The role of physical activity in the molecular regulation of colon cancer risk

by

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Abstract

A strong body of observational evidence supports the notion that physical activity (PA) is inversely associated with colon cancer (CC) risk. Epigenetic alterations, such as aberrant DNA methylation, are apparent in the early stages of carcinogenesis. Recent evidence has suggested that PA can alter DNA methylation patterns in a variety of tissues. It is not known whether PA can affect DNA methylation patterns in genes implicated in CC risk. Study 1 was a longitudinal investigation of 253 females (70.7 ± 4.0 yrs) and 137 males (71.4 ± 4.3 yrs) for whom DNA from peripheral blood leukocytes were available. Participants showed differential DNA methylation of relevant genes depending on whether they had increased, decreased, or maintained their PA level over eight years. Study 2 was a randomised controlled trial of sedentary colon adenoma patients (n = 31; 68.5 ± 3.2 yrs) who were randomised to a 6-12 month Active Lifestyle Programme or Usual Care. DNA methylation patterns of CC risk genes in buccal cells were unaffected by taking part in the Active Lifestyle Programme. Low recruitment probably resulted in the study being underpowered, and buccal cells may not have been a suitable surrogate marker of colon DNA methylation. A lack of colon biopsies prevented any analysis of changes in methylation in this tissue. Due to the absence of colon biopsies, Study 3 tested whether serum from physically active or inactive volunteers affected the proliferation of *in vitro* models of colon epithelia, in the fasted state and after a single bout of treadmill running. A randomised controlled trial with a crossover design was conducted in 20 male participants (59 ± 6.0 yrs) free of metabolic or cardiovascular disease, and data pooled together with 20 male colon adenoma patients from the previous investigation. This study showed that male adenoma patients' cardiorespiratory fitness was inversely correlated with serum-stimulated growth of the colorectal cancer cell line Caco-2 *in vitro*, but no relationship was observed in disease-free volunteers at rest or after exercise. In conclusion, this series of studies demonstrated that whilst PA is associated with beneficial changes in DNA methylation longitudinally, this might not be realised in a trial setting with sedentary older persons. It remains unclear whether increasing PA is an effective strategy for reducing CC risk via changes to DNA methylation patterns. Furthermore, it has suggested that

cardiorespiratory fitness might be important in modulating the growth of the Caco-2 cell line, but more investigation is needed.

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List of Abbreviations

ACF	Aberrant crypt foci
ACS	American Cancer Society
AICR	American Institute of Cancer Research
ALP	Active Lifestyle Programme
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AOM	Azoxymethane
ATCC	American Type Culture Collection
APC	Adenomatous polyposis coli
ATCC	American Type Culture Collection
BBSRC	Biotechnology and Biological Sciences Research Council
BME	Beta-mercaptoethanol
BMI	Body mass index
BORICC	Biomarkers of Risk in Colorectal Cancer
BSA	Bovine serum albumin
CC	Colon cancer
CGI	CpG island
CHS	CpG island methylator phenotype
CIMP	Cardiovascular Health Study
CIN	Chromosomal instability
COBRA	Combined bisulphite restriction analysis
CONSORT	Consolidated Standards of Reporting Trials
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CpG	5’ – cytosine – phosphate – guanine – 3’
CRC	Colorectal cancer
CRF	Cardiorespiratory fitness
CRUK	Cancer Research UK
CT	Cycle threshold
CVD	Cardiovascular disease
DCA	Deoxycholate

DMEM	Dulbecco's Modified Eagle Medium
DMH	Dimethylhydrazine
DNMT	DNA methyltransferase
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EGF	Endothelial growth factor
EPIC	European Prospective Investigation into Cancer and Nutrition
FAP	Familial adenomatous polyposis
FOBT	Faecal occult blood test
FXR	Farnesoid x receptor
GCP	Good Clinical Practice
GITT	Gastrointestinal Transit Time
GP	General Practitioner
HAPAQ	Historical Adulthood Physical Activity Questionnaire
HDAC	Histone deacetylase
HIIT	High-intensity interval training
HNPCC	Hereditary non-polyposis colorectal cancer
HSE	Health Survey for England
HST	HotStarTaq
IBD	Inflammatory bowel disease
IFR	Institute of Food Research
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IPAQ	International Physical Activity Questionnaire
Kb	kilobase
LOH	Loss of heterozygosity
LOI	Loss of imprinting
LUMA	Luminometric methylation assay
MCP	Monocyte chemoattractant protein
MEM	Membrane desalting buffer
MET	Metabolic equivalent of task
MMR	Mismatch repair

MSI	Microsatellite instability
MT	Mutant
MVPA	Moderate to vigorous physical activity
NaOAc	Sodium acetate
ncRNA	non-coding RNA
NHLBI	National Heart, Lung and Blood Institute
NHS	National Health Service
NIA	National Institute of Aging
NINDS	National Institute of Neurological Disorders
NNUH	Norfolk and Norwich University Hospital
NPS	National Polyp Study
NSAID	Non-steroidal anti-inflammatory drug
ONS	Office of National Statistics
PA	Physical activity
PAEE	Physical activity energy expenditure
PARC	Physical Activity and Risk of Colon Cancer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PL	Phospholipase
PS	Penicillin-streptomycin
QMSP	Quantitative methylation-specific polymerase chain reaction
RCF	Relative centrifugal force
RCT	Randomised controlled trial
REC	Research ethics committee
ReGro	Regular Exercise and colon cell Growth
RER	Respiratory exchange ratio
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
RR	Risk ratio
RT-PCR	Real-time polymerase chain reaction
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences

TBE	Tris-borate EDTA buffer
TEMED	Tetramethylethylenediamine
TET	Ten-eleven translocation
TNF	Tumour necrosis factor
TNM	Tumour, node, metastasis
TSG	Tumour suppressor gene
UC	Ulcerative colitis
UC	Usual care
WCRF	World Cancer Research Fund
WST	Water-soluble tetrazolium
WT	Wild type

Chapter 1

Review of Literature

1.1 Aetiology of colon cancer

Cancer is characterised by unchecked cell growth, including, but not limited to, inhibition of programmed cell death (apoptosis) and acceleration of cellular proliferation. In the UK, approximately two thirds of colorectal cancers (CRC) are colon cancer (CC) (CRUK, 2015). Colon cancer (CC) is a progressive, non-communicable disease affecting the large colon, and the third most common form of cancer in the United Kingdom. Over 90% of cases occur in individuals over the age of 50, with two thirds of these being in men. There are three principal methods of determining the severity of the disease in the clinical setting: Duke's stage (A-D), TNM (tumour, node, metastasis) and Stage (0 – IV). Briefly, a cancer at stage 0-1 is contained within the colon. A Stage II tumour has penetrated the outer lining and perhaps spread to other nearby tissue. At Stage III, the morphology of the tumour may be similar to 0 – II, but it has spread to regional lymph nodes. Finally, stage IV tumours have metastasized to other organs via the lymph or circulatory system, which leads to organ failure and ultimately death. The chances of survival are inversely correlated to the stage, so early diagnosis and treatment improves patient outcomes.

CC in the absence of a known hereditary cancer syndrome, accounts for > 90% of cases (Watson and Collins, 2011; Weitz *et al.*, 2005). The age of onset is usually 60 years and above and although lacking a definitive inherited component, it is estimated that > 20% of CC cases are related to family history (de la Chapelle, 2004; Weitz *et al.*, 2005). Its aetiology broadly follows the adenoma-carcinoma sequence model described by Fearon & Vogelstein, whereby mutations can inactivate tumour suppressor genes and concurrently activate oncogenes associated with tumour development (Fearon and Vogelstein, 1990). This can lead to the formation of benign abnormal tissue, known as an adenoma. Adenomas usually take the form of polyps (small extrusions on the lining of the large intestine) which can eventually become cancerous should somatic mutations continue to accumulate (Figure 1.1) (Fearon,

2011). The adenomatous polyp is a physical indicator of deregulated cell growth, differentiation and apoptosis in the early stages of CC (Figure 1.1).

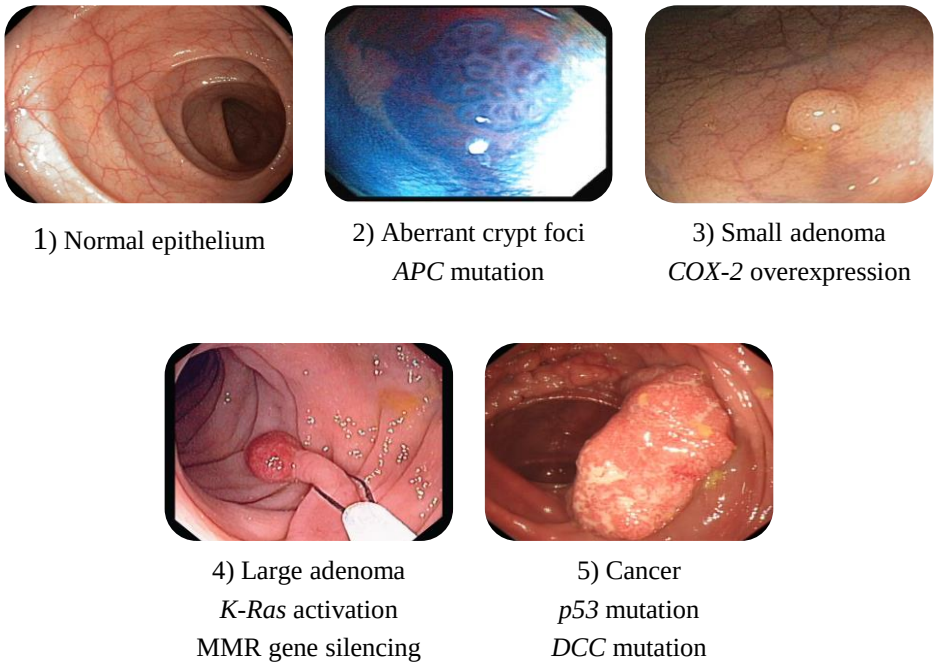


Figure 1.1 Transition from normal epithelium to cancer via the 'classic' sequence model and some of the common molecular perturbations at each stage.

1.2 Global incidence of colorectal cancer

In developed countries, colorectal cancer (CRC) is estimated to be the third most prevalent by absolute incidence in males and females combined, accounting for approximately 727,400 new cases and 320,100 deaths in 2008 (Jemal *et al*., 2011). Globally, its incidence and mortality is markedly higher in males than females, although this varies by region (Center *et al*., 2009b). Alarmingly, the incidence of these cancers appears to be increasing in several countries with developing economies, notably in Eastern Europe and South East Asia (Center *et al*., 2009b). For example, CRC incidence in Slovakia per 100,000 persons has increased by 54% and 31% in males and females, respectively, between the periods of 1983-87 to 1998-2002 (Center *et al*., 2009b). This is reflected in mortality rates from such regions, with seven of the ten countries with the highest CRC mortality rates as of 2005 being located in Central and Eastern Europe (Center *et al*., 2009a). Mortality from the disease has decreased or stabilised in developed economies such as the United States (Edwards *et al*., 2010), UK, and Japan. For instance, mortality in Japan has decreased

year-on-year by 0.9% and 5% in males and females, respectively, from 1996 – 2005 (Center *et al*., 2009a), despite incidence in the northern Miyagi and Yamagata prefectures increasing two-fold in men and by approximately half in women between the years 1983 – 2002 (Center *et al*., 2009b). This might reflect improving treatment and/or detection of CRC in economically developed nations; many of whom have national bowel cancer screening programmes in place (Center *et al*., 2009a). The same cannot be said of some developing countries, particularly in Eastern Europe, where the increase in CRC incidence over the last three decades is associated with increased mortality.

Rates of CRC are typically low in less industrialised world regions, and are highest in developed and transitional economies. It has been observed that migrants to Western nations typically adopt the CRC risk profile of the host country (Key *et al*., 2002). As a case in point, Japanese migrants to the western United States – whilst initially maintaining a similar risk of CRC mortality as their nation of birth (Haenszel and Kurihara, 1968) – adopted the risk profile of the resident white population within two generations (Shimizu *et al*., 1987), and even exceeded the risk of the latter (Flood *et al*., 2000). Furthermore, > 90% of CRC cases occur in the absence of a known heritable disease (Watson and Collins, 2011; Weitz *et al*., 2005). Thus, it has been postulated that CRC is a disease of 'Westernisation' (Watson and Collins, 2011). That is, aspects of a 'Western' lifestyle e.g. increased life expectancy, smoking, and diet composed of surplus energy, meat and processed foods and deficient in fruit, fibre and vegetables, and a lack of physical activity (PA) contribute to the incidence of CRC.

1.3 Colon cancer risk factors

1.3.1 Age

Advancing age is the single biggest risk factor for CRC and CC (Armitage and Doll, 1954). The authors predicted that this was due to a sequential change in cell phenotype in response to chronic stimuli some 36 years before Fearon and Vogelstein published their adenoma-carcinoma sequence model (Fearon and Vogelstein, 1990) Indeed, CC was responsible for 8248 deaths in England and Wales in 2010, of which 95% were in persons aged 60 or over (ONS, 2011). This, in part, is due to the

increased probability of replicating cells acquiring a mutation over time (Kelly, 2012). Lifestyle factors appear to be able to influence this rate of acquisition, which in turn alter risk.

1.3.2 Smoking

Whilst strongly implicated in a host of cancers, particularly lung and nasopharyngeal, its relationship with CRC is less straightforward. A recent meta-analysis reported that current or former smokers, compared with those who have never smoked, were 18% more likely to develop CRC. In addition, the likelihood of developing CRC accumulated by 9.5% per 10 years of smoking, which posed a statistically significant risk after 30 years (Botteri *et al.*, 2008a) Moreover, another meta-analysis from the same group reported that current smokers were more than twice as likely to develop adenomatous polyps (the pre-cancerous lesion underpinning the majority of CRC cases), than never-smokers (Botteri *et al.*, 2008b). One explanation for an increase in risk is a greater prevalence of smoking in developed nations.

1.3.3 Gender

Men are at greater risk than women for contracting CRC (Watson and Collins, 2011). The mechanisms by which this occurs are not known, however, it has been observed that peak incidence rates in developed countries amongst women is delayed by 4-8 years compared with men (Brenner *et al.*, 2007a). The author suggests that this could be related to the onset of menopause, as age-matched premenopausal women are at reduced risk for CC than postmenopausal women (Franceschi *et al.*, 2000b). Furthermore, risk behaviours for CC are more frequent amongst men than women, such as obesity and/or abdominal adiposity, higher alcohol intake, higher red meat consumption and smoking (Johnson *et al.*, 2013).

1.3.4 Anthropometry

Five meta-analyses of body mass index (BMI) and CC incidence and mortality have indicated that there is strong association between increasing BMI and disease risk, particularly in men (Dai *et al.*, 2007; Harriss *et al.*, 2009b; Larsson and Wolk, 2007; Moghaddam *et al.*, 2007; Ning *et al.*, 2010). The latest of these reported pooled data

from 56 studies of BMI and CRC, and the authors surmised that there is an 18% increase in risk with every 5 kg/m² increment in BMI. The risk increase amounted to 41% in obese individuals (BMI ≥ 30 kg/m²) (Ning *et al.*, 2010). Given that the correlation between rectal cancer and BMI is less strong than for CC (Larsson and Wolk, 2007; Ning *et al.*, 2010) it might be that these alarming estimations are too conservative for CC alone. Indeed, other reviewers have calculated that the incremental risk per 5 kg/m² for CC specifically is 30% and 12% for males and females, respectively (Larsson and Wolk, 2007). However, it is possible that the effect of BMI might have been overestimated due to the tendency to understate BMI in self-report measures, and many of the cohort studies included for analysis by Larsson & Wolk (2007) employed this technique (Larsson and Wolk, 2007). Waist circumference and waist-hip ratio, as proxy measures of visceral adiposity, are considered stronger risk factors for CC than BMI (Pischon *et al.*, 2006). Per added inch of waist circumference, RR increases by 0.04 (0.01-0.07), and a 0.1 point increment in waist-hip ratio is associated with a RR increase of 0.27 (0.15-0.41) (World Cancer Research Fund/American Insitute of Cancer Research; WCRF/AICR, 2011). In addition, there is compelling evidence emerging for obesity increasing risk of the precursor for CC, colon adenomas (Omata *et al.*, 2013), particularly in obese, white men (Comstock *et al.*, 2014a).

1.3.5 Diet

The foods considered most likely to increase risk of CC are red and processed meat and alcohol (WCRF/AICR, 2011). Indeed, according to the WCRF/AICR Systematic Literature Review Continuous Update Project Report, a dose-response relationship is observed for processed meat (Risk Ratio (RR) = 1.24 per 50 g/day consumed), red meat (RR = 1.17 per 100 g/day) and alcohol (RR = 1.11 per drink/day) (WCRF/AICR, 2011). The mechanisms by which this might occur are beyond the scope of this review, but briefly it is posited that higher red and processed meats consumption might encourage neoplasia through the pro-carcinogenic effects of heterocyclic amines, and through DNA and tissue damage by surplus iron and N-nitroso compound formation (for review, see Lund *et al.*, 2011b).

