome assay to measure the genotoxic potential of faecal water could potentially be a productive approach for assessing the effect of diet on risk of colorectal cancer. Furthermore, current research has shown that caecal water

collected from rats following a high fat, low calcium and fibre with barbequed red meat as the protein source diet (known high colon cancer risk diet in rats) was much more genotoxic than caecal water collected from rats fed a high fibre and calcium, low fat with casein as the protein source diet (low colon cancer risk diet in rats) measured using the CBMN cytome assay in the WIL2-NS cell line (Benassi *et al* 2007) lending strong support to the predictive power of this assay to determine the impact of diet on faecal water genotoxicity and identify diets that are carcinogenic in the colon. The other important consideration for a good biomarker is the magnitude of the fold increase relative to the %CV (i.e. the variation). This gives us an arbitrary unit ratio which takes into account both the strength of response for each biomarker as well as the background variation of the biomarker which could be due to both experimental and individual variation. Using this system it can be suggested that the genome instability biomarkers (micronuclei, nucleoplasmic bridges and nuclear buds) are the better biomarkers within this assay for measuring adverse effects of faecal water using the CBMN cytome assay in the WIL2-NS cell line for both within and between individuals.

In the current study, the *in vitro* genotoxicity and cytotoxicity of faecal water was assessed in the WIL2-NS cell line, a human B lymphoblastoid cell line. It may be suggested that the assessment of the DNA damage and cytotoxic potential of faecal water in a colonic cell line would be a better reflection of what may occur *in vivo*. The WIL2-NS cell line has been validated for use in the in vitro MN assay due to its low background MN frequency and excellent cellular morphology, which facilitates cell scoring [122, 123], and also the mutation in the p53 gene allows cells to express genome damage, making it easy to assess genotoxic load of a test agent [141, 142]. There is however no standard cell line used in genotoxicity testing. There is no evidence to suggest that the WIL2-NS cell line would respond differently compared with a colonic cell line in the determination of faecal water genotoxicity and cytotoxicity using the CBMN cytome assay, however a further study comparing the WIL2-NS cell line with colonic cell lines would be ideal.

In conclusion, this study provides preliminary validation data for the CBMN cytome assay using the WIL2-NS cell line as a comprehensive and reliable method to measure the genotoxic potential of faecal water within and between individuals. The results support the hypotheses that human faecal water induces genotoxic and cytotoxic damage in the WIL2-NS cell line in a dose-dependent manner and the

variation is smaller within one individual than between individuals. The faecal water CBMN cytome assay can be used to study the effect of different dietary patterns on genotoxicity of the contents in the bowel and the associated risk with colorectal cancer. Further experiments in this thesis aim to evaluate the performance of this novel faecal water CBMN cytome assay test with faecal samples from individuals who are consuming different diets designed for weight loss in overweight individuals.

Publications arising from this chapter:

Benassi B., LeLeu R., Bird A., Clifton P., Fenech M. Cytokinesis-block micronucleus cytome assays for the determination of genotoxicity and cytotoxicity of caecal water in rats and faecal water in humans, accepted for publication: Cancer Epidemiology, Biomarkers and Prevention.

# 6. THE EFFECT OF HIGH PROTEIN-HIGH RED MEAT VS HIGH CARBOHYDRATE WEIGHT LOSS DIETS ON BIOMARKERS OF BOWEL HEALTH

## *AIM*

To investigate whether a high protein weight loss diet, specifically high in red meat, influences the genome damage rate in colonic cells, the genotoxic and/or cytotoxic potential of faecal water and other conventional bowel health biomarkers differently compared to a high carbohydrate weight loss diet.

## *HYPOTHESES*

A high protein-high red meat dietary pattern does not increase the genotoxic/cytotoxic potential of faecal water compared with a high carbohydrate weight loss diet.

A high protein-high red meat dietary pattern does not have a negative impact on conventional bowel health biomarkers compared with a high carbohydrate weight loss diet.

The influence of dietary patterns on the genotoxic/cytotoxic potential of faecal water is correlated with changes seen with conventional bowel biomarkers.

A high protein-high red meat dietary pattern does not increase the micronucleus index in colonic cells compared with a high carbohydrate weight loss diet.

## *6.1 Introduction*

Since colorectal cancer is considered to be largely attributed to environmental factors, assessment of the carcinogenic load of the contents of the colonic lumen is of great importance due to its direct potential to transform the cells lining the bowel to cancerous cells [126]. The faecal stream does not consist of a single agent, but a complex mixture of agents, that may be carcinogenic and/or anti-carcinogenic in nature. These agents can arise directly from the foods consumed in the diet, both naturally occurring or as a product of food processing and also as products of the digestion of food within the bowel. From this, it can be suggested that nutritionally related cancers, such as colorectal cancer, occur as a result of an imbalance of carcinogenesis and anti-carcinogenesis [42]. This highlights the importance of the dietary pattern as it has the potential to influence the carcinogenic profile of the faecal stream and hence influence colorectal cancer risk. The impact of different dietary patterns commonly used for weight loss on bowel health however has not been assessed to date.

It has been suggested that the relevance of DNA damage biomarkers in measuring risk of carcinogenesis depends on the tissue in which DNA damage is measured relative to the cancer studied [102, 103, 145]. Therefore, a more direct marker of colorectal cancer risk, although more invasive, may be in the assessment of the effect of diet on DNA damage directly in cells obtained from a mid-rectal biopsy sample. As human faeces represent the outcome of the digestion process as well as the metabolic products of colonic bacteria, the assessment of the faecal contents provides a non-invasive approach for studying exposure of the colon to dietary factors and determination of the contribution of the diet to increase or decrease the risk of colorectal cancer [45, 127]. There are a number of biomarkers that are considered indicative of the health status of the bowel and hence risk of colorectal cancer.

Short chain fatty acids (SCFAs), produced by the fermentation of carbohydrates in the proximal colon, are considered to be associated with a reduced risk of colorectal cancer [25, 146]. The major SCFAs produced in humans are, in order of magnitude; acetate > proprionate ≥ butyrate. SCFAs can produce effects in the bowel as a result of their presence in the lumen and also from their uptake and metabolism by colonocytes [25, 146]. The presence of SCFAs in the lumen results in lowering of

luminal pH which is thought to prevent the overgrowth of pH-sensitive pathogenic bacteria for example *E.Coli*. SCFAs, specifically butyrate, are important for colonic function and are absorbed and metabolised by colonocytes, suppling 60-70% of the energy needs of colonocytes [25]. SCFAs are also thought to increase colonic blood flow which enhances tissue oxygenation and nutrient uptake and transport and promotes a normal colonic phenotype through butyrate-induced apoptosis of cells with abnormal genomes. One study showed that increasing consumption of fermentable carbohydrate by 10-30 g/day increases faecal SCFAs [147], however there was also an increase in faecal bulk and reduced transit time which may have raised faecal SCFAs without increasing production [148, 149]. Other studies which had no change in transit or laxation have shown an increase in faecal SCFAs with increased carbohydrate intake [150, 151]. However, assessment of SCFAs in faecal samples does not necessarily reflect total SCFA production considering that >95% of SCFAs are absorbed for use by the colon [25].

Phenolic compounds, such as phenol and p-cresol, are products formed by colonic bacteria degradation of amino acids tyrosine, phenylalanine and tryptophan from dietary protein [152-154]. Phenolic compounds are largely absorbed by the colon, detoxified by the liver and excreted in the urine as p-cresol [152]. Phenols and cresols have been suggested to be potentially carcinogenic and are elevated in humans eating high meat diets, however the relationship between phenols and bowel cancer remains unclear [152-156]. In vitro work suggests that phenol may enhance N-nitrosation and also react with nitrite to produce p-diazoquinone which is mutagenic [157].

It has been suggested that the faecal water or aqueous phase of the faeces is of particular importance in studying the effect of diet on colorectal cancer risk as this faecal fraction is more likely to exert an effect on the colonic cells than compounds which are bound to food residues and bacterial mass [128]. From this, it has been demonstrated that the genotoxic potential of faecal water may be a more suitable biomarker in studying the impact of diet on colorectal cancer risk [158, 159]. Consumption of a diet high in fat, meat and sugar and low in vegetables and wholemeal products was shown to increase the genotoxic potential of faecal water assessed using single gel electrophoresis (comet assay) [132]. A further study using the comet assay however failed to observe an increase in faecal water genotoxicity with a high meat dietary pattern [129]. Another study which did detect high DNA

damage levels using the comet assay with a high red meat dietary pattern, however with no control diet for comparison, failed to see an reduction in DNA damage with the addition of factors thought to provide a protective effect such as vegetables or tea [160]. A study of dairy-rich diet compared with a dairy-free diet failed to see a difference in faecal water genotoxicity with the comet assay [161]. The comet assay has also been used to demonstrate the genotoxic activity of bile acids which are thought to be promoters of colorectal cancer [133]. The comet assay is limited by confounding of DNA strand breaks in early apoptotic and necrotic cells, along with the inability to detect mis-repair of DNA strand breaks and chromosome malsegregation events or telomere end fusions, all of which are important events in the colorectal carcinogenic process [135]. Consequently, it remains unclear whether the comet assay data reported to date reflects cell death events or genome damage.

The cytokinesis-block micronucleus (CBMN) cytome assay is an alternative comprehensive approach to measure the genotoxicity and cytotoxicity human faecal water [98, 136-138]. Genomic instability, induced by exposure to genotoxins or as a result of acquired or inherited defects in genome maintenance, is a fundamental property of cancer cells and therefore biomarkers of genomic instability are often used as reliable predictors of cancer risk [102]. The CBMN cytome assay is a well established test for genotoxicity measurement at the chromosomal level using three distinct biomarkers: Micronuclei (MN), a biomarker of chromosome breakage and/or loss ; Nucleoplasmic bridges (NPBs), a biomarker of DNA strand break mis-repair and/or telomere end fusion; Nuclear buds (Nbuds), a biomarker of gene amplification and/or elimination of DNA repair complexes. Furthermore in the same assay one can measure frequency of apoptotic and necrotic cells (biomarkers of cell death) and Nuclear Division Index (NDI), a measure of cytostatic effects [98, 136-138]. From this it can be suggested that the use of the CBMN cytome assay for the measurement of faecal water genotoxicity will provide a more comprehensive assessment of the overall carcinogenic potential of the faecal fraction most likely to exert an effect on the colonic cells and hence influence risk of colorectal cancer. Furthermore, the MN index in the CBMN cytome assay has been validated as a biomarker of cancer risk in a major prospective study [108] and MN, NPB and NBud biomarkers have been strongly associated with lung cancer risk in smokers [162]. Also, the CBMN assay genome damage biomarkers using the WIL2-NS cell line have recently been shown to be strongly related to diet with increased caecal water genotoxicity in rats following a high-risk (low fibre, high fat) colorectal cancer diet

compared with a low-risk (high fibre, low fat) colorectal cancer diet (Benassi *et al*, 2008), providing further support to the value of CBMN assay genome damage markers as biomarkers of dietary associated genotoxicity and cancer risk.

The impact of weight loss diets with different macronutrient profiles on the genotoxic potential of faecal water measured using the CBMN cytome assay has not been previously assessed. In this chapter, the assessment of the DNA damage potential of faecal water using the CBMN cytome assay, along with the assessment of other biomarkers which are suggested to be indicative of bowel health, is used to assess the induction of chromosome damage events associated with increased risk of cancer in overweight men following a weight loss diet that is either high protein-high red meat or high carbohydrate.

## *6.2 Methods*

As previously described (Chapter 4), 55 overweight male subjects were recruited and randomised to one of two dietary interventions, a high protein–high red meat (HP) diet or a high carbohydrate-low red meat (HC) diet. The study was a parallel design, comprising of a 12 week intensive weight loss phase and follow-up at one year with interim monthly visits.

Bowel actions from a 24 hour period (typically from midnight Saturday to midnight Sunday) were collected by volunteers at weeks 0, 12 and 52, stored in an esky with frozen ice bricks until they were brought to the clinic the following morning. The samples were weighed and then stored at -20°C until further processing. Faecal water was isolated and stored at -20°C as previously described in Chapter 4.5.2.

### 6.2.1 Cytokinesis block micronucleus cytome assay protocol for faecal water genotoxicity and cytotoxicity testing in the WIL2-NS cell line

The schematic protocol for the testing of faecal water using the CBMN cytome assay in the WIL2-NS cell line is shown in Figure 6.1 and outlined in detail in Chapter 5.2.3. CBMN cytome assay slides were scored according to criteria described in Chapter 3.5.

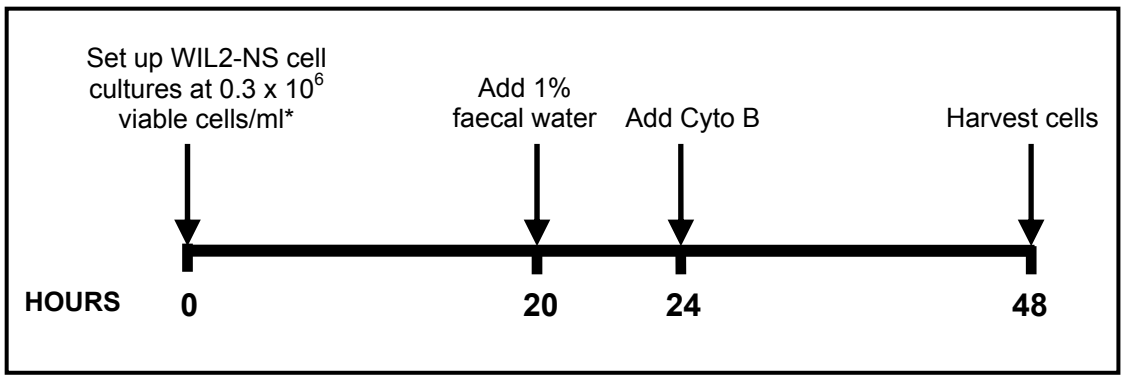


**Figure 6.1 Schematic protocol of faecal water CBMN cytome assay.**

### 6.2.2 Determination of faecal moisture

Approximately 2 grams of each faecal sample was weighed and recorded as 'wet weight faeces' and then placed at -20°C for 4 hours before being placed in the freeze dryer overnight. The dried faecal sample was weighed and recorded as 'dried weight faeces'. Faecal moisture was then calculated using the following equation:

**% faecal moisture = [1 – ('dried weight faeces'/'wet weight faeces')] x 100**

Faecal moisture was measured by Debbie Davies and Paul Orchard, Research Officers at CSIRO Human Nutrition, Adelaide.

### 6.2.3 Short chain fatty acids and faecal pH

Approximately 1 gram of faecal sample was placed into a 10ml centrifuge tube and diluted 3 times the amount of faecal sample with internal standard (1.68MM heptanoic acid) and kept at 4°C. The mixture was vortexed until homogenised, the pH measured and the mixture centrifuged at 3000rpm for 10 minutes at 5°C. The supernatant was removed and 150ul of supernatant was transferred to a 5ml flask containing 30ul of 10% phosphoric acid and distilled by shell freeze in -80°C ethanol. SCFAs were then analysed by gas chromatography using the Agilent GC 6890. SCFA and faecal pH was measured by Debbie Davies and Paul Orchard, Research Officers at CSIRO Human Nutrition, Adelaide.

### 6.2.4 Phenol and p-cresol measurement

Approximately 1 gram of faecal sample was placed into a 10ml centrifuge tube and diluted in three times the amount of 0.5mg/ml 4-ethylphenol. The mixture was then vortexed until homogenised and centrifuged at 3000rpm for 10 minutes at 5°C. 150ul of supernatant was transferred to a clean eppendorf containing 30ul of 1M phosphoric acid and distilled by shell freeze in -80°C ethanol. Samples were analysed using a GBC automated high performance liquid chromatography system. Phenol/p-cresol was measured by Debbie Davies and Paul Orchard, Research Officers at CSIRO Human Nutrition, Adelaide.

### 6.2.5 Micronucleus assay in pig and human colorectal biopsy tissue

Pig biopsy studies were performed in order to develop and optimise the assay prior to use with human mid-rectal biopsy samples.

#### *Collection and storage of pig colonic cell biopsy tissue*

Pig intestine was collected from Chapman's abattoirs in Murray Bridge, South Australia by Debbie Davies (Research Officer, CSIRO Human Nutrition) and transported to CSIRO Human Nutrition in Hanks Balanced Salt Solution (Thermo Electron, Melbourne, Australia) on ice. Prior approval was received from the Animal Ethics Committee of the CSIRO.

The intestine was rinsed with pure water and biopsies collected with forceps. Each sample was then transferred to 1.8ml cryovials (Nunc, Roskilde, Denmark) containing 900µl foetal bovine serum (Thermo Electron, Melbourne, Australia). 100µl of dimethyl sulphoxide (DMSO; Sigma, Sydney, Australia) was added and mixed well and placed on ice. Within 15 minutes of adding DMSO, cryovials were transferred to a cold (4°C) Stratacooler® cryopreservation module (Stratagene, CA, USA) and placed at -80°C. After 24 hours, cryovials were transferred to storage boxes (Nunc, Roskilde, Denmark) for storage at -80°C until required.

#### *Collection and storage of human mid-rectal cell biopsy tissue*

Mid-rectal cell biopsies were obtained from volunteers at weeks 0, 12 and 52 at the CSIRO Clinical Research Unit by a qualified gastroenterologist (Dr P. Clifton). Participants need to empty their bowel before the procedure. The biopsies were taken with rigid forceps through a sigmoidoscope at 10cm from the anal verge. Biopsy forceps were introduced through the sigmoidoscope to take a sample of tissue. The tissue was collected in 3ml of RPMI 1640 culture medium (Thermo Electron, Melbourne, Australia) and placed on ice and transported to the laboratory.

The sample was rinsed with pure water and transferred to a new sterile tube with 3ml RPMI 1640 culture medium. The tube was gently shaken and sample transferred to a new sterile tube with 3ml RMPI culture medium and repeated 3 times. Sample was then transferred to 1.8ml cryovials (Nunc, Roskilde, Denmark) containing 900µl foetal bovine serum (Thermo Electron, Melbourne, Australia). 100µl of dimethyl sulphoxide

(DMSO; Sigma, Sydney, Australia) was added and mixed well and placed on ice. Within 15 minutes of adding DMSO, cryovials were transferred to a cold (4°C) Stratacooler® cryopreservation module (Stratagene, CA, USA) and placed at -80°C. After 24 hours, cryovials were transferred to storage boxes (Nunc, Roskilde, Denmark) for storage at -80°C until required.

***Isolation and slide preparation of biopsy tissue***

Numerous methods were trialled using pig intestine biopsies. The method that provided the best slide preparations for the pig intestine biopsies is described below.

Cryovials were removed from liquid nitrogen and rapidly thawed by agitation in 37 °C pre-warmed pure water. The contents of the cryovial were pipetted into 5 ml of cold RPMI 1640 culture medium (Thermo Electron, Melbourne, Australia). Tubes were centrifuged at 1500 g for 10 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 1 ml RPMI 1640 culture medium. The sample was vortexed using a benchtop vortex machine for 1-2 minutes or until the tissue sample appeared well disaggregated. The tissue sample was further disaggregated manually using a glass pipette. Cells were transferred to slides by cytocentrifugation using a Shandon Cytospin Cytocentrifuge (Shandon Scientific, Chesire, England). 120 µl of each sample were transferred to cytocentrifuge chambers (consisting of a slide, filter card and cytocentrifuge sampling cup) and spun in the cytocentrifuge at 600 rpm for 5 minutes, to give a spot of cells on the slide. Slides were then rotated and a second 120 µl sample transferred, using the same conditions, to generate a second spot of cells. Slides were removed from the cytocentrifuge chamber, laid flat and left to airdry for 10 minutes.

Slides were stained using the commercial kit 'Diff Quik' (Lab Aids, Narrabeen, Australia). This involved fixing the cells by immersing the slides in fixative for 10 minutes. Slides were then removed and immediately transferred first in the Diff-Quik solution 1 (red stain) for approximately 10 seconds and then in Diff-Quik solution 2 (blue stain) for a further 6 seconds. Staining time was adjusted slightly depending on the age of the stain. Slides were then washed thoroughly under running water to remove excess stain. Slides were then left to airdry completely for at least 30 minutes. Slides were then coverslipped using Depex (BDH Laboratory Supplies, Poole, England) and left to set overnight. Slides were then stored in a slide box until required.

### 6.2.6 Statistical analysis

Data is shown as mean ± standard error of the mean (SEM) for all Figures and Tables. Two-way (mixed between-within subjects) ANOVA was used to compare the main effect for time and diet and any interaction effect. Independent *t*-tests were used to compare the diets at baseline. A correlation matrix of all data was determined using Pearsons test. Significance for all tests was accepted at $P<0.05$. All statistical analyses were performed using SPSS 14.0 for Windows and all graphs produced using GraphPad Prism version 4.00 software.

Results for faecal water genotoxicity and cytotoxicity using the CBMN cytome assay were calculated as the faecal water challenge result minus the baseline 'background' result recorded for the WIL2-NS cell line for each assay run to minimise any potential confounding effect from a change in the background DNA damage/cytotoxicity frequency of the cell line. Therefore results for the CBMN cytome assay are reported as induced effect of faecal water. The background values for the DNA damage biomarkers in the WIL2-NS cell line are shown in Appendix 6.1.

## *6.3 Results*

### 6.3.1 Short term intensive weight loss phase

55 male subjects completed the 12 week intensive weight loss phase; with n=24 in the high protein (HP) group and n=31 in the high carbohydrate (HC) group. Weight loss was not significantly different between diets. Average weight loss was 8.4 ± 0.5 kg after the 12 weeks, as described in Chapter 4.4.

#### *Faecal water genotoxicity and cytotoxicity*

Figure 6.2 and Table 6.1 show the frequency of MN, NPB, NBuds and total DNA damage at baseline (week 0) and after 12 weeks intensive weight loss on either a HP or HC diet. Two-way ANOVA shows no significant difference with time or between diet for NPB. A significant reduction with time but not diet was seen for MN (P=0.017), NBuds (P<0.001) and total DNA damage (P=0.013). There were no significant time by diet interactions.

Figure 6.3 and Table 6.2 show rates of necrosis, apoptosis and nuclear division cytotoxicity index (NDCI) at week 0 and after 12 weeks weight loss with either a HP of HC diet. There was no significant effect of time or diet for necrosis or NDCI. Two-way ANOVA suggests a significant effect of time (P=0.042) but not diet for apoptosis. There were no significant time by diet interactions.

#### *Conventional bowel health biomarkers*

Effect on faecal pH, moisture, weight, short chain fatty acids (SCFA) excretion, phenol and p-cresol after 12 weeks intensive weight loss are shown in Figures 6.4 and 6.5 and Tables 6.3 and 6.4. Two-way ANOVA indicated no significant change over time or between diets for faecal pH, weight, moisture, SCFAs or p-cresol excretion. An effect of time but not diet was seen for phenol levels (P=0.023). There were no significant time by diet interactions.

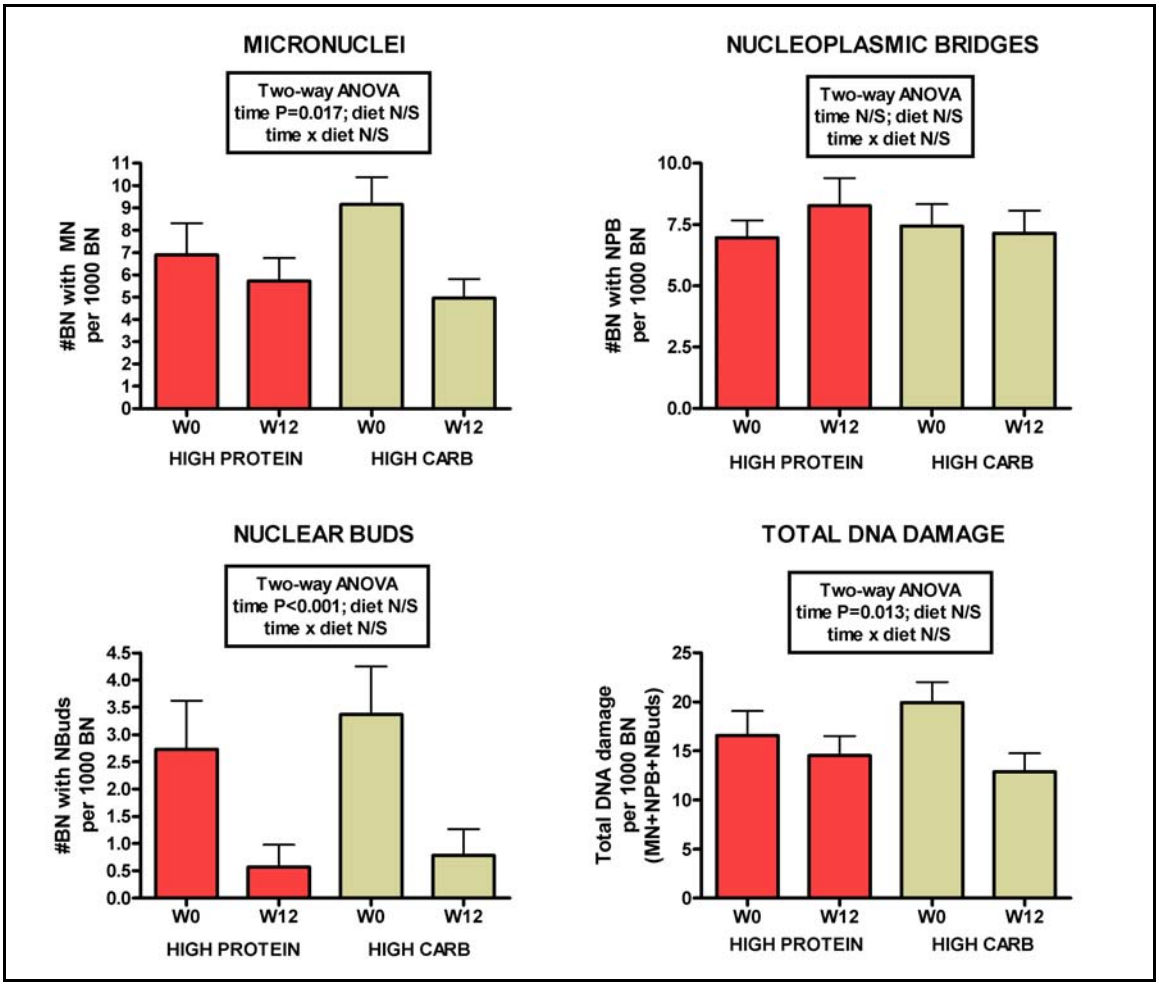


**Figure 6.2 Induced* DNA damage by faecal water assessed using the CBMN cytome assay with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.** HP n=24; HC n=31. BN=binucleate cells, MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, W=week, N/S=not significant. *Induced indicates the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch.