Conversely, the foodstuffs with the most evidence of decreasing risk are dietary fibre, garlic, milk and calcium (WCRF/AICR, 2011: Table 2.1). However, it is

apparent that studies into the effects of isolated dietary constituents on risk have not attempted to account for the confounding effects of obesity and physical activity (or lack thereof). Hence, it is difficult to extricate the risk posed by, for example, meat consumption alone (Alexander *et al.*, 2011; Huxley *et al.*, 2009). This is a common problem across the cohort studies included in the WCRF/AICR Expert Report for a variety of foods. Interestingly, some inverse associations between physical activity and CC risk appear not to be confounded by diet (Slattery and Potter, 2002) Importantly, the authors observed that a 'high risk' diet was associated with the greatest relative risk increase in sedentary persons (Slattery and Potter, 2002). This suggests that the relative importance of dietary risk factors is tempered by physical activity.

Table 1.1 Lifestyle factors associated with risk of colon cancer (adapted from WCRF/AICR, 2011).

Level of evidence	**Lifestyle factors associated with risk of colon cancer**	
	Increases risk:	**Decreases risk:**
Convincing	Smoking, alcohol drinks (men), red and processed meat, abdominal fatness, obesity	Physical activity, dietary fibre
Probable	Alcoholic drinks (women)	Garlic, milk
Limited-suggestive	Foods containing iron, cheese, foods containing animal fats, foods containing sugars	Non-starchy vegetables, fruits, foods containing Vitamin D

1.3.6 Physical activity

1.3.6.1 Epidemiological studies

The first investigation to propose a link between PA and cases of CC was conducted by Garabrant and colleagues, who discovered that the disease tended to afflict men in long-term sedentary jobs. From the findings, they calculated that such individuals, irrespective of ethnicity and socioeconomic status, had 1.6 times the risk of peers in labour-intensive occupations (Garabrant *et al.*, 1984). Subsequent observational studies found similar associations between occupational PA (Brownson *et al.*, 1989; Vena *et al.*, 1985) and total PA (Albanes *et al.*, 1989; Fredriksson *et al.*, 1989; Gerhardsson *et al.*, 1988; Severson *et al.*, 1989; Slattery *et al.*, 1988; Wu *et al.*, 1987). Since then, many more studies have been produced in the field, and a host of systematic reviews have supported the association between PA and reduced risk of CC (Friedenreich *et al.*, 2010a; Kushi *et al.*, 2006; Samad *et al.*, 2005; Slattery, 2004); although the strength of this relationship has been disputed by others (Harriss *et al.*, 2009a; Spence *et al.*, 2009). Indeed, the work of Harriss and colleagues only included those studies that collected leisure-time PA data before a cancer diagnosis, thereby reducing the impact of recall bias. Whilst it still indicated that leisure-time PA shows a modest inverse association with CC, it suggested that PA interventions targeting leisure-time PA are unlikely to bring about CC risk reduction owing to the modesty of the relationship (Harris *et al*., 2009a). Similarly, in a systematic review of cohort studies the latter group found that despite a non-linear trend between CC risk and PA, it did not amount to a substantial association (Spence *et al*., 2009).

A meta-analysis of case-control and cohort studies published after the pioneering work of Garabrant *et al*. (1984) estimated that persons participating in the greatest volume and duration of PA had a 24% decrease in CC risk, compared with those engaging in the least amount on average per study (Wolin *et al.*, 2009). However, it should be noted that the definition of ‘greatest’ and ‘least’ PA was different across studies, and that the 24% average might not be an adequate reflection of the state of any relationship. Currently, the most recent WCRF/AICR continuous update report states that the evidence is ‘convincing’ that increased lifetime PA is inversely associated with CC risk, (WCRF/AICR, 2011).

1.3.6.2 Intervention studies

1.3.6.2.1 Rodent models

In male Wistar or Fischer 344 rats treated with a cancer-causing agent (dimethylhydrazine (DMH) or azoxymethane (AOM)) numerous modes of regular (> 4 d/wk) exercise training resulted in consistently reduced indices of cancer progression i.e. colonic tumour size, multiplicity, and pre-cancerous lesions (Andrianopoulos *et al.*, 1987; Demarzo *et al.*, 2008; Fuku *et al.*, 2007; Lunz *et al.*, 2008; Thorling *et al.*, 1994). On the other hand, results have been less consistent in the Adenomatous Polyposis Coli with Multiple intestinal neoplasia (APC^{Min}) mouse, which mimics the symptoms of familial adenomatous polyposis (FAP; see 2.3 Aetiology of Colon Cancer) in humans, and seem to be affected by gender, mode of exercise, energy balance and diet composition (Baltgalvis *et al.*, 2009; Basterfield and Mathers, 2010; Colbert *et al.*, 2000; Colbert *et al.*, 2003; Colbert *et al.*, 2006; Ju *et al.*, 2008; McClellan *et al.*, 2014; Mehl *et al.*, 2005). Arguably the most consistent finding in this mouse model is that exercise training might reduce the number of large polyps in male APC^{Min} mice.

Although these lend support to the current paradigm of PA and exercise having a preventative effect against CC, the applicability of these models to an aged human is questionable. Indeed, the use of highly pernicious carcinogens in young rats (Andrianopoulos *et al.*, 1987; Demarzo *et al.*, 2008; Fuku *et al.*, 2007; Lunz *et al.*, 2008; Thorling *et al.*, 1994) and mice (Aoi *et al.*, 2010; Ju *et al.*, 2008) is probably not reflective of the protracted time course of CC development in humans. In addition, to the best of the author's knowledge, all of the investigations which successfully reduced the number of colon tumours in rats also reduced body fat in the exercisers (Andrianopoulos *et al.*, 1987; Demarzo *et al.*, 2008; Fuku *et al.*, 2007; Thorling *et al.*, 1994). This confounds any insight into the role of exercise alone, since increased body fat is independently associated with CC and adenoma risk in humans (Comstock *et al.*, 2014a; Ning *et al.*, 2010; Omata *et al.*, 2013). Also, the aforementioned rat studies utilised wheel/treadmill running for a minimum of 2 km or 60 min per day on 5 days per week. It is not clear whether the biological impact of such extreme levels of exercise in rodents would be equivocal in humans who perform sufficient activity for maintenance of health.

1.3.6.2.2 Human intervention studies

The mechanisms through which PA might exert these effects upon risk, in both rodent models and in humans, are not clear. In the main, there has been a focus on circulating growth factors, sex hormones and chronic inflammation, which have been associated with cancer risk with varying degrees of evidence (Friedenreich *et al.*, 2010a). What is also not clear is whether an increase in PA or fitness in sedentary individuals affects risk. To date, only one randomised controlled trial has investigated whether an increase in PA affects any biological markers associated with risk. Amongst the findings, sedentary male polyp patients who increased their levels of moderate to vigorous PA (MVPA) to ≥ 250 min/wk or their cardiorespiratory fitness (CRF) by ≥ 5% through a 12 month exercise training programme displayed reduced proliferation of colon crypt cells (McTiernan *et al.*, 2006), and a more 'pro-apoptotic' protein profile in crypt bases (Campbell *et al.*, 2007). This suggests that an increase in PA reduces crypt cell growth (particularly in men), which might reduce the likelihood of a polyp forming. However, no data were published pertaining to any genetic or epigenetic effects in the crypts that could underlie the findings. Indeed, it has been known for decades that CC progression is fundamentally fuelled by molecular changes (Fearon and Vogelstein, 1990).

1.4 The molecular features of colon cancer

Whilst the majority of CC cases do not have purely genetic causes, it is important to acknowledge that the somatic mutations observed in colorectal tumours are often mirrored in hereditary cases. Indeed, the two most common types of hereditary CC, familial adenomatous polyposis (FAP) and hereditary nonpolyposis CRC (HNPCC/Lynch syndrome) share common genetic features with non-hereditary cases. > 90% of FAP is caused by germ-line (i.e. in sperm or egg cells) autosomal mutations on the adenomatous polyposis coli (*APC*) tumour suppressor gene (TSG). These are overwhelmingly attributed to frameshift mutations (atypical nucleotide insertions or deletions on the *APC* DNA sequence, altering the eventual amino acid formed *and* subsequent amino acids produced) or nonsense mutations (premature appearance of a stop codon on the DNA sequence, preventing full transcription), which result in non-functioning or truncated protein production (Fearon, 2011). Although not causing

carcinoma *per se*, it dramatically increases the appearance of benign colon polyps in afflicted individuals. These adenomatous growths occasionally become cancerous with additional mutations – as per the progression of sporadic CC – but such is the extreme number of polyps (> 100 by middle age, often thousands) the likelihood of untreated FAP patients developing CC increases indefinitely with age (Fearon, 2011). At present, prophylactic removal of the colon is the only treatment option to prevent CC.

On the other hand, HNPCC does not cause an abnormal production of polyps as seen in FAP. Rather, once an adenoma has formed, its progression to a carcinoma can be rapid. Indeed, the timescale for the 'classic' sporadic adenoma-carcinoma sequence described by Fearon & Vogelstein (Fearon and Vogelstein, 1990) is between 20 – 40 years; whereas in HNPCC this can be as little as 3-5 years (Fearon, 2011). This explains why the age of onset of HNPCC is significantly earlier than sporadic CC i.e. by ten years or more. The molecular basis of HNPCC is proposed to be due to germ-line mutations in DNA mismatch repair (MMR) genes, for example *MSH2* and *MLH1*. Consequently, the proteins produced by these genes are less able to recognise mismatch errors at microsatellite repeats on the genome, resulting in increased copying errors in daughter strands. This is known as microsatellite instability (MSI). Although there is no loss of heterozygosity (LOH), the unaffected allele can be inactivated by somatic (i.e. not in germ-line cells) mutations or epigenetic changes. Once this occurs, the accumulation of mutations in TSGs can be markedly faster than sporadic CC. These cancers are often associated with secondary carcinomas in other regions (e.g. endometrium and stomach), multiple CRCs at surgery (synchronous cancer), and tendency for development of a consecutive CRC unrelated to the initial lesions (metachronous cancer) after 6 months of surgery (Lynch and de la Chapelle, 2003). Typically, these cancers arise in individuals aged 40-50 years, and together they account for approximately 5 – 10% of CC cases (Lynch and de la Chapelle, 2003). A small fraction of hereditary CC is attributed to related syndromes, such as Gardner syndrome, Turcot's syndrome, and Cowden disease (Fearon, 2011).

There are other genotypic aberrations that occur in somatic incidences of the disease, as evidenced by cell lines that have been harvested and cultured from individual cancer patients. These specimens are used for *in vitro* research, and have been especially useful in the absence of being able to reliably obtain colon tissue. Each has a number of shared and different molecular features, which highlights the

heterogeneity of the disease. Some of these commonly used in our laboratory are summarised in Table 1.2.

Table 1.2 Cell lines commonly used in laboratory setting with common mutations and molecular phenotypes. Key: wt = wild type; mt = mutant; CIMP: CpG island methylator phenotype; CIN: chromosomal instability; MSI: microsatellite instability; + = positive for this phenotype; - = negative for this phenotype (adapted from Ahmed *et al*., 2013).

	Gene					**Molecular phenotype**		
Cell line	***BRAF***	***KRAS***	***PIKC3A***	***PTEN***	***TP53***	**CIMP**	**CIN**	**MSI**
Caco-2	Wt	wt	Wt	wt	mt	+	+	_
DLD-1	Wt	mt	mt	wt	mt	+	+	+
HCT-116	Wt	mt	Mt	wt	wt	+	+	_
HT-29	Mt	wt	Mt	mt	mt	+	+	_
LoVo	Wt	mt	Wt	wt	wt	_	_	+

There is accumulating evidence that alternative molecular processes are responsible for creating a 'tumour-friendly' environment, allowing the pernicious mutations seen in CC to occur. The following section will discuss the potential role of 'epigenetic' factors in this phenomenon.

1.4.1 An introduction to epigenetics

Since Conrad Waddington provided the first working description of epigenetics (Waddington, 1942), it now may be described as processes that produce a "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence." (Berger *et al*., 2009). Thus far, three of these processes have been discovered, namely interference from non-coding RNA (ncRNA), histone modifications, and DNA methylation. It is now recognised that aberrant expression of one class of ncRNAs, microRNAs, is apparent at each stage of CC (Schetter *et al*., 2012). Additionally, the modification of histones (proteins around which the DNA strand is 'wound') has been implicated in human colon carcinogenesis, notably via aberrant activation of histone deacetylases (HDAC) (Wilson *et al*., 2006). Whilst a detailed discussion of all of the epigenetic influences on CC progression is beyond the

scope of this review, it should be acknowledged that they play a pivotal role in the aetiology of the disease.

The third, and most intensively researched, epigenetic marker of CC is aberrant DNA methylation. DNA methylation is perhaps the best researched epigenetic phenomenon because the discovery of 5-methylcytosine in bacterial cultures occurred early in the 20th century (Johnson and Coghill, 1925) and it was subsequently detected as a separate residue by paper chromatography (Hotchkiss, 1948). Its relevance to human cancer was first shown in the early 1980s, owing to the finding that methylated cytosine is not cleaved by restrictive endonucleases, allowing for separation of methylated and unmethylated fragments by gel electrophoresis (Bird and Southern, 1978). Using this technique, it was observed that a genomic loss of 5-methylcytosine distinguishes colon cancers and adenomas from normal tissue (Feinberg and Vogelstein, 1983; Goelz *et al.*, 1985). It has been postulated that this process of 'hypomethylation' allows colon tumours to rapidly alter their phenotype, which could be why it is associated with progression and metastatic potential (Hoffmann and Schulz, 2005). The mechanisms by which this occurs are not completely understood. Recent work has implicated the oxidation of 5-methylcytosine by ten-eleven translocation (TET) enzymes in this process, resulting in the methyl attachment not being not being copied in daughter cells either passively or via base-excision repair (Kohli and Zhang, 2013).

With the advent of bisulphite sequencing in 1992, it was made possible to quantify the extent of methylation at specific genomic loci (Frommer *et al*., 1992). At the turn of the 21st century, a landmark study used this method to identify that aberrant methylation in gene promoter regions is a ubiquitous occurrence in a range of tumours (Esteller *et al.*, 2001). Indeed, cancer occurs in the context of gene-specific methylation in TSGs, but global loss of methylation (hypomethylation) across the methylome (Esteller, 2005). Regarding the former, *de novo* methylation of is typically facilitated by DNMT (DNA methyltransferase) enzymes 3a and 3b (Okano *et al.*, 1999). Whilst the understanding of the numerous mechanisms by which methylation represses transcription is not yet complete, there is plentiful evidence for a role of methylated binding proteins (MeBP), HDAC complexes and co-repressors recruited to the methylated region (Esteller, 2005; Jones and Baylin, 2002; Jones *et al.*, 1998). The action of HDAC results in the deacetylation of histones, ultimately causing chromatin compaction and prevention of transcription (Figure 1.2).

A cytosine-guanine pair linked by a phosphodiester bond (5' – cytosine – phosphate – G – 3') is universally known as a CpG. Many gene promoters contain regions rich in these pairs, and are known as CpG islands (CGI). The addition of a methyl group to the 5-carbon position of cytosine (forming methylcytosine) is called methylation. The DNA methylation process is vital for normal cell differentiation and development, since the process can transcriptionally silence genes that do not need to be expressed (Jones *et al.*, 1998). However, inappropriate activation and deactivation of this process has been linked to cancer (Ehrlich, 2009; Jones and Baylin, 2002). Indeed, aberrant methylation of CGIs is a hallmark of cancerous and pre-cancerous tissue, and can be used to distinguish between normal and malignant tissue (Ahuja *et al.*, 1998; Belshaw *et al.*, 2008; Chan *et al.*, 2002; Horvath, 2013). Intriguingly, more recent work has suggested that variation in methylation at CpG sites 2 kilobases (kb) upstream from the promoter makes this distinction more accurately, and is more conserved among tissue types independent of the species from which it originated (Irizarry *et al*., 2009).

1.4.2 DNA methylation patterns in humans

There is ongoing debate as to how well DNA methylation patterns are conserved across different tissues. However, it appears that there is greater conservancy of DNA methylation patterns in the same tissues from different persons than there is between different tissues from the same person (Rakyan *et al*., 2008; Byun *et al*., 2009; Slieker *et al*., 2013; Lokk *et al*., 2014). All human investigations sampling multiple organs and tissues have come from autopsies. Initial work in these samples have suggested that across sixteen tissue types, including lung, colon, pancreas and whole blood, 18% of the genomic loci investigated could be classified as differentially methylated. Interestingly, there was a tendency for this variation to occur in promoters that were relatively less rich in CpGs (Rakyan *et al*., 2008). Later work, which analysed the methylation status of 1505 CpG loci in sixteen tissues from six autopsy specimens, found that 29% were differentially methylated (Byun *et al*., 2009). Both of these works were limited by the available technology, in that only relatively small, select

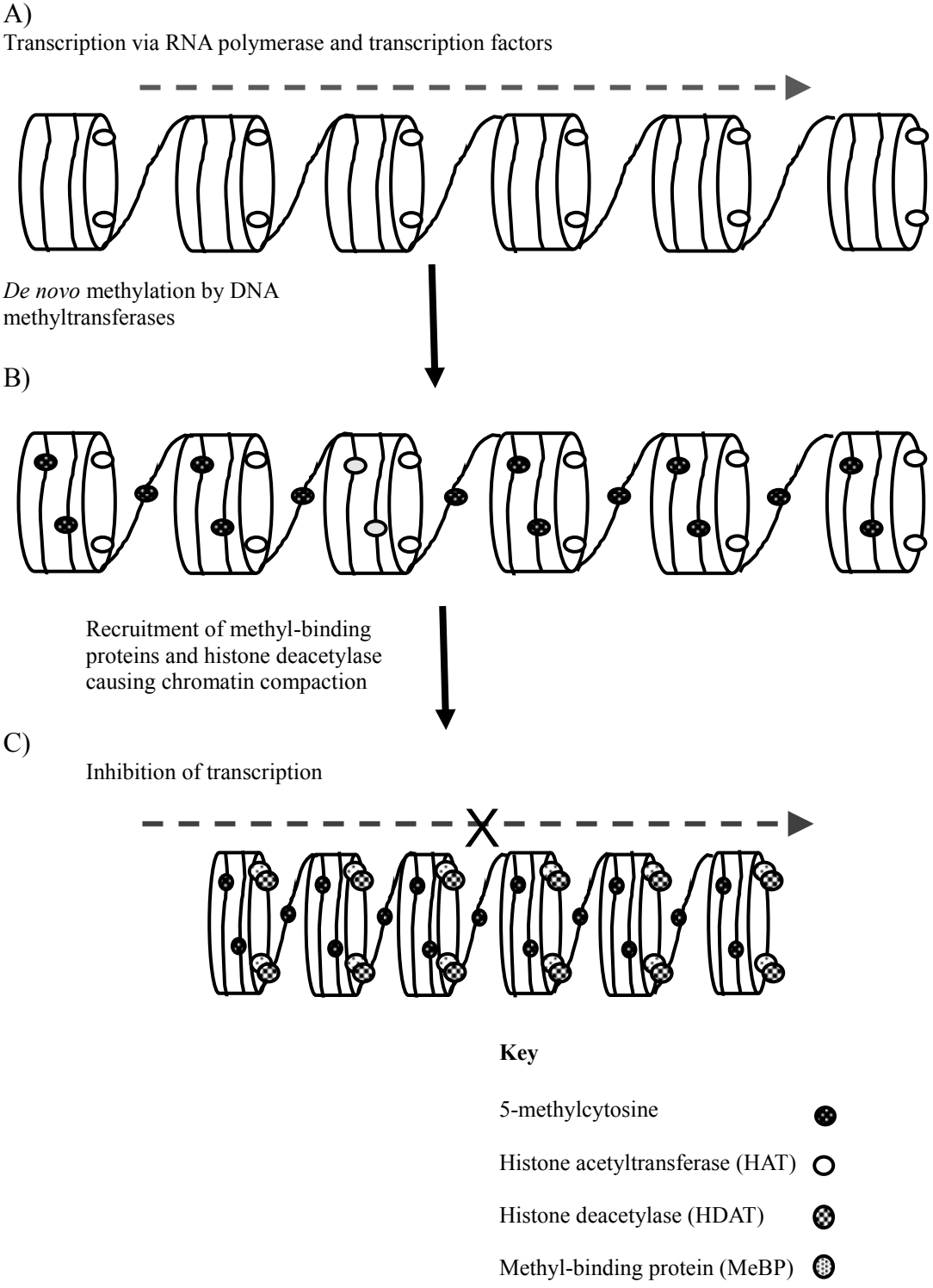


Figure 1.2 Methylation-induced transcriptional repression at a CpG island. A) depicts chromatin in its 'open' state with histones acetylated allowing for transcription. *De novo* methylation of cytosine residues by DNMTs occurs in B), recruiting histone deacetylase and associated methyl-binding proteins to cause chromatin compaction and suppression of transcription in C).

regions of the 'methylome' were investigated. More recent studies that took advantage of an Illumina 450k array, that interrogates > 450,000 CpG sites on the genome, found

that the DNA methylation patterns vary greatly by tissue type. Indeed, in one investigation, 14,441 differentially methylated regions were identified, of which 79% were in the same locale as genes. Perhaps surprisingly, 41.7% of these were in the promoter region and a mere 36.5% were in CGIs (Lokk *et al*., 2014). The second such study using this method showed there is a very strong correlation between whole blood DNA methylation marks and that of omentum and liver tissue, and that there is overlap of DNA methylation marks between tissues (Slieker *et al*., 2013). This is of particular interest to research into finding surrogate indices of disease risk (e.g. whole blood DNA methylation) to predict or track health in less accessible tissues. However, it should be noted that that study only used blood/organ samples from two individuals, and that it is possible that the correlation could be explained by genetic variations i.e. SNPs (Slieker *et al*., 2013), and are as such not modifiable by lifestyle factors.

1.4.3 Role of DNA methylation in colon cancer development

Some colorectal tumours have been identified as having particularly high levels of CGI methylation, and have been dubbed 'CGI methylator phenotype' (CIMP; see Table 1.2) (Jass, 2007). However, there is emerging evidence to suggest that aberrant age-related CGI methylation is an early event in CC development, irrespective of the eventual phenotype of the tumour (Belshaw *et al*., 2010). The lining of the colon consists of billions of small indentations that secrete mucus and absorb faecal water, known as crypts. CGI methylation in a panel of genes known to regulate colon crypt cell homeostasis is dramatically raised in colon tumour cells compared with normal intestinal mucosa from cancer patients (Belshaw *et al.*, 2008). Yet this study also demonstrated that, compared with neoplasm-free and polyp patients, the normal mucosa from cancer patients had identifiably altered CGI methylation in the same genes (Belshaw *et al.*, 2008). This suggests that aberrant CGI methylation is a global phenomenon that consists of epigenetic modification of the entire mucosal field. This so called 'field effect' is also observed in mucosal protein expression, which is altered in normal epithelia of cancer patients compared with healthy controls (Polley *et al.*, 2006). Given that aberrant CGI methylation is apparent in 'normal' mucosa of CC patients, this could signify that such epigenetic changes occur early in cancer development. These changes might 'prime' the affected mucosa for the formation of aberrant crypt foci (ACF) that are thought to be the initial precursor lesions in the

neoplastic pathway (Nucci *et al.*, 1997; Roncucci *et al.*, 1991). Certainly, CGI methylation is a frequent finding in ACF (Chan *et al.*, 2002). Aberrant methylation, therefore, could increase susceptibility to CC by creating an environment that selects for tumour propagation.

1.5 How might physical activity affect DNA methylation in the colon?

PA is purported to reduce the risk of CC through several biologically plausible mechanisms of action, including effects on gastrointestinal transit time (GITT), bile acid production, localised gut inflammation, chronic inflammation, steroid hormones, insulin action, immune function, and adipokines (Friedenreich *et al*., 2010). The next section of this review will focus on the available evidence for these biomarkers of risk affecting DNA methylation processes, and whether PA has been shown to play a role in modifying them.

1.5.1 Gastrointestinal Transit Time (GITT)

The authors of the initial investigations into PA and CC risk (Garabrant *et al.*, 1984) proposed that accelerated movement of the faecal stream in the large bowel encouraged by physical exertion (Holdstock *et al.*, 1970; Koffler *et al.*, 1992; Oettle, 1991) is the principal mechanism of combating neoplasia. In essence, the contact time between dietary carcinogens and the lining of the colon is theoretically reduced, hence decreasing the likelihood of a cancer. Although this explanation is attractively simple, there is now sufficient evidence to confirm that reduced gastrointestinal transit time is unlikely to have a bearing on CC development (Chan and Giovannucci, 2010; Harriss *et al.*, 2007; Rogers *et al.*, 2008). Also, it is likely that any changes in gut motility brought about by exercise have a negligible effect on risk (Friedenreich *et al.*, 2010a).

1.5.2 Bile acids

Identified as an endogenous carcinogen 75 years ago (Cook, 1940) bile acids have long been suspected as a risk factor for CC. Indeed, an early epidemiological study found that faecal content of deoxycholic acid (DCA) is higher in CC and adenoma patients than colorectal disease-free controls (Reddy and Wynder, 1977). More recent

studies showed that human and mouse colon crypt cells and CC cell lines exposed to bile acids consistently induced reactive oxygen species (ROS) with evidence of DNA damage (Bernstein *et al.*, 2009). These observations have led to the hypothesis that prolonged exposure to bile acids, which have a pro-apoptotic effect on normal colonic epithelial cells, promotes the selection of cells that are resistant to apoptosis, thereby increasing the likelihood of cancer (Crowley-Weber *et al.*, 2002; Magnuson *et al.*, 1994). This process might be mediated in part by epigenetic processes, as studies in rodents have found that feeding supraphysiological concentrations of deoxycholate in Fischer 344 rats and B6C3F1 mice induced aberrant DNA hypomethylation in the colon (Pereira *et al.*, 2004). More recently, the expression of the bile acid receptor, Farnesoid X receptor (FXR), in human colon adenomas and tumours was found to be partially regulated by DNA methylation (Bailey *et al.*, 2014).

It is not known whether PA affects bile acid production in humans. To date, only two studies have examined this possibility. Compared with sedentary controls, male distance runners produced less faecal bile acids, but this observation was non-significant when adjusted for diet composition (Sutherland *et al.*, 1991). In another study, the number of self-reported minutes of recreational PA (which consisted of a host of moderate to vigorous intensity activities) performed daily by colon adenoma patients aged 40-80 was inversely related to faecal bile acid presence, except in patients who exhibited 'high' (> 136 mg/dl) levels of circulating triglycerides (Wertheim *et al.*, 2009). However, these results must be viewed with caution, as the mean number of minutes patients reported as exercising was 75 min/day, which is highly improbable given that this would equate to 525 min per week of moderate to vigorous PA. For reference, according to the 2008 Health Survey for England (HSE) self-report measures of PA, only 33% of males and 29% of females aged 16 or over are achieving the 150 min per week minimum, and according to accelerometry data, this is as low as 6% and 4% in males and females, respectively (Aresu *et al.*, 2009). As such, there is a need for more robust observational studies, with better measurements of PA, before any associations between PA and bile acids can be confirmed.

1.5.3 Cardiorespiratory fitness

Low cardiorespiratory fitness (CRF) is a powerful predictor of all-cause mortality (Lee *et al.*, 1999; Lee *et al.*, 2011a; Myers *et al.*, 2002); and research suggests that cardiorespiratory fitness predicts cancer-specific mortality irrespective of BMI in men and women (Evenson *et al.*, 2003; Farrell *et al.*, 2007; Farrell *et al.*, 2011) and digestive cancer mortality in men (Peel *et al.*, 2009). It should be acknowledged that these studies did not derive the risk of CC specifically, and the association between CC and CRF (rather than amount of PA) is yet to be investigated. Nonetheless, a Finnish study found a 3.5 $ml \cdot kg \cdot min^{-1}$ increase in baseline maximal aerobic capacity (VO_{2max}) conferred a 12% reduced risk of gastrointestinal cancers in a cohort of men aged 42-60 years during ~17 years of follow-up, after adjusting for age, BMI and other lifestyle behaviours (Laukkanen *et al.*, 2010). At present it is unclear whether VO_{2max} directly affects risk; or merely appears to do so, as those who are younger and/or do more PA tend to have a higher VO_{2max}. This is an important point, as it is apparent that some individuals do not improve their VO_{2max} in response to exercise training (Bouchard *et al.*, 2011; Timmons *et al.*, 2010). The available, albeit limited, evidence suggests that increased PA in the absence of improving VO_{2max} might be sufficient to reduce risk. Indeed, sedentary male adenoma patients who increased their levels of moderate PA to ≥ 250 min/wk *or* their VO_{2max} by ≥ 5% through a 12 month exercise training programme, displayed reduced indices of proliferation in colon crypts (McTiernan *et al.*, 2006).

The physiological mechanisms underpinning any possible relationships are, again, not clear. It might be that CRF is associated with the maintenance of 'healthy' levels of other markers linked to risk. It might also be argued that enhanced CRF reflects a greater capacity to 'cope' with oxidative stress. Oxidative stress is purportedly associated with degenerative diseases, such as ageing and cancer (Ames *et al.*, 1993), and might be one mechanism by which chronic inflammation could promote tumorigenesis (Federico *et al.*, 2007; Tudek and Speina, 2012). It has been reported that 8-oxo-7,8-dihydroguanosine (8-oxodG), a marker of oxidative DNA damage, is more prevalent in leukocytes and urine of colorectal adenoma patients compared with healthy controls. This was associated with a plasma reduction of the antioxidants retinol and α-tocopherol (Obtulowicz *et al.*, 2010). Similar findings were reported in the EPIC cohort, where plasma reactive oxygen metabolites were

positively associated with CC risk (Leufkens *et al.*, 2012). Such changes could be indicative of risk; but equally might reflect disease progression, since these changes are exacerbated in CC (Obtulowicz *et al.*, 2010). Indeed, researchers in the EPIC investigation concluded that pre-clinical tumours were most likely to have been responsible for the associations between oxidative stress biomarkers and CC (Leufkens *et al.*, 2012).

Furthermore, particular single nucleotide polymorphisms (SNPs) in genes associated with oxidative stress (e.g. eoisinophil peroxidise (*EPX*) and nitric oxide synthase (*NOS2*)) have been associated with greater risk of CC, and that the risk increases with number of 'risky' SNPs. Interestingly, the authors noted that this risk was reduced by dietary antioxidant intake, including Vitamin D, lycopene and folate, even in those with the 'riskiest' SNP profile (Slattery *et al.*, 2012). If exercise has an 'anti-oxidant' effect similar to that of dietary antioxidants, it might reduce oxidative stress and, possibly, CC risk. There is accumulating evidence that oxidative stress can exert effects on gene-specific DNA methylation (Rang and Boonstra, 2014). Specific examples include the findings that exposure to ROS induces CpG methylation of *Oct-1* in liver carcinoma cells *in vitro* (Quan *et al.*, 2011), and that DNA adducts caused by ROS impair *de novo* methylation by DNMT3a in human placental (Turk *et al.*, 1995) and murine tissue (Maltseva *et al.*, 2009). However, to the author's knowledge this is yet to be found in human colon or colon cancer models.

Although aerobic exercise training probably reduces post-exercise oxidative stress after a single bout of exercise (Finaud *et al.*, 2006), studies into the effects of regular exercise on basal systemic oxidative stress and/or the capacity to deal with local, pathological stressors are lacking. In men over 70 years of age, chronic training was associated with increased plasma oxidative stress biomarkers, but improved health, compared with sedentary controls (de Gonzalo-Calvo *et al.*, 2012a). On the other hand, aerobically fit individuals aged 60-80 displayed significantly less urinary stress biomarkers than their less fit counterparts and were better able to cope with ischaemia/reperfusion injury, as measured by plasma F2-isoprostanes. These findings were not related to plasma or urinary antioxidant status (Traustadottir *et al.*, 2012).

One RCT in overweight postmenopausal women found a reduction in F2-isprostanes after a 12-month exercise intervention, but only when CRF increased by >15% (Campbell *et al.*, 2010b). This strategy might therefore only be of use to the minority whose CRF responds greatly to exercise training, given the heterogeneity in

ability to increase CRF in response to exercise training in a given population (Bouchard *et al.*, 2011; Timmons *et al.*, 2010). Taken together, it is unclear whether systemic oxidative stress is a risk factor for CC, since the associations that have been found can be explained by disease progression (Leufkens *et al.*, 2012; Obtulowicz *et al.*, 2010). Plus, there is limited evidence that long-term exercise training affects systemic oxidative stress or the ability to cope with a localised inflammatory insult that can induce increased production of ROS, as is the case in colon adenoma formation (McLean *et al.*, 2011).

1.5.4 Insulin-IGF axis

1.5.4.1 Insulin

Fasting insulin levels and glucose tolerance have long been known to be associated with habitual PA (Helmrich *et al.*, 1991), and PA interventions have improved these parameters in individuals with impaired glucose tolerance (Knowler *et al.*, 2002; Pan *et al.*, 1997). Whilst studies have been conflicting as to whether hyperinsulinaemia *per se* increases CC risk (Giovannucci, 2007), it has been argued that this creates an environment that promotes cancer cell growth (Gallagher and LeRoith, 2011; Giouleme *et al.*, 2011). For example, HT-29 and Caco-2 human CC cell lines and the rat intestinal epithelial cell line IEC-6 showed increased proliferation in media supplemented with insulin (Sun and Jin, 2008). At present, there is no evidence to suggest that insulin alone contributes to the generation of ACF. For example, hyperinsulinaemia via regular insulin injections after AOM administration in Fischer 344 rats increased colon ACF crypt multiplicity but did not affect ACF number (Corpet *et al.*, 1997). In this model at least, insulin does not seem to contribute to the transition from normal epithelia to ACF, but might accelerate the stepwise progression to a carcinoma in affected areas. This is potentially through downstream activation of Akt and forkhead transcription factors, resulting in induction of cell proliferation (Gallagher and LeRoith, 2011).