**Table 6.1 CBMN cytome DNA damage biomarkers induced* by faecal water with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 |
|---|---|---|---|---|
| **micronuclei** | *HP* | *n=24* | 6.89 ± 1.43 | 5.73 ± 1.04 |
| | *HC* | *n=31* | 9.15 ± 1.24 | 4.95 ± 0.86 |
| | | ***t*-test^^** | P=0.24 | |
| **nucleoplasmic bridges** | *HP* | *n=24* | 6.98 ± 0.69 | 8.27 ± 1.13 |
| | *HC* | *n=31* | 7.44 ± 0.89 | 7.15 ± 0.91 |
| | | ***t*-test^^** | P=0.70 | |
| **nuclear buds** | *HP* | *n=24* | 2.73 ± 0.89 | 0.57 ± 0.41 |
| | *HC* | *n=31* | 3.37 ± 0.88 | 0.79 ± 0.48 |
| | | ***t*-test^^** | P=0.62 | |
| **total DNA damage** | *HP* | *n=24* | 16.60 ± 2.47 | 14.55 ± 1.96 |
| | *HC* | *n=31* | 19.95 ± 2.06 | 12.89 ± 1.87 |
| | | ***t*-test^^** | P=0.30 | |

Data represented as mean ± SEM
^^ independent t-test, comparing the diets at baseline
*values in table equal to the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch

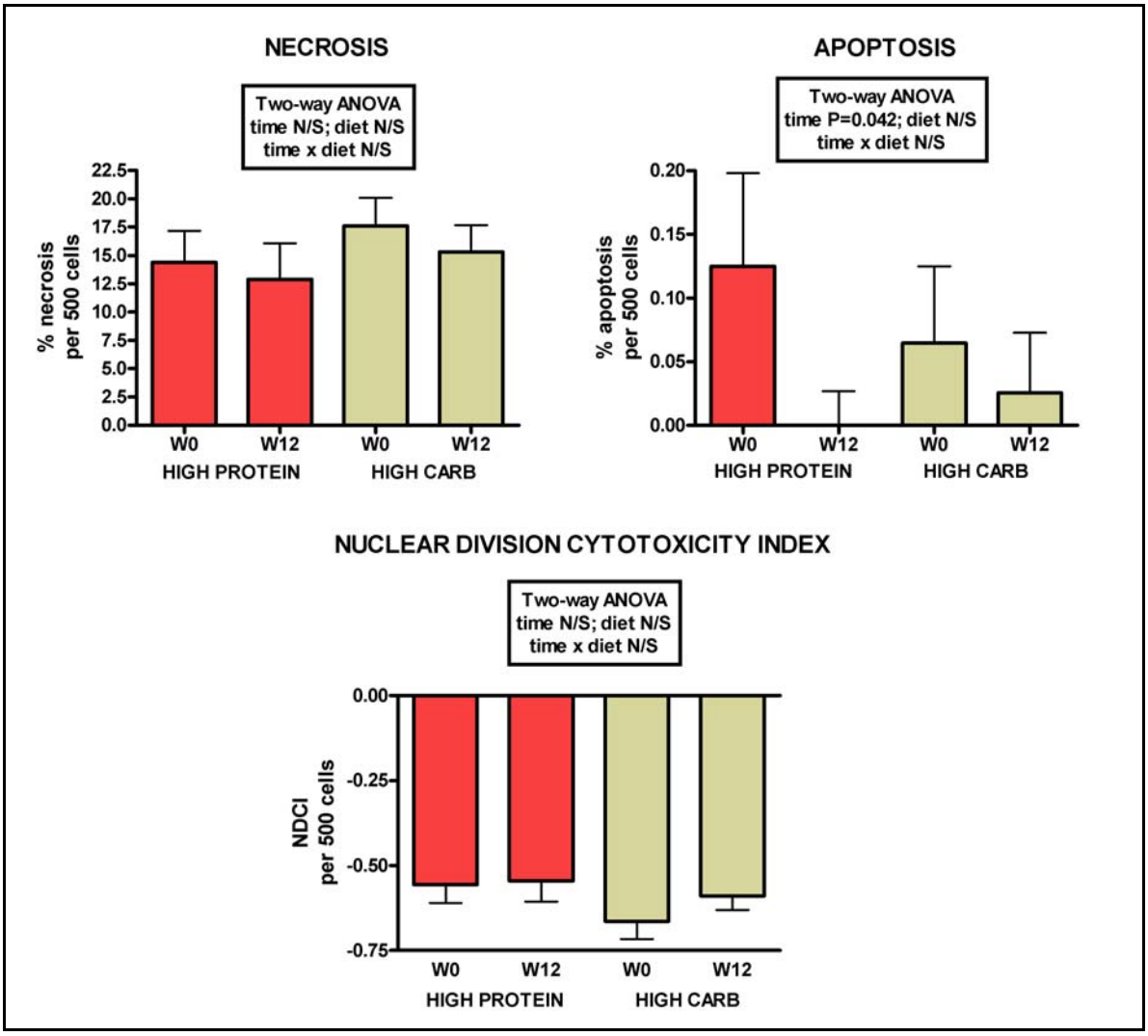


**Figure 6.3 Induced* cytotoxicity by faecal water assessed using the CBMN cytome assay with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.** HP n=24; HC n=31. BN=binucleate cells, W=week, N/S=not significant. *Induced indicates the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch.

**Table 6.2 CBMN cytome cytotoxicity biomarkers induced* by faecal water with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 |
|---|---|---|---|---|
| **necrosis** (%) | *HP* | *n=24* | 14.40 ± 2.75 | 12.89 ± 3.16 |
| | *HC* | *n=31* | 17.60 ± 2.49 | 15.29 ± 2.38 |
| | | ***t*-test^^** | P=0.39 | |
| **apoptosis** (%) | *HP* | *n=24* | 0.13 ± 0.07 | 0.00 ± 0.03 |
| | *HC* | *n=31* | 0.06 ± 0.06 | 0.03 ± 0.26 |
| | | ***t*-test^^** | P=0.52 | |
| **NDCI** | *HP* | *n=24* | -0.56 ± 0.05 | -0.55 ± 0.06 |
| | *HC* | *n=31* | -0.66 ± 0.05 | -0.59 ± 0.04 |
| | | ***t*-test^^** | P=0.16 | |

Data represented as mean ± SEM
^^ independent t-test, comparing the diets at baseline
*values in table equal to the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch

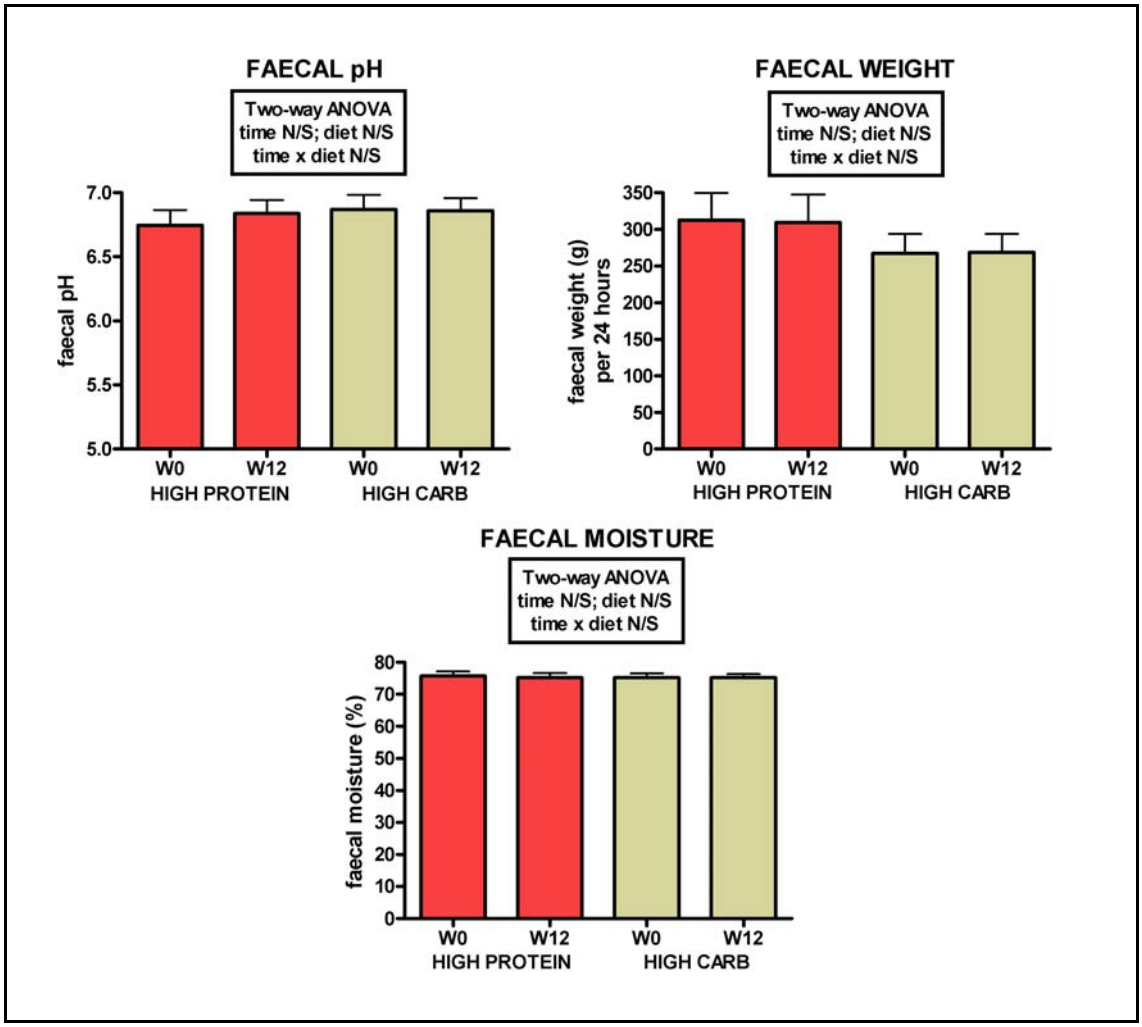


**Figure 6.4 Faecal pH, weight and moisture with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.** HP n=24; HC n=31. W=week, N/S=not significant.

**Table 6.3. Change in faecal pH, weight and moisture with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 |
|---|---|---|---|---|
| **faecal pH** | *HP* | *n=24* | 6.75 ± 0.12 | 6.84 ± 0.10 |
| | *HC* | *n=31* | 6.87 ± 0.11 | 6.86 ± 0.10 |
| | | ***t*-test^^** | P=0.45 | |
| **faecal weight** (g/24hrs) | *HP* | *n=24* | 312.84 ± 36.97 | 309.65 ± 37.89 |
| | *HC* | *n=31* | 267.24 ± 26.60 | 268.51 ± 25.20 |
| | | ***t*-test^^** | P=0.31 | |
| **faecal moisture** (%) | *HP* | *n=24* | 75.72 ± 1.41 | 75.25 ± 1.36 |
| | *HC* | *n=31* | 75.24 ± 1.32 | 75.16 ± 1.12 |
| | | ***t*-test^^** | P=0.80 | |

Data represented as mean ± SEM
^^ independent t-test, comparing the diets at baseline

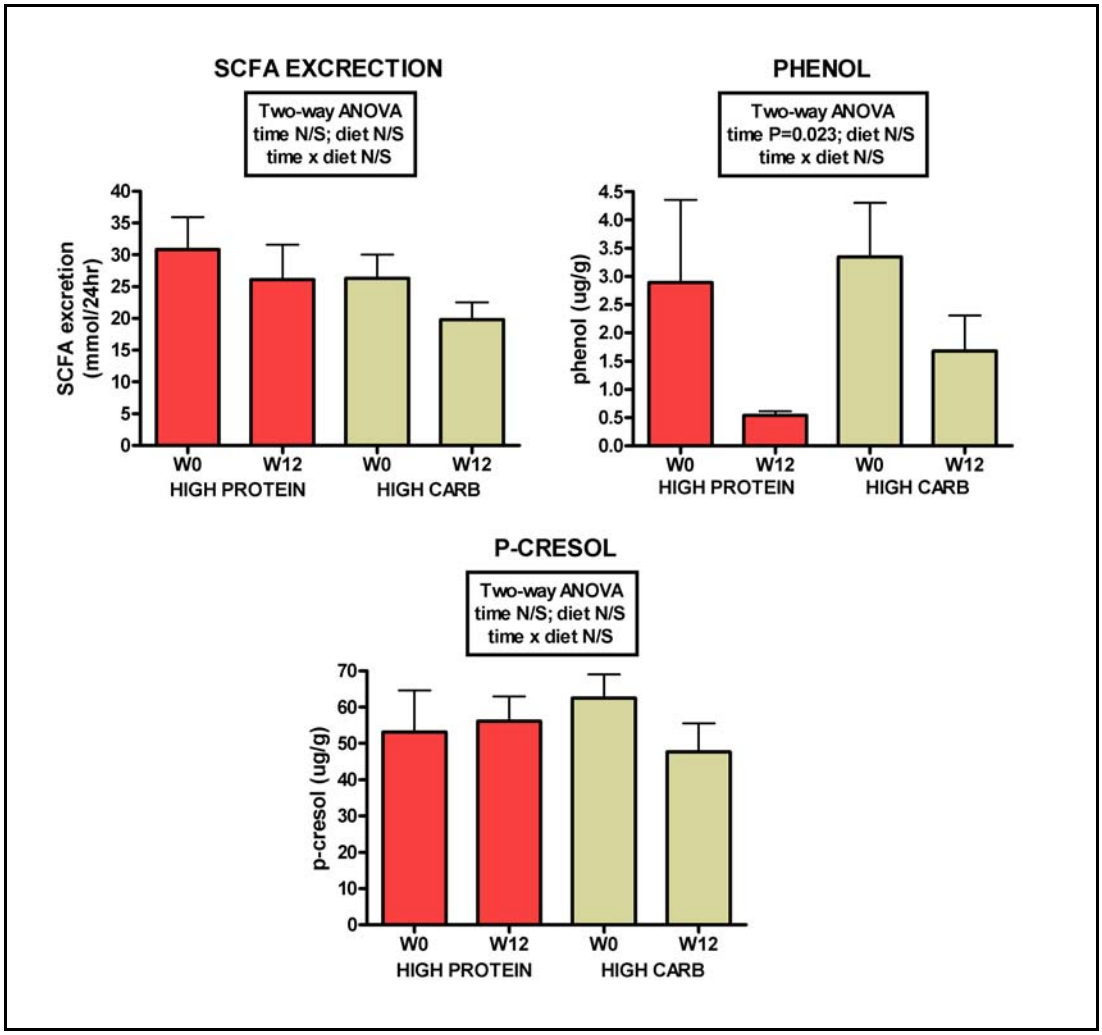


**Figure 6.5 Faecal SCFA, phenol and p-cresol with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.** W=week, N/S=not significant.

**Table 6.4. Excretion rate of faecal SCFA, phenol and p-cresol with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 |
|---|---|---|---|---|
| **SCFA** (mmol/24hr) | *HP* | *n=24* | 30.82 ± 5.11 | 26.13 ± 5.49 |
| | *HC* | *n=31* | 26.28 ± 3.76 | 19.83 ± 2.68 |
| | | ***t*-test^^** | P=0.47 | |
| **phenol** (ug/g) | *HP* | *n=24* | 2.89 ± 1.46 | 0.54 ± 0.08 |
| | *HC* | *n=30* | 3.35 ± 0.96 | 1.68 ± 0.63 |
| | | ***t*-test^^** | P=0.79 | |
| **p-cresol** (ug/g) | *HP* | *n=24* | 53.12 ± 11.47 | 56.17 ± 6.76 |
| | *HC* | *n=30* | 62.48 ± 6.56 | 47.70 ± 7.81 |
| | | ***t*-test^^** | P=0.46 | |

Data represented as mean ± SEM
^^ independent t-test, comparing the diets at baseline

### 6.3.2 Long term weight maintenance phase

33 male subjects completed the 12 week intensive weight loss phase and following 9 months weight maintenance; with n=16 in the high protein (HP) group and n=17 in the high carbohydrate (HC) group. Weight loss was not significantly different between diets at week 12 or week 52. Average weight loss was 9.3 ± 0.7 kg after 12 weeks intensive weight loss with no further significant change in weight from week 12 to week 52, as described in Chapter 4.4.

***Faecal water genotoxicity and cytotoxicity***

MNi, NPBs, NBuds and total DNA damage frequency after 12 weeks intensive weight loss and 9 months weight maintenance is shown in Figure 6.6 and Table 6.5. A significant effect of time but not diet, or time by diet interactions, was seen for all biomarkers (Two-way ANOVA P=0.001; P=0.011, P<0.001 and P<0.001 respectively). Bonferroni post hoc test indicates there is a significant reduction for MNi and NBuds from week 0 to week 12 (P=0.007 and P=0.001 respectively) and then a subsequent significant increase from week 12 to 52 (P=0.001 and P=0.004) to frequencies not significantly different to baseline. Bonferroni post hoc test shows no significant change in NPBs from week 0 to 12 but a significant increase at week 52 compared with week 0 (P=0.015) and week 12 (P=0.011). Total DNA damage was found to decrease with 12 weeks intensive weight loss (Bonferroni P=0.006) and then increase at week 52 to levels not significantly different to week 0 for both diets.

Rates of necrosis, apoptosis and NDCI after 12 weeks intensive weight loss and 9 months weight maintenance are shown in Figure 6.7 and Table 6.6. Two-way ANOVA shows no significant effect between the HP and HC diets, nor any significant effect over time from week 0 to week 12 for any of these biomarkers. There were no significant time by diet interactions.

It can be suggested that fold change of faecal water challenge result from baseline ‘background’ result recorded for the WIL2-NS cell line for each assay run may prove more informative than an induced effect of faecal water. The graphs for fold change of faecal water challenge result relative to the control ‘background’ result are shown in Appendix 6.8. The only difference seen was for NPBs which no longer significantly changed with time when analysed by fold change compared with significant changes in induced frequencies of faecal water genotoxicity as reported above.

### *Conventional bowel health biomarkers*

Figure 6.8 and Table 6.7 show changes in faecal pH, weight and moisture with 12 weeks intensive weight loss and 9 months weight maintenance. Two-way ANOVA shows no significant effect of time or diet, nor any time by diet interaction effect for any of these biomarkers. Likewise, for SCFA excretion, phenol and p-cresol levels shown in Figure 6.9 and Table 6.8, two-way ANOVA indicates no significant effect of time, diet or time by diet interaction effect for any of the biomarkers.

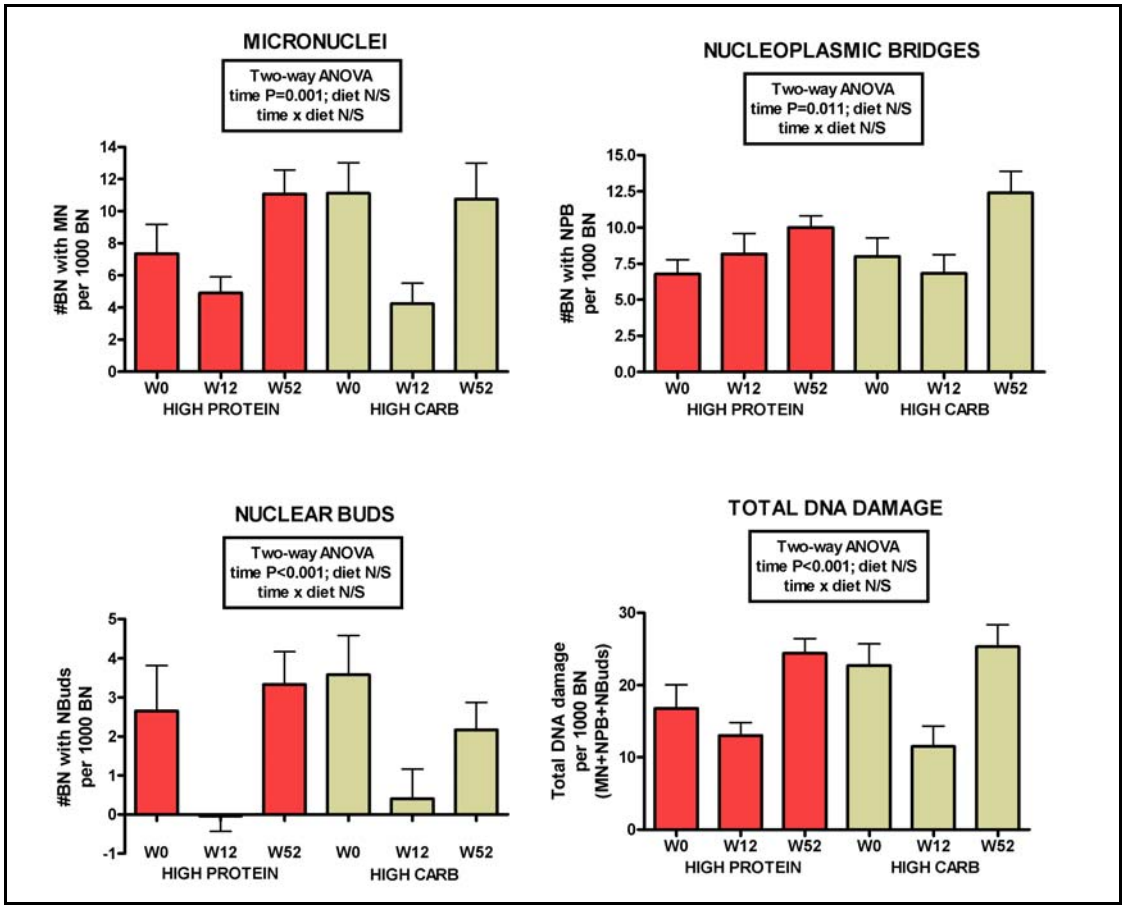


**Figure 6.6 Induced* DNA damage by faecal water using the CBMN cytome assay with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.** HP n=16; HC n=17. BN=binucleate cells, MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, W=week, N/S=not significant. *Induced indicates the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch.

**Table 6.5 CBMN cytome DNA damage biomarkers induced* by faecal water using the CBMN cytome assay with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 | Week 52 |
|---|---|---|---|---|---|
| **micronuclei** | *HP* | *n=16* | 7.35 ± 1.84 | 4.91 ± 0.98 | 11.08 ± 1.49 |
| | *HC* | *n=17* | 11.13 ± 1.88 | 4.25 ± 1.26 | 10.76 ± 2.25 |
| | | ***t*-test^^** | P=0.16 | | |
| **nucleoplasmic bridges** | *HP* | *n=16* | 6.79 ± 0.98 | 8.16 ± 1.42 | 10.01 ± 0.80 |
| | *HC* | *n=17* | 8.01 ± 1.29 | 6.84 ± 1.30 | 12.41 ± 1.49 |
| | | ***t*-test^^** | P=0.46 | | |
| **nuclear buds** | *HP* | *n=16* | 2.65 ± 1.17 | -0.04 ± 0.40 | 3.33 ± 0.84 |
| | *HC* | *n=17* | 3.58 ± 1.00 | 0.40 ± 0.76 | 2.17 ± 0.70 |
| | | ***t*-test^^** | P=0.55 | | |
| **total DNA damage** | *HP* | *n=16* | 16.79 ± 3.26 | 13.04 ± 1.78 | 24.41 ± 2.02 |
| | *HC* | *n=17* | 22.72 ± 3.02 | 11.48 ± 2.85 | 25.34 ± 3.06 |
| | | ***t*-test^^** | P=0.19 | | |

Data represented as mean ± SEM
^^independent t-test, comparing the diets at baseline
*values in table equal to the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch

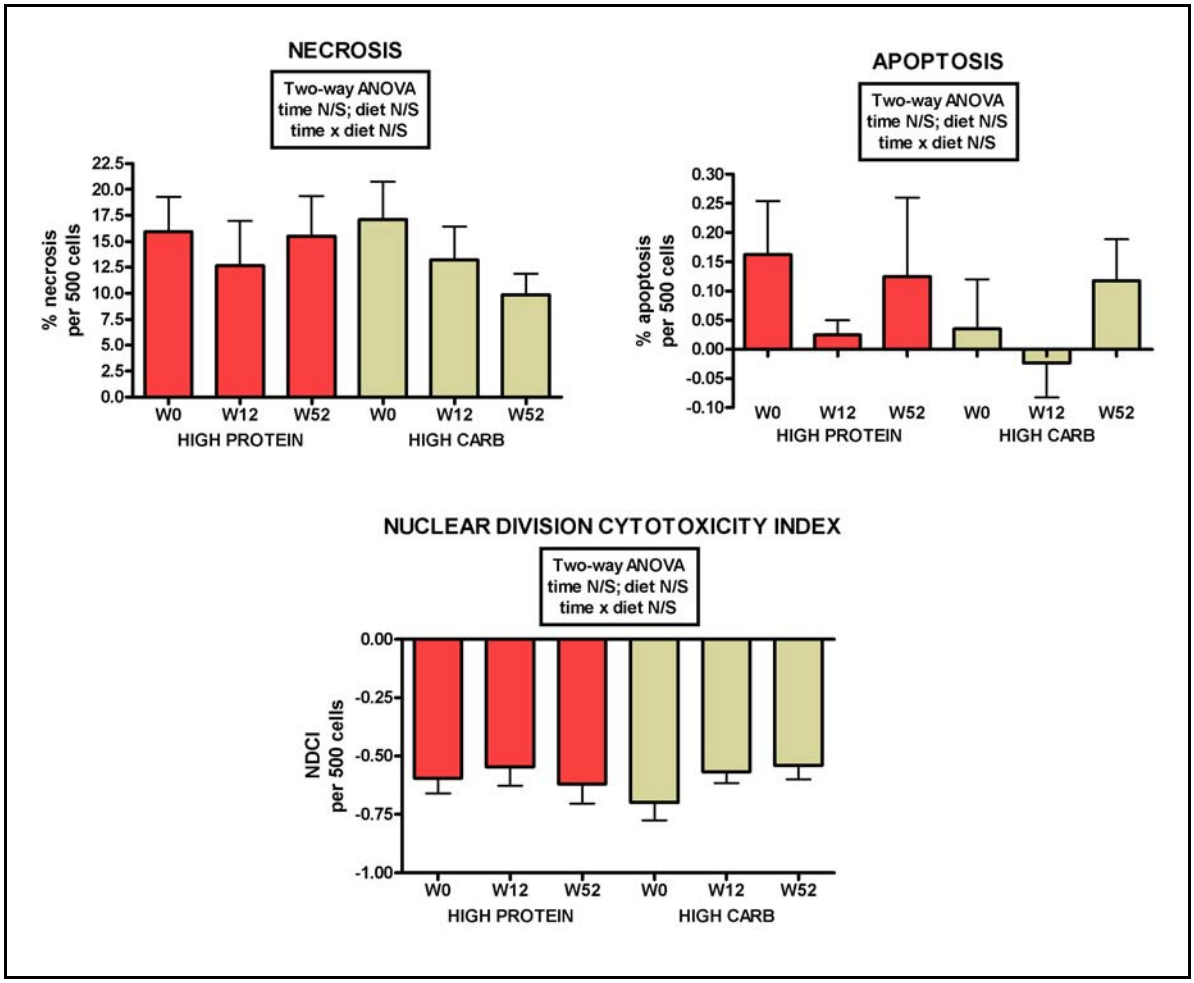


**Figure 6.7 Induced* cytotoxicity by faecal water using the CBMN cytome assay with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.** HP n=24; HC n=31. BN=binucleate cells, W=week, N/S=not significant.
*Induced indicates the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch.