Despite insulin not being a *de facto* mutagen (Godsland, 2010), it might serve to ‘prime’ cells for subsequent mutagenesis. Rat and human colon epithelia express insulin receptors (Drew *et al.*, 2007; Silviera *et al.*, 2012) and Fischer 344 rat colon epithelial cells show a dose-dependent increase in proliferation in response 10 h of

hyperinsulinaemia (Tran *et al.*, 2006). Recent work has also demonstrated that neonate Wistar rat overfeeding results in silencing, by methylation, of the hypothalamic insulin receptor promoter (*IRP*) (Plagemann *et al.*, 2010), showing that hyperinsulinaemia could modulate the epigenetic programming of a metabolically responsive cell. Indeed, CpG methylation in the insulin gene promoter (*INS*) in apparently normal colon mucosa from bowel cancer patients is significantly increased compared with controls without cancer (Silviera *et al.*, 2012). The significance of this with respect to neoplasia formation is not clear (especially given that the normal mucosa from cancer patients in this particular study expressed significantly *more* INS despite being more frequently methylated), but suggests that epigenetic modification of pathways related to insulin action might be related to cancer risk. Since circulating insulin is modified by PA, both by stimulating glucose uptake during a bout of exercise and increasing muscle insulin sensitivity thereafter (Goodyear and Kahn, 1998) its effect upon epigenetic modifications is worthy of further investigation.

1.5.4.2 Insulin-like growth factors (IGF)

Circulating IGF-1 has been associated with colorectal cancer risk (Ma *et al.*, 1999; Renehan *et al.*, 2004). IGF-1 is a growth factor for LNCaP prostate cancer cells (Ngo *et al.*, 2003) and both IGF-1 and 2 induce cell proliferation in a number of colorectal cancer cell lines (Lahm *et al.*, 1992). CC cells express the IGF receptor (Guo *et al.*, 1992) and its expression level directly correlates with the stage of the disease (Hakam *et al.*, 1999). IGF-1, in particular, might encourage neoplasia by deregulating cell apoptosis (Pollak *et al.*, 2004). Similarly to insulin, these pathways are subject to epigenetic modification. Promoter methylation of IGF-2 results in increased IGF-2 expression in ageing and cancer (Issa *et al.*, 1996). Conversely, loss of imprinting (LOI) in the *IGF2* gene locus (caused by loss of methylation of the usually methylated maternal allele) is associated with a 5-fold increase in risk (Cui *et al.*, 2003). In *IGF2* LOI positive (+/+) mice AOM treatment resulted in 60% more colon ACF than in LOI negative (-/-) mice. Remarkably, when this was accompanied by IGF-1 receptor blockade in both strains, the number of ACF was reduced by 30% in the LOI +/+ mice only (Kaneda *et al.*, 2007). This suggests that the pathway downstream of the IGF-1 receptor becomes accustomed to higher levels of IGF-2, and is thus highly sensitive to perturbations in IGF-2 signalling. Therefore, if exercise has the capacity to reduce

circulating IGF-1, particularly if it is at a highly elevated level to which the receptor has become accustomed, this could drastically reduce cell proliferation via aberrant IGF-1 signalling. This might be a mechanism by which PA, via epigenetic modifications, regulates cell proliferation and reduces risk of adenoma development, and its subsequent progression to carcinoma.

The evidence regarding PA and regulation of IGF is mixed. Serum IGF-1 was reduced by four weeks of military training in young, healthy men (Rosendal *et al.*, 2002), an intensive two-week diet and aerobic exercise programme in overweight, sedentary men aged 50+ (Ngo *et al.*, 2002) and a 15 week cycling intervention in post-menopausal breast cancer survivors (Fairey *et al.*, 2003). However, an aerobic exercise-induced effect on serum IGF-1 or IGF binding proteins (IGFBP) in the long-term is not a ubiquitous finding (Frystyk, 2010; Nindl and Pierce, 2010). For example, the Alberta Physical Activity and Breast Cancer Prevention Trial found that a 12-month aerobic exercise intervention had no impact on IGF-1 or IGFBP-3 concentrations (Friedenreich *et al.*, 2011). Paradoxically, it is also reported that circulating IGF-1 increases with sustained endurance training in older persons (Poehlman *et al.*, 1994) and resistance exercise programmes (Kraemer and Ratamess, 2005). It appears, then, that an exercise-induced effect on IGF and IGFBP is highly variable, and probably depends upon age, adiposity, diet, mode of exercise and circulating insulin, which suppresses IGFBP-1 production, thereby increasing bioavailable IGF-1 (Sandhu *et al.*, 2002; Yu and Rohan, 2000). Given the potential involvement of IGFs in the epigenetic origins of CC, it is of great interest to investigate solutions to reduce IGF-1 in high-risk populations. However, it seems unlikely that increasing exercise or PA behaviour will be an effective means of doing so.

1.5.5 Adipose tissue regulation

1.5.5.1 Adipose tissue physiology

Regular PA can help to prevent the accumulation of adipose (fat) tissue, which far from being an inert energy depot, is now considered to be a physiologically significant endocrine organ (Kershaw and Flier, 2004) whose function is, at least transiently, altered by exercise (Thompson *et al.*, 2012). An accumulation of visceral

adipose tissue resulting from positive energy balance can lead to several metabolic derangements in adipocytes (fat cells) (van Kruijsdijk *et al.*, 2009). Indeed, adipocytes enlarge in response to positive energy balance (Björntorp and Sjostrom, 1971; Hirsch and Batchelor, 1976; Salans *et al.*, 1973) and larger human adipocytes display a more 'pro-inflammatory phenotype' *ex vivo* (Skurk *et al.*, 2007). In addition, such cellular hypertrophy (and hyperplasia in the most extreme cases of truncal obesity) could reduce adipose tissue blood flow *in vivo*; resulting in adipocyte hypoxia, which some have argued is the basis of aberrant cytokine release (Trayhurn *et al.*, 2008). Indeed, several studies have demonstrated that hypoxic stress is a trigger for adipocyte dysfunction (Hosogai *et al.*, 2007; O'Rourke *et al.*, 2011; Wang *et al.*, 2007). This might affect circulating levels of cytokines secreted by adipocytes in the vicinity of the colon (cytokines or adipokines), and which are emerging as potential risk factors for CC.

1.5.5.2 Adipose-derived cytokines

Obesity is also associated with adipose tissue infiltration by macrophages (Weisberg *et al.*, 2003; Wellen and Hotamisligil, 2003; Xu *et al.*, 2003), which could be responsible for the release of cytokines that contribute to systemic inflammation in Type II diabetes and cardiovascular disease (Kershaw and Flier, 2004; Ye, 2009) and perhaps CC. IL-6 is dramatically raised in morbid obesity (Fontana *et al.*, 2007), and serum levels of IL-6 are also linked to risk of colorectal adenoma (Kim *et al.*, 2008). Interestingly, one study found the mean concentrations of TNFα and IL-1β in morphologically 'normal' colon mucosa from obese, premenopausal women was approximately equivalent to that found in inflammatory bowel disease (IBD) patients (Pendyala *et al.*, 2011; Reimund *et al.*, 1996). After these women underwent diet-induced weight loss of 10% body mass over 7 weeks, colon tissue expression of IL-1β, TNFα, and IL-6 decreased, and expression of genes implicated in inflammatory processes was reduced (Pendyala *et al.*, 2011).

IBD patients are typically at 2-3 fold greater risk for CC (Terzic *et al.*, 2010). IBD-associated cancer has been linked with increased age-related CGI methylation in colon biopsies (Issa *et al.*, 2001) and IL-6 exposure induces DNA methyltransferase-1 (*DNMT1*) expression in HCT116 colorectal cancer cells (Foran *et al.*, 2010). Since IBD increases CC risk, it is noteworthy that substantial reductions in gut

inflammation can be achieved through a change in energy balance, although it is not clear whether these observations were a result of the change in diet, fat loss, or both. Given that obese, insulin-resistant humans secrete more tumour necrosis factor α (TNFα), interleukin-6 (IL-6) and interleukin-1 β (IL-1β) from visceral adipocytes than lean controls (Barbarroja *et al.*, 2010; Tilg and Moschen, 2008), it is tempting to speculate that inflammatory cytokines, perhaps secreted from adipose tissue, could be one mechanistic link between lack of PA, obesity and CC risk via an effect on DNA methylation.

How a high fat diet and/or adipose tissue inflammation might be related to gut inflammation in humans is not known. This may be mediated through systemic inflammation, as evidenced by the reductions in serum IL-8, TNFα and macrophage chemoattractant protein 1 (MCP-1) after weight loss (Pendyala *et al.*, 2011). Alternatively, the author suggests that this could be related to changes in colon and adipose tissue 'cross-talk'. For example, a high fat diet increases the permeability of the proximal colon in mice (Lam *et al.*, 2012). One possible ramification of this is that the fat surrounding the colon is exposed to more enteric bacteria, triggering an inflammatory response. This appears to occur in rats fed a high-fat diet (Ding *et al.*, 2010). Also, such increased permeability could allow for inflammatory mediators from visceral adipose tissue to reciprocally activate inflammatory processes in the gut lumen as part of a vicious cycle between a high fat diet and central obesity. Indeed, in female APC^{Min} mice, visceral fat removal but not calorie restriction was associated with a reduction in adenoma formation (Huffman *et al.*, 2013). This was not associated with any changes in systemic cytokines or adipokines, which lends credence to the author's postulation, that products of visceral fat can bypass this route and alter the gut environment directly.

Regular exercise might be able to alleviate any effects of dysfunctional visceral fat on CC risk by preventing accrual of visceral adipose tissue, and by metabolising existing fat as part of a weight-loss intervention, limiting adipose-derived cytokine secretion (Thompson *et al.*, 2012). It has been argued that PA-only interventions in previously sedentary older persons, without reducing body fat, are unlikely to affect adipose-derived cytokine secretion (You and Nicklas, 2008). Independent of weight loss, perhaps exercise is beneficial by increasing adipose tissue blood flow, and hence oxygen availability to adipocytes. Blood flow to abdominal fat appears to increase during exercise and for (at least) several hours afterwards

(Thompson *et al.*, 2012). This could have important implications for the metabolic regulation of adipocytes, even if the effects are transient. If adipose tissue hypoxia is a trigger for localised inflammation and release of inflammatory cytokines into the systemic circulation (Trayhurn *et al.*, 2008), overcoming the oxygen 'deficit' through regular PA might restore deranged visceral adipocytes to normal function. However, as Thompson and colleagues have outlined in their recent review, it is unclear whether exercise stimulates long term changes in adipose tissue blood flow. Also, most measures express blood flow per unit of fat mass, so one cannot determine whether any increases in blood flow due to exercise training are a reflection of a reduction in fat mass (Thompson *et al.*, 2012).

1.5.5.3 Adipokines (Leptin and adiponectin)

Increased central fat leads to an increase in localised and circulating leptin production from adipose tissue (Maffei *et al.*, 1995), which could serve to promote colon cell proliferation and mutagenesis. Although an association between serum leptin and CC risk is not firmly established in population studies (Garofalo and Surmacz, 2006; Joshi *et al.*, 2014), a recent meta-analysis of case-control studies has suggested that it could be linked with risk of adenoma (Gialamas *et al.*, 2013). Serum leptin might be predictive of tubular adenomas in asymptomatic Caucasian men, as one cross-sectional study observed that serum leptin > 9 ng/ml increased risk of polyps three-fold (Comstock *et al.*, 2014a). Leptin is a growth factor in human cancer cell lines (Aparicio *et al.*, 2005; Hardwick *et al.*, 2001; Ogunwobi and Beales, 2007) and in mouse colon epithelia through induction of the *IL-6* promoter (Fenton *et al.*, 2006; Fenton *et al.*, 2007). Although AOM administration in mice does not affect the number of colon ACF between wild-type, leptin-deficient (ob/ob obese), or hyperleptinaemic (db/db obese) mice (Ealey *et al.*, 2008), leptin increased pro-inflammatory cytokine gene expression in mouse colon, specifically *CXCL1*, *IL-6* and *IL1-β* (Padidar *et al.*, 2011). Curiously, it has also been found that leptin mRNA (*LEP*) is greatly overexpressed in colonic mucosa from cancer patients compared with controls (Silviera *et al.*, 2012). It is possible; therefore, that leptin overproduction might contribute to an inflammatory milieu in the colon, both indirectly via the systemic circulation and perhaps more directly in colonic mucosa. It has been observed that the extent of promoter methylation of *LEP* in adipose tissue predicts

weight loss response to a very low calorie diet over 8 weeks in obese women (Cordero *et al.*, 2011). However, there is no evidence of differential methylation of *LEP* in the colon contributing to cancer risk, despite being differentially expressed (Silviera *et al.*, 2012).

There is emerging work that strongly implicates obesity and insulin resistance in the differential methylation of *LEP* and *ADIPOQ* (adiponectin) in peripheral blood of adolescents (Garcia-Cardona *et al.*, 2014), but it is not known whether this could mediate the known associations between metabolic syndrome, obesity and CC risk (Giovannucci, 2007; Ning *et al.*, 2010). *In vitro*, adiponectin suppresses colorectal cancer cell growth via the AMPK/mTOR pathway (Kim *et al.*, 2010a; Sugiyama *et al.*, 2009), and co-treatment of MC38 CC cells with adiponectin decreased IL-6-induced cell proliferation via inhibition of STAT3 (Fenton and Birmingham, 2010). Also, in the Health Professionals Follow-up Study, the lowest quintiles of plasma total adiponectin were associated with an increase in CRC risk in men (Wei *et al.*, 2005). This was also observed in the European Prospective Investigation into Cancer and Nutrition (EPIC) and the Nurses' Health Study cohort, although neither of these studies reported significant associations when plasma adiponectin was corrected for waist circumference (Aleksandrova *et al.*, 2012; Song *et al.*, 2013). A meta-analysis of case-control and cohort studies also found that adiponectin levels were lower in colorectal cancer and adenoma patients (An *et al.*, 2012; Joshi *et al.*, 2014). It is not conclusive; however, that exercise independent of any effects on fat mass meaningfully alters systemic adiponectin or leptin levels (Bouassida *et al.*, 2010; Simpson and Singh, 2008) l*et al*one whether it is via alterations to the methylome. An isolated study found that a one-year diet and exercise intervention decreased serum leptin independently of body composition in obese, insulin resistant men and women, and was associated with improvements in insulin sensitivity (Corpeleijn *et al.*, 2007). It is generally agreed that a lifestyle intervention that reduces fat mass reduces circulating leptin and increases adiponectin (Bouassida *et al.*, 2010), but the evidence for PA exerting an independent effect is lacking.

1.5.6 Inflammation

1.5.6.1 Chronic inflammation

Age-related increases in chronic inflammation have been linked with cancer development (Caruso *et al.*, 2004; Franceschi *et al.*, 2000a; Il'yasova *et al.*, 2005) and risk of colon adenoma (Kim *et al.*, 2008). Chronic inflammation is purported to play a key role in the initial stages of the carcinogenic process (Shacter and Weitzman, 2002). With respect to CC, levels of circulating C-reactive protein (CRP) and IL-6 have been associated with increased risk in the Health, Aging and Body Composition cohort, although the association was stronger for risk of mortality than incidence (Il'yasova *et al.*, 2005), and IL-6 in one study was linked with increased adenoma risk (Kim *et al.*, 2008). On the other hand, a recent meta-analysis concluded that CRP, but not IL-6, was associated with CC risk (Zhou *et al.*, 2014), and follow-up of patients in the Polyp Prevention Trial showed no associations between inflammatory biomarkers and adenoma recurrence (Bobe *et al.*, 2010b). However, the risk of advanced adenomas in that study was directly related to IL-6 concentrations (Bobe *et al.*, 2010a) pointing to a potential role of IL-6 in adenoma progression, but not initiation.

Numerous observational studies have reported reduced markers of chronic inflammation with increasing PA in older persons (Brinkley *et al.*, 2009; Cesari *et al.*, 2004; Colbert *et al.*, 2004; de Gonzalo-Calvo *et al.*, 2012b; Geffken *et al.*, 2001; Jankord and Jemiolo, 2004; King *et al.*, 2003; McFarlin *et al.*, 2006; Reuben *et al.*, 2003; Verdaet *et al.*, 2004) suggesting that a change in PA might reduce chronic inflammation, which could be implicated in CC risk. To date, treatments that have been effective in reducing systemic inflammation concomitant with apparently beneficial effects on colon health include rapid weight loss in obese women (Pendyala *et al.*, 2011) and Vitamin D supplementation in adenoma patients (Ahearn *et al.*, 2012; Fedirko *et al.*, 2009; Hopkins *et al.*, 2011). Nonetheless, it is important to state that whilst these studies have shown a correlation between some reductions in systemic inflammation and improved colon health, including reduced colon inflammation in the case of Pendyala and colleagues (Pendyala *et al.*, 2011), the relationship might not be causal. For example, recent work in obese male and female APC^{Min} mice demonstrated that visceral fat removal or caloric restriction reduced the number of

colon macroadenomas without affecting any measured serum inflammatory cytokines including IL-10, IL-6, IL-12 or IL-1β (Huffman *et al.*, 2013).