**Table 6.6 CBMN cytome cytotoxicity biomarkers induced* by of faecal water using the CBMN cytome assay with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 | Week 52 |
|---|---|---|---|---|---|
| **necrosis** (%) | *HP* | *n=16* | 15.94 ± 3.33 | 12.66 ± 4.29 | 15.49 ± 3.86 |
| | *HC* | *n=17* | 17.10 ± 3.63 | 13.19 ± 3.20 | 9.86 ± 2.03 |
| | | ***t*-test^^** | P=0.82 | | |
| **apoptosis** (%) | *HP* | *n=16* | 0.16 ± 0.09 | 0.03 ± 0.03 | 0.13 ± 0.14 |
| | *HC* | *n=17* | 0.04 ± 0.08 | -0.02 ± 0.06 | 0.12 ± 0.07 |
| | | ***t*-test^^** | P=0.32 | | |
| **NDCI** | *HP* | *n=16* | -0.59 ± 0.07 | -0.55 ± 0.08 | -0.62 ± 0.08 |
| | *HC* | *n=17* | -0.70 ± 0.08 | -0.57 ± 0.05 | -0.54 ± 0.06 |
| | | ***t*-test^^** | P=0.32 | | |

Data represented as mean ± SEM
^^independent t-test, comparing the diets at baseline
*values in table equal to the observed response of faecal water exposure in the cell line minus the baseline control response of the same cell line batch

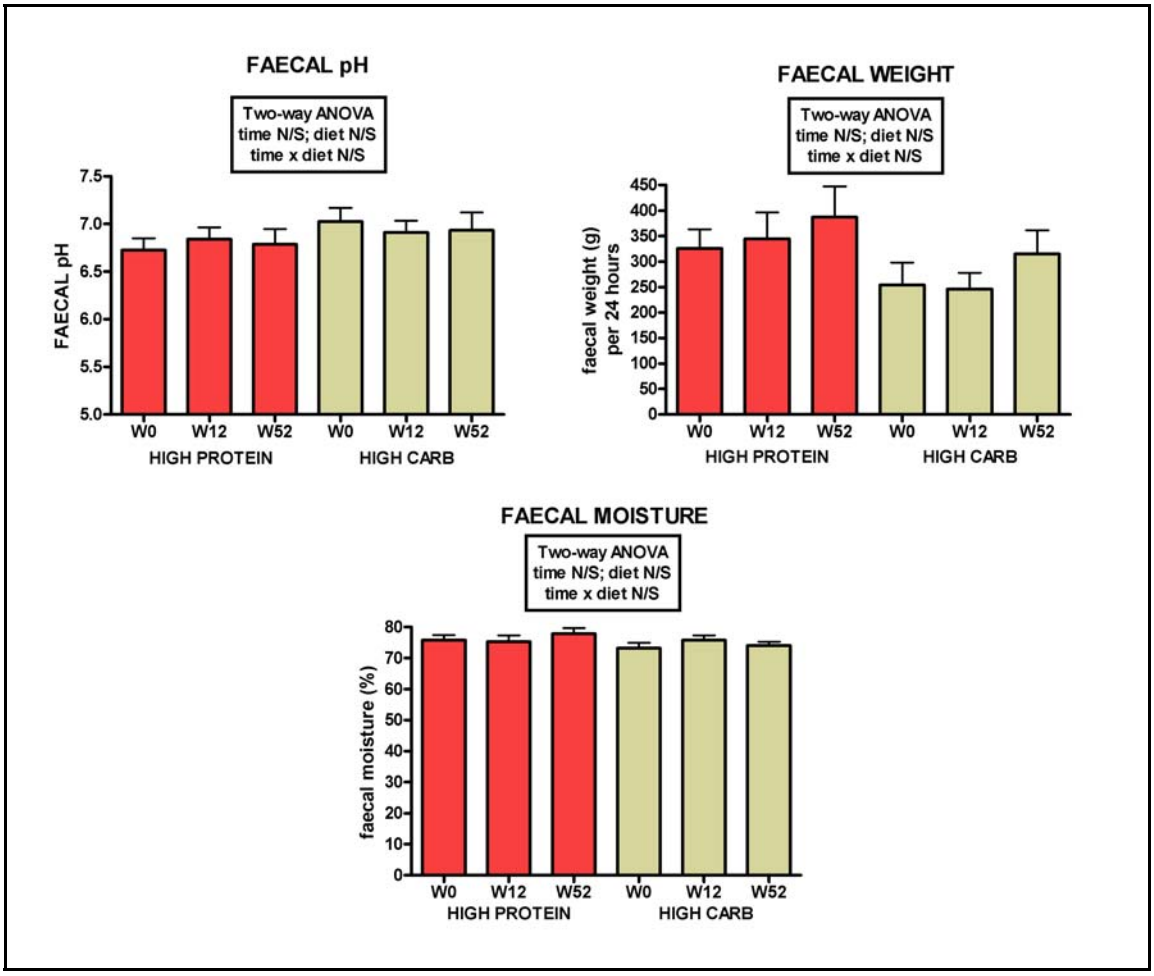


**Figure 6.8 Faecal pH, weight and moisture with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.** W=week, N/S= not significant.

**Table 6.7 Change in faecal pH, weight and moisture with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 | Week 52 |
|---|---|---|---|---|---|
| **faecal pH** | *HP* | *n=16* | 6.73 ± 0.12 | 6.84 ± 0.12 | 6.79 ± 0.16 |
| | *HC* | *n=17* | 7.03 ± 0.14 | 6.91 ± 0.13 | 6.94 ± 0.19 |
| | | ***t*-test^^** | P=0.12 | | |
| **faecal weight** (g/24hrs) | *HP* | *n=16* | 325.16 ± 38.33 | 344.44 ± 51.94 | 387.22 ± 59.98 |
| | *HC* | *n=17* | 254.03 ± 44.00 | 246.20 ± 31.79 | 314.72 ± 46.88 |
| | | ***t*-test^^** | P=0.23 | | |
| **faecal moisture** (%) | *HP* | *n=16* | 75.80 ± 1.59 | 75.21 ± 2.02 | 77.79 ± 1.86 |
| | *HC* | *n=17* | 73.19 ± 1.79 | 75.73 ± 1.58 | 73.99 ± 1.29 |
| | | ***t*-test^^** | P=0.29 | | |

Data represented as mean ± SEM
^^independent t-test, comparing the diets at baseline

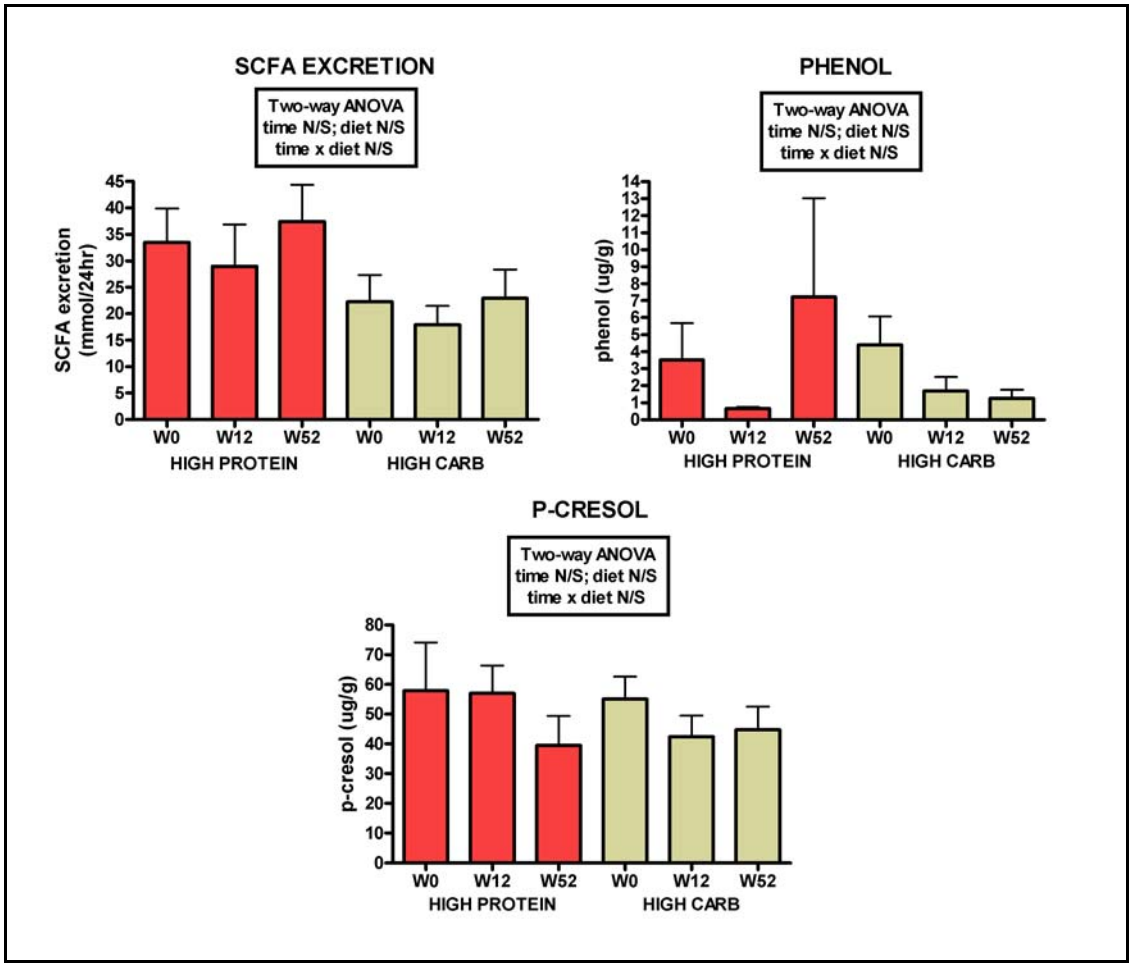


**Figure 6.9 Faecal SCFA, phenol and p-cresol with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.** W=week, N/S=not significant.

**Table 6.8. Excretion rate of faecal SCFA, phenol and p-cresol with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 | Week 52 |
|---|---|---|---|---|---|
| **SCFA** (mmol/24hr) | *HP* | *n=16* | 33.47 ± 6.38 | 28.97 ± 7.90 | 37.36 ± 6.99 |
| | *HC* | *n=17* | 22.25 ± 5.06 | 17.92 ± 3.54 | 22.89 ± 5.47 |
| | | ***t*-test^^** | P=0.18 | | |
| **phenol** (ug/g) | *HP* | *n=16* | 3.52 ± 2.15 | 0.65 ± 0.10 | 7.22 ± 5.80 |
| | *HC* | *n=16* | 4.04 ± 1.67 | 1.69 ± 0.82 | 1.26 ± 0.51 |
| | | ***t*-test^^** | P=0.75 | | |
| **p-cresol** (ug/g) | *HP* | *n=16* | 57.85 ± 16.17 | 56.97 ± 9.34 | 39.53 ± 9.92 |
| | *HC* | *n=16* | 55.10 ± 7.54 | 42.36 ± 7.14 | 44.75 ± 7.72 |
| | | ***t*-test^^** | P=0.88 | | |

Data represented as mean ± SEM
^^independent t-test, comparing the diets at baseline

### 6.3.3 Correlations

Correlation matrices for all measures at week 0, 12 and 52 are shown in Appendix 6.2, 6.4 and 6.6 respectively. Correlation matrices for faecal water induced genome damage markers and the individual SCFAs at week 0, 12 and 52 (acetic, propionic, butyric, valeric and caproic acids) are shown in Appendix 6.3, 6.5 and 6.7 respectively. Significant correlations (P<0.05) are highlighted in yellow.

***Week 0***

There was a positive correlation with MN and NPB (r=0.501; P<0.001) and NBuds (r=0.294; P=0.029). Necrosis was negatively correlated with NDCI (r=-0.767; P<0.001). P-cresol was negatively associated with NDCI (r=-0.313; P=0.021), faecal weight (r=-0.322; P=0.018), moisture (r=-0.422; P=0.001) and SFCAs (r=-0.337; P=0.013). SCFAs were also associated negatively with faecal pH (r=-0.676; P<0.001) and positively with faecal weight (r=0.831; P<0.001) and moisture(r=0.752; P<0.001). Faecal moisture was negatively associated with faecal pH (r=-0.547; P<0.001) and faecal weight (r=0.608; P<0.001). Faecal weight correlated negatively with faecal pH (r=-0.411; P=0.002).

Acetic, propionic, butyric and valeric acids were all positively correlated with each other. Caprioc acid was positively correlated with valeric acid only. NBuds were positively correlated with propionic acid (r=0.292; P=0.031) and valeric acid (r=0.300, P=0.026).

***Week 12***

There was a positive correlation of MN with NPB (r=0.496; P<0.001) and NBuds (r=0.485; P<0.001). NPB were negatively associated with NDCI 9r=-0.311, P=0.021). Necrosis was also negatively associated with NDCI (r= -0.738; P<0.001) and faecal moisture (r= -0.318; P=0.018). Faecal weight and faecal pH were negatively correlated (r=-0.469 ; P<0.001). Faecal moisture was also correlated negatively with faecal pH (r= -0.439; P=0.001) and p-cresols (r= -0.397; P=0.003) and positively with NDCI (r= 0.400; P=0.002), faecal weight (r= 0.534; P<0.001), SCFAs (r= 0.540; P<0.001) and phenols (r= 0.272; P=0.047). SCFAs were positively associated with faecal weight ((r= 0.833; P<0.001) and negatively with faecal pH (r= -

0.669; P<0.001) and p-cresols (r= -0.290; P=0.033). P-cresols and faecal pH were also positively correlated (r= 0.363; P=0.007).

Acetic, propionic, butyric, valeric and caprioc acids were all positively correlated with each other. There were no correlations with individual SCFAs and faecal water genotoxicity CBMN cytome assay biomarkers.

***Week 52***

NBuds were positively correlated with faecal weight (r=0.442; P=0.010), faecal moisture (r=0.425; P=0.014), SCFAs (r=0.510; P=0.002) and negatively with p-cresols (r=-0.461; P=0.007). NDCI was negatively associated with necrosis (r=-0.793; P<0.001) and positively associated with apoptosis (r=0.442; P=0.010). Faecal pH was correlated negatively with faecal weight (r=-0.564; P=0.001), faecal moisture (r=-0.561; P=0.001) and SCFAs (r=-0.714; P<0.001) and positively with p-cresols (r=0.605; P<0.001). Faecal moisture and faecal weight were positively associated (r=0.575; P<0.001). SCFAs were also positively associated with faecal weight (r=0.915; P<0.001) and moisture (r=0.752; P<0.001). P-cresols was also correlated negatively with faecal weight (r=-0.490; P=0.004), moisture (r=-0.663; P<0.001) and SCFAs (r=-0.593; P<0.001).

Acetic, propionic, butyric, valeric and caprioc acids were all positively correlated with each other. NBuds were positively associated with acetic acid (r=0.502; P=0.003), propionic acid (r=0.423; P=0.014) and butyric acid (r=0.526; P=0.002).

### 6.3.4 Micronucleus assay in pig and human colorectal biopsy tissue

Isolation and preparation of slides for use in the micronucleus assay was successfully produced from pig colon biopsy samples. Images are shown in Figure 6.10. The following scoring criteria was developed for the analysis of colonocyte cell slides and based on that described for scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures in Fenech *et al* 2003 for scoring [124]:

***Criteria for selecting colonocyte cells which can be scored for the frequency of micronuclei***

1. The colonocyte cells should be simple columnar in shape.
2. The colonocyte cell should have an intact nuclear membrane and be within the cytoplasmic boundary.
3. The cytoplasmic boundary must be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

***Criteria for scoring micronuclei (MNi) in colonocytes***

1. The diameter of MNi are between 1/16 and 1/3 the size of the mean diameter of the main nuclei.
2. Are round or oval in shape.
3. Not linked or connected to the main nuclei.
4. May touch but not overlap the main nuclei such that the micronuclear boundary is distinguishable from the nuclear boundary.
5. Have the same staining characteristics of the main nuclei.

This assay method proved unsuccessful in producing scorable slides suitable for the micronucleus assay with the human mid-rectal biopsy samples. Images are shown in Figure 6.11. Slides appeared to be covered in dead cell debris with very few intact scorable cells present. Further work in slide development is required to make this a useful assay.

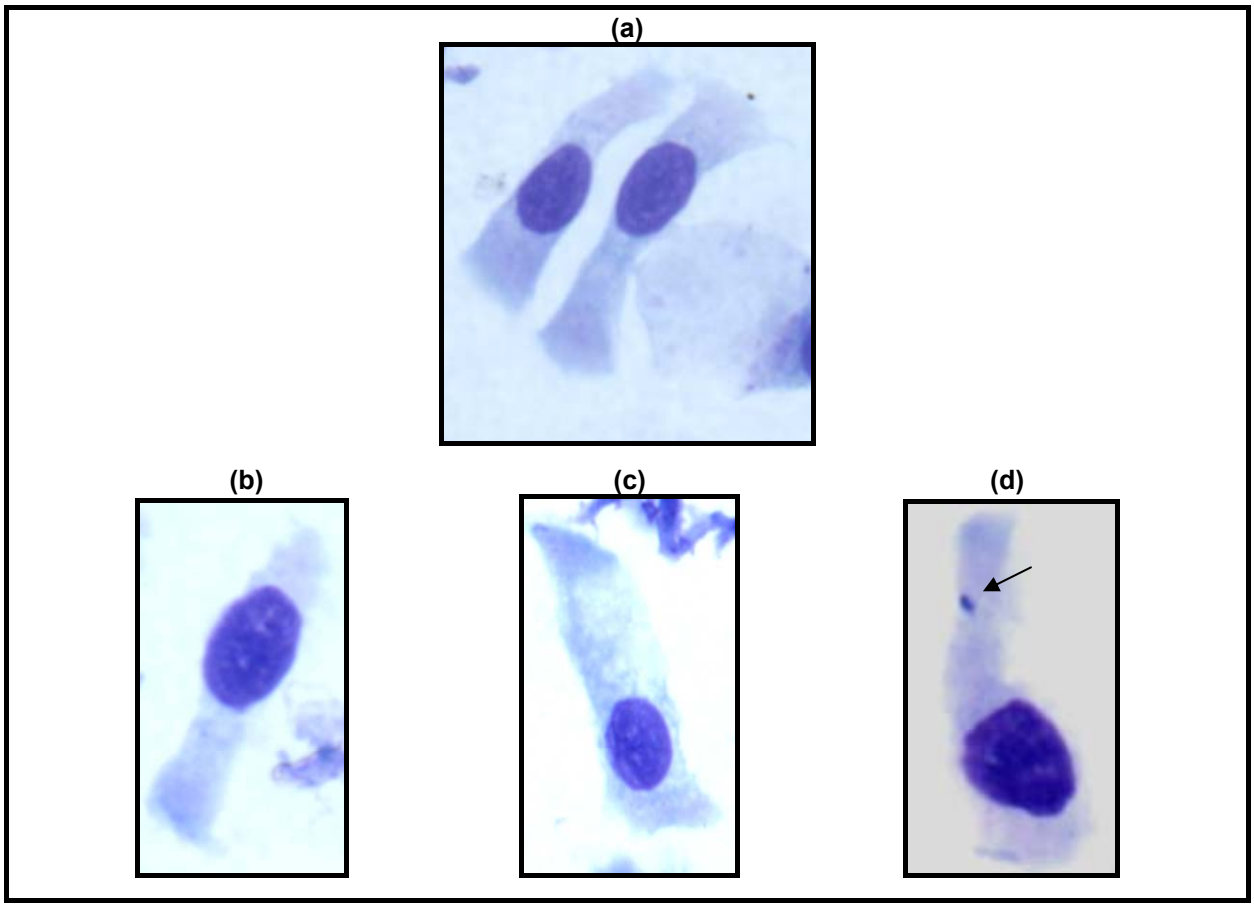


**Figure 6.10 Images of slides prepared from pig intestine biopsy samples**
(a) two colonocytes, (b)-(c) single colonocytes (d) single colonocytes containing one micronuclei indicated by arrow

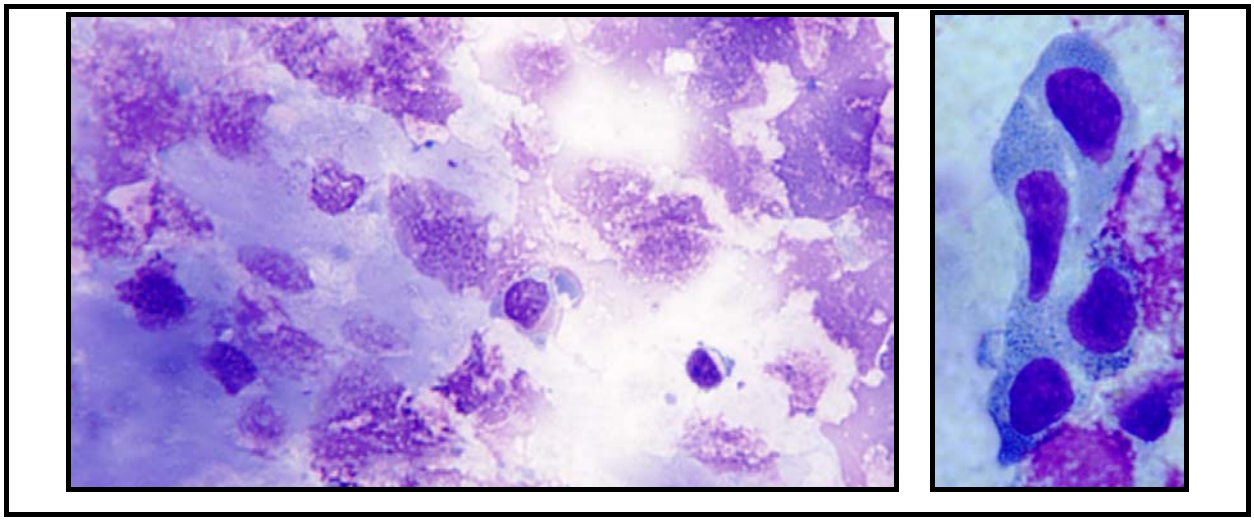

**Figure 6.11 Images of slides prepared from human mid-rectal biopsy samples**
(a) example of images observed with human mid-rectal biopsy sample, no clear intact cells, appears to be a heavy mucus layering, (b) example of two colonocytes, nuclear boundaries unclear, rare observation.

## *6.4 Discussion*

The cytokinesis block micronucleus (CBMN) cytome assay was used in this study to assess the in vitro genotoxic potential of faecal water in overweight men following one of two weight loss diets with different macronutrient profiles, one high protein - high red meat (HP) and the other high carbohydrate (HC). Conventional biomarkers thought to be indicative of bowel health were also assessed. To date, the effect of different weight loss diets on faecal water genotoxicity assessed with the CBMN cytome assay has not been studied. Results from this study are important for assessment of the impact of weight loss and calorie restriction on bowel health and considering the increasing concern with high red meat consumption and increased risk of colorectal cancer, the faecal water genotoxicity response with a HP diet compared with a HC diet.

In this study, the effect of 12 weeks intensive weight loss on faecal water genotoxicity assessed in the WIL2-NS cell line, resulted in a significant decrease in micronuclei (MN) and nuclear buds (NBuds) for both the HP and HC diets and no change in nucleoplasmic bridges (NPBs). In the subjects who completed the 12 weeks intensive weight loss and 9 months weight maintenance, there was a reduction in faecal water induced MN following 12 weeks weight loss, and then a subsequent increase at week 52 to levels not significantly different to that seen at baseline. A similar pattern was seen for NBuds, with a reduction at week 12 compared to week 0 and then an increase at week 52 to levels not significantly different to week 0. For faecal water induced NPBs, there was no change from week 0 to week 12 but a significant increase was seen at week 52 compared with week 0 and 12. Faecal water genotoxicity analysed using 'fold change' from the background DNA damage rate (control), as opposed to the above induced frequencies, did not show any significant differences in response except for NPBs which were no longer shown to significantly change with time.

NPBs provide a measure of chromosome rearrangement and DNA misrepair in the CBMN cytome assay. They arise from dicentric chromosomes, whose centromeres are pulled to opposite spindle poles of the cell during cell division, resulting in a nucleoplasmic connection [93, 97]. An increase in the occurrence of NPBs is usually associated with a simultaneous increase in MNi, as the formation of a dicentric chromosome (NPB) as a result of misrepair of DNA strand breaks will also result in

the formation of an acentric chromosome fragment (MN) [123]. However, it is also possible for NPBs to arise from dicentric chromosomes due to telomere end fusion that can result in the production of a NPB without the accompanying acentric DNA strand break fragment (MN) [97, 98, 163]. However, as NPB frequencies did not significantly change when assessed using fold increase genotoxicity from baseline frequencies as opposed to induced genotoxicity frequencies, it is probable the change in NPBs is likely to be marginal if at all present.

To provide an estimate of the overall genome damage potential of faecal water as a result of the dietary intervention we have presented 'total DNA damage', which simultaneously takes into account all of the CBMN cytome assay DNA damage biomarkers (MN, NPB and NBuds). This may provide one possible method that could be utilised when assessing the overall or net effect on faecal water genotoxicity, particularly as a result of different dietary patterns. Using this measure, both the HP and HC diets showed a significant reduction in total DNA damage potential of faecal water with 12 weeks intensive weight loss (significant only for the HC group), and then a subsequent increase after 9 months weight maintenance to damage frequencies seen at baseline. From this, it could be proposed that it may be active weight loss and/or caloric restriction that has reduced the genotoxic potential of the faecal water, however any effect on genome stability in this study cannot be attributed as a direct result of weight loss and/or caloric restriction as there was no free-living control group for comparison. Future studies would require a non-weight loss control group to be included, using coded slides, to determine unequivocally whether weight loss reduces faecal water genotoxicity.

There was no effect seen for time or diet on rates of necrosis and apoptosis, cytotoxicity markers in the CBMN cytome assay, in this study. From this it could be suggested that neither a HP nor HC dietary pattern resulted in the production of cytotoxic agents in the faecal water that had the ability to damage cellular membranes or modify mechanisms which control the apoptotic response.

Faecal water in this study was diluted to 1%, an amount found to be required for *in vitro* testing in the WIL2-NS cell line while maintaining sufficient viability and that a 10% dilution resulted in complete cytotoxicity (see Chapter 5.). It can be suggested from this that the effective concentration of faecal water solutes reaching the colonic cells *in vivo* is likely to be of an order of approximately 1%, however this has yet to be

tested directly. It may be possible that the process of dilution of faecal water for testing *in vitro* may result in the dilution of any toxic or carcinogenic agents present to levels that will not induce a detectable genome damage effect in the WIL2-NS cell line, however may induce damage in colonocytes *in vitro*. Further studies are required to identify the likely concentration of faecal water solutes reaching colonic cells *in vivo* to optimise and validate the dilution of faecal water for *in vitro* testing and validate the extrapolation of *in vitro* results to the *in vivo* situation. Furthermore, it will be necessary to determine whether WIL2-NS cells behave in a similar manner to colonocytes with regards to sensitivity to faecal water toxicity. Future studies may also benefit from the use of a positive control to assess fluctuation in genotoxic response of the cell line to the faecal water test agent over time instead of the use of a negative 'baseline' control as used in the present study. The positive control should be relevant to faecal water genotoxicity, for example a known highly toxic caecal/faecal water sample from a rat or human.

There was no significant change in levels of faecal total short chain fatty acid (SCFA) or phenolic compounds, phenol and p-cresol, in this study. SCFA results trended in similar pattern to faecal water total DNA damage, where there was a trend for a reduction after 12 weeks weight loss and then a subsequent increase after 9 months weight maintenance to levels similar to week 0. There was no correlation of SCFAs with MN and NPB genome damage biomarkers. There was however some positive correlations were seen for SCFAs and NBuds. This could suggest that SCFAs either facilitate the elimination of amplified DNA by nuclear budding or that they increase the rate of gene amplification, however further studies are required to determine whether this is a causal or simply coincidental relationship. Considering SCFAs are thought to be protective in the bowel it could be suggested that if faecal water genome damage potential is lowered, as seen after 12 weeks weight loss in this study, then levels of SCFAs would have increased. The trend for a reduction in total genome damage along with a reduction in SCFAs as found in this study therefore may suggest that SCFAs are not associated with the genotoxic potential of faecal water and perhaps therefore not an important factor for the determination bowel health and risk of colorectal cancer. However, the WIL2-NS cell line is p53 deficient and hence cells do not tend to follow the apoptosis pathway when DNA damage occurs. This may explain the lack of relationship seen with SCFAs and micronuclei assessed by the CBMN cytome assay in the WIL2-NS cell line. Apoptosis induction by butyrate, if it were to have occurred, would have resulted in cell death of cells with

DNA damage and hence MN frequency would have reduced. The lack of an inverse relationship between SCFAs and WIL2-NS MN frequency suggests lack of sensitivity of this cell line to SCFA induced apoptosis.

No significant changes were found for phenol and p-cresol with time or diet type, in this study. Considering phenol and p-cresol are believed to be detrimental to bowel health and increased in humans who consume high meat diets, it was reasonable to expect that phenol and p-cresol level would have increased in the HP diet group; however this was not the case for the present study. Phenol and p-cresol levels can be measured in the faecal output as was done with this study and also in the urine as phenols are detoxified in the liver and excreted in the urine. It is possible that phenol and p-cresol concentration in the urine could have differed between diet groups if this approach is more sensitive to dietary protein differences. However the relevance of phenol levels in the urine as opposed to the faecal sample (i.e. level of phenols exposed to the bowel) may be questionable. Direct evidence for a causative role of phenol and p-cresol in colon carcinogenesis is not strong and results from the present study suggest that they may not be an important factor in the determination of bowel health and genotoxic risk in the bowel.

It may be suggested that a lack of effect seen for faecal water genotoxicity or cytotoxicity or SFCA or phenolic compound production could be an effect of an increase in faecal moisture, where increasing the moisture content of the faecal matter may have a dilution effect on any carcinogenic compounds present in the faecal stream, reducing the carcinogenic potential of the faecal water. However, in this study, no significant change in faecal moisture with either diet was observed which supports the conclusion that the genome damaging effect of faecal water seen *in vitro* was not a result of a change in faecal water concentration of the sample. Similarly, *in vivo*, increasing faecal bulk increases the rate of transit of faecal matter through the bowel and hence, reduces the time of exposure of the colonic cells to potentially toxic or carcinogenic compounds in the faecal stream [3, 23]. In this study there were no significant changes in faecal weight with either diet which suggests that the observed effect of the dietary intervention on faecal water genotoxicity in this study was not confounded by differences in faecal weight.

Although this study focuses on dietary patterns and not individual components in the diet, it is interesting to note that although fruits and vegetables, which are considered

to be cancer-protective due to their high levels of micronutrients with antioxidant properties, were prescribed at different amounts for the HP and HC diets (HP 66% less total fruit than HC diet), there was no difference in faecal water genotoxicity. In addition, an increase in haem content with a HP compared with HC diet also did not result in any difference in faecal water genotoxicity. Previous research suggested that increased haem in the diet is associated with promotion of colorectal cancer however the relevance is debatable considering either the majority of studies are performed in animal models, red meat content was typically very high in human studies that showed genotoxicity (>300g/day), different DNA damage biomarker endpoints are used and no studies have assessed HP weight loss diets [50, 59, 60, 164-166]. This may lend support to the notion that the study of the total dietary pattern rather than individual components of the diet may be more relevant to assessing genotoxic risk.

Although concerns have been raised regarding the risk of colorectal cancers with weight loss diets that are high in red meat, the impact of weight loss diets with different macronutrient profiles on the genotoxic potential of faecal water assessed using the CBMN cytome assay has not been previously assessed. Results from this study supports the hypothesis that a high protein-high red meat diet does not appear to influence the faecal water genotoxicity differently to a high carbohydrate diet, when assessed using the CBMN cytome assay in overweight men. There was also no impact of diet on faecal water levels of short chain fatty acids or phenolic compounds, other common biomarkers of bowel health. The results from this study does not support the hypothesis that faecal water genotoxicity/cytotoxicity is correlated with changes in conventional bowel biomarkers. Any effect of weight loss and/or caloric restriction on genome damage biomarkers observed in this study needs to be confirmed with a study design that includes a non-weight loss control. The hypothesis that a HP diet does not increase the micronucleus index in colonic cells could not be tested and further work in the development of scorable slides with intact colonocytes cells is required for the establishment of the use of the micronucleus index in human mid-rectal biopsy samples.

# 7. THE EFFECT OF HIGH PROTEIN-HIGH RED MEAT VS HIGH CARBOHYDRATE WEIGHT LOSS DIETS ON GENOME STABILITY IN LYMPHOCYTES

## *AIM*

To investigate whether a high protein weight loss diet, specifically high in red meat, influences the genome stability profile in peripheral blood lymphocytes differently compared to a high carbohydrate weight loss diet.

## *HYPOTHESES*

A high protein-high red meat dietary pattern does not increase genome instability in peripheral blood lymphocytes compared with a high carbohydrate weight loss diet.

A high protein-high red meat dietary pattern results in an improved status of micronutrients required for genome stability compared with a high carbohydrate weight loss diet.

## *7.1 Introduction*

It is well established that a large number of micronutrients are required for the maintenance of genome stability and this highlights the importance of the diet in the assessment of cancer risk [62, 104, 116, 117]. A recent study by Fenech *et al* (2005) [115] showed significant associations between dietary intake data (from food frequency questionnaires) and DNA damage rates measured using the cytokinesis block micronucleus (CBMN) cytome assay, supporting the hypothesis that genome damage is to a significant degree a result of dietary and micronutrient imbalance. *In vitro* and *in vivo* studies show that folate deficiency, vitamin B12 deficiency and elevated plasma homocysteine levels are associated with DNA damage including chromosome breaks, incorporation of uracil into DNA, micronucleus formation and DNA hypomethylation [118-120, 167-174]. Cross-sectional and intervention studies with folate and/or vitamin B12 in humans show that genome damage is minimised when plasma vitamin B12 is above 300 pmol/L and plasma homocysteine is below 7.5 µmol/L [120, 171-174].

A limited number of studies have compared different dietary patterns and genome damage rates. The comparison of vegetarian vs. non-vegetarian diets have been assessed as it was hypothesised that different dietary intakes may alter micronutrient status and therefore could affect DNA damage rates. A study by Fenech and Rinaldi (1995) [172] found no significant difference in micronucleus (MN) frequency (a biomarker of chromosome breakage or loss) between vegetarians and non-vegetarians. However, vegetarians were found to have increased plasma levels of folate and vitamin C and decreased levels of vitamin B12 compared with non-vegetarians and vitamin B12 was associated with an decrease in MN frequency in both men and women, folate with a reduced MN index in women and vitamin C with an increase in MN frequency in men [172]. This finding was supported in a study by Kazimirova *et al* [175] who also found no difference in MN frequency between vegetarians and non-vegetarians. Another study by Kazimirova *et al* [176] also found no effect of vegetarianism compared with non-vegetarianism on micronuclei (MNi), however found a small yet significant decrease with DNA strand breaks and FPG-sites (a marker of oxidative damage) assessed using the comet assay.

An association between BMI and genome damage in lymphocytes has also been reported; however to date, the evidence is conflicting. Two studies have shown an association of high BMI with an increased frequency of MNi [177, 178]. Another study found that DNA damage, assessed with the comet assay, was significantly higher in obese subjects compared with those that were of normal weight [179]; however Giovannelli *et al* [180] did not report a significant association of BMI with DNA damage assessed with the comet assay in a group of healthy subjects. Studies of oxidative DNA damage, which is thought to be associated with cancer induction, found that urinary levels of 8-hydroxydeoxyguanosine (8-OHdG); a marker of cellular oxidative stress, to be inversely correlated with BMI [181, 182].

Evidence for a role of total caloric intake, rather than particular nutrients, and genome damage is emerging. Caloric restriction has been associated with an increase in maximum life span and various mechanisms have been proposed, including protection against oxidative damage, enhanced apoptosis and an increase in DNA repair capacities [183-186]. It is suggested that the level of energy available (i.e. the amount of food intake) may determine the activity of proteins involved in pathways which influence longevity and senescence [184]. In yeast cells, in times of low energy levels (ie low food intake - caloric restriction), there are 2 cellular consequences, first a decrease in nicotinamide adenine dinucleotide (NAD), which results in less glycolysis and less requirement of NAD, allowing for adequate presence of NAD to function as a cofactor for the protein Sir2 and maximise Sir2-dependant histone deacetylase activity, causing chromatin to compact at specific genome locations such as the telomeres and hence prolong life span [184]. Second, less reactive oxygen species (ROS), natural by-products of metabolism, are produced in the calorie-restricted stae, minimising DNA damage and as such senescence. Conversely, during times of high energy levels (i.e. high food intake - caloric abundance) NAD is more heavily converted to NADH and as such histone deacetylase activity of Sir2 is reduced, chromatin is less compact and life span is reduced [184]. A moderate reduction in DNA damage levels with caloric restriction and subsequently weight loss in non-obese subjects, assessed using the comet assay, was shown by Heilbronn *et al*, however this was not markedly different to observed effects in the control group [187]. A reduction in urinary excretion of oxidative damage marker 8-OHdG, following 8 weeks of an energy restricted diet was observed in a study of normal weight atopic dermatitis patients [188]. However, this finding was not supported in a study by Loft *et al* [189], who saw no effect of 10

weeks caloric restriction in non-obese subjects on urinary excretion of 8-OHdG. Dandona *et al* [190] demonstrated that oxidative damage and reactive oxygen species (ROS) generation was significantly greater in obese than non-obese subjects and that they significantly reduced following 4 weeks dietary restriction of 1000 calories/day and resultant weight loss. This reduction in ROS generation and oxidative damage was evident after one week of dietary restriction, before significant weight loss occurred, and increased back to baseline levels following the 4 week intervention despite the maintained reduction in body weight. This suggests that changes in oxidative stress may only be transient during weight loss.

To our knowledge, the impact of weight loss diets with different micronutrient and macronutrient profiles on genomic stability has not been previously assessed. In this chapter, the assessment of the DNA damage and cytotoxicity profile of peripheral blood lymphocytes using the CBMN cytome assay, along with the assessment of micronutrients required for genome stability, is used to assess cancer risk in overweight men following a weight loss diet that is either high protein-high red meat or high carbohydrate.

## *7.2 Methods*

As previously described (Chapter 4), 55 overweight male subjects were recruited and randomised to one of two dietary interventions, a high protein–high red meat (HP) diet or a high carbohydrate-low red meat (HC) diet. The study was a parallel design, comprising of a 12 week intensive weight loss phase and follow-up at one year with interim monthly visits.

Venous blood was collected from fasted (overnight) volunteers at baseline (week 0), week 12 and week 52 in Vacuette®-Heparin tubes (9 ml) and lymphocytes were isolated and stored in cryovials in liquid nitrogen as previously described (Chapter 4.4.1).

### 7.2.1 Cytokinesis block micronucleus cytome assay 72 hour protocol for isolated lymphocytes stored in liquid nitrogen

Cryovials were removed from liquid nitrogen and rapidly thawed by agitation in 37 °C pre-warmed pure water. The contents of the cryovial were pipetted into 4 ml of RPMI 1640 culture medium (Thermo Electron, Melbourne, Australia) containing 10% foetal bovine serum (FBS; Thermo Electron, Melbourne, Australia), 1% penicillin-streptomycin solution (Thermo Electron, Melbourne, Australia), 1% L-glutamine (Sigma, Sydney, Australia) and 1% sodium pyruvate solution (Thermo Electron, Melbourne, Australia). Tubes were centrifuged at 180 g for 10 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 5 ml culture medium and centrifuged at 180 g for 10 minutes at room temperature. The supernatant was discarded and the pellet resuspended in 1 ml culture medium. Cell count and percentage viability tests were performed to determine the number of viable cells. 15 µl of the cell suspension was transferred into 20 ml vials containing 15 ml Isoton-II solution (Beckman Coulter, Miami, USA) and cell number determined using a Coulter Counter (Beckman Coulter, Miami, USA) according to the manufacturer's instructions. Cell viability was determined by mixing 50 µl of cell suspension to 50 µl trypan blue (Sigma, Sydney, Australia) which was loaded onto a haemocytometer (ProSciTech, Kirwan, Australia) and a minimum of 100 cells were manually counted using a Leica DMLB light microscope on 400X magnification. Viable (live) cells remained unstained whilst non-viable (dead) cells stained dark blue. Average cell viability after thawing lymphocytes was 93%. Viable cell growth was calculated by multiplying cell number by percentage viability. Duplicate 0.5 ml

cultures per sample were set up in 5 ml culture tubes at a concentration of $1x10^6$ viable cells/ml and placed in a humidified atmosphere with 5% $CO_2$ ($CO_2$ incubator; Quantum Scientific, Brisbane, Australia) for 1 hour.

Cultures were removed from the $CO_2$ incubator and 6.7 µl of phytohaemagglutinin (2.25 mg/ml; Remel, Kent, UK) at room temperature was added to each 0.5ml culture to stimulate cell division. Cultures were then returned to the $CO_2$ incubator for 44 hours.

Cultures were removed from the $CO_2$ incubator and 38 µl of cytochalasin-B (final concentration of 4.5 µg/ml in cultures; Sigma, Sydney, Australia) was added to each 500 µl culture to block cytokinesis. Cultures were then returned to the $CO_2$ incubator for a further 28 hours.

Cultures were removed from the $CO_2$ incubator and 200 µl of culture medium was removed without disturbing the cells. 30 µl of dimethyl sulfoxide (DMSO; 75 µl/ml; Sigma, Sydney, Australia) was added to minimise cell clumping and the cells were thoroughly resuspended by pipette. Cells were harvested by cytocentrifugation using a Shandon Cytospin Cytocentrifuge (Shandon Scientific, Chesire, England). 120 µl of each sample were transferred to cytocentrifuge chambers (consisting of a slide, filter card and cytocentrifuge sampling cup) and spun in the cytocentrifuge at 600 rpm for 5 minutes, to give a spot of cells on the slide. Slides were then rotated and a second 120 µl sample transferred, using the same conditions, to generate a second spot of cells. Slides were removed from the cytocentrifuge chamber, laid flat and left to airdry for 10 minutes. The experimental design is shown in Figure 7.1.

Slides were stained using the commercial kit 'Diff Quik' (Lab Aids, Narrabeen, Australia). This involved fixing the cells by immersing the slides in fixative for 10 minutes. Slides were then removed and immediately transferred first in the Diff-Quik solution 1 (red stain) for approximately 10 seconds and then in Diff-Quik solution 2 (blue stain) for a further 6 seconds. Staining time was adjusted slightly depending on the age of the stain. Slides were then washed thoroughly under running water to remove excess stain. Slides were then left to airdry completely for at least 30 minutes. Slides were then coverslipped using Depex (BDH Laboratory Supplies, Poole, England) and left to set overnight. Slides were then stored in a slide box until required.

CBMN cytome assay slides were scored according to criteria described in Chapter 3.5.

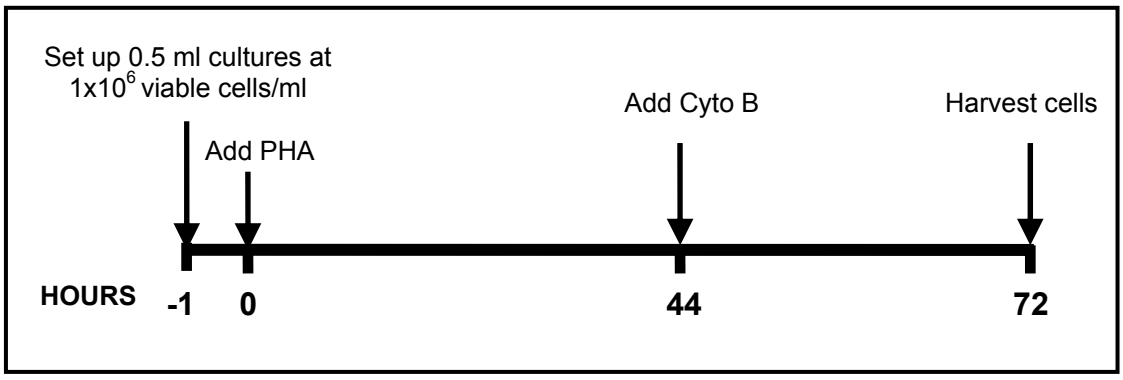


**Figure 7.1 Schematic protocol of 72 hour CBMN cytome assay experiment.**

### 7.2.2 Quantification of folate in plasma

Plasma folate was quantified using the ARCHITECT® folate assay, a chemiluminescent microparticulate folate binding protein assay (Abbott Laboratories, Abbot Park, IL, USA), on the ARCHITECT® *i* System. The assay has a total imprecision of <10% (within the calibration range of 0-45.3 nmol/L) and an analytical sensitivity of ≤1.8 nmol/L. Plasma folate was quantified by the Institute of Medical and Veterinary Science (IMVS), Adelaide.

Briefly, there are two pre-treatment steps which mediate the release of folate from endogenous folate binding protein. First, the sample and dithiothreitol (DTT) were aspirated and dispensed into a reaction vessel. Second, an aliquot of this mixture and potassium hydroxide were aspirated and dispensed into a second reaction vessel. Next, an aliquot of the mixture was transferred into a third reaction vessel and mouse anti-folate binding protein coupled to microparticles affinity bound with bovine folate binding protein, TRIS buffer and protein stabilisers (human albumin) were added. Folate present in the sample binds to the folate binding protein coated microparticles. After washing with phosphate buffered saline (PBS), pteroic acid-acridinium labelled conjugate was added and this binds to the unoccupied sites on the microparticles. Pre-trigger (1.32% w/v hydrogen peroxide) and trigger (0.35N sodium hydroxide) solutions was added and the resultant chemiluminescent reaction is measured in relative light units by the ARCHITECT® *i* optical system. There is an

inverse relationship between the amount of folate in the sample and the relative light units measured.

### 7.2.3 Quantification of vitamin B12 in plasma

Plasma vitamin B12 was quantified using the ARCHITECT® B12 assay (Abbott Laboratories, Abbot Park, IL, USA), a chemiluminescent microparticle intrinsic factor assay, on the ARCHITECT® *i* System. The assay has a total imprecision of <10% (within the calibration range of 0-1476 pmol/L) and an analytical sensitivity of ≤44.3 pmol/L. Plasma vitamin B12 was quantified by the Institute of Medical and Veterinary Science (IMVS), Adelaide.

In brief, the sample was combined with 3 pre-treatment reagents; 1.0 N sodium hydroxide with 0.005% potassium cyanide, alpha monothioglycerol and EDTA and cobinamide dicyanide in borate buffer with protein (avian) stabilisers. An aliquot of this mixture was aspirated and transferred to a new reaction vessel and combined with a diluent of borate buffer with ETDA and intrinsic factor (porcine) coated microparticles in borate buffer with protein (bovine) stabilisers. Vitamin B12 present in the sample binds to the intrinsic factor coated microparticles. The mixture was washed with PBS and then B12 acridinium-labelled conjugate, pre-trigger solution containing 1.32% (w/v) hydrogen peroxide and trigger solution containing 0.35 N sodium hydroxide was added. The resultant chemiluminescent reaction is measured in relative light units by the ARCHITECT® *i* optical system. There is an inverse relationship between the amount of vitamin B12 in the sample and the relative light units measured.

### 7.2.4 Quantification of total L-homocysteine in plasma

L-homocysteine was quantified using the AxSYM® homocysteine assay (Abbott, Wiedbaden, Germany), a fluorescence polarization immuno assay, on the AxSYM system. The assay has a total imprecision of <6% (within the calibration range of 0-50 µmol/L) and an analytical sensitivity of ≤0.8 µmol/L. Plasma L-homocysteine was quantified by the Institute of Medical and Veterinary Science (IMVS), Adelaide.

In the assay, homocystine, mixed disulfide and protein-bound forms of homocysteine are reduced by dithiothreitol (DTT) to form free homocysteine. Free homocysteine is then converted to S-adenosylhomocysteine (SAH) by the use of SAH hydrolase and

excess adenosine. Under physiological conditions, SAH hydrolase converts SAH to homocysteine. Excess adenosine in the pre-treatment solution drives the conversion of homocysteine to SAH by SAH hydrolase.

Briefly, the sample was pre-treated in a reaction vessel with 0.1M phosphate buffer, bovine SAH hydrolase in phosphate buffer with bovine protein stabilisers and a pre-treatment solution of DTT and adenosine in citric acid. An aliquot of this mixture was mixed with monoclonal mouse anti-S-adenosyl-L-homocysteine (in phosphate buffer with bovine protein stabilisers) and 0.1M phosphate buffer and transferred to the cuvette of the reaction vessel. A second aliquot of the mixture was mixed with S-adenosyl-L-cysteine fluorescence tracer (in phosphate buffer with bovine protein stabilisers) and 0.1 M phosphate buffer and transferred to the cuvette. SAH and the labelled fluorescent tracer compete for sites on the monoclonal antibody. The intensity of the resultant polarised fluorescent light was measured by the Fluorescence Polarisation Immunoassay (FPIA) optical assembly. Total L-homocysteine concentrations of the samples were determined from the standard calibration curve.

### 7.2.5 Statistical analysis

Data is shown as mean ± standard error of the mean (SEM) for all Figures and Tables. Two-way (mixed between-within subjects) ANOVA was used to compare the main effect for time and diet and any interaction effect. Independent *t*-tests were used to compare the diets at baseline. A correlation matrix of all data was performed using Pearsons test. Significance for all tests was accepted at $P<0.05$. All statistical analyses were performed using SPSS 14.0 for Windows and all graphs produced using GraphPad Prism version 4.00 software.

## *7.3 Results*

### 7.3.1 Short term intensive weight loss phase

55 male subjects completed the 12 week intensive weight loss phase; with n=24 in the high protein (HP) group and n=31 in the high carbohydrate (HC) group. Weight loss was not significantly different between diets. Average weight loss was 8.4 ± 0.5 kg after the 12 weeks, as described in Chapter 4.4.

#### *Genome damage biomarkers*

Figure 7.2 and Table 7.1 show the frequency of MN, NPBs, NBuds and total DNA damage at baseline (week 0) and after 12 weeks of weight loss on either a HP or HC diet. Two-way ANOVA indicates there was no significant difference over time from week 0 to week 12 for any of the genome damage biomarkers nor was there any significant difference between the diets. There were no significant time by diet interactions.

#### *Cytotoxicity biomarkers*

Rates of necrosis, apoptosis and nuclear division cytotoxicity index (NDCI) at baseline (week 0) and after 12 weeks of weight loss on either a HP or HC diet are shown in Figure 7.3 and Table 7.2. There was no significant effect with time or diet for necrosis or NDCI. There was a significant effect of time (P=0.037) but not for diet for a reduction in apoptosis after 12 weeks weight loss. There were no significant time by diet interactions.

#### *Plasma folate, vitamin B12 and homocysteine*

Plasma folate, vitamin B12 and homocysteine levels following 12 weeks intensive weight loss on either a HP or HC diet are displayed in Figure 7.4 and Table 7.3. There was no significant effect of time or diet on plasma homocysteine levels, however there was a significant effect of time but not diet for plasma folate and vitamin B12. There were no significant time by diet interactions for plasma folate, vitamin B12 or homocysteine.