There is very mixed evidence that an increase in PA affects systemic inflammation in previously sedentary middle-aged and older persons. Some have reported reduced CRP (Campbell *et al.*, 2009; Kohut *et al.*, 2006; Stewart *et al.*, 2007; Vieira *et al.*, 2009) and IL-6 (Kohut *et al.*, 2006; Nicklas *et al.*, 2008) after moderate-vigorous aerobic exercise interventions (65-80% estimated aerobic capacity) over 10-12 months, although the findings of Vieira and colleagues were influenced by the degree of loss of central adipose tissue (Vieira *et al.*, 2009). Similarly, fat loss over 18 months in obese knee osteoarthritis patients has been demonstrated to reduce TNFα, IL-6 and CRP, with no effect of increasing PA via brisk walking for 3 h per week (Nicklas *et al.*, 2004). Other studies have found no effect on serum inflammatory biomarkers (Beavers *et al.*, 2010; Hammett *et al.*, 2004; Nicklas *et al.*, 2004; Thompson *et al.*, 2010) including a 12 month intervention in a large (N = 424) community-dwelling older cohort aged 70-89 (Beavers *et al.*, 2010) (for review, see Woods *et al.*, 2012).

A role for inflammation in tumorigenesis is clear (Coussens and Werb, 2002), and in CC this could be at least partly mediated via DNA methylation (Issa *et al.*, 2001). *In vitro* studies have shown that a supra-physiological dose of IL-6 upregulates DNMT activity in HCT-116 cells (Foran *et al.*, 2010), although whether such a mechanism operates at the lower concentrations of IL-6 present in serum over the course of several years, or decades, is not known. In summary, it appears that chronic inflammation could serve to promote CC progression from adenoma, but might not directly influence adenoma risk. A role for increasing PA alone in previously sedentary persons as a means of moderating chronic inflammation in the absence of weight loss is inconclusive.

1.5.6.2 Localised inflammation

1.5.6.2a Prostaglandins

Prostaglandins (PG) are a marker of great clinical interest with regard to gut inflammation. Produced by cyclooxygenase (COX) enzyme activity, PGs have been the focus of much attention with regard to cancer progression since it was discovered

that adenomas and 'normal' mucosa from colorectal cancer patients produced more prostaglandin E2 (PGE2) than control tissue (Pugh and Thomas, 1994). This process might be regulated in part by DNA methylation, as PGE2 increases DNA methyltransferase -1 and -3 (DNMT1/3) enzyme expression in three human CC cell lines and in colonic mucosa from the APC^{Min} mouse (Xia *et al.*, 2012). Research has demonstrated that consumption of the non-steroidal anti-inflammatory (NSAID) aspirin, a COX-2 inhibitor, has a mild preventative effect on new adenoma formation in adenoma patients (Baron *et al.*, 2003). Moreover, the recent Colorectal Adenoma/Carcinoma Prevention 2 (CAPP2) study reported a 63% reduction in cancer incidence in Lynch syndrome patients consuming 600 mg of aspirin daily for at least 25 months (Burn *et al.*, 2011). However, this did not translate into any reductions in initial adenoma formation.

Whether exercise exerts a similar protective effect via inhibition of COX is uncertain. Treadmill running for 30 min/day in AOM-treated mice had no effect on colon cyclooxygenase-2 (COX-2) expression after 6 weeks compared with sedentary controls (Aoi *et al.*, 2010). However, rats treated with dimethylhydrazine (DMH) and subjected to an intensive exercise protocol (50-90 min swimming on 5 days per week for 8 weeks) reduced *COX-2* expression in colon crypts compared with controls who did not exercise (Demarzo *et al.*, 2008). It is possible that an exercise-induced decrease in *COX-2* is particular to the rat-DMH model of CC and/or very intense exercise; equally the lack of effect reported by Aoi and colleagues could have occurred due to the less intense exercise mode and/or AOM-mouse model. Furthermore, it might be that exercise reduces COX-2 recruitment at the site of an adenoma, thereby slowing the adenoma-carcinoma progression. This could be the case with chronic NSAID use, which might explain why Burn and colleagues found no difference in number of colon adenomas but a significantly reduced incidence of cancer (Burn *et al.*, 2011). In partial support of this interpretation, the original investigations into PGE2 and CC did not find any difference in concentrations between normal mucosa from control participants and polyp patients (Pugh and Thomas, 1994) suggesting a role for COX-2 in the adenoma-carcinoma progression but not from normal mucosa to adenoma. However, this remains to be tested.

A lack of PA might be associated with PG production via an increase in systemic and localised inflammation as observed in physically inactive individuals (Woods *et al.*,, 2012). Specifically, IL-1β up-regulates *COX-2* expression in colon

epithelial cancer cells *in vitro* (Duque *et al.*, 2006) although a consistent link between systemic and localised gut inflammation has not been demonstrated in rodents or humans (Huffman *et al.*, 2013; Pendyala *et al.*, 2011). Additionally, exercise training in rats significantly reduces the colon mucosal gene expression of calcium-independent phospholipase-2 (*ipL-A2*) (Buehmeyer *et al.*, 2008). This could be important, since the phospholipase A2 (PLA2) enzyme it encodes is responsible for the release of arachidonic acid from phospholipids, which COX-2 converts into prostaglandins. Exercise could therefore limit prostaglandin production by decreasing the PLA2-dependent supply of arachidonic acid.

The single randomised controlled trial in humans investigating an increase in PA and changes in colonic inflammation found that a 12 month aerobic exercise programme had no effect on colon PG levels (which are elevated in adenoma and CC patients (Pugh and Thomas, 1994)) in untrained polyp patients (Abrahamson *et al.*, 2007). However, this does not completely rule out a role for exercise in cancer prevention via an effect on PGs. Indeed, whilst mucosal PGs were unaffected, this was measured in only one biopsy and it has been shown that COX-2 expression is highly variable throughout the colon (Wiese *et al.*, 2003), which the authors of the study acknowledge could have masked any exercise-induced changes (Abrahamson *et al.*, 2007). Also, the range of PGE2 concentrations in individual biopsies recorded by Abrahamson and colleagues had enormous variation. In male controls, for instance, the mean was 1,143 pg/mg, but the range was 61-7,803 pg/mg (Abrahamson *et al.*, 2007). Given that the importance of PGE2 and COX-2 seems to be more apparent in adenoma to carcinoma progression as opposed to polyp risk (Burn *et al.*, 2011; Pugh and Thomas, 1994) it is questionable whether PGE2 is a suitable marker for the effectiveness of an intervention designed to reduce adenoma incidence. This could partly explain why the association between PA and reduced incidence of adenoma is stronger for larger, more advanced polyps (Wolin *et al.*, 2011).

1.5.6.2b Cytokines

Localised inflammation is a facet of the colon tumour microenvironment (McLean *et al.*, 2011), and inflammatory bowel disease (IBD) is unequivocally linked with CC risk (Terzic *et al.*, 2010). Limited evidence in rodents has suggested that such localised inflammation might be amenable to exercise training. Indeed, in aged mice,

reduced expression of *TNF* in intestinal lymphocytes has been observed after 4 months of daily wheel running (~4 km per day) (Packer and Hoffman-Goetz, 2012).A lifestyle intervention in humans that has significantly reduced gut inflammation was achieved by Pendyala and colleagues, via very low calorie diet induced weight loss in obese women (Pendyala *et al.*, 2011). Since IBD increases CC risk, and obesity is a risk factor for CC and adenoma (Comstock *et al.*, 2014a; Ning *et al.*, 2010; Omata *et al.*, 2013), it is noteworthy that substantial reductions in gut inflammation can be achieved through a change in energy balance. This finding provides evidence that rapid weight loss in obese persons could mitigate colon inflammation, which in turn might reduce CC risk. Ongoing work is investigating whether sustained weight loss affects future adenoma risk. For example, in the BeWEL study, overweight adenoma patients randomised to a weight loss intervention lost a mean of 3.5 kg, or 3.9% of body weight, over 12 months, which resulted in significant improvements in blood pressure, blood glucose and glycated haemoglobin concentration (Anderson *et al.*, 2014). The results of this trial with respect to future adenoma risk are awaited.

A role for localised inflammation in DNA methylation and CC is clear. For example, inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) and Crohn's disease increase risk of CC by 2-3 fold (Terzic *et al.*, 2010). Localised inflammation is a hallmark of colorectal tumours (McLean *et al.*, 2011), and it accelerates normal age-related DNA methylation in the colon (Issa *et al.*, 2001; Lobaton *et al.*, 2014). Although the mechanism of action is not clear, it has been observed *in vitro* in CRC cell lines that inflammatory stimuli increase DNA methyltransferase (DNMT) activity (Foran *et al.*, 2010; Xia *et al.*, 2012).

Regular PA has been frequently associated with lower levels of some serum inflammatory cytokines and a relative increase in anti-inflammatory cytokines in older persons; whereas the converse is found with physical inactivity (Brinkley *et al.*, 2009; Cesari *et al.*, 2004; Colbert *et al.*, 2004; de Gonzalo-Calvo *et al.*, 2012b; Geffken *et al.*, 2001; Jankord and Jemiolo, 2004; King *et al.*, 2003; McFarlin *et al.*, 2006; Reuben *et al.*, 2003; Verdaet *et al.*, 2004) and in chronic disease states (Brinkley *et al.*, 2009). Interestingly, in randomised exercise intervention studies in disease-free sedentary middle aged and older persons, there is inconsistent evidence of an effect on inflammatory biomarkers (Woods *et al.*, 2012). Given that inflammatory cytokine concentrations are resilient to abrupt (1 wk) changes in PA habits (Lund *et al.*, 2011a), it might be that any subtle effects of PA on chronic

inflammation take place over many years, masking any effects of increased PA in intervention studies. In addition, it could be that levels of inflammation in epidemiological studies reflect disease states which impact upon PA participation, confounding the associations with PA. Another possibility is that PA induces changes to the inflammatory methylome, which regulates production of inflammatory cytokines in response to a stimulus (Fitzpatrick and Wilson, 2003; Miranda and Jones, 2007).

1.5.7 Which mechanisms have the best evidence?

Overall, the epidemiological and randomised trial evidence is limited that PA, independent of diet and fat mass, over the life course affects GITT, bile acid production, IGF-1, and adipokine secretion. However, the epidemiological evidence is convincing that PA over the life course beneficially affects chronic inflammation and insulin sensitivity. A chronic lack of PA over the life course induces numerous well-characterised cardiovascular and metabolic perturbations (Blair, 2009), which could plausibly impact cancer risk in the ageing gut via DNA methylation. With respect to CC, there is abundant evidence that age-related DNA methylation has a pivotal role to play in the gut ageing process and possibly CC risk (Ahuja *et al.*, 1998; Bailey *et al.*, 2014; Belshaw *et al.*, 2008; Belshaw *et al.*, 2010; Chan *et al.*, 2002; Esteller *et al.*, 2001; Horvath, 2013; Issa *et al.*, 2001; Issa *et al.*, 1994; Issa *et al.*, 1996; Silviera *et al.*, 2012; Tapp *et al.*, 2013). To date, only one study in rodents has demonstrated that hyperinsulinaemia might induce DNA methylation in peripheral tissues (Plagemann *et al.*, 2010), but this has not yet been demonstrated in humans. On the other hand, inflammatory stimuli are known to induce aberrant DNA methylation in a range of human tissues (Niwa and Ushijima, 2010). Equally, there is plentiful evidence that chronic inflammation, particularly cytokine expression, is regulated by DNA methylation (Fitzpatrick and Wilson, 2003).

1.6 Can physical activity beneficially affect DNA methylation patterns in older persons at risk of colon cancer?

Recent evidence has pointed to a role of exercise in affecting global and gene-specific CGI methylation in a range of tissues. Transient DNA hypomethylation is a means of increasing the expression of genes associated with exercise metabolism (e.g. citrate synthase and pyruvate dehydrogenase kinase) during exercise in muscle tissue (Barres *et al.*, 2012). In addition, increased methylation of long interspersed repetitive elements on the genome (LINE-1: a surrogate of global DNA methylation) was associated with higher levels of physical activity in the Sister Study cohort (White *et al.*, 2013). Furthermore, there was a trend toward increased LINE-1 methylation with increasing PA as measured by accelerometer in the North Texas Healthy Heart Study, despite the overwhelmingly sedentary nature of the study population (~70% physically inactive) (Zhang *et al.*, 2011). Conversely, increased PA was associated with decreased global methylation as determined by Luminometric Methylation Assay (LUMA) in a cohort of elderly Swedish men (Luttropp *et al.*, 2013). However, the LUMA assay is an inappropriate measure of global methylation as it does not interrogate hypermethylated regions of the genome, which might explain the conflicting result.

With regard to gene-specific methylation, it has been documented that 6 months of aerobic exercise training was associated with increased methylation of *ASC* (Apoptosis-associated Speck-like protein with CARD domain), which encodes a protein that is responsible for the conversion of the inflammatory cytokine interleukin 1β (IL-1β) from its inactive form, in the serum of men aged 40-87 (Nakajima *et al.*, 2010). In gastric cancer patients, the methylation of *CACNA2D3* in tumour samples was inversely associated with physical activity level (Yuasa *et al.*, 2009). In addition, aberrant methylation of the 'gatekeeper' *APC* (Adenomatous Polyposis Coli) gene implicated in the development of breast and colon cancer, was inversely correlated with 5-year physical activity levels of women aged 43 ± 7 yrs in benign breast biopsies (Coyle *et al.*, 2007). More recent work has identified that methylation of the breast cancer tumour suppressor gene *L3MBTL1* in serum is reduced by 6 months of exercise training in breast cancer survivors, which was associated with improved

disease prognosis and survival (Zeng *et al.*, 2012). One group demonstrated that three hours of high-intensity aerobic exercise per week for six months in 23 previously sedentary middle-aged men with or without a family history of type 2 diabetes affected methylation status of 134 genes in muscle and >7,500 genes in subcutaneous adipose tissue (Nitert *et al.*, 2012; Ronn *et al.*, 2013). In overweight, premenopausal women, it has also been shown that cardiorespiratory fitness and/or minutes per week of PA are associated with reduced gene-specific and overall methylation of 45 CGIs in known breast cancer suppressor genes in saliva (Bryan *et al.*, 2013). Furthermore, voluntary treadmill running for 12 weeks was associated with a decline in betaine-homocysteine S-methyltransferase-2 (*BHMT2*) gene expression in rat colon (Buehmeyer *et al.*, 2008). This raises the possibility that exercise alters methyl group availability, which might impact colon CGI methylation. Taken together, this suggests that PA has the capacity to alter CGI methylation in tissues distant from the exercising skeletal muscles, and in genes known to be implicated in CC development and inflammation. With this in mind, it is possible that PA can induce changes in age-related CGI methylation, particularly in inflammation-related genes, which might be a means by which it affects CC risk.

1.7 How much physical activity is required to demonstrate a change in DNA methylation?

There are currently no longitudinal studies in the extant literature examining changes in DNA methylation patterns with changes in PA. It would be of great interest if PA data and genetic material from a previous cohort was available for such an investigation. Furthermore, only two non-randomised, and no randomised, trials have explored how much physical activity is necessary to induce a significant change in DNA methylation in target tissues. One of which was a six-month walking intervention in an aged Japanese cohort (142 men, 294 women, mean age 65 ± 7 years). The exercisers (n = 274) completed sessions of interval walking (3 minutes 40% VO2 max; followed by 3 min at 70% VO2 max for several sets) for a mean of 52.2 ± 18.5 minutes per day on 3.9 ± 1.2 days per week. Compared with the control group, methylation of *ASC* in peripheral blood leukocytes was 1% greater (Nakajima *et al.*, 2010). However, whilst the results were intriguing there was no evidence of

between-group matching or balancing, nor were the PA levels of the control group recorded. Also, the blood samples were taken only after the completion of the intervention. It is therefore distinctly possible that the between-group differences in *ASC* methylation at the end of the intervention were present to begin with.