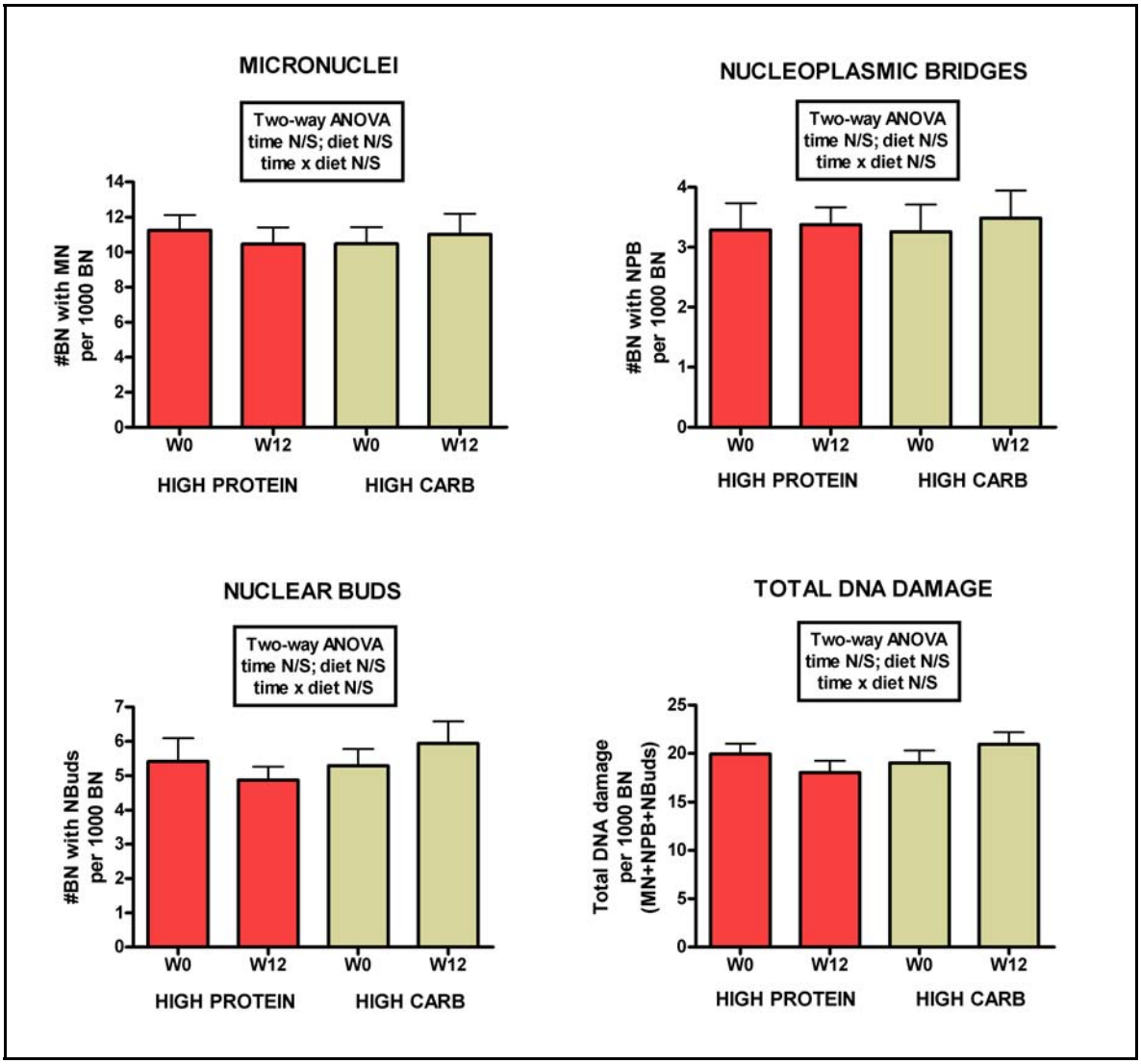


**Figure 7.2 Effect of 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet on CBMN cytome assay genome damage biomarkers in peripheral blood lymphocytes.** HP n=24; HC n=31. BN=binucleate cells, MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, W=week, N/S=not significant.

**Table 7.1 Frequency of CBMN cytome assay genome damage biomarkers in peripheral blood lymphocytes with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 |
|---|---|---|---|---|
| **micronuclei** | *HP* | *n=24* | 11.25 ± 0.86 | 10.46 ± 0.96 |
| | *HC* | *n=31* | 10.48 ± 0.94 | 11.03 ± 1.15 |
| | | ***t*-test^^** | P=0.56 | |
| **nucleoplasmic bridges** | *HP* | *n=24* | 3.29 ± 0.44 | 3.38 ± 0.29 |
| | *HC* | *n=31* | 3.26 ± 0.45 | 3.48 ± 0.46 |
| | | ***t*-test^^** | P=0.96 | |
| **nuclear buds** | *HP* | *n=24* | 5.42 ± 0.68 | 4.88 ± 0.38 |
| | *HC* | *n=31* | 5.29 ± 0.49 | 5.94 ± 0.64 |
| | | ***t*-test^^** | P=0.88 | |
| **total DNA damage** | *HP* | *n=24* | 19.96 ± 1.07 | 18.04 ± 1.20 |
| | *HC* | *n=31* | 19.03 ± 1.28 | 20.97 ± 1.21 |
| | | ***t*-test^^** | P=0.60 | |

Data represented as mean ± SEM
^^independent *t*-test, comparing the diets at baseline

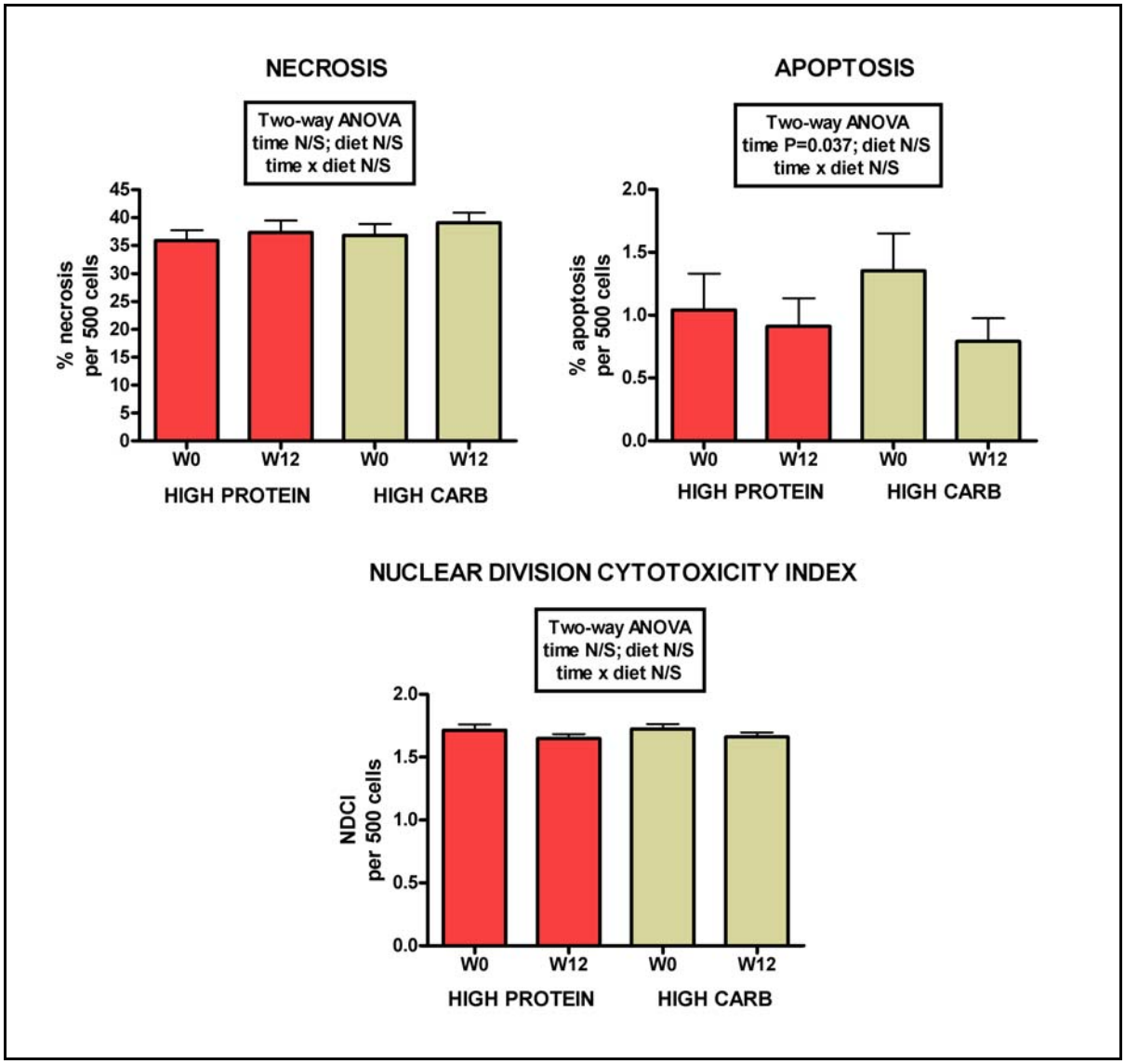


**Figure 7.3 Effect of 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet on CBMN cytome assay cytotoxicity biomarkers in peripheral blood lymphocytes.** HP n=24; HC n=31. BN=binucleate cells, NDCI=nuclear division cytotoxicity index. W=week, N/S=not significant.

**Table 7.2 Frequency of CBMN cytome assay cytotoxicity biomarkers in peripheral blood lymphocytes with 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 |
|---|---|---|---|---|
| **necrosis (%)** | *HP* | *n=24* | 35.92 ± 1.82 | 37.34 ± 2.14 |
| | *HC* | *n=31* | 36.85 ± 1.98 | 39.09 ± 1.77 |
| | | ***t*-test^^** | P=0.74 | |
| **apoptosis (%)** | *HP* | *n=24* | 1.04 ± 0.29 | 0.91 ± 0.23 |
| | *HC* | *n=31* | 1.35 ± 0.30 | 0.79 ± 0.18 |
| | | ***t*-test^^** | P=0.46 | |
| **NDCI** | *HP* | *n=24* | 1.72 ± 0.05 | 1.65 ± 0.04 |
| | *HC* | *n=31* | 1.73 ± 0.04 | 1.65 ± 0.04 |
| | | ***t*-test^^** | P=0.87 | |

Data represented as mean ± SEM
^^independent *t*-test, comparing the diets at baseline

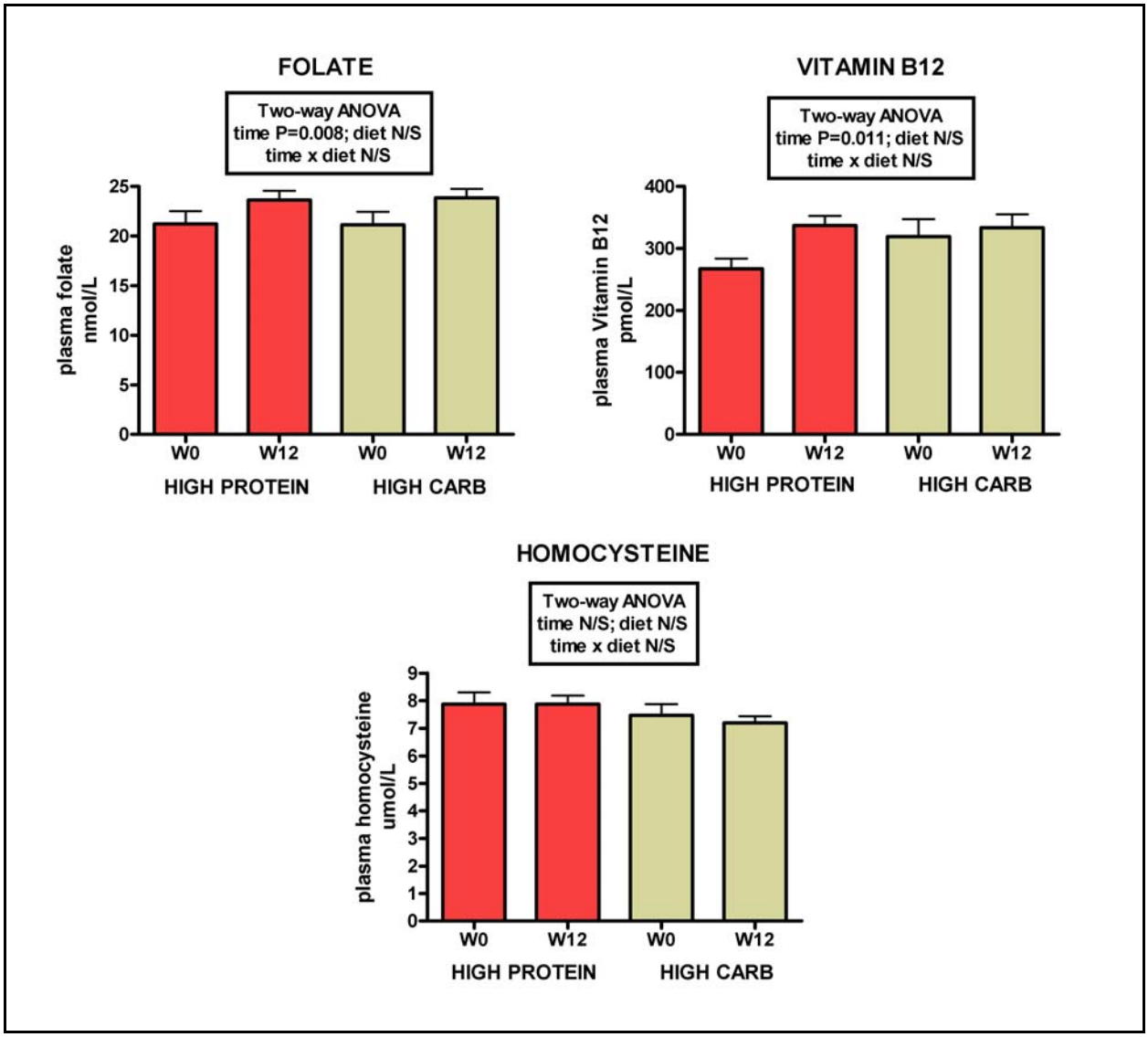


**Figure 7.4 Effect of 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet on plasma micronutrients and homocysteine.** W=week; N/S=not significant.

**Table 7.3 Mean plasma micronutrients and homocysteine after 12 weeks intensive weight loss on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 |
|---|---|---|---|---|
| **plasma folate** | *HP* | *n=24* | 21.23 ± 1.29 | 23.66 ± 0.90 |
| | *HC* | *n=31* | 21.14 ± 1.31 | 23.88 ± 0.89 |
| | | ***t*-test^^** | P=0.96 | |
| **plasma vitamin B12** | *HP* | *n=23* | 267.17 ± 16.81 | 337.09 ± 15.62 |
| | *HC* | *n=31* | 319.06 ± 28.52 | 333.45 ± 21.79 |
| | | ***t*-test^^** | P=0.16 | |
| **plasma homocysteine** | *HP* | *n=24* | 7.87 ± 0.44 | 7.89 ± 0.31 |
| | *HC* | *n=30* | 7.47 ± 0.40 | 7.19 ± 0.24 |
| | | ***t*-test^^** | P=0.48 | |

Data represented as mean ± SEM
^^independent *t*-test, comparing the diets at baseline

### 7.3.2 Long term weight maintenance phase

33 male subjects completed the 12 week intensive weight loss phase and 9 month weight maintenance phase; with n=16 in the high protein (HP) group and n=17 in the high carbohydrate (HC) group. Weight loss was not significantly different between diets at week 12 or week 52. Average weight loss was 9.3 ± 0.7 kg after 12 weeks intensive weight loss with no further significant change in weight from week 12 to week 52, as described in Chapter 4.4.

#### *Genome damage biomarkers*

The frequency of MNi, NPBs, NBuds and total DNA damage after 12 weeks intensive weight loss and 9 months weight maintenance are shown in Figure 7.5 and Table 7.4. There was no significant effect of time or diet for MNi and total DNA damage. A significant effect of time but not diet was seen for a reduction in NBuds (P=0.03) and significance was almost met for an increase in NPB (P=0.051). There were no significant time by diet interactions.

#### *Cytotoxicity biomarkers*

Necrosis, apoptosis and NDCI after 12 weeks intensive weight loss and 9 months weight maintenance are displayed in Figure 7.6 and Table 7.5. No significant effect was found for NDCI with time or diet. There was a significant effect of time only for reduced necrosis (P=0.037) and reduced apoptosis (P=0.007). There were no significant time by diet interactions.

#### *Plasma folate, vitamin B12 and homocysteine*

Plasma folate, vitamin B12 and homocysteine status following 12 weeks intensive weight loss and 9 months weight maintenance are shown in Figure 7.7. Two-way ANOVA suggests a significant main effect of time but not diet for both micronutrients measured; i.e. plasma folate (P<0.001), plasma vitamin B12 (P<0.001). Two-way ANOVA also suggests a significant main effect of time but not diet for homocysteine (P=0.002). There were no significant time by diet interactions.

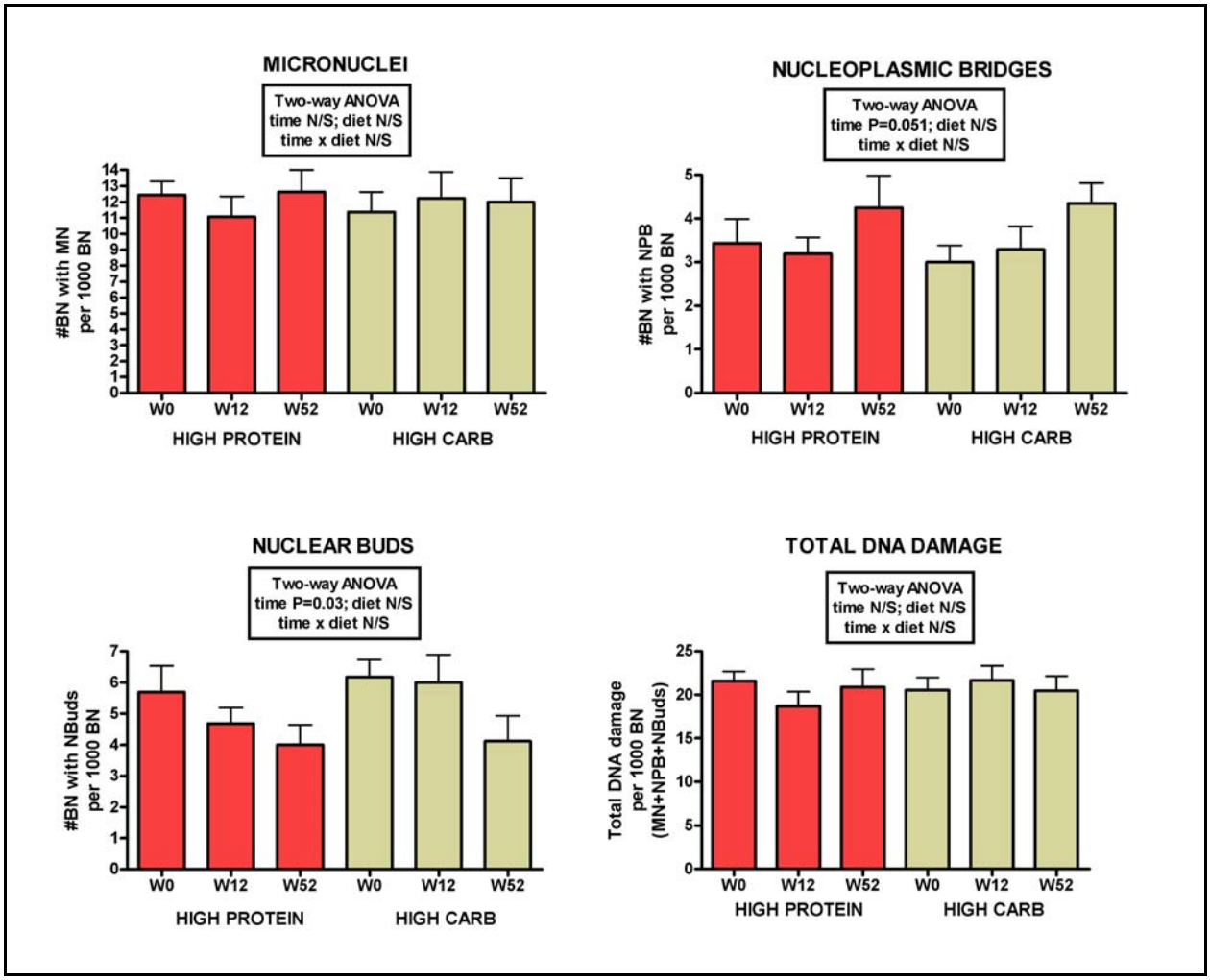


**Figure 7.5 Effect of 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet on CBMN cytome assay genome damage biomarkers in peripheral blood lymphocytes.** HP n=16; HC n=17. BN=binucleate cells, MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, W=week, N/S=not significant.

**Table 7.4 Frequency of CBMN cytome assay genome damage biomarkers in peripheral blood lymphocytes with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 | Week 52 |
|---|---|---|---|---|---|
| **micronuclei** | *HP* | *n=16* | 12.44 ± 0.88 | 11.06 ± 1.29 | 12.63 ± 1.38 |
| | *HC* | *n=17* | 11.35 ± 1.27 | 12.24 ± 1.64 | 12.00 ± 1.51 |
| | | ***t*-test^^** | P=0.49 | | |
| **nucleoplasmic bridges** | *HP* | *n=16* | 3.44 ± 0.55 | 3.19 ± 0.38 | 4.25 ± 0.74 |
| | *HC* | *n=17* | 3.00 ± 0.38 | 3.29 ± 0.53 | 4.35 ± 0.46 |
| | | ***t*-test^^** | P=0.52 | | |
| **nuclear buds** | *HP* | *n=16* | 5.69 ± 0.86 | 4.69 ± 0.50 | 4.00 ± 0.64 |
| | *HC* | *n=17* | 6.18 ± 0.56 | 6.00 ± 0.89 | 4.12 ± 0.81 |
| | | ***t*-test^^** | P=0.63 | | |
| **total DNA damage** | *HP* | *n=16* | 21.56 ± 1.11 | 18.69 ± 1.66 | 20.88 ± 2.10 |
| | *HC* | *n=17* | 20.53 ± 1.48 | 21.65 ± 1.69 | 20.47 ± 1.69 |
| | | ***t*-test^^** | P=0.58 | | |

Data represented as mean ± SEM
^^independent *t*-test, comparing the diets at baseline

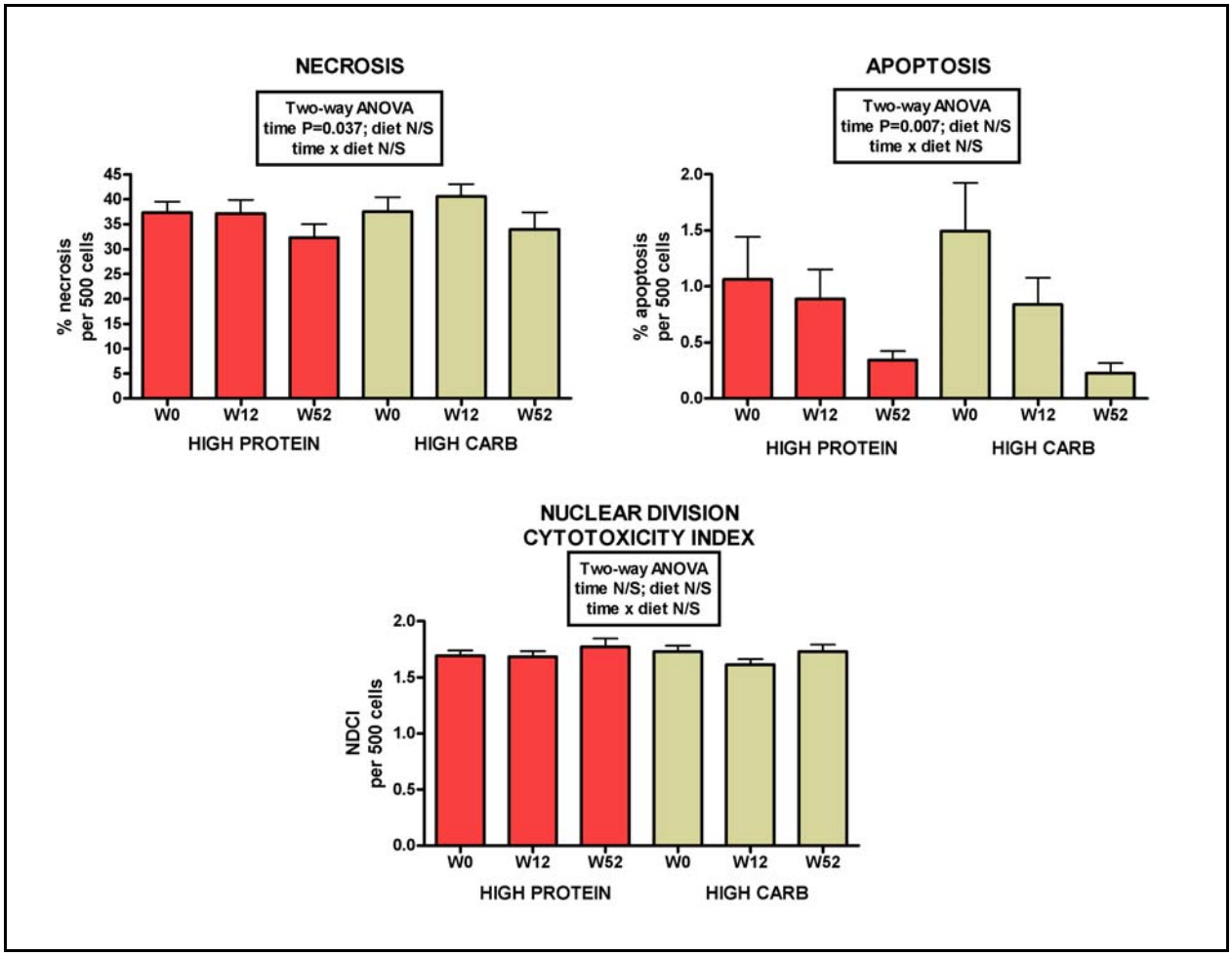


**Figure 7.6 Effect of 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet on CBMN cytome assay cytotoxicity biomarkers in peripheral blood lymphocytes.** NDCI=nuclear division cytotoxicity index. W=week, N/S=not significant.

**Table 7.5 Frequency of CBMN cytome assay cytotoxicity biomarkers in peripheral blood lymphocytes with 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 | Week 52 |
|---|---|---|---|---|---|
| **necrosis (%)** | *HP* | *n=16* | 37.34 ± 2.16 | 37.09 ± 2.80 | 32.32 ± 2.69 |
| | *HC* | *n=17* | 37.51 ± 2.94 | 40.56 ± 2.46 | 33.95 ± 3.43 |
| | | ***t*-test^^** | P=0.96 | | |
| **apoptosis (%)** | *HP* | *n=16* | 1.06 ± 0.38 | 0.89 ± 0.27 | 0.34 ± 0.08 |
| | *HC* | *n=17* | 1.49 ± 0.43 | 0.84 ± 0.24 | 0.22 ± 0.09 |
| | | ***t*-test^^** | P=0.46 | | |
| **NDCI** | *HP* | *n=16* | 1.69 ± 0.05 | 1.68 ± 0.05 | 1.77 ± 0.07 |
| | *HC* | *n=17* | 1.73 ± 0.05 | 1.61 ± 0.05 | 1.73 ± 0.06 |
| | | ***t*-test^^** | P=0.60 | | |

Data represented as mean ± SEM
^^independent *t*-test, comparing the diets at each baseline

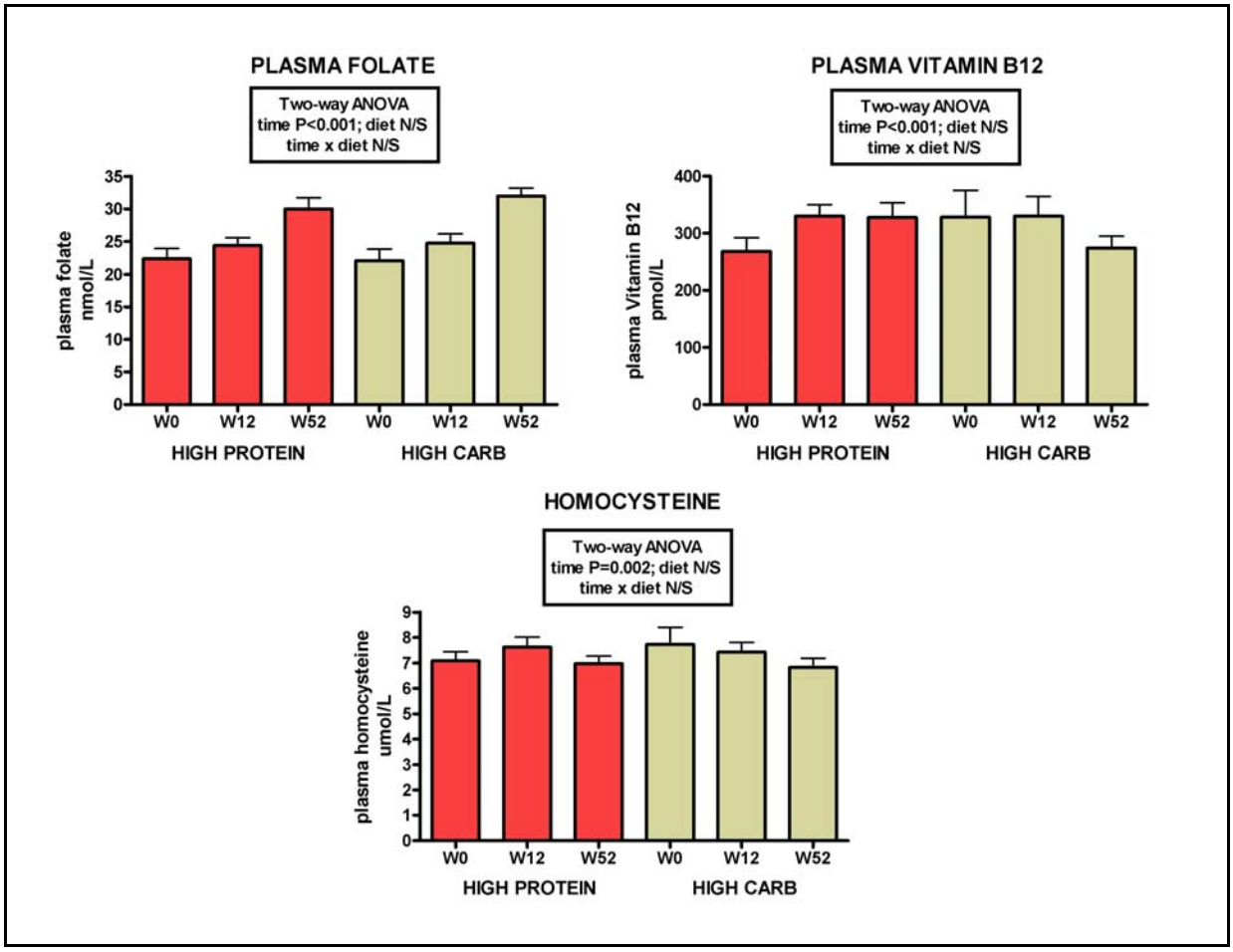


**Figure 7.7 Effect of 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet on plasma micronutrients and homocysteine.**
W=week, N/S=not significant.

**Table 7.6 Mean plasma micronutrients and homocysteine after 12 weeks intensive weight loss and 9 months weight maintenance on a high protein-high red meat or high carbohydrate diet.**

| | | | Week 0 | Week 12 | Week 52 |
|---|---|---|---|---|---|
| **plasma folate** | *HP* | *n=16* | 22.37 ± 1.55 | 24.39 ± 1.16 | 29.96 ± 1.80 |
| | *HC* | *n=17* | 22.02 ± 1.82 | 24.77 ± 1.41 | 31.99 ± 1.17 |
| | | ***t*-test^^** | P=0.89 | | |
| **plasma vitamin B12** | *HP* | *n=15* | 268.47 ± 23.56 | 329.73 ± 20.78 | 327.40 ± 26.36 |
| | *HC* | *n=17* | 328.59 ± 46.54 | 329.88 ± 34.89 | 274.18 ± 20.65 |
| | | ***t*-test^^** | P=0.28 | | |
| **plasma homocysteine** | *HP* | *n=16* | 7.09 ± 0.34 | 7.63 ± 0.40 | 6.98 ± 0.30 |
| | *HC* | *n=16* | 7.73 ± 0.68 | 7.43 ± 0.39 | 6.82 ± 0.36 |
| | | ***t*-test^^** | P=0.41 | | |

Data represented as mean ± SEM
^^independent *t*-test, comparing the diets at baseline

### 7.3.3 Correlations

Correlation matrices for all measures at week 0, 12 and 52 are shown in Appendix 7.1, 7.2 and 7.3 respectively. Significant correlations (P<0.05) are highlighted in yellow.

***Week 0***

There was a negative correlation of necrosis with NDCI (r=-0.694; P<0.001) and a positive association with Vitamin B12 (r=0.310, P=0.021). NPB was negatively associated with apoptosis (r=-0.268; P=0.048). Folate was negatively associated with homocysteine (r=-0.364; P=0.006).

***Week 12***

NDCI was negatively correlated with NBuds (r=-0.341; P=0.011) and necrosis (r=-0.665; P<0.001). MN were positively associated with NPB (r=0.285; P=0.035) and NBuds were positively correlated with necrosis (r=0.291; P=0.031). Folate was positively associated with necrosis (r=0.341; P=0.011), Vitamin B12 (r=0.280; P=0.039) and homocysteine (r=0.289; P=0.032). Vitamin B12 was also positively correlated with homocysteine (r=0.998; P<0.001).

***Week 52***

A positive correlation was seen for necrosis and NDCI (r=0.571; P=0.001) and NBuds (r=0.548; P=0.001).

## *7.4 Discussion*

The cytokinesis block micronucleus (CBMN) cytome assay was used in this study to assess the impact of two popular weight loss diets with different macronutrient profiles, one high protein - high red meat (HP) and the other high carbohydrate (HC), on the genome stability profile in peripheral blood lymphocytes of overweight men. Micronutrients known to be involved in the maintenance of genome stability (i.e. folate and vitamin B12 and associated metabolite homocysteine) were also assessed. To date, the effect of different weight loss diets on genome stability in peripheral blood lymphocytes has not been studied. Results from this study are important for assessment of the impact of weight loss and calorie restriction on genome damage. Specifically, considering the increasing concern with high red meat consumption and increased risk of colorectal cancer, the genome damage response as a result of following a HP diet compared with a HC diet is important.

In this study, 12 weeks intensive weight loss on either a HP or HC diet showed no effect on the rate of any of the genome damage biomarkers assessed in peripheral blood lymphocytes. In the subjects who completed the 12 weeks weight loss and 9 months weight maintenance, there was no change in the rate of micronuclei (MNi) however there was a significant decrease in nuclear buds (NBuds) and a trend for an increase in nucleoplasmic bridges (NPBs). Considering the apparent inconsistent result found for genome damage, whereby one DNA damage marker, NPB, has increased while another, NBuds, has decreased, it is difficult to ascertain whether the intervention had a positive or negative overall effect on genome stability. One possibility would be to assess the net result of DNA damage. In this study we have presented 'total DNA damage'. This has the advantage in that it takes into account all markers of DNA damage assessed by the CBMN cytome assay simultaneously, effectively looking at the overall picture. This could be justified considering that the significant effects seen were fairly weak associations. Using this measure, neither a HP nor a HC weight loss diet has any observable effect on genome stability, suggesting that neither diet aggravates the incidence of genomic events associated with an increased risk of cancer. Alternatively, results from a recent study by El Zein *et al* [162] which assessed the association of MNi, NPBs and NBuds occurrence with lung cancer found that NPBs were associated with a 29-fold increase in cancer risk whereas NBuds had a 6-fold increase. This suggests that NPBs may be more strongly associated with cancer risk than NBuds. The trend in this study for an

increase in NPBs and the possible association of NPBs with cancer risk requires further investigation.

NPBs are scored in the CBMN cytome assay as they provide a measure of chromosome rearrangement and DNA misrepair. They arise from dicentric chromosomes, whose centromeres are pulled to opposite spindle poles of the cell during cell division, resulting in a nucleoplasmic connection [93, 97]. An increase in the occurrence of NPBs is usually associated with a simultaneous increase in MNi, as the formation of a dicentric chromosome (NPB) as a result of misrepair of DNA strand breaks will also result in the formation of an acentric chromosome fragment (MN) [123]. In the present study however there was a trend for a small increase in NPB without any significant increase in MNi. It is possible that this may be due to NPBs arising from dicentric chromosomes due to telomere end fusion [97, 98]. Telomere end-fusion as a result of telomere shortening or dysfunction could result in the production of a NPB without the accompanying acentric fragment. This is supported by a study from Rudolph *et al* [163] in models of rodent and human intestinal cancer *in vivo*, who observed a positive correlation with NPB formation and telomere shortening, further suggesting that NPB may be used as a surrogate marker of critically short telomeres.

It is important to note that any effect on genome stability in this study cannot be attributed to or refuted as a direct result of weight loss and/or caloric restriction as there was no free-living control group included for comparison. It could be suggested that the trend for an increase in NPB and decrease in NBuds detected was simply due to fluctuations that would also have occurred in the normal population, possibly as a result of seasonal variation of the biomarkers and not as a result of the intervention. However, there is no published evidence to date for change in NPB and/or NBuds to be related to season. A number of studies that assessed DNA damage using the comet assay have reported an effect of season, with an apparent increase in DNA damage in summer as opposed to winter and it has been suggested that this may be due to sunlight exposure in the warmer months [191-194]. Only one study has reported on the effect of season on the micronucleus assay index [195]. In this study, blood samples were taken 4 times per subject over a 12 month period with the sampling phases occurring between April - June, July - September, October - December and January - March. No significant differences were found for micronucleus frequency, suggesting season is not a variable in the micronucleus

assay. There is no evidence to date for the response of NPB and NBuds with season. In the current study, there was no significant change in MNi at week 0, 12 or 52. This of course primarily suggests that there is no impact of the intervention however, also illustrates that there was no effect over time. The first blood sample collection (week 0) occurred during May – June, prior/beginning winter and week 12 during August – September, end winter/beginning spring. This therefore supports results of Fenech *et al* [195] in that the effect of season did not impact micronucleus frequency.

Necrosis and apoptosis, markers of cytotoxicity in the CBMN cytome assay, both decreased significantly over time with no difference seen for either diet. However, considering the percentage of cells that were apoptotic in this study was small, in the range of 0.2-1.5%, although statistical significance was reached, the effect is probably not biologically significant. Although the statistical significance was not as strong, there was a larger percentage of cells that were necrotic, ranging between 32 and 41%. It is possible that the reduction in the percentage of necrotic cells over time in the present study may be due to a reduction in oxidative events that damage the cellular membranes rather than DNA. We cannot exclude the possibility that frozen storage of lymphocytes may have affected necrosis rates, however previous studies have not found an effect of cryopreservation on DNA damage levels with the comet or CBMN cytome assay [196, 197].

There was a significant effect of time for plasma folate and plasma vitamin B12 during the intervention. An increase in plasma folate was seen over time for both diets. Mean values for plasma vitamin B12 suggest an increase at week 12 and 52 compared with baseline for the HP group, with a trend for reduced plasma vitamin B12 over time in the HC group. This provides some evidence for adherence to the dietary protocol by the participants and that the HP group were actually consuming higher levels of protein, as the main sources for vitamin B12 in the diet is red meat, as well as dairy products [198-200]. A significant effect of time was seen in plasma homocysteine levels during the intervention with a trend for a reduction in mean plasma homocysteine levels at week 52. It is important to note however that the average plasma homocysteine levels at baseline were in the range of 7.1-7.8umol/L, with a slight improvement during the study to levels under 7umol/L. Similarly, plasma vitamin B12 was found to be in the range of 269-328pmol/L at baseline which did not change for the HC group and rose to around 330pmol/L for the HP group at the end

of the intervention. Results from previous studies indicated that genome damage measured as MNi with the CBMN assay is minimal when plasma vitamin B12 is more than 300pmol/L and plasma homocysteine is below 7.5umol/L [120, 171]. In the present study, although there was a general improvement in plasma homocysteine and vitamin B12 levels, baseline values were already near-optimal, and this may provide a reason for the minimal changes found with MN frequency in peripheral blood lymphocytes.

The potential impact of the relationship between genetic polymorphisms in genes involved in DNA repair and folate metabolism and micronucleus formation on the results found in this study cannot be excluded. It is possible that differences in genotype may have affected the outcome of this study however the study did not have sufficient power to assess genotype. Future larger studies may benefit from the inclusion of genotyping for polymorphisms associated with micronucleus formation and specifically associated with colorectal cancer such as methylenetetrahydrofolate (*MTHFR*), glutathione *S*-transferases (e.g. *GSTM1*), *N*-acetyl transferases (e.g. *NAT2*) and NAD(P)H quinone oxidoreductase (*NQO1*) [201].

Although concerns have been raised regarding the risk of certain cancers with weight loss diets that are high in red meat, the impact of weight loss diets with different macronutrient profiles on genome damage has not to our knowledge been previously assessed. Results from this study supports the hypothesis that a high protein-high red meat diet does not appear to influence the genome stability profile of peripheral blood lymphocytes differently to a high carbohydrate diet, when assessed using the CBMN cytome assay in overweight men who are not folate of vitamin B12 deficient. The study provides some evidence to support the hypothesis that a high protein-high red meat diet may have some advantages in improving status of micronutrients required for genome maintenance compared with a high carbohydrate diet. Any effect of weight loss and/or caloric restriction on genome damage biomarkers needs to be confirmed with a study design that includes a non-weight loss control.

# 8. RELATIONSHIP BETWEEN PERIPHERAL BLOOD LYMPHOCYTE GENOME STABILITY AND FAECAL WATER INDUCED GENOTOXICITY

### *AIM*

To investigate whether the genotoxic potential of faecal water is correlated with the genome stability profile of peripheral blood lymphocytes.

### *HYPOTHESIS*

The genotoxic potential of faecal water is a risk factor for increased genome damage in peripheral blood lymphocytes.

## *8.1 Introduction*

Considering cancer is likely to be caused by gene and chromosomal mutations and can take years to develop, numerous studies use DNA damage biomarkers for an assessment of cancer risk [102]. The induction of the genomic instability phenotype is considered a crucial early event in carcinogenesis, enabling a cell to evolve into a cancer cell [102]. The CBMN cytome assay provides a measure of genomic instability, as the result of DNA damage, and is therefore used as a biomarker of cancer risk [103]. The CBMN cytome assay provides a comprehensive picture of the genotoxic, cytotoxic and cytostatic effects of a particular cellular insult [98, 136-138].

The current methodology whereby lymphocytes from a blood sample are cultured *ex vivo* and once divided cells distinguished by blocking of cytokinesis with cytochalasin-B, allows for a measure of DNA damage that has accumulated while lymphocytes circulate throughout the body, and with a half life of approximately 6-12 months, integrating genotoxic events across body tissues [102, 104]. The strongest evidence for the association of MN frequency and early events in carcinogenesis in humans comes from a recent large international cohort study, where individuals were tested for MN frequency between 1980 and 2002 and were free of cancer at the time of testing. A significant increase for all cancers were found in subjects in the medium to high MN frequency groups (relative risk of 1.84 and 1.53 respectively) after adjusting for confounders such as age, gender and smoking status and were strongest for urogenital and gastro-intestinal cancers [108]. Findings from this study support the use of MNi in peripheral blood lymphocytes as a predictive biomarker of cancer risk.

The CBMN cytome assay has also become one of the standard cytogenetic measurements used in the genetic toxicology testing of chemicals and radiation *in vitro* [121]. Since colorectal cancer is considered to be largely attributed to environmental factors, assessment of the carcinogenic load of the contents of the colonic lumen, due to its direct potential to transform the cells lining the bowel to cancerous cells, is also often assessed as a biomarker of risk [126]. It has been suggested that the faecal water or aqueous phase of the faeces is of particular importance in studying the effect of diet on colorectal cancer risk as this faecal fraction is more likely to exert an effect on the colonic cells than compounds which are bound to food residues and bacterial mass [128]. It has been demonstrated that

the genotoxic potential of faecal water may be a suitable biomarker in studying the impact of diet on colorectal cancer risk [158, 159].

Considering the vast blood supply of the small and large intestine and the presence of lymphoid tissue along the small intestine (termed Peyer's patches or gut-associated lymphoid tissue), along with the primary role of the small intestine for nutrient absorption and the large intestine for water absorption, it could further be plausible to suggest that genotoxins present within the bowel contents may have the potential to escape or leak from the lumen into the bloodstream [202]. As such, this may provide an alternative suggestion for a potential association between the genotoxicity of faecal water with peripheral blood lymphocyte DNA damage.

To our knowledge, whether genotoxicity of faecal water is correlated with genome damage rate of peripheral blood lymphocytes, using the CBMN cytome assay, has not been previously assessed. In this chapter the genotoxicity of faecal water in overweight men following 12 weeks intensive weight loss and 9 months weight maintenance is compared with the genome damage rate of peripheral blood lymphocytes.

## *8.2 Methods*

As previously described (Chapter 4), 55 overweight male subjects were recruited and randomised to one of two dietary interventions, a high protein–high red meat (HP) diet or a high carbohydrate-low red meat (HC) diet. The study was a parallel design, comprising of a 12 week intensive weight loss phase and follow-up at one year with interim monthly visits. 55 subjects completed the short term 12 weeks intensive weight loss phase and 33 subjects completed the short term intensive weight loss and long term 9 months weight maintenance.

### 8.2.1 Cytokinesis block micronucleus cytome assay for faecal water genotoxicity and cytotoxicity testing in the WIL2-NS cell line

Bowel actions from a 24 hour period (typically from midnight Saturday to midnight Sunday) were collected by volunteers at weeks 0, 12 and 52, stored in an esky with frozen ice bricks until they were brought to the clinic the following morning. The samples were weighed and then stored at -20°C until further processing. Faecal water was isolated and stored at -20°C as previously described in Chapter 4.5.2. The schematic protocol for the testing of faecal water using the CBMN cytome assay in the WIL2-NS cell line is shown in Figure 8.1 and outlined in detail in Chapter 5.2.3. CBMN cytome assay slides were scored according to criteria described in Chapter 3.5.

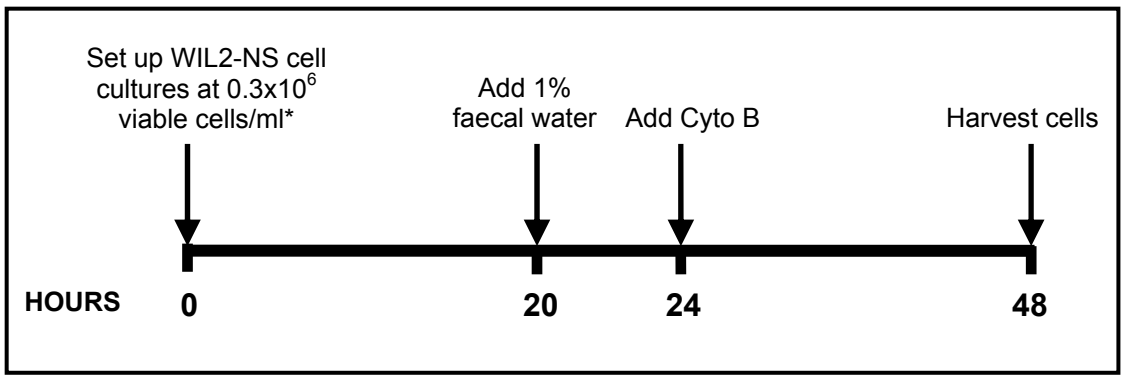


**Figure 8.1 Schematic protocol of faecal water CBMN cytome assay.**

### 7.2.1 Cytokinesis block micronucleus cytome assay for isolated lymphocytes stored in liquid nitrogen

Venous blood was collected from fasted (overnight) volunteers at baseline (week 0), week 12 and week 52 in Vacuette®-Heparin tubes (9 ml) and lymphocytes were isolated and stored in cryovials in liquid nitrogen as previously described (Chapter 4.4.1). The schematic 72 hour protocol for isolated lymphocytes is shown in Figure 8.2 and outlined in detail in Chapter 7.2.1. CBMN cytome assay slides were scored according to criteria described in Chapter 3.5.

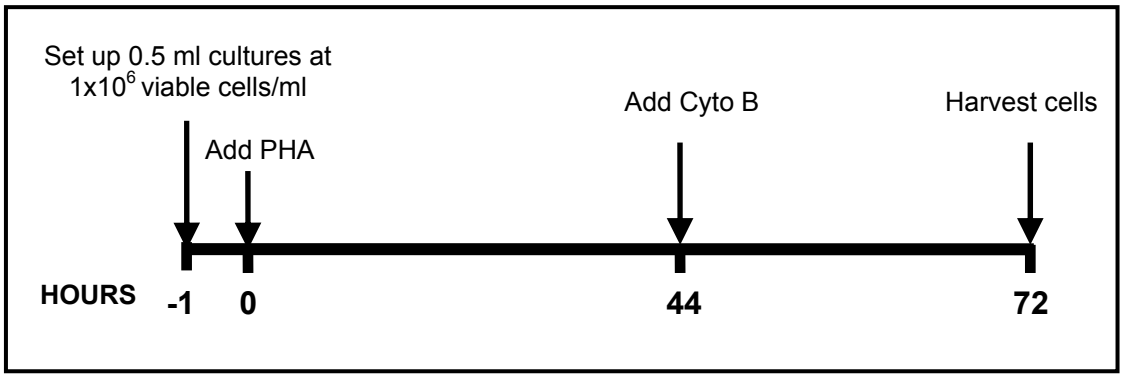


**Figure 8.2 Schematic protocol of 72 hour CBMN cytome assay experiment.**

### 7.2.3 Statistical analysis

A correlation matrix of all data at each time point was performed using Pearsons test. Significance for all tests was accepted at $P<0.05$. All statistical analyses were performed using SPSS 14.0 for Windows.

Results for faecal water genotoxicity and cytotoxicity were calculated as the faecal water result obtained minus the baseline 'background' result recorded for the WIL2-NS cell line for each assay run to minimise any potential confounding effect from a change in the background DNA damage/cytotoxicity frequency of the cell line and reported as induced effect of faecal water. Data shown in Appendix 6.1.

## *8.3 Results*

### 8.3.1 Week 0

55 male subjects began the study; with n=24 in the high protein (HP) group and n=31 in the high carbohydrate (HC) group. A correlation matrix comparing faecal water genotoxicity and genome damage in peripheral blood lymphocytes at week 0 is shown in Table 8.1. Significant correlations ($P<0.05$) are highlighted in yellow. Faecal water induced NPB were positively correlated with peripheral blood lymphocyte apoptosis rate ($r=0.352$; $P=0.008$). Faecal water induced necrosis was negatively associated with MN in peripheral blood lymphocytes ($r=-0.411$; $P=0.002$).

### 8.3.2 Week 12

55 male subjects completed the 12 week intensive weight loss phase; with n=24 in the high protein (HP) group and n=31 in the high carbohydrate (HC) group. Weight loss was not significantly different between diets. Average weight loss was 8.4 ± 0.5 kg after the 12 weeks, as described in Chapter 4.4.

A correlation matrix comparing faecal water genotoxicity and genome damage in peripheral blood lymphocytes at week 12 is shown in Table 8.2. No correlations were seen between any faecal water genotoxicity and peripheral blood lymphocyte genome damage biomarkers.

### 8.3.3 Week 52

33 male subjects completed the 12 week intensive weight loss phase and 9 month weight maintenance phase; with n=16 in the high protein (HP) group and n=17 in the high carbohydrate (HC) group. Weight loss was not significantly different between diets at week 12 or week 52. Average weight loss was 9.3 ± 0.7 kg after 12 weeks intensive weight loss with no further significant change in weight from week 12 to week 52, as described in Chapter 4.4.

A correlation matrix comparing faecal water genotoxicity and genome damage in peripheral blood lymphocytes at week 52 is shown in Table 8.3. Significant correlations ($P<0.05$) are highlighted in yellow. Faecal water induced MN was positively correlated with peripheral blood lymphocyte MN ($r=0.387$; $P=0.026$).