The second intervention was reported in two publications in 2012-2013 (Nitert *et al*., 2012; Ronn *et al*., 2013). 23 overweight men (BMI 28.2 ± 2.9 kg/m2; 37.3 ± 4.4 yrs) with or without a family history of type 2 diabetes had a fat biopsy from the right thigh, and a muscle biopsy from the vastus lateralis, before and after a six-month high-intensity aerobic exercise intervention. The intervention consisted of a one hour spinning class and two hour-long aerobics sessions per week, with a mean of 1.8 sessions per week being attended. DNA was bisulphite-modified and subjected to Illumina 450K array. In adipose tissue, 45 CpGs in 21 genes were differentially methylated after the programme, including *FTO* and *KCNQ1*, which are both implicated in obesity (Ronn *et al*., 2013). In muscle tissue, differential methylation was observed at 134 genes, including reduced methylation of *THADA*, *RUNX1*, *MEF2A* and *NDUFC2* (Nitert *et al*., 2012). In each case of differential methylation, there was a concomitant change in mRNA expression in the associated gene i.e. greater methylation was observed with reduced expresssion and vice-versa (Nitert *et al*., 2012; Ronn *et al* 2013).

It might be argued that a similar intervention to Nitert and colleagues would be sufficient to induce changes in DNA methylation patterns in an aged cohort at risk of colon cancer. However, the age of those at greatest risk of the disease is typically > 55 years. It is questionable that such a vigorous regimen would be suitable for this group, given that a recent report indicated that two-thirds of health service users aged over 65 in parts of the United Kingdom are living with more than one comorbidity (Barnett *et al*., 2012). It is arguably of greater relevance to investigate whether long-term changes in more moderate activities could induce changes to DNA methylation patterns in an older persons at risk. There is a need for longitudinal and randomised trial evidence in this arena.

1.8 Rationale for present investigations

There is a distinct lack of longitudinal and randomised trial data regarding changes in PA and concomitant changes in DNA methylation patterns in persons at risk of CC. As such, this series of studies will seek to fill that gap in knowledge to better understand the relationship between PA and DNA methylation. To this end, this series of studies will attempt to discern whether a change in PA, in a longitudinal and randomised trial setting, can affect DNA methylation patterns in older persons who might be at elevated risk for colon cancer. Since it is evident that these patterns differ by tissue type (Rakyan *et al*., 2008; Byun *et al*., 2009; Slieker *et al*., 2013; Lokk *et al*., 2014) the best efforts will be made to interrogate the source tissue i.e. colon epithelia. Where this is not possible, surrogate epithelial tissues will be utilised, and where that is not possible peripheral blood will be collected instead. Furthermore, these investigations will use *in vitro* models of colon epithelia which will be treated with serum before and after a short-or-long term exercise intervention. This will help provide insight as to what might be occurring at the level of the colon in response to systemic changes brought about by exercise when biopsy material from participants is not available.

Chapter 2
General Methods

This section details the general methods used over the course of the investigations presented in this thesis based at the Norfolk and Norwich University Hospital (NNUH) University of East Anglia Exercise Physiology Laboratory and the Institute of Food Research (IFR). Where appropriate, abridged versions of the detailed methods presented here can be found within the ensuing chapters.

2.1 Participants

Participants in the intervention studies detailed in Chapters 5 and 6 were generally aged between 50 and 75, and recruited via nurse referral, direct contact with the research team at the Norfolk and Norwich University Hospital, or via online and printed flyers placed at the University of East Anglia, Norfolk and Norwich University Hospital, Norwich Evening News, and sent via the University of East Anglia alumni association. All methods of recruitment received approval from the Norfolk NHS Research Ethics Committee (REC) and the University of East Anglia School for Medicine and Health Sciences Research Ethics Committee where appropriate. All members of the research team who had direct contact with patients and participants obtained an enhanced CRB, were Good Clinical Practice (GCP) trained and had undertaken site-specific courses in Information Governance and Data Protection. All members also obtained a Research Passport for activities based on hospital premises.

All participants who had consented to taking part in the research studies after reading the provided Patient/Participant Information Sheet attended an information session at the Clinical Exercise Science laboratory. Here, the team ensured that participants understood the demands of taking part and were encouraged to ask questions regarding the research. Once the research team and participant were satisfied that they wished to continue, both parties signed two copies of an approved consent form. The participants were reminded that they were free to withdraw from the study without giving reason.

2.2 Tissue collection

2.2.1 Colonoscopy referral process

Patients were referred for flexible sigmoidoscopy or colonoscopy via their General Practitioner due to unexplained bowel symptoms or through the National Bowel Cancer Screening Programme. Briefly, patients over the age of 60 registered with the NHS were offered to take a home-based faecal occult blood test (FOBT). If occult blood was detected in 5-6 instances over the course of three separate bowel movements, the patient was invited for a consultation with a bowel cancer screening nurse at the Gastroenterology Department at the NNUH. At this point, the screening nurse provided materials regarding the research study, including a Patient Information Sheet and Patient Consent Form for registration of interest and provision of contact details. These details were collected by the research team and the potential participants contacted (typically by telephone) by the research team. Verbal interest in taking part was confirmed, with the view to provide written informed consent for the collection of research biopsies with a member of the research team present prior to colonoscopy.

2.2.2 Colon biopsies

Approximately twenty-four hours before the procedure, patients were instructed to take the osmotic laxative Klean-Prep® (Helsinn Healthcare S.A., Lugano, Ticino, Switzerland) according to manufacturers' instructions. On occasion, some participants were instructed to take Picolax® (Ferring Pharmaceuticals Ltd, West Drayton, Middx, UK). The preparation method was recorded by the research team. Written informed consent for the collection of research biopsies was obtained on the day of colonoscopy, prior to preparation of the patient for the procedure. A member of the research team was present in the theatre with the clinical staff. Most patients requested a sedative but others declined. The endoscopist screened the entire bowel. Where polyps were detected by the endoscopist at any point in the bowel, they were removed via snare and/or cauterisation. If this was the case, five pinch biopsies (~2-5 mm in diameter) were obtained from the sigmoid colon, at least 10 cm from any polyp margin. Where abnormal growths in the lining of the colon that could not be

definitively identified as large polyps or cancer were found, research biopsies were taken pending histological confirmation. Two of these were placed in 1 ml of RNAlater® (Life Technologies), one was placed in 1 ml 10% formalin, one was placed in Carnoy's fixative (75% EtOH, 25% acetic acid) and one was frozen on dry ice. The samples in RNAlater® and Carnoy's fixative were refrigerated for 48 hours to allow full fixation, after which the former was stored at -75 °C and the latter transferred into 1 ml 70% EtOH and refrigerated at 4 °C. Formalin-fixed samples were stored at 4 °C, and frozen samples stored at -75 °C. Where the bowel was not sufficiently clean to investigate the epithelium and could not be adequately cleaned with saline solution *in situ*, the patient was invited to attend another colonoscopy. The investigation was generally very well-tolerated and lasted from 45 to 80 minutes.

2.2.3 Blood and buccal cells

On experimental days where human tissue collection occurred, all participants arrived at the Clinical Exercise Science laboratory after an overnight fast and having refrained from moderate-vigorous PA for 72 h, and from consumption of alcohol for 24 h. Venous blood was obtained by venepuncture of the left or right antecubital vein by a trained phlebotomist. Venous blood was transferred to serum collection tubes using the Vacutainer® technique (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA), inverted gently several times and allowed to clot for 30 min at ambient temperature. They were then centrifuged at 3600 rpm at 4 °C for 15 min (Thermo Fisher Scientific 1-SR Multifuge, Thermo Fisher Scientific, Waltham, MA, USA). Serum and plasma was extracted, pipetted in 200 µl aliquots into 1.5 ml Eppendorf tubes and frozen on dry ice. Buccal cells were collected using an Epicentre® Catch-All™ Sample Collection Swab (Cambio Ltd, Cambridge, Cambs, UK) according to the manufacturer's instructions and stored at -20 °C. All samples were subsequently transferred on dry ice to the Institute of Food Research and stored at -75 °C.

2.3 Anthropometric measures

Height (m) and mass (kg) were measured using a calibrated stadiometer and balance (Seca 711, Seca UK, Birmingham, UK). Resting blood pressure (DINAMAP v1000, GE Healthcare, Bucks, UK) was taken after the participant was in a semi-supine

position for five minutes, was recorded and body composition obtained via bioelectrical impedance analysis (AKERN BIA 101, SMT Medical GmbH & Co., KG, Germany) and integrated software (BodyGram Pro 3.0, SMT Medical GmbH & Co., KG, Germany).

2.4 Cardiorespiratory fitness

Maximal aerobic capacity ($\dot{V}O_{2\,max}$; $ml \cdot kg \cdot min^{-1}$) was determined using a Medgraphics™ Ultima Cardio2 suite (Medical Graphics Corporation, St. Paul, MN, USA). Prior to the test, the unit was calibrated according to manufacturers' instructions. In short, the system was switched on and allowed to warm up and stabilise for ~40 min. In the software, the vacuum pump was switched on and allowed to warm up for two minutes until the 'READY' sign was displayed. Calibration of expired and inspired air volume was performed using a known volume of room air (3 litres) at ambient temperature, pressure and humidity, using a syringe moved at progressively slower speeds to simulate breathing. This was connected to the unit via bi-directional Pitot tubing and flow sensor. The volume of air detected by the flow sensor was accurate to true volume to ± 3% or 50 ml, whichever was greater. Calibration failed if the volume detected deviated by ± 2% from the syringe volume. Air composition was calibrated against supplied calibration gas (12% O_2, 5% CO_2) and reference gas (21% O_2, 0% CO_2) with cylinders opened and set to 5 psi. It was ensured that the same Pitot tubing and flow sensor used for calibration was also used for testing. All tests that required a follow-up measurement utilised a mouthpiece and nose-clip apparatus to ensure standardisation between tests.

Participant information including name, gender, age, ethnicity and trial number was recorded and imputed into the software. Height (m) and mass (kg) was measured using a calibrated stadiometer and balance (Seca 711, Seca UK, Birmingham, UK) immediately prior to the test, and 10 lead ECG attached by an accompanying medical professional. The research team explained the nature of the test to the participant, emphasising the need for a maximal effort, using the Borg scale of perceived exertion (Borg, 1970) and demonstrating how to insert the mouthpiece. Cardiorespiratory fitness (CRF) was measured using a 20-watt incremental ramp protocol on an electrically braked cycle ergometer (Lode Excalibur Sport, Lode B.V.

Groningen, NL) or with a Balke graded treadmill protocol. The cycle protocol was used for the participants in Chapter 5 of this thesis, whose mean age was 68.5 years with several suffering from musculoskeletal ailments that would have made a treadmill protocol uncomfortable, if not intolerable. The treadmill protocol was used for the younger (mean age 55 years) and more physically active participants in Chapter 6 of this thesis, in preparation for the treadmill exercise session on the study days. Briefly, the ergometer test started with a 2 min 'freewheel' period against no resistance so that the participant was accustomed to the protocol. On the treadmill, approximately 2 minutes was spent selecting a comfortable brisk walking/running speed which the participant could maintain for the duration of the test. Thereafter, the resistance/gradient increased every minute by 20 watts until $\dot{V}O_{2\,max}$ or volitional exhaustion was reached. The result was accepted if the respiratory exchange ratio (RER) was > 1.15 and a plateau in $\dot{V}O_2$ on the graphical and numerical displays were evident. Where appropriate, approximate attainment of age-predicted maximum heart rate was also used as a criterion, but not when participants were taking medication that could affect heart rate (e.g. beta-blockers). During the test, a continuous 10 lead ECG trace (Mortara XScribe 5, Mortara Dolby UK Ltd, Stirling, UK) was monitored by the medical professional, and the test stopped immediately where any abnormalities arose during the exercise bout. The test ceased once the participant reached their $\dot{V}O_{2\,max}$ and/or was unable to continue. The medical professional continued to monitor the ECG trace to ensure that any abnormalities did not manifest in the post-exercise epoch. Participants then had the opportunity to shower and change.

2.5 Current physical activity

Objective free-living PA level was assessed over 7 days using accelerometry (ActiGraph® GT3X, ActiGraph Corps, Pensacola, FL, USA). The small unobtrusive accelerometer was worn on the hip and collected data including estimated activity counts, step counts, minutes of MVPA per day and per week, bouts of MVPA of ten minutes or more, and total estimated exercise energy expenditure. Data collection occurred at one-minute epochs. Wear time was considered sufficient if it met standardised criteria i.e. at least 10 hours of wear time on at least five days including

one weekend day by an inbuilt algorithm (Choi *et al.*, 2011) and visually examined by the researcher to ensure that days of data collection corresponded to the activity diary provided. Data was analysed using integrated software (ActiLife version 6.9.1, ActiGraph Corps, Pensacola, FL, USA). The activity count cut off points used in all intervention studies is displayed in Table 2.1.

Table 2.1 Actigraph physical activity accelerometer counts per ten-minute bout and corresponding estimated activity intensity.

Activity count range	**Estimated activity intensity**
≤ 100	Sedentary
101 – 759	Lifestyle
760 – 1952	Light
1953 – 5724	Moderate
5725 – 9498	Vigorous
≥ 9499	Very vigorous

Self-reported PA was assessed using a seven-day physical activity diary completed by the participants. Participants were instructed to record time spent in all physical activity behaviour during all waking hours, including sitting, walking, chores, and leisure-time PA. Participants were also provided with the Borg scale of perceived exertion (Borg, 1970) to complement the record, to aid the researchers in calculating activity intensity. The intensity of physical activities in metabolic equivalent of task (MET) was determined using the Compendium of Physical Activities (Ainsworth *et al.*, 2000). 'Moderate' activities were deemed to be > 3 METS, and 'vigorous' activities > 6 METS. Amount of exercise in MET-hours per week was calculated by multiplying hours and intensity of specific activity undertaken. Self-report PA was also assessed using the International PA Questionnaire (IPAQ) (Friedenreich *et al.*, 1998) and the Godin Leisure Time Exercise Questionnaire (Boyle *et al.*, 2015; Godin and Shephard, 1985). Both questionnaires were self-administered and use a 7-day recall period. The IPAQ is designed to measure four domains of PA: 1) Job-related; 2) Transportation; 3) House work; and 4) Recreation, sport and leisure-time. An additional question asks for the time spent sitting. The validity of the IPAQ has been rated as acceptable for the different activity domains (Friedenreich *et al.*, 1998). The Godin Leisure Time Exercise Questionnaire is a short four-item questionnaire that assesses the number of times that strenuous, moderate or mild exercise was performed

for more than 15 min over the last 7 days. Research team members read aloud the questionnaire questions verbatim at follow-up visits to maintain consistency, with participants providing a verbal response. The questionnaires took approximately 30 minutes to complete.

2.6 Historical physical activity

Historical physical activity was assessed using the Historical Adulthood Physical Activity Questionnaire (HAPAQ) (Besson *et al.*, 2010). In sum, the questionnaire operates by allowing participants to recall PA behaviour over several decades by 'pegging' those memories to significant world and life events in a 'Life Calendar'. Uniquely, this approach has been validated against objectively measured PA via accelerometer (Besson *et al.*, 2010) unlike other questionnaires which often exhibit poor correlation and agreement with actual PA (Boyle *et al.*, 2015). Based on the sample (incomplete) Life Calendar provided by Besson and colleagues in their original work (Besson *et al.*, 2010) the author updated the Calendar to encompass life and historical events for the potential eldest participant (75 yrs) from the age of 21 years. Participants completed the 'Life Calendar' section (which was not analysed by the researcher) followed by the formal HAPAQ questionnaire in line with the original authors' instructions. Similarly to previous questionnaires, the questions were asked verbatim with the researcher recording the response. A PA 'prompt card' was also used to identify the specific types of occupational or leisure time PA performed by the participants. In total this questionnaire took between 45 and 80 minutes to complete.

2.7 Preparation of samples for methylation analysis

2.7.1 Buccal swabs

Buccal swabs were defrosted on ice, placed in 500 µl of DNA extraction buffer (0.2M Tris, 0.25mM EDTA, 0.5% SDS, pH 8.5) with 5 µl proteinase K (20 mg/ml) and 5 µl RNAse A (10 mg/ml: Sigma Aldrich) in a 1.5 ml Eppendorf and incubated in a water bath at 50 °C for two hours. Upon removal, buccal swabs were pressed firmly against the sides of the Eppendorf to release as much of the solution as possible. 500 µl phenol-chloroform (Sigma Aldrich) was added to the clear extract under a fume hood,

shaken vigorously until a homogenous mixture was formed (~ 1 minute) and centrifuged at 13.2 x 10^3 RCF for five minutes. The upper, aqueous phase was removed with a pipette and added to 500 µl chloroform, shaken vigorously, and the homogenate again centrifuged at 13.2 x 10^3 RCF for five minutes. The DNA was precipitated by adding the upper aqueous phase to 500 µl iPrOH and 50 µl 3M NaOAc (pH 5.5). This was mixed by flicking and centrifuged at 13.2 x 10^3 RCF at 4 °C for 30 min. The supernatant was removed carefully by pipette and discarded, leaving the DNA pellet undisturbed. 500 µl of ice-cold 70% EtOH was added to the pellet, mixed by flicking and centrifuged at 13.2 x 10^3 RCF for 10 minutes. The majority of supernatant was then removed by pipette, and the pell*et al*lowed to air-dry overnight. This was then resuspended in 20 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The amount of DNA was then quantified by NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific).