**Table 8.1 Pearsons Correlation Matrix - Week 0.**

| | | | PERIPHERAL BLOOD LYMPHOCYTE GENOME DAMAGE | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **MN** n=55 | **NPB** n=55 | **NBUDS** n=55 | **NDCI** n=55 | **NECROSIS** n=55 | **APOPTOSIS** n=55 |
| **FAECAL WATER GENOTOXICITY** | **MN** n=55 | *r* | -0.060 | -0.102 | 0.069 | -0.099 | 0.144 | 0.221 |
| | | P | 0.665 | 0.457 | 0.619 | 0.472 | 0.294 | 0.105 |
| | **NPB** n=55 | *r* | -0.114 | -0.222 | 0.019 | 0.031 | -0.008 | 0.352 |
| | | P | 0.408 | 0.103 | 0.892 | 0.823 | 0.952 | 0.008 |
| | **NBUDS** n=55 | *r* | 0.140 | 0.122 | -0.165 | -0.171 | 0.094 | 0.166 |
| | | P | 0.310 | 0.376 | 0.230 | 0.213 | 0.496 | 0.227 |
| | **NDCI** n=55 | *r* | 0.229 | -0.133 | -0.081 | 0.075 | 0.030 | -0.205 |
| | | P | 0.092 | 0.332 | 0.556 | 0.585 | 0.825 | 0.133 |
| | **NECROSIS** n=55 | *r* | -0.411 | 0.158 | 0.148 | 0.022 | 0.042 | 0.170 |
| | | P | 0.002 | 0.250 | 0.280 | 0.874 | 0.763 | 0.216 |
| | **APOPTOSIS** n=55 | *r* | 0.098 | -0.082 | 0.085 | -0.099 | -0.072 | -0.032 |
| | | P | 0.476 | 0.554 | 0.538 | 0.472 | 0.601 | 0.814 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index

**Table 8.2 Pearsons Correlation Matrix - Week 12.**

| | | | PERIPHERAL BLOOD LYMPHOCYTE GENOME DAMAGE | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **MN** n=55 | **NPB** n=55 | **NBUDS** n=55 | **NDCI** n=55 | **NECROSIS** n=55 | **APOPTOSIS** n=55 |
| **FAECAL WATER GENOTOXICITY** | **MN** n=55 | *r* | 0.095 | 0.088 | -0.123 | -0.088 | 0.062 | 0.240 |
| | | P | 0.488 | 0.525 | 0.372 | 0.522 | 0.652 | 0.078 |
| | **NPB** n=55 | *r* | 0.067 | -0.020 | -0.078 | -0.188 | 0.038 | 0.202 |
| | | P | 0.629 | 0.887 | 0.573 | 0.170 | 0.780 | 0.140 |
| | **NBUDS** n=55 | *r* | 0.153 | 0.168 | -0.111 | -0.011 | 0.091 | 0.075 |
| | | P | 0.265 | 0.220 | 0.420 | 0.937 | 0.510 | 0.589 |
| | **NDCI** n=55 | *r* | 0.154 | 0.111 | 0.226 | -0.023 | 0.135 | -0.034 |
| | | P | 0.262 | 0.422 | 0.097 | 0.870 | 0.326 | 0.804 |
| | **NECROSIS** n=55 | *r* | -0.255 | 0.038 | -0.063 | -0.038 | -0.070 | 0.027 |
| | | P | 0.061 | 0.782 | 0.647 | 0.783 | 0.613 | 0.846 |
| | **APOPTOSIS** n=55 | *r* | 0.000 | -0.039 | 0.186 | -0.175 | 0.061 | 0.125 |
| | | P | 0.998 | 0.780 | 0.173 | 0.201 | 0.660 | 0.364 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, NDCI=nuclear division cytotoxicity index

**Table 8.3 Pearsons Correlation Matrix – Week 52.**

| | | | PERIPHERAL BLOOD LYMPHOCYTE GENOME DAMAGE | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **MN** n=33 | **NPB** n=33 | **NBUDS** n=33 | **NDCI** n=33 | **NECROSIS** n=33 | **APOPTOSIS** n=33 |
| **FAECAL WATER GENOTOXICITY** | **MN** n=33 | *r* | 0.387 | 0.179 | 0.250 | 0.041 | 0.017 | 0.127 |
| | | P | 0.026 | 0.318 | 0.160 | 0.823 | 0.924 | 0.480 |
| | **NPB** n=33 | *r* | 0.230 | 0.112 | 0.072 | -0.165 | 0.105 | -0.266 |
| | | P | 0.197 | 0.536 | 0.692 | 0.359 | 0.561 | 0.135 |
| | **NBUDS** n=33 | *r* | 0.055 | 0.258 | 0.078 | -0.102 | 0.118 | -0.014 |
| | | P | 0.763 | 0.147 | 0.667 | 0.572 | 0.513 | 0.940 |
| | **NDCI** n=33 | *r* | 0.138 | 0.265 | -0.193 | 0.071 | -0.207 | -0.139 |
| | | P | 0.444 | 0.136 | 0.281 | 0.693 | 0.248 | 0.440 |
| | **NECROSIS** n=33 | *r* | -0.235 | -0.203 | 0.111 | -0.156 | 0.207 | 0.021 |
| | | P | 0.189 | 0.257 | 0.537 | 0.387 | 0.247 | 0.906 |
| | **APOPTOSIS** n=33 | *r* | 0.007 | 0.091 | -0.181 | 0.108 | -0.182 | -0.102 |
| | | P | 0.967 | 0.616 | 0.313 | 0.551 | 0.311 | 0.573 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index

## *8.4 Discussion*

The cytokinesis block micronucleus (CBMN) cytome assay was used in this study to assess the impact of two weight loss diets with different macronutrient profiles; one high in protein, specifically high in red meat (HP) and the other high carbohydrate (HC), on (1) the genome stability profile in peripheral blood lymphocytes and (2) the in vitro genotoxic potential of faecal water in overweight men. To our knowledge, to date, the effect of different weight loss diets on the genome stability profile in peripheral blood lymphocytes or the genotoxicity of faecal water assessed with the CBMN cytome assay has not been studied. Results from this study are important for assessment of the impact of dietary patterns used for weight loss on genomic damage and bowel health, which is of particular importance considering the concerns of high red meat consumption and increased risk of colorectal cancer. In this chapter, we have assessed whether the genotoxic potential of the contents of the bowel is reflected in the genome damage rate of peripheral blood lymphocytes.

In this chapter, the impact of 12 weeks intensive weight loss and 9 months weight maintenance on genome damage rate in peripheral blood lymphocytes and genotoxicity of faecal water using the CBMN cytome assay was compared. At baseline (week 0), 2 correlations were seen. There were no correlations seen at week 12 and only 1 correlation at week 52. Of these 3 correlations, the strongest found was a negative correlation of faecal water genotoxicity induced necrosis with peripheral blood lymphocyte micronucleus (MN) frequency, with an r value of -0.411, therefore explaining only 16.9% of variance. From this, it could be suggested that there is very minimal, if any, correlation between the genome damage rate of peripheral blood lymphocytes and the genotoxic potential of faecal water. Therefore, the results from the present study do not suggest that peripheral blood lymphocyte genome damage reflects the genotoxic potential of the environment of the bowel or vice-versa.

A lack of association of peripheral blood lymphocyte genome damage with faecal water genotoxicity may indicate that the assessment of both end points are important and are required in the evaluation of the impact of dietary patterns on colorectal cancer risk. The colonic cells which line the bowel are exposed to the luminal contents of the bowel but also consist of a vast blood supply, and as such, have the potential to be exposed to DNA damaging agents that can be either faecal or blood

borne [126]. Alternatively, it may be possible for peripheral blood lymphocytes to be exposed to genotoxins that have leaked from the bowel into the bloodstream. This supports the suggestion that the assessment of DNA damage of both peripheral blood and faecal origin biomarkers may provide a more comprehensive assessment of colorectal cancer risk. Ideally, these biomarkers would be compared with the MN index of colonic cells to determine which one is a more suitable surrogate biomarker.

It has been proposed that the relevance of DNA damage biomarkers as an indicator of cancer risk is dependant on the tissue studied and its location relative to the carcinogen exposure route studied [145]. Studies of smokers and MN frequency have shown that smoking is only associated with an increase in MN frequency in peripheral blood lymphocytes in heavy smokers (≥30 cigarettes per day) but not at lower smoking levels [203]. One suggestion is that this may be a result of the concentration of genotoxic agents of cigarette smoke being lower in the blood stream than in the lung tissue [203]. From this, it could be surmised that the genotoxicity of faecal water may prove to be a better indicator of colorectal cancer risk than genome damage of peripheral blood lymphocytes. The CBMN assay genome damage biomarkers have recently been shown to be strongly related to diet with increased caecal water genotoxicity in rats following a high-risk (low fibre, high fat) colorectal cancer diet compared with a low-risk (high fibre, low fat) colorectal cancer diet (Benassi *et al*, 2008), providing further support to the value of CBMN assay genome damage markers as biomarkers of dietary associated genotoxicity and cancer risk. However, we must bear in mind that the genotoxicity of faecal water in this study is measured *in vitro* in the WIL2-NS cell line, a human B lymphoblastoid cell line, and these results may not necessarily accurately reflect the human *in vivo* situation.

The lining of the bowel is characterised by crypts that are approximately 50 cells deep, and this, in association with an ‘upward and outward’ pressure exerted by secreted mucus, is thought to protect the progenitor cells at the base of the crypt from the mutagenic environment of the colon lumen, such that any direct interaction of the faecal contents and replicating colonic cells is minimised [126]. This therefore could alternatively suggest that any mutagenic effects that do occur as a result of faecal water exposure would be in colonic cells that are differentiated and not replicating, and may be beginning to undergo apoptosis and be sloughed away,

having no impact on the integrity of the colonic stem cell population [126]. This indicates that for a mutated cell to give rise to an adenoma, a replicating cell must undergo the 'first hit' and as such this mutagen may be blood borne rather than arise from the faecal contents [126]. From this, it could be suggested that the DNA damage rate of peripheral blood lymphocytes is more important than the genotoxic potential of faecal water in regards to colorectal cancer risk. This is also supported by a recent major prospective study which has shown the MN assay in peripheral blood lymphocytes is predictive of cancer risk in human cohorts followed over a 15-20 year period [108]. For colorectal cancer specifically, it was shown that subjects with a medium/high MN frequency had a relative risk of colorectal cancer incidence and mortality of 1.75 ($P = 0.14$) compared with the low MN frequency group [108].

Results from this chapter show that overall, the genome stability profile of peripheral blood lymphocytes does not correlate with the genotoxic potential of faecal water and therefore does not support the hypothesis of the genotoxic potential of faecal water being a risk factor for increased genome damage in peripheral blood lymphocytes. Further studies which assess the MN index of colonic cells are required to determine whether one of these biomarkers is a more suitable surrogate biomarker of colorectal cancer risk.

# 9. GENERAL SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Since the 1960s, diets that are high in protein and restrictive of carbohydrate intake have been popular among dieters [5]. The replacement of dietary carbohydrate with protein, along with a low intake of fat, has been shown to induce larger weight loss in overweight subjects when compared with conventional high carbohydrate/low fat diets, suggesting this is a favourable weight loss regime for overweight people [6-9]. Despite the popularity and effectiveness of high protein diets for weight loss, there have been no long term studies to determine their safety, raising concerns over their long term use in the general public [5, 16, 17].

It has been recognised since the 1970s that the development of colorectal cancer is not entirely genetic in basis and that diet is an important risk factor. From this, it can be suggested that colorectal cancer can to some extent, be preventable by dietary changes [23]. Although this suggests that the effect of diet on colorectal cancer risk are of great public importance, the influence of specific dietary factors on colorectal cancer risk remains largely uncertain [3, 23]. The inter-relation of food components in the diet makes it difficult to define the role of any specific dietary factor on colorectal cancer risk [89]. It has been suggested that the study of the overall dietary and lifestyle pattern may prove more important than any individual component when assessing the impact on risk of colorectal cancer [89, 90]. Although a high protein diet may be associated with increasing the risk of colorectal cancer if it contains a higher content of red meat, such a diet is also associated with weight loss and improved insulin sensitivity, both risk factors which are also associated with reduced risk of colorectal cancer. From this it may be suggested that a dietary pattern used for weight loss that is higher in protein but remains low in fat and high in foods rich in fibre and micronutrients that are required for prevention of DNA damage may not increase the risk or may decrease the net risk of colorectal cancer, thus providing a safe and effective dietary method of weight loss in obese or overweight subjects. The aim of this thesis was to compare the impact of a weight loss diet that is high protein, specifically high in red meat, with a conventional high carbohydrate diet on various biomarkers of colorectal cancer in overweight men.

The first aim of this thesis was to establish a novel *in vitro* assay which assessed the genotoxic potential of faecal water using the cytokinesis block micronucleus (CBMN) cytome assay (Chapter 5.). Faecal water is considered to be the most important faecal fraction with regards to colorectal cancer risk as it is more likely to be able to exert an effect on the colonic cells than compounds which are bound to the food residues and bacterial mass [45, 128]. The genotoxic effects of faecal water have been assessed using other methods such as the Comet assay however this assay is limited due to a confounding by DNA strand breaks in early apoptotic and necrotic cells, the lack of detection of mis-repair of DNA strand breaks and the fact that chromosome malsegregation events or telomere end fusions are not detectable, all of which are important events in the colorectal carcinogenic process [131, 134, 135]. As such it is suggested that the CBMN cytome assay may provide a more comprehensive approach to measure the genotoxicity and cytotoxicity of faecal water [98, 136-138]. This assay is a well established test for genotoxicity measurement at the chromosomal level using three distinct biomarkers: micronuclei (MN), a biomarker of chromosome breakage and/or loss; nucleoplasmic bridges (NPBs), a biomarker of DNA strand break mis-repair and/or telomere end fusion; nuclear buds (NBUDs), a biomarker of gene amplification and/or elimination of DNA repair complexes. The same assay also allows separate measurement of apoptosis and necrosis, biomarkers of cell death, and nuclear division index (NDI), a measure of cytostatic effects [98, 136-138].

The pilot study of *in vitro* faecal water genotoxicity testing (Chapter 5.) showed that a 1% dilution of faecal water was required for use in the WIL2-NS cell line with sufficient viability and that a 10% dilution resulted in complete cytotoxicity. Previous studies have shown that undiluted faecal water was cytotoxic to HT-29 cells [143] and it seems reasonable to assume that colonic cells *in vivo* would not be exposed to the same level of faecal water as in the lumen, due to a concentration gradient (decreasing from lumen to cells) which is the result of the excretion or exocytosis of compounds such as bile acids from the colonic cells into the enterohepatic circulation and also the mucin layer that coats the epithelial cells, which makes the diffusion of compounds from the lumen to the cells more difficult [143].

As this is, to our knowledge, the first study to use the CBMN cytome assay for *in vitro* faecal water genotoxicity testing the sensitivity and variation of the biomarkers was unknown. Work shown in Chapter 5. suggests that the CBMN cytome assay provides a good measure of variation of faecal water between and also within individuals. The assay was also shown to be reliable and reproducible with the CV being smaller for repeated measures from one individual as compared to samples from different individuals. Further, the results also suggest that the genotoxicity of faecal water from an individual who maintains a relatively unchanged dietary pattern is not significantly different from one week to the next, and as such a single faecal sample will be largely indicative of the genotoxic potential of the individuals dietary pattern. The fold increase relative to the %CV was then assessed to give an arbitrary unit ratio that takes into account both the sensitivity of the biomarker as well as the background variation of the biomarker. Using this ratio, it was determined that the genome damage biomarkers (MN, NPB and NBuds) were the best biomarkers within this assay for measuring adverse effects of faecal water within and between individuals using the CBMN cytome assay in the WIL2-NS cell line.

Future directions in this field of research includes further work in identifying whether 1% dilution of faecal water is appropriate for the larger general population. In this study, faecal water diluted to 1% resulted in a statistically significant genotoxic insult in the WIL2-NS cell line, however, with larger study numbers and/or different subject characteristics eg sex, age, health status, it may be that a lower degree of dilution of faecal water is optimal, however this is likely to not be greater than 10% which resulted in complete cytotoxicity for all subjects. Further, it should be defined whether the percentage of diluted faecal water used for *in vitro* genotoxicity testing is reflective of that which is seen in the *in vivo* situation. Although it has been suggested that the effective concentration of faecal water solutes reaching the colonic cells is in the order of 1-10% [143], this is yet to be tested directly. Furthermore, it would be important to ascertain whether susceptibility to genotoxicity of faecal water is dependant on inherited characteristics relating to detoxification and DNA repair mechanisms.

A further question arising from the pilot faecal water genotoxicity using the CBMN cytome assay work in Chapter 5. surrounds the use of the WIL2-NS cell line for *in vitro* faecal water genotoxicity testing with the CBMN cytome assay as it may be suggested that a colonic cell line could provide a closer reflection of what may occur *in vivo*. The WIL2-NS cell line was selected as it has been validated for use in the *in vitro* MN assay, due to the low background MN frequency and excellent cellular morphology [122, 123] and also the mutation in the p53 gene allows cells to express genome damage, making it easy to assess genotoxic load of a test agent [141, 142]. There is however no standard cell line used for genotoxicity testing and a further study comparing the WIL2-NS cell line with colonic cells lines with a range of DNA repair defects would be warranted.

Further to determining the genome damage response and sensitivity of the WIL2-NS cell line in comparison to the *in vitro* situation, there is also a need to define the potential genotoxic and/or cytotoxic agents and the quantity in which they are present in human faecal water. This would then allow for the determination of whether diluting faecal water for *in vitro* genotoxicity testing alters the type of genomic damage or sensitivity of agents that would actually have a damaging effect *in vivo*.

The novel i*n vitro* faecal water genotoxicity test using the CBMN cytome assay described in Chapter 5. was then utilised to assess the genotoxicity of faecal water from overweight men consuming either a high protein–high red meat (HP) diet or a high carbohydrate-low red meat (HC) diet. The weight loss study comprised of a 12 week intensive weight loss phase and 9 months weight maintenance. There was no significant difference in weight loss between diets. The total DNA damage response, which takes into account all CBMN cytome assay genome damage biomarkers (MN, NPB and NBuds), showed a significant reduction in faecal water genotoxicity potential after 12 weeks weight loss, with no difference between dietary patterns, and a subsequent increase after 9 months weight maintenance to DNA damage frequencies seen at baseline. From this, it could be proposed that active weight loss and/or caloric restriction may reduce the genotoxic load of faecal water. However, to be able to attribute a lowered genome damage potential of faecal water to weight loss and /or caloric restriction, a further study that includes a non-weight loss control group for comparison is needed.

Conventional bowel health biomarkers of faecal total short chain fatty acids (SCFAs) or phenolic compounds phenol or p-cresol did not change significantly in this study (Chapter 6.). SCFAs levels trended in a similar pattern to total DNA damage levels, with a downward trend following 12 weeks weight loss and a subsequent increase after 9 months weight maintenance to levels similar to that seen at baseline. Considering SCFAs such as butyrate are believed to have a protective effect in the bowel through promotion of apoptosis [25, 146] it could be suggested that a reduction in faecal water induced total genome damage would coincide with an increase in SCFA levels. As such, a trend for a reduction in genome damage occurring with a similar reduction in SCFAs, as seen in this study, may suggest that SFCAs are not associated with faecal water genotoxicity and therefore not an important factor for the determination of bowel health and colorectal cancer risk. However, the WIL2-NS cell line is p53 deficient and hence is unable to initiate apoptosis when DNA damage occurs. This may also explain the lack of relation seen with SCFAs (which induce apoptosis) and micronuclei assessed by the CBMN cytome assay in the WIL2-NS cell line. It is also possible that SCFAs may be more relevant to alterations in proliferation of cells than to chromosomal instability.

The impact of a HP and HC weight loss diet on the genome stability profile in peripheral blood lymphocytes is described in Chapter 7. In this study, there was no effect of 12 weeks weight loss or 9 months weight maintenance with either a HP or HC diet on total DNA damage rate in peripheral blood lymphocytes. It should be noted however that previous studies have shown that genome damage measured as MNi with the CMBN cytome assay is minimal when plasma vitamin B12 levels are above 300pmol/L and plasma homocysteine levels below 7.5umol/L [120, 171]. In the present study, there was a slight general improvement in plasma vitamin B12 and homocysteine levels however baseline values were already near-optimal and this may provide a reason for the lack of detectable changes seen with MN frequency. As such, it can be concluded that a HP diet does not appear to influence the genome stability profile of peripheral blood lymphocytes differently to a HC diet, when assessed using the CBMN cytome assay, in overweight men who are not vitamin B12 deficient. As previously mentioned, any effect of weight loss and /or caloric restriction needs to be tested with a study design that includes a non-weight loss control group.

The final aim for this thesis was to assess whether the genotoxic potential of faecal water is reflected in the genome stability profile of peripheral blood lymphocytes (Chapter 8.). These results suggest that peripheral blood lymphocyte genome damage rate does not reflect the genotoxic potential of the environment of the bowel or vice-versa. Taking into account that colonic cells that line the bowel are exposed to both the luminal bowel environment as well as a vast blood supply, they may have the potential to be exposed to both faecal and/or blood borne DNA damaging agents. As such, a lack of correlation seen between faecal water genotoxicity and peripheral blood lymphocyte genome damage may suggest that the assessment of both end points are important in the evaluation of dietary patterns on colorectal cancer risk. However, ultimately further studies which assess the genomic damage in colonic cells are required to determine whether one of these biomarkers is more suitable for the assessment of the impact of dietary patterns on risk of colorectal cancer.

The influence of dietary patterns, in particular a weight loss diet that is high in red meat, on risk of colorectal cancer is a complex issue that has been highly debated. It may be suggested that the study of the overall dietary and lifestyle pattern may prove more important than any individual dietary component in the assessment of colorectal cancer risk. The work from this thesis suggests that, the genotoxic potential of faecal water can be assessed *in vitro* using the CBMN cytome assay and that a high protein-high red meat weight loss diet does not increase faecal water genotoxicity or peripheral blood genome damage, measured with the CBMN cytome assay, differently compared with a conventional high carbohydrate weight loss diet. Weight loss and/or caloric restriction following either a high protein or high carbohydrate diet may beneficially modify the carcinogenic load of the colon in the short term. A lack of relationship between these two biomarkers may suggest that the assessment of both the genome damage potential of the bowel contents and the assessment of the genome stability profile of peripheral blood lymphocytes may be important in comprehensively assessing the impact on genome damage by different dietary patterns.

Epilogue:

Subsequent to the initial completion and submission of this thesis the 2nd WCRF & AICR Expert Report - Food, Nutrition, Physical Activity and the Prevention of Cancer: a Global Perspective has been issued. It is of relevance to note that the view on red meat intake is reported as "convincing increased risk" with regards to colorectal cancer and that intake of red meat should be limited to less than 500g per week. This is increased from the 'probable' status it was given in the 1st WCRF & AICR report in 1997. The 2nd report also makes comment however on the inability to make recommendations on general patterns of food and nutrition, or 'whole diets' as there are relatively few epidemiological studies to examine diet in an integrated form and studies are performed differently such that they cannot be compared. The panel recommended that protocols be agreed upon to enable epidemiological studies of food patterns, nutrition and physical activity to allow for comparison. This lends support for the suggestion that the study of the overall dietary and lifestyle pattern may prove more important than any individual dietary component in the assessment of colorectal cancer risk and that the impact of high protein-high red meat weight loss diets on colorectal cancer risk remains unclear and further research is necessary.

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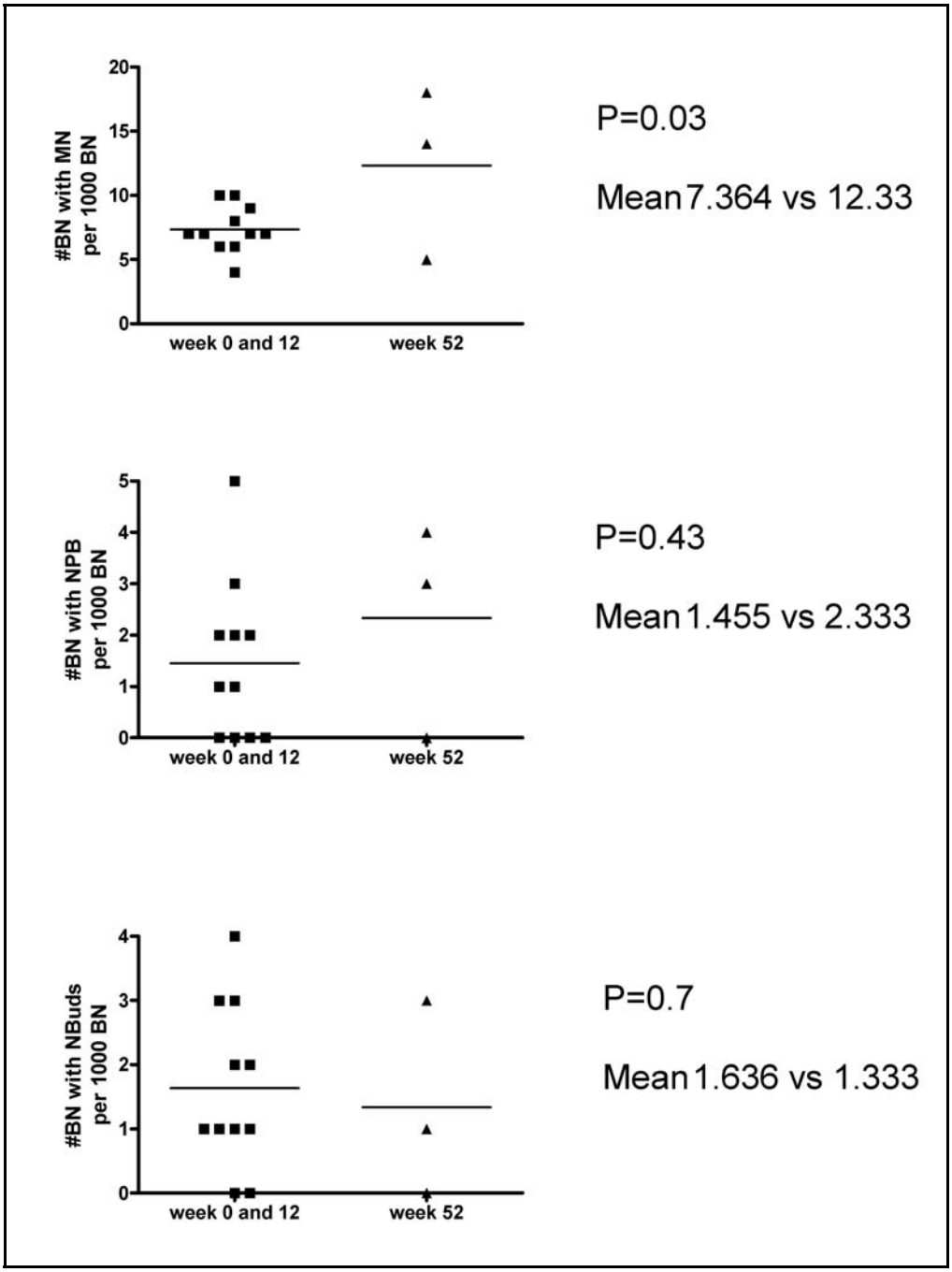
# APPENDIX

***A 6.1 Baseline WIL2-NS data for faecal water genotoxicity using the CBMN assay***




BN=binucleate cells, MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds

### *A 6.2 Pearsons Correlation Matrix – Faecal water genotoxicity and conventional bowel biomarkers week 0*

| | | NPB | NBuds | necrosis | apoptosis | NDCI | faecal pH | faecal weight | faecal moisture | SCFAs | phenols | p-cresols n=54 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MN** | *r* | 0.501 | 0.294 | 0.083 | 0.150 | -0.248 | -0.135 | 0.058 | -0.011 | 0.026 | -0.093 | 0.089 |
| n=55 | P | 0.000 | 0.029 | 0.546 | 0.274 | 0.068 | 0.327 | 0.672 | 0.934 | 0.850 | 0.504 | 0.524 |
| **NPB** | *r* | | 0.028 | 0.011 | -0.007 | -0.223 | -0.027 | 0.002 | -0.181 | -0.021 | -0.205 | 0.244 |
| n=55 | P | | 0.841 | 0.936 | 0.958 | 0.102 | 0.848 | 0.989 | 0.186 | 0.880 | 0.137 | 0.075 |
| **NBuds** | *r* | | | 0.146 | 0.030 | -0.217 | -0.240 | 0.219 | 0.136 | 0.167 | 0.178 | 0.034 |
| n=55 | P | | | 0.287 | 0.830 | 0.122 | 0.078 | 0.108 | 0.322 | 0.222 | 0.197 | 0.810 |
| **necrosis** | *r* | | | | 0.090 | -0.767 | 0.042 | -0.194 | -0.130 | -0.158 | -0.118 | 0.221 |
| n=55 | P | | | | 0.515 | 0.000 | 0.759 | 0.157 | 0.343 | 0.250 | 0.394 | 0.109 |
| **apoptosis** | *r* | | | | | -0.141 | 0.024 | -0.073 | 0.041 | -0.078 | 0.015 | 0.068 |
| n=55 | P | | | | | 0.305 | 0.860 | 0.594 | 0.768 | 0.570 | 0.915 | 0.623 |
| **NDCI** | *r* | | | | | | -0.060 | 0.149 | 0.208 | 0.084 | 0.039 | -0.313 |
| n=55 | P | | | | | | 0.665 | 0.279 | 0.128 | 0.544 | 0.782 | 0.021 |
| **faecal pH** | *r* | | | | | | | -0.411 | -0.547 | -0.676 | -0.258 | 0.249 |
| n=55 | P | | | | | | | 0.002 | 0.000 | 0.000 | 0.060 | 0.070 |
| **faecal weight** | *r* | | | | | | | | 0.608 | 0.831 | 0.009 | -0.322 |
| n=55 | P | | | | | | | | 0.000 | 0.000 | 0.949 | 0.018 |
| **faecal moisture** | *r* | | | | | | | | | 0.752 | 0.172 | -0.422 |
| n=55 | P | | | | | | | | | 0.000 | 0.212 | 0.001 |
| **SCFAs** | *r* | | | | | | | | | | 0.123 | -0.337 |
| n=55 | P | | | | | | | | | | 0.375 | 0.013 |
| **phenol** | *r* | | | | | | | | | | | -0.124 |
| n=54 | P | | | | | | | | | | | 0.370 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index, SCFAs=short chain fatty acids

### *A 6.3 Pearsons Correlation Matrix – Faecal water genotoxicity and short chain fatty acids week 0*

| | | acetic acid | propionic acid | butyric acid | valeric acid | caproic acid n=55 |
|---|---|---|---|---|---|---|
| **MN** | *r* | 0.019 | -0.033 | 0.095 | -0.053 | -0.076 |
| n=55 | P | 0.892 | 0.813 | 0.488 | 0.700 | 0.582 |
| **NPB** | *r* | -0.010 | -0.139 | 0.046 | -0.042 | -0.046 |
| n=55 | P | 0.943 | 0.311 | 0.741 | 0.760 | 0.737 |
| **NBuds** | *r* | 0.129 | 0.292 | 0.125 | 0.300 | 0.118 |
| n=55 | P | 0.349 | 0.031 | 0.364 | 0.026 | 0.392 |
| **necrosis** | *r* | -0.162 | -0.152 | -0.123 | -0.057 | -0.169 |
| n=55 | P | 0.236 | 0.267 | 0.372 | 0.680 | 0.217 |
| **apoptosis** | *r* | -0.113 | -0.025 | -0.021 | 0.011 | 0.013 |
| n=55 | P | 0.413 | 0.854 | 0.878 | 0.938 | 0.925 |
| **NDCI** | *r* | 0.101 | 0.103 | 0.022 | -0.038 | 0.114 |
| n=55 | P | 0.464 | 0.454 | 0.872 | 0.785 | 0.406 |
| **acetic acid** | *r* | | 0.865 | 0.885 | 0.354 | 0.148 |
| n=55 | P | | 0.000 | 0.000 | 0.008 | 0.282 |
| **propionic acid** | *r* | | | 0.743 | 0.569 | 0.190 |
| n=55 | P | | | 0.000 | 0.000 | 0.165 |
| **butyric acid** | *r* | | | | 0.341 | 0.146 |
| n=55 | P | | | | 0.011 | 0.287 |
| **valeric acid** | *r* | | | | | 0.473 |
| n=55 | P | | | | | 0.000 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index

## *A 6.4 Pearsons Correlation Matrix – Faecal water genotoxicity and conventional bowel biomarkers week 12*

| | | NPB | NBuds | necrosis | apoptosis | NDCI | faecal pH | faecal weight | faecal moisture | SCFAs | phenols | p-cresols n=54 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MN** | *r* | 0.496 | 0.485 | 0.168 | -0.095 | -0.170 | -0.200 | 0.017 | -0.105 | 0.022 | 0.087 | 0.010 |
| n=55 | P | 0.000 | 0.000 | 0.220 | 0.492 | 0.214 | 0.143 | 0.904 | 0.445 | 0.872 | 0.531 | 0.945 |
| **NPB** | *r* | | 0.254 | 0.238 | -0.043 | -0.311 | 0.196 | -0.066 | -0.195 | -0.097 | 0.021 | 0.156 |
| n=55 | P | | 0.062 | 0.080 | 0.754 | 0.021 | 0.152 | 0.632 | 0.153 | 0.482 | 0.883 | 0.261 |
| **NBuds** | *r* | | | 0.161 | -0.147 | -0.125 | -0.123 | 0.049 | -0.025 | 0.074 | -0.160 | 0.041 |
| n=55 | P | | | 0.241 | 0.285 | 0.364 | 0.369 | 0.722 | 0.857 | 0.593 | 0.247 | 0.770 |
| **necrosis** | *r* | | | | 0.024 | -0.738 | -0.031 | -0.057 | -0.318 | -0.008 | -0.048 | 0.152 |
| n=55 | P | | | | 0.864 | 0.000 | 0.822 | 0.680 | 0.018 | 0.955 | 0.731 | 0.272 |
| **apoptosis** | *r* | | | | | -0.040 | 0.008 | 0.224 | 0.051 | 0.138 | 0.131 | 0.092 |
| n=55 | P | | | | | 0.771 | 0.951 | 0.101 | 0.710 | 0.313 | 0.354 | 0.506 |
| **NDCI** | *r* | | | | | | -0.057 | 0.113 | 0.400 | 0.081 | 0.027 | -0.195 |
| n=55 | P | | | | | | 0.677 | 0.411 | 0.002 | 0.559 | 0.848 | 0.157 |
| **faecal pH** | *r* | | | | | | | -0.469 | -0.439 | -0.669 | -0.064 | 0.363 |
| n=55 | P | | | | | | | 0.000 | 0.001 | 0.000 | 0.647 | 0.007 |
| **faecal weight** | *r* | | | | | | | | 0.534 | 0.833 | -0.054 | -0.263 |
| n=55 | P | | | | | | | | 0.000 | 0.000 | 0.698 | 0.055 |
| **faecal moisture** | *r* | | | | | | | | | 0.540 | 0.272 | -0.397 |
| n=55 | P | | | | | | | | | 0.000 | 0.047 | 0.003 |
| **SCFAs** | *r* | | | | | | | | | | -0.032 | -0.290 |
| n=55 | P | | | | | | | | | | 0.818 | 0.033 |
| **phenol** | *r* | | | | | | | | | | | -0.091 |
| n=54 | P | | | | | | | | | | | 0.512 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index, SCFAs=short chain fatty acids

### *A 6.5 Pearsons Correlation Matrix – Faecal water genotoxicity and SCFAs week 12*

| | | acetic acid | propionic acid | butyric acid | valeric acid | caproic acid n=55 |
|---|---|---|---|---|---|---|
| **MN** | *r* | 0.003 | 0.036 | 0.055 | 0.083 | 0.170 |
| n=55 | P | 0.985 | 0.794 | 0.690 | 0.549 | 0.214 |
| **NPB** | *r* | -0.112 | -0.071 | -0.082 | 0.072 | 0.026 |
| n=55 | P | 0.418 | 0.607 | 0.553 | 0.600 | 0.848 |
| **NBuds** | *r* | 0.054 | 0.066 | 0.104 | 0.176 | 0.196 |
| n=55 | P | 0.697 | 0.632 | 0.450 | 0.199 | 0.152 |
| **necrosis** | *r* | -0.036 | -0.030 | 0.053 | 0.173 | 0.053 |
| n=55 | P | 0.795 | 0.829 | 0.702 | 0.206 | 0.700 |
| **apoptosis** | *r* | 0.110 | 0.148 | 0.178 | 0.014 | 0.214 |
| n=55 | P | 0.425 | 0.280 | 0.192 | 0.921 | 0.116 |
| **NDCI** | *r* | 0.097 | 0.104 | 0.033 | -0.047 | 0.161 |
| n=55 | P | 0.483 | 0.448 | 0.810 | 0.734 | 0.242 |
| **acetic acid** | *r* | | 0.941 | 0.927 | 0.755 | 0.309 |
| n=55 | P | | 0.000 | 0.000 | 0.000 | 0.022 |
| **propionic acid** | *r* | | | 0.867 | 0.815 | 0.326 |
| n=55 | P | | | 0.000 | 0.000 | 0.015 |
| **butyric acid** | *r* | | | | 0.733 | 0.290 |
| n=55 | P | | | | 0.000 | 0.032 |
| **valeric acid** | *r* | | | | | 0.464 |
| n=55 | P | | | | | 0.000 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index

### *A 6.6 Pearsons Correlation Matrix – Faecal water genotoxicity and conventional bowel biomarkers week 52*

| | | NPB | NBuds | necrosis | apoptosis | NDCI | faecal pH | faecal weight | faecal moisture | SCFAs | phenols | p-cresols n=33 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MN** | *r* | 0.041 | 0.200 | -0.151 | -0.151 | 0.184 | -0.059 | 0.152 | -0.112 | 0.037 | 0.176 | -0.076 |
| n=33 | P | 0.820 | 0.264 | 0.403 | 0.402 | 0.305 | 0.743 | 0.400 | 0.535 | 0.840 | 0.327 | 0.676 |
| **NPB** | *r* | | 0.083 | 0.050 | 0.147 | -0.224 | -0.108 | 0.068 | -0.020 | 0.054 | -0.044 | 0.063 |
| n=33 | P | | 0.648 | 0.784 | 0.414 | 0.210 | 0.548 | 0.706 | 0.910 | 0.764 | 0.807 | 0.726 |
| **NBuds** | *r* | | | 0.278 | -0.069 | -0.159 | -0.274 | 0.442 | 0.425 | 0.510 | -0.048 | -0.461 |
| n=33 | P | | | 0.118 | 0.703 | 0.375 | 0.122 | 0.010 | 0.014 | 0.002 | 0.790 | 0.007 |
| **necrosis** | *r* | | | | -0.314 | -0.793 | -0.194 | 0.120 | -0.122 | 0.126 | 0.033 | -0.063 |
| n=33 | P | | | | 0.075 | 0.000 | 0.280 | 0.507 | 0.498 | 0.485 | 0.853 | 0.730 |
| **apoptosis** | *r* | | | | | 0.442 | 0.122 | 0.201 | 0.180 | 0.134 | -0.226 | -0.097 |
| n=33 | P | | | | | 0.010 | 0.498 | 0.262 | 0.317 | 0.458 | 0.206 | 0.592 |
| **NDCI** | *r* | | | | | | 0.191 | 0.106 | 0.071 | -0.012 | -0.184 | 0.007 |
| n=33 | P | | | | | | 0.287 | 0.559 | 0.695 | 0.947 | 0.306 | 0.970 |
| **faecal pH** | *r* | | | | | | | -0.564 | -0.561 | -0.714 | -0.020 | 0.605 |
| n=33 | P | | | | | | | 0.001 | 0.001 | 0.000 | 0.912 | 0.000 |
| **faecal weight** | *r* | | | | | | | | 0.575 | 0.915 | -0.199 | -0.490 |
| n=33 | P | | | | | | | | 0.000 | 0.000 | 0.268 | 0.004 |
| **faecal moisture** | *r* | | | | | | | | | 0.752 | -0.108 | -0.663 |
| n=33 | P | | | | | | | | | 0.000 | 0.549 | 0.000 |
| **SCFAs** | *r* | | | | | | | | | | -0.113 | -0.593 |
| n=33 | P | | | | | | | | | | 0.531 | 0.000 |
| **phenol** | *r* | | | | | | | | | | | 0.006 |
| n=33 | P | | | | | | | | | | | 0.975 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index, SCFAs=short chain fatty acids

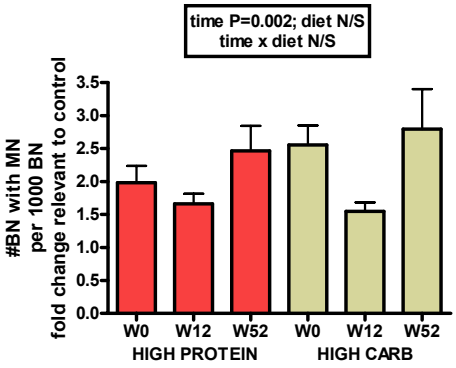
### *A 6.7 Pearsons Correlation Matrix – Faecal water genotoxicity and SCFAs week 52*

| | | acetic acid | propionic acid | butyric acid | valeric acid | caproic acid n=33 |
|---|---|---|---|---|---|---|
| **MN** | *r* | 0.017 | 0.006 | 0.097 | 0.090 | 0.023 |
| n=33 | P | 0.924 | 0.973 | 0.593 | 0.620 | 0.899 |
| **NPB** | *r* | 0.024 | 0.135 | 0.058 | 0.099 | -0.022 |
| n=33 | P | 0.896 | 0.454 | 0.747 | 0.584 | 0.905 |
| **NBuds** | *r* | 0.502 | 0.423 | 0.526 | 0.216 | 0.340 |
| n=33 | P | 0.003 | 0.014 | 0.002 | 0.227 | 0.053 |
| **necrosis** | *r* | 0.157 | -0.012 | 0.127 | -0.134 | 0.169 |
| n=33 | P | 0.382 | 0.948 | 0.483 | 0.457 | 0.347 |
| **apoptosis** | *r* | 0.119 | 0.111 | 0.147 | 0.169 | 0.293 |
| n=33 | P | 0.510 | 0.540 | 0.414 | 0.347 | 0.098 |
| **NDCI** | *r* | -0.045 | 0.102 | -0.022 | 0.293 | 0.068 |
| n=33 | P | 0.802 | 0.573 | 0.903 | 0.097 | 0.708 |
| **acetic acid** | *r* | | 0.836 | 0.941 | 0.540 | 0.478 |
| n=33 | P | | 0.000 | 0.000 | 0.001 | 0.005 |
| **propionic acid** | *r* | | | 0.728 | 0.859 | 0.434 |
| n=33 | P | | | 0.000 | 0.000 | 0.012 |
| **butyric acid** | *r* | | | | 0.445 | 0.425 |
| n=33 | P | | | | 0.010 | 0.008 |
| **valeric acid** | *r* | | | | | 0.514 |
| n=33 | P | | | | | 0.002 |

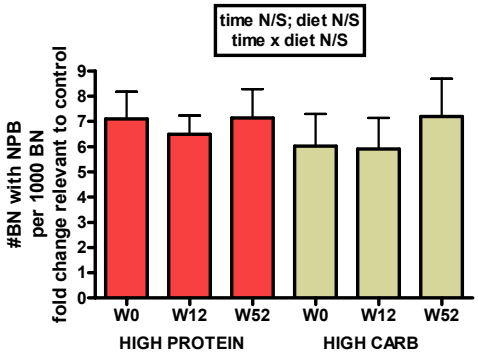
MN=micronuclei, NPB=nucleoplasmic bridges, NBud=nuclear buds, NDCI=nuclear division cytotoxicity index

## *A 6.8 FAECAL WATER GENOME DAMAGE BIOMARKERS – FOLD CHANGE*

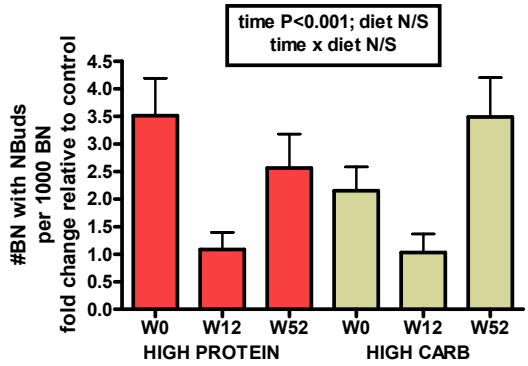
MICRONUCLEI - FOLD CHANGE



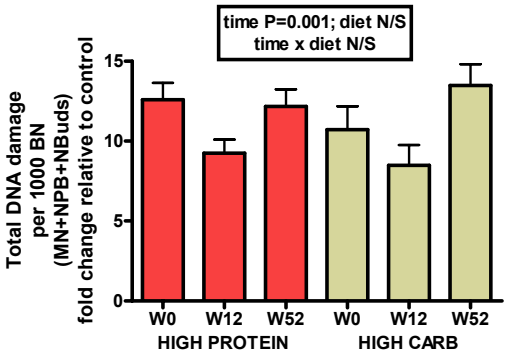

NUCLEOPLASMIC BRIDGES - FOLD CHANGE



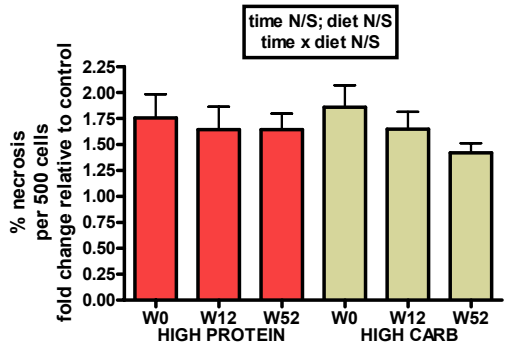

NUCLEAR BUDS - FOLD CHANGE



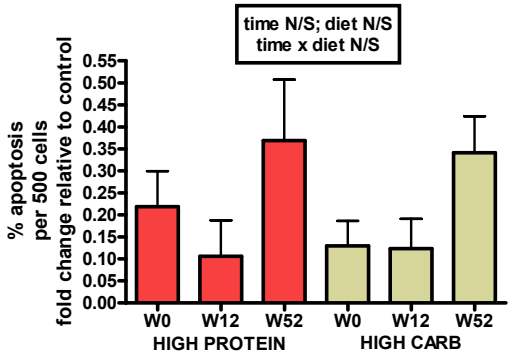

TOTAL DNA DAMAGE- FOLD CHANGE



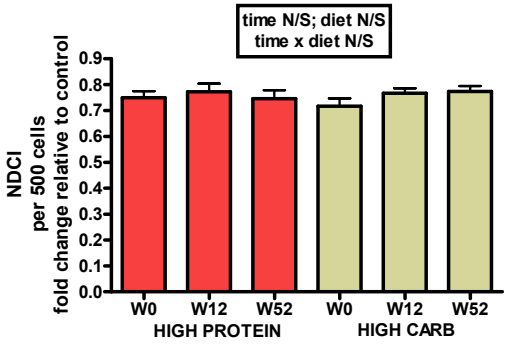

NECROSIS - FOLD CHANGE




APOPTOSIS - FOLD CHANGE




NUCLEAR DIVISION CYTOTOXICITY INDEX
FOLD CHANGE



### *A 7.1 Pearsons Correlation Matrix – Peripheral blood lymphocytes week 0*

| | | MN | NPB | NBuds | NECROSIS | APOPTOSIS | VITAMIN B12 | FOLATE | HOMOCYSTEINE n=55 |
|---|---|---|---|---|---|---|---|---|---|
| **NDCI** | *r* | -0.203 | -0.012 | 0.070 | -0.694 | -0.086 | -0.142 | -0.168 | 0.053 |
| n=55 | P | 0.137 | 0.929 | 0.612 | 0.000 | 0.530 | 0.300 | 0.221 | 0.699 |
| **MN** | *r* | | 0.057 | 0.108 | 0.003 | -0.130 | -0.050 | -0.199 | 0.071 |
| n=55 | P | | 0.678 | 0.434 | 0.980 | 0.343 | 0.715 | 0.146 | 0.605 |
| **NPB** | *r* | | | -0.107 | -0.069 | -0.268 | -0.127 | -0.098 | 0.098 |
| n=55 | P | | | 0.438 | 0.616 | 0.048 | 0.355 | 0.475 | 0.476 |
| **NBuds** | *r* | | | | 0.062 | 0.193 | -0.031 | 0.118 | -0.117 |
| n=55 | P | | | | 0.653 | 0.159 | 0.822 | 0.392 | 0.396 |
| **NECROSIS** | *r* | | | | | 0.170 | 0.310 | 0.201 | 0.082 |
| n=55 | P | | | | | 0.215 | 0.021 | 0.141 | 0.552 |
| **APOPTOSIS** | *r* | | | | | | 0.128 | -0.055 | 0.041 |
| n=55 | P | | | | | | 0.352 | 0.693 | 0.764 |
| **VITAMIN B12** | *r* | | | | | | | 0.112 | -0.012 |
| n=55 | P | | | | | | | 0.417 | 0.928 |
| **FOLATE** | *r* | | | | | | | | -0.364 |
| n=55 | P | | | | | | | | 0.006 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, NDCI=nuclear division cytotoxicity index

### *A 7.2 Pearsons Correlation Matrix – Peripheral blood lymphocytes week 12*

| | | MN | NPB | NBuds | APOPTOSIS | NECROSIS | VITAMIN B12 | FOLATE | HOMOCYSTEINE n=55 |
|---|---|---|---|---|---|---|---|---|---|
| **NDCI** | *r* | -0.041 | -0.161 | -0.341 | -0.066 | -0.665 | -0.058 | -0.229 | -0.060 |
| n=55 | P | 0.764 | 0.240 | 0.011 | 0.634 | 0.000 | 0.673 | 0.093 | 0.664 |
| **MN** | *r* | | 0.285 | 0.150 | 0.173 | -0.077 | -0.029 | 0.149 | -0.010 |
| n=55 | P | | 0.035 | 0.273 | 0.207 | 0.574 | 0.835 | 0.278 | 0.943 |
| **NPB** | *r* | | | 0.136 | -0.021 | -0.057 | -0.039 | -0.058 | -0.040 |
| n=55 | P | | | 0.323 | 0.882 | 0.677 | 0.779 | 0.673 | 0.772 |
| **NBuds** | *r* | | | | 0.171 | 0.291 | -0.031 | -0.045 | -0.031 |
| n=55 | P | | | | 0.212 | 0.031 | 0.823 | 0.747 | 0.822 |
| **APOPTOSIS** | *r* | | | | | 0.238 | -0.122 | 0.239 | -0.119 |
| n=55 | P | | | | | 0.080 | 0.376 | 0.079 | 0.388 |
| **NECROSIS** | *r* | | | | | | 0.119 | 0.341 | 0.117 |
| n=55 | P | | | | | | 0.388 | 0.011 | 0.394 |
| **VITAMIN B12** | *r* | | | | | | | 0.280 | 0.998 |
| n=55 | P | | | | | | | 0.039 | 0.000 |
| **FOLATE** | *r* | | | | | | | | 0.289 |
| n=55 | P | | | | | | | | 0.032 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, NDCI=nuclear division cytotoxicity index

### *A 7.3 Pearsons Correlation Matrix – Peripheral blood lymphocytes week 52*

| | | MN | NPB | NBuds | APOPTOSIS | NECROSIS | VITAMIN B12 | FOLATE | HOMOCYSTEINE n=32 |
|---|---|---|---|---|---|---|---|---|---|
| **NDCI** n=33 | *r* | 0.202 | 0.032 | -0.199 | -0.061 | 0.571 | -0.042 | 0.280 | 0.016 |
| | P | 0.259 | 0.858 | 0.267 | 0.734 | 0.001 | 0.818 | 0.115 | 0.930 |
| **MN** n=33 | *r* | | 0.115 | 0.158 | -0.026 | -0.337 | -0.078 | -0.201 | -0.052 |
| | P | | 0.523 | 0.380 | 0.885 | 0.055 | 0.673 | 0.262 | 0.778 |
| **NPB** n=33 | *r* | | | -0.003 | -0.041 | -0.066 | -0.015 | -0.010 | -0.316 |
| | P | | | 0.988 | 0.822 | 0.714 | 0.936 | 0.956 | 0.078 |
| **NBuds** n=33 | *r* | | | | 0.235 | 0.548 | -0.247 | 0.036 | 0.259 |
| | P | | | | 0.187 | 0.001 | 0.173 | 0.843 | 0.152 |
| **APOPTOSIS** n=33 | *r* | | | | | 0.217 | -0.051 | 0.266 | 0.304 |
| | P | | | | | 0.224 | 0.780 | 0.134 | 0.091 |
| **NECROSIS** n=33 | *r* | | | | | | -0.235 | -0.170 | 0.283 |
| | P | | | | | | 0.196 | 0.343 | 0.116 |
| **VITAMIN B12** n=32 | *r* | | | | | | | 0.194 | -0.290 |
| | P | | | | | | | 0.289 | 0.113 |
| **FOLATE** n=33 | *r* | | | | | | | | 0.076 |
| | P | | | | | | | | 0.681 |

MN=micronuclei, NPB=nucleoplasmic bridges, NBuds=nuclear buds, NDCI=nuclear division cytotoxicity index