2.7.2 Colon biopsies

Genomic DNA was extracted from frozen biopsies using the Genelute Mammalian Genomic DNA Kit (Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK) according to manufacturers' instructions. The biopsies were placed in 180 µl of cell lysis solution for tissue and 20 µl Proteinase K and incubated in a water bath (Grant SUB14, Grant Instruments, Cambridge, Cambs UK) at 55 °C for ~ 36 hours until fully digested, leaving a clear solution. 20 µl of provided RNAse A was added to each solution, vortexed and left to incubate at room temperature for 15 min. 200 µl of cell lysis solution was added, and incubated for 10 min at 70 °C. 200 µl of 100% EtOH was added and mixed by vortexing, and the solution transferred to a binding column and centrifuged for one minute at 13.2 x 10^3 RCF. This was followed by two wash and centrifugation steps using the wash solution provided, and centrifugation at 13.2 x 10^3 RCF for five minutes to dry the column. DNA was eluted in 200 µl of elution solution and quantified by NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific).

2.7.3 Bisulphite modification

DNA (~ 100 ng) was bisulphite-modified using an EZ 96 DNA Methylation-Gold™ Kit (Zymo Research Corp, Irvine, CA, USA) according to the manufacturer's instructions. The kit converts >99% of unmethylated cytosines to uracil, with 75%

DNA recovery. The protocol consists of a conversion/sulphonation step, a binding step, and two wash steps either side of a desulphonation step prior to elution. ~ 100 ng of DNA sample (made up to 20 µl with RNAse free water) was added to a 96-well conversion plate containing 130 µl of conversion reagent, mixed by pipetting up and down, sealed, and placed on a 96-well PCR block for 10 minutes at 98 °C, 2.5 hours at 64 °C, and stored at 4 °C for 18 hours. This comprised the sulphonation and deamination step, where free cytosine was converted to cytosine sulphonate through the addition of a sulphonate group to the 2 – carbon position, followed by bisulphite-mediated deamination to form uracil sulphonate. The samples were then transferred to a supplied 96-well binding plate, containing 600 µl of binding buffer per well, and placed on top of a collection plate. This was covered and centrifuged at 2.25 x 10^3 RCF for five minutes (Eppendorf 5804R). The flow-through was discarded, 400 µl of wash buffer added to each well of the binding plate, and the centrifugation repeated. Afterwards, 200 µl of desulphonation buffer was added to each well and allowed to stand at ambient temperature for 20 minutes. This process cleaves the sulphonate group from the 2 – carbon position, leaving uracil. This was then centrifuged and the flow through discarded. Two more wash steps were completed by adding 400 µl to each well, and centrifuging for five minutes and ten minutes, discarding the flow-through each time. The binding plate was then placed on a 96-well plate (Thermo Fisher Scientific) and the converted DNA was eluted with 40 µl of elution buffer per well. This was allowed to stand for 15 minutes, and centrifuged for five minutes. The eluted DNA was stored at - 20 °C until analysis.

2.8 Methylation analyses

2.8.1 Combined bisulphite restriction analysis (COBRA) and gel electrophoresis

This method was used to quantify the extent of DNA methylation in bisulphite-modified DNA extracted from buccal cells and colon biopsies as described previously. Gene-specific DNA methylation was measured using combined bisulphite and restriction analysis (COBRA) and subsequent enzyme digestion and gel electrophoresis (Xiong and Laird, 1997). Gene-specific primers free of CpGs were designed so as to maintain specificity of the primer to the target sequence after

bisulphite modification (Table 2.2). For the buccal and biopsy bisulphite-modified DNA, reactions were performed in volumes of 5 µl, comprising 1.5 µl of sample, and 3.5 µl of master mix (2.5 µl HotStarTaq (Qiagen, Hilden, Germany), 0.5 µl bovine serum albumin (BSA; 1 mg/ml-1) (Sigma-Aldrich), 0.05 µl $MgCl_2$ (0.5 mM)(Qiagen), 0.01 µl of both forward and reverse primer at 4 pmol concentration (Sigma-Aldrich), and RNAse-free water (Qiagen) up to 5 µl). Master mix batches were made up to 110% of the volume required and dispensed and mixed with samples using a PIRO® robot (Dornier-LTF GmbH, Lindau, Germany) into a 384 well PCR plate (Life Technologies). Three control samples of unmethylated DNA, methylated DNA and RNAse free water (Qiagen) were included in each sample batch. This was sealed, mixed again by vortexing, centrifuged, and placed in a 384 well PCR block (Bioer GenePro, Binjiang, China). The COBRA PCR consisted of a 15 minute 'hot start' at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s and extension at 72 °C for one minute. A final extension step of 72 °C for five minutes was also included. After amplification, samples were EtOH-precipitated by adding 1 part NaOAc (pH 5.5) to 25 parts EtOH in volumes of 13 µl per sample. This was mixed and centrifuged at 2.25 x 10^3 RCF at 4 °C for 30 min. The supernatant was then removed by removing the seal and inverting the plate onto a clean paper towel, and centrifuging at 50 RCF for ten seconds. 25 µl of 70% EtOH was added to each (invisible) pellet and centrifuged for five minutes at 2.25 x 10^3 RCF. The supernatant was removed using the method described above, and the pellets allowed to air-dry overnight. 10 µl of restriction digest solution was added to each sample pellet, containing 0.1 µl restriction enzyme (1-2 units) (New England Biolabs, Ipswich, MA, USA) 1 µl of 10 x cut smart buffer (New England Biolabs, Ipswich, MA, USA) and 8.9 µl of RNAse-free water. This was incubated in a 384 well PCR block for > 4 hours to ensure full digestion. Enzymes and restriction digest temperatures for each gene can be found in (Table 2.2). The enzyme is specific for a base pair sequence containing CpGs (i.e. methylated CpGs unaffected by bisulphite modification), thus cleaving methylated sequences into two smaller fragments, whilst leaving unmethylated sequences unaffected. After digestion, the samples were centrifuged and refrigerated until use.

Table 2.2 Gene panel with combined bisulphite restriction analysis (COBRA) primers and corresponding restriction enzymes

Gene	COBRA primers	Tm	Enzyme (incubation temperature)	Fragment sizes (unmethylated methylated)
SOX17	**F:**GGGGATATGAAG GTGAAG **R:**CACCCAACATCT TACTCAACT	61	BstU1 (60)	193, 135+58
WIF1	**F:**GTGATGTTTTAGG GGTTT **R:**ACCTAAATACCA AAAAACCTA	53	Hinf1 (37)	413, 328+85
BHMT2	**F:**GGGGTTAAGAAG GTGAGTTT **R:**AACCTCAATTTC CTCACCTAT	57	BstU1 (60)	282, 245+37
APC	**F:**GTTAGGGTTAGG TAGGTTGT **R:**CCATAATAACTCC AACACCTA	59.5	Sau3A1 (37)	267, 166+101
TNF	**F:**GGAAGTTAGAAG GAAATAGATTATAG ATT **R:**ACAAACATCAAA AATACCCCT	58	Hinf1 (37)	187, 138+49
IL10	**F:**AGTTAGTAAGGA GAAGTTTTGGGTAT **R:**CTCCTCCTTCTCT AACCTCTCTAA	59	HpyCH41 (37)	331, 237+94
HPP1	**F:**AGAGTTTTTTTTT TATGGTAGTAGTT **R:**ACTCCCACAACA CCATAACTA	56	Taq1 (65)	291, 182+109
IL6	**F:**GTTAAGATATGTT AAAGTGTTGAGTT **R:**CTCAAACATCTC CAATCCTA	56	HpyCH41 (37)	299,141+158
ASC	**F:**GGAGGGGATTAA GGGTGTAGTAA **R:**AAATCTCCAAAT AAAAACTAACCAA CTTA	59	BssHII (50)	372, 188+184

Gel electrophoresis was performed using the polyacrylamide 'mega-gel' method described previously (Wang *et al.*, 2003). A high-throughput vertical mega gel stand (C.B.S Scientific, San Diego, CA, USA) was assembled according to the manufacturer's instructions. The rounded borosilicate back plate was sealed, with spacers and an accompanying gel wrap plate placed on top. This was secured by eight clips and stood upright. 5X Tris-borate-EDTA (TBE) stock buffer (4 g Tris; 37.5 g boric acid, 4.68 g EDTA made up to 1 l with deionised water) was made up, and 100 ml stock was added to 900 ml deionised water to make 0.5X TBE. 40 ml of 0.5X TBE was mixed with 30 ml of acrylamide (Sigma-Aldrich) and 130 ml deionised water in a conical flask. 100 mg of ammonium persulfate (APS) (Sigma-Aldrich) dissolved in 1 ml deionised water and 250 µl tetramethylethylenediamine (TEMED; Sigma Aldrich) was then added and mixed briefly by swirling. This solution was poured smoothly between the glass plates, rapping the apparatus to remove air bubbles where necessary. A 100-well comb was then inserted and the gel left to set for ~30 min. 1 µl of 5X Loading Dye (Bioline Reagents, London, UK) was added to each completed enzyme digestion, centrifuged, and left to stand for five minutes. During this time, the clips were removed from the gel plate sandwich and attached to the stand with gaskets. The upper and lower reservoirs were filled with 0.5X TBE running buffer such that the gel wells were covered and the bottom of the gel was submerged (NB the lower reservoir was filled before inserting the gel sandwich to avoid formation of air bubbles). The gel comb was removed and wells cleared of any debris by pipetting ~ 20 µl of reservoir buffer into each one.

Dyed samples were loaded left-to-right with a multi-channel pipette. Two wells were left blank in the centre of each gel to avoid cutting through samples. 1 µl of HyperLadder™ IV (100 bp) standard (Bioline Reagents) was loaded into the final well after the controls. The gel was run at ~ 200V (Biometra Standard Power Pack P25, Biometra GmbH, Göttingen, Germany) for 1 hour 45 minutes. When the run had completed, the gel was separated from the wrap plates and sliced in half. Both gels were folded, placed into ~ 250 ml of TBE running buffer in a large, shallow container, and unfurled. 10 µl of 100 x SYBR Green reporter dye (Life Technologies) was added to the buffer and agitated to ensure even distribution. The gels were placed on an orbital shaker for > 1 hour and allowed to stain. After staining, the gels were inserted into a PharosFX Molecular imager (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and visualised with QuantityOne Software (Bio-Rad Laboratories). The protocol

'Nucleic Acid Stain - SYBR Green' was selected, and DNA fragments observed at 530 nm. A high quality TIFF image (50 µm resolution) was acquired and analysed using the TotalLab programme (Non-linear dynamics, Newcastle-upon-Tyne, UK). In the 1D analysis suite, lanes were created and sized manually. Background subtraction was performed using the rolling ball method and bands detected automatically. Where appropriate, the pixel position of the band was moved so that it corresponded with the peak signal intensity. These were checked against the standard and methylated and unmethylated control DNA, which had also been bisulphite-modified, to ensure the correct fragment size. The volume of the bands detected in each lane was calculated, and percentage methylation derived by dividing 100 by the total volume of all three bands, and multiplying by the total volume of the methylated fragments.

2.8.2 Quantitative methylation-specific PCR (QMSP)

2.8.2.1 Preparation of Standard plasmids for QMSP assay

Control DNA consisting of a mixture of DNA from the HCT-116, DLD-1, HT-29 and Caco-2 cell lines (American Type Culture Collection (ATCC), Manassas, VA, USA) was bisulphite-modified. After optimisation of COBRA primers, 0.5 µl of sample was PCR amplified (10 µl reaction volume) and diluted to 100 µl with RNase free water (Qiagen). 1 µl of this product was used to optimise the methylation-specific primers. 0.5 µl of the product was ligated into pCR 2.1 vector and inserted into TOP10 e-coli cells (Life Technologies). Colonies were resuspended in RNase free water and screened by PCR using methylation-specific primers. Suspensions containing correct clones identified by gel electrophoresis were cultured and purified using a QiaPrep mini kit (Qiagen). Purified DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and 50 ng of sample made up to 5 µl RNase free water was sequenced using a BigDye terminator cycle sequencing kit (Life Technologies) and an ABI 3100 Avant sequencer (Applied Biosystems®, Life Technologies).

Methylated fragments were released from purified DNA by restriction digest using HindIII and Spe1, and unmethylated fragments released using HindII and Xba1. These were purified from agarose gel as above and ligated using Quick Ligase and inserted into TOP10 e-coli cells (Life Technologies). Colonies were picked and resuspended, plasmids were PCR amplified using M13 primers (Sigma Aldrich),

screened and purified from agarose gel and quantified as described previously. Standards were created by adding purified plasmid DNA to 100 µl of EB at a concentration of 1 ng/µl (first standard) and serially diluted ten-fold to make four standards. These were frozen at -75 °C until use.

2.8.2.2 QMSP assay

DNA from peripheral blood leukocytes was isolated by the Cardiovascular Health Study team and shipped to our laboratory on dry ice. ~ 500 ng of DNA was bisulphite modified (see 3.7.3 Bisulphite modification). 1.5 µl of sample, methylated and unmethylated control DNA, and water, was PCR amplified using a COBRA protocol in a 384 well PCR block (see 3.8.1 Combined bisulphite restriction analysis (COBRA) and gel electrophoresis). The PCR product was diluted with 20 mM Tris-HCl to a concentration ~ 1.6 x 10^{-3} of the original (i.e. 1 µl diluted in 25 µl Tris-HCl, and repeated).

1.5 µl of diluted sample and four plasmid standards were added to a master mix containing 2.5 µl Immomix, 0.5 µl BSA (0.5 $mg \cdot ml^{-1}$), 0.2 µl $MgCl_2$ (0.5mM), 0.1 µl ROX reference dye, 0.03 µl 100 x diluted SYBR Green, 0.01 µl forward and reverse methylation-specific primers (both 4 pmol/l) (Table 2.3), made up to 5 µl with RNase free water, in triplicate. This was RT-PCR amplified in a Viia™ 7 Real-Time PCR System (Applied Biosystems) using the following protocol: 10 minute 'hot start' at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing for 30 s and extension at 72 °C for 30 s. The plate was read during the extension step. The cycle threshold (CT) was detected automatically but adjusted manually where necessary and the results exported to a .csv file. Means were calculated for each triplicate and outliers eliminated. The mean CT of each triplicate standard was plotted against the expected number of log copies. The equation of the fit line ($y = mx + c$) was used to calculate the inverse log copy number for the mean of triplicate samples (i.e. 10^ (mean CT – c) / - m)). This was performed for both the methylated and unmethylated reactions. Percentage methylation was calculated using the formula 100 × M copies / (U copies + M copies). The experimental work for the QMSP assay was performed by Dr Wing Leung.

2.9 Cell culture experiments

2.9.1 Cell handling

All reagents were warmed to 37 °C and sprayed with 70% EtOH before use. All experiments were conducted in a Herasafe™ Class II Biological Safety Cabinet (Thermo Fisher Scientific). Colorectal cancer cell lines (HT29, Caco-2, LoVo and HCT-116; American Type Culture Collection (ATCC), Manassas, VA, USA) stored at -196 °C were placed on dry ice and thawed by gently mixing with 1 ml of Gibco® Dulbecco's Modified Eagle Medium F12 + GlutaMAX™ (DMEM/F12 + GlutaMAX™, Life Technologies Ltd, Paisley, UK) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin-streptomycin solution (PS; Sigma-Aldrich Company Ltd, Gillingham, Dorset, UK). This was added to 5 ml of DMEM/F12 in a T25 Nunc™ cell culture flask (Thermo Fisher Scientific) and cells were allowed to adhere in an incubator for 24h at 37 °C and 5% CO_2 (RS Biotech Galaxy S+, CMS Scientific, West Lothian, Scotland, UK).

Cells were passaged by removing the DMEM/F12, rinsing with ~ 3ml of chilled sterile phosphate buffered saline (PBS), adding 3 ml of 0.05% Trypsin-EDTA solution (Life Technologies) and incubating for 5-10 min until cells had were no longer adherent. The solution was then transferred into 7 ml of fresh DMEM/F12 in a 15 ml Falcon™ conical collection tube (Thermo Fisher Scientific) and centrifuged at ambient temperature at 300g/1179 rpm for 4 min (Heraeus™ Labofuge™ 400; Thermo Fisher Scientific). The supernatant was poured off, and the remaining cells resuspended thoroughly in 10 ml of fresh DMEM/F12. 2ml of this resuspension (or 1 ml in the case of HCT-116 which proliferate more quickly than the other cell lines) was added to 10 ml of fresh DMEM/F12 in a Nunc™ T75 and incubated. Media was replaced every 2-3 days and cells passaged every 5-8 days when they had reached 80% confluence. All experiments were performed within 10 passages.

2.9.2 Cell seeding

For preparation of cells for treatment, the passage procedure was halted at the resuspension step once the cells had reached 80% confluence. Here, the number of cells present in the 10 ml resuspension was estimated by extracting 20 µl and

manually counting the number using a haemocytometer, microscope and click-counter. The numbers of cells in each quadrant were counted twice, with the average number taken to represent the number of cells present per 0.1 µl. This was then multiplied by 1 x 10^6 to return the total number of cells in the suspension. 30 ml of solution was made up with a mixture of fresh DMEM/F12 containing FBS and PS and cell resuspension, such that ~ 10,000 cells per 100 µl were present (e.g. in a 10 ml cell resuspension containing 1.4 x 10^7 cells, 2.14 ml was added to 27.86 ml of fresh media). This cell density was used for Caco-2, HT-29, and LoVo cells. Owing to the more rapid proliferative capacity of HCT-116 cells (Ahmed *et al.*, 2013), the cell density was halved (i.e. 5000 cells per 100 µl). These were gently mixed in a 50 ml Falcon tube by a motorized pipette (Eppendorf Easypet™, Hamburg, Germany). Plates were seeded by adding 100 µl of the mixture per well. To maintain an even distribution of cells in the mixture, the tube was swirled gently after every six wells, and inverted gently after each plate. Vigorous mixing was avoided to prevent foaming. All plates were seeded for 24 hours in an incubator set at 37 °C and 5% CO_2 concentration (RS Biotech Galaxy S+, CMS Scientific, West Lothian, Scotland, UK).

2.9.3 Cell proliferation assay (WST-1)

The water soluble tetrazolium salt (WST-1) assay (Roche Daignostics Corporation, Indianapolis, IN, USA) works on the principle that the glycolytic product, nicotinamide adenine dinucleotide phosphate ($NADP+$), cleaves the salt into a yellow formazan dye. The amount of dye produced is therefore proportional to cellular metabolic activity and is an accurate proxy measure of the number of viable cells. Optimisation work with FBS control serum demonstrated that proliferation of cell lines was in its exponential phase after three hours. Cells were therefore incubated for three hours before assay. 3 ml of cell proliferation reagent WST-1 (Roche Diagnostics Corporation, Indianapolis, IN, USA) was thawed from - 20 °C and mixed with 30 ml DMEM/F12. The media from each well of a 96 well plate (Nunc™, Thermo Fisher Scientific) was removed with a multi-channel pipette and replaced with 100 µl of DMEM/F12 + WST-1. An additional well which had remained free of cells and media during incubation was filled with 100 µl of DMEM/F12 + WST-1 to act as a negative control. The time of complete replacement of media on each plate was recorded. The plates were then incubated for three hours at 37 °C and 5%

CO_2.The colorimetric assay was performed with a microplate reader using a single read Endpoint protocol (Bio-Rad Benchmark Plus, Bio-Rad Laboratories, Hercules, CA, USA). Dual absorbance was set between 450nm and 690nm as per manufacturers' instructions, with a five second mix time at medium speed prior to plate read. Approximately two minutes was allowed between plate reads so each plate received three hours of incubation before analysis. Mean absorbance of replicate sample wells and the six control wells treated with FBS on the same plate was calculated. The absorbance of the negative control was subtracted, and the results expressed as a percentage of the FBS control.

2.9.4 Gene expression from cultured cells

RNA was isolated from cultured cells in 24 well plates (Nunc™, Thermo Fisher Scientific) using an Isolate II RNA Mini Kit (Bioline) according to manufacturers' instructions. Cell culture media was removed, and 350 µl RLY lysis buffer and 3.5 µl beta-mercaptoethanol (β-ME; Sigma Aldrich) was added to each well and left for five minutes. The lysate was extracted and passed through a filter column by centrifugation at 13.2 x 10^3 RCF. 350 µl 70% EtOH was added to the lysate and mixed. This was centrifuged at 13.2 x 10^3 RCF in a binding column and desalted by centrifugation with 350 µl membrane desalting buffer (MEM). DNA was digested for 15 min at ambient temperature by adding 100 µl pre-prepared DNA reaction buffer to the centre of the membrane. Three wash steps were performed and RNA eluted in 60 µl RNase free water (Qiagen) and quantified by NanoDrop Spectrophotometer (Thermo Fisher Scientific).

4 µl (~ 200 ng) of eluted RNA was placed in a 96-well PCR plate (Thermo Fisher Scientific) and added to 4 µl qScript™ cDNA Supermix (Quanta Biosciences, Gaithersburg, MD, USA) and made up to 20 µl with RNase free water (Qiagen). The contents were sealed, vortexed and centrifuged at 2.25 x 10^3 RCF for one minute. The samples were then reverse-transcribed in a 96-well PCR block (Biometra T-Gradient, Biometra GmbH, Göttingen, Germany) with the following steps: 25 °C for five minutes, 42 °C for 30 minutes, 85 °C for five minutes, and 4 °C overnight. This was then diluted in 80 µl RNase free water and frozen at - 20 °C until use.

1.5 µl of cDNA sample were added to a master mix containing 2.5 µl Immomix, 0.5 µl BSA (1 mg/ml-1), 0.2 µl $MgCl_2$ (0.5 mM), 0.1 µl ROX reference

dye, 0.03 µl 100 x diluted SYBR Green, 0.01 µl forward and reverse primers (both 4 pmol concentration) (Table 2.4), made up to 5 µl with RNase free water, per sample, in triplicate. This was RT-PCR amplified in a Viia™ 7 Real-Time PCR System (Applied Biosystems) using the following protocol: 10 minute 'hot start' at 95 °C, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The plate was read during the extension step. The cycle threshold (CT) was detected automatically but adjusted manually where necessary and the results exported to a .csv file. Eight genes were analysed, including the ribosomal DNA sequence *18S* which was used as reference gene (Table 2.4). Mean CTs were calculated from triplicate values and outliers eliminated. Gene expression relative to *18S* was calculated using the ΔΔCT method described previously (Schmittgen and Livak, 2008).

2.9.5 Global methylation in cultured cells

DNA was extracted and purified from 24 well culture plates using the phenol-chloroform method and bisulphite-modified using the techniques described previously (see 3.7 Preparation of samples for methylation analysis). LINE-1 methylation, which has been recognized as a suitable surrogate for global DNA methylation (Weisenberger *et al.*, 2005) was analysed by an adapted form of the QMSP assay described previously (Iacopetta *et al.*, 2007) (see 3.8.2.2 QMSP assay), which forwent the COBRA PCR step. LINE-1 primer sequences can be viewed in Table 2.3.

Table 2.3 Primer sequences for combined bisulphite restriction analysis (COBRA) and quantitative methylation specific PCR assay (QMSP).

Gene	COBRA primers	QMSP-U primers	QMSP-M primers
ABCA1	**F:**GGAAAGTAGGATTTAGAGGAAG **R:**ATAAAAAATACAACAAATATCTTAAAATC	**F:**TTTTTGGTTGTTGGGAAT **R:**AAAAACACTAAACTCAAAACTACA	**F:**TGTTTTTGGTTGTCGGGAAC **R:**AAAAACGCTAAACTCGAAACTACG
SNCG	**F:**GTTGGGTTGAGTTAGTAGGAGTTT **R:**AAACCCTTCTTAAAAACATCCATA	**F:**TGGTTTTTGTATTAATATTTTATTGGT **R:**AAAACTAAATCTCCCTACAAACTACA	**F:**GGTTTTCGTATTAATATTTTATCGGC **R:**GAAACTAAATCTCCCTACGAACTACG
NOD2	**F:**TGATGTAGTTGTTGGGAGGATA **R:**CTCCCTTACACACCAAACCTA	**F:**GAGTTTTGAGTTATGTGGTTTGGGT **R:**ATATAAACCATACACACCCCAACTAATAACA	**F:**TCGAGTTACGTGGTTTGGGC **R:**GTATAAACCATACACACCCCAACTAATAACG

SOD3	**F:**GTTGTGTGTTGAAGGTTATTGGTTA **R:**ACCTTTCCAACTCCTCCAAA	**F:**TGTGGAATGGAATTTTTGGTTT **R:**ACCTCCACCTCCTCCCA	**F:**CGCGGAATGGAATTTTTGGTTC **R:**GCCTCCACCTCCTCCCG
TNF	**F:**GGAAGTTAGAAGGAAATAGATTATAGATT **R:**ACAAACATCAAAAATACCCCT	**F:**GGTTTTGAGGGGTATGGGGAT **R:**CATCCTCCCTACTCCAATTCCA	**F:**GGTTTTGAGGGGTATGGGGAC **R:**ATCCTCCCTACTCCGATTCCG
IL10	**F:**AGTTAGTAAGGAGAAGTTTTGGGTAT **R:**CTCCTCCTTCTCTAACCTCTCTAA	**F:**TGTTATTGTGATTTAGGAATATGT **R:**TCCTAAAAAAACAACTATTCTATACA	**F:**CGTTATTGTGATTTAGGAATACGC **R:**AATTTCCTAAAAAAACAACTATTCTATACG
UGT1A1	**F:**TGTGGATTGATAGTTTTTTATAGTT **R:**CAACAATATCTTCCCAACAT	**F:**TGTGATATAGTTAAATATTAATTTGGTGTATT **R:**CTAAAACTCCACAACCATAACA	**F:**CGTGATATAGTTAAATATTAATTTGGTGTATC **R:**CTAAAACTCCACAACCATAACG
SERPINA5	**F:**GGTTTAGTTGGTGGTGTAAGGTTT **R:**AACCCCAAAACTCTAAAATCCAT	**F:**TGTTTGTAGGTAGGTTTGTTGGTT **R:**AAAAACCTCCCTTTACAACATCA	**F:**CGTTTGTAGGTAGGTTTGTTGGTC **R:**AAACCTCCCTTTACGACATCG
LINE-1	n/a	**F:**TGTGTGTGAGTTGAAGTAGGGT **R:**ACCCAATTTTCCAAATACAACCATCA	**F:**CGCGAGTCGAAGTAGGGC **R:**ACCCGATTTTCCAATACGACCG

Table 2.4 Primer sequences for gene expression experiments in cultured cells. All Tm 60 °C.

Gene	**Forward primers**	**Reverse primers**
MUC2	TGTAGGCATCGCTCTTCTCA	GACACCATCTACCTCACCCG
COX-2	CCGGGTACAATCGCACTTAT	GGCGCTCAGCCATACAG
Ki-67	TGACTTCCTTCCATTCTGAAGAC	TGGGTCTGTTATTGATGAGCC
DNMT3b	TCTCCATTGAGATGCCTGGT	GAGATTCGCGAGCCCAG
E-Cad	GACCGGTGCAATCTTCAAA	TTGACGCCGAGAGCTACAC
LGR-5	GTTTCCCGCAAGACGTAACT	CAGCGTCTTCACCTCCTACC
C-Myc	CACCGAGTCGTAGTCGAGGT	TTTCGGGTAGTGGAAAACCA
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

2.10 Statistical analysis

All numerical values in tables and within the body text are presented as mean (SD) unless otherwise stated. In all figures with error bars, these are displayed as mean (SE), excepting the box-plots for baseline methylation data in Figure 3.2, Figure 4.4, and Figure 4.5, which show medians and 95% confidence intervals. For exploratory analysis of the longitudinal data in the first of these studies, initial investigations were performed using SPSS v.20 (IBM, Armonk, NY, USA). Hypothesis generation was conducted based on the outcome of these investigations. Subsequent to this, ANOVA-based models were constructed and figures created using Matlab (Mathworks Inc., Cambridge, UK) by Dr Henri Tapp. These are detailed in Chapter 3 of this thesis.

Analysis of the intervention data was performed using SPSS v.20 (IBM, Armonk, NY, USA). All ratio data was assessed for normality using the Kolmogorov-Smirnov test. Where normality assumptions were violated, non-parametric tests were used. The Physical Activity and Risk of Colon cancer (PARC) trial, whose design was a pre-test post-test randomised group comparison, was analysed using n-way ANCOVA. The primary outcome measures for each test were DNA methylation status of target genes, self-report physical activity, accelerometer derived physical activity, and cardiorespiratory fitness by group allocation (control vs intervention) after 6 months and after 12 months. The baseline value of the primary outcome measure was added as a covariate to correct for the potential confounding effect of group differences at baseline. Results from the Regular Exercise and colon cell Growth (ReGro) study were analysed using a repeated-measures ANOVA. Sphericity was assessed using Mauchley's Test of Sphericity. Where mild sphericity was evident the Huyhn-Feldt correction was used for any significant F values, and Greenhouse-Geisser corrected in cases of more severe asphericity. Where significance was reached, post-hoc T-tests with a Bonferroni correction were used to locate any differences.

Chapter 3

Study 1: Associations between physical activity and differential methylation of genes in peripheral blood leukocytes: results from The Cardiovascular Health Study (CHS)

3.1 Introduction

Epigenetic mechanisms, defined as heritable changes in gene function that are not dependent on DNA sequence, are increasingly recognised as playing an important role in the development of a number of chronic diseases including colon cancer (CC) (Robertson, 2005). Perhaps the best understood epigenetic modification is DNA methylation, which affects cytosines in cytosine-guanine (CpG) dinucleotides and is generally associated with transcriptional repression (Robertson, 2005). DNA methylation patterns are established in early life and are generally stable in somatic cells. However, aging has been shown to induce changes to the methylation status of numerous genomic loci across a broad spectrum of human tissues. Importantly, the role of environmental stimuli as modulators of age-related DNA methylation has been demonstrated (Christensen *et al.*, 2009; Talens *et al.*, 2012; Tapp *et al.*, 2013).

CC is an age-related disorder whose risk is associated with several lifestyle factors including physical activity (PA) (Wolin *et al.*, 2009). Numerous observational studies have demonstrated an inverse association between PA level and CC incidence and mortality (Wolin *et al.*, 2010; Wolin *et al.*, 2009). Increased PA, for the most part, beneficially modulates adiposity, blood pressure, lipid and glucose metabolism (Nelson *et al.*, 2007) and has been associated with apparently beneficial effects on chronic inflammation in older persons (Hamer *et al.*, 2012; Woods *et al.*, 2012). However, the molecular mechanisms underpinning the long-term health benefits of regular PA in relation to CC risk are poorly understood.

Recently both the Sister Study (White *et al.*, 2013) and North Texas Healthy Heart Study (Zhang *et al.*, 2011) showed a positive association between PA and LINE-1 methylation in leukocytes from healthy participants. LINE-1 methylation is a surrogate marker for global DNA methylation, which has been shown to decrease with ageing and CC in colon tissue (Sunami *et al.*, 2011; Tapp *et al.*, 2013). In addition to effects on global DNA methylation, studies have reported altered DNA methylation of genes implicated in chronic inflammation, type 2 diabetes and breast cancer in saliva, blood, skeletal muscle and adipose tissue after short-term exercise programmes (Bryan *et al.*, 2013; Nakajima *et al.*, 2010; Nitert *et al.*, 2012; Ronn *et al.*, 2013). This raises the question of whether age-related methylation of genes implicated in inflammatory processes that are linked to CC risk can be modulated by healthy lifestyle behaviours, such as regular PA, in later life.

Given the potential role of habitual PA in modifying CC risk, and evidence of changes in DNA methylation patterns following short-term exercise interventions, the methylation status of genes known to be implicated in chronic inflammation, together with LINE-1, was determined in leukocytes collected from a sample of older adults participating in the Cardiovascular Health Study (CHS) (Fried *et al.*, 1991). Associations between self-reported levels of leisure-time PA collected at two clinic visits eight years apart and corresponding leukocyte DNA methylation were investigated.

Although most studies investigating disease-specific epigenetic events have focused on the tissues affected, several previous studies have demonstrated age-related epigenetic changes in DNA from whole blood cells including those involving twins and, therefore, implicating the environment/lifestyle in 'driving' epigenetic drift (Bjornsson *et al.*, 2008; Boks *et al.*, 2009; Christensen *et al.*, 2009; Fraga *et al.*, 2005; Kaminsky *et al.*, 2009; Shen *et al.*, 2007). Of significant importance in this respect is the very recent demonstration that both aging and environmental exposures linked to risk of diseases in other tissues are reflected by alterations to gene-specific methylation in blood-derived DNA (Christensen *et al.*, 2009) indicating the utility of this readily-available material for investigating the impact of lifestyle on biological aging and the risk of associated diseases. Such analyses, despite the lack of availability of colon epithelia from CHS participants, could therefore provide valuable insight into CC risk.

3.2 Methods

3.2.1 Study participants

The CHS is a multisite observational cohort study designed to investigate risk factors for CVD in the elderly (Fried *et al.*, 1991). The CHS recruited 5,201 participants during 1989/90 from Medicare eligibility lists in four U.S. communities: Forsyth County, NC, Washington County, MD, Sacramento County, CA, and Pittsburgh, PA. In 1992/93 an additional 687 African-Americans were recruited into the study. Participants were aged 65 or older at the baseline examination. All participants signed an informed consent form at entry into the study and at specified intervals during the course of the study.

From 1989/90 until 1999/00, annual clinic visits were completed by study participants. Data were collected at these annual examinations including vital signs, anthropometry, medical history and behaviours, physical function, and psychosocial interviews. Demographics collected at baseline included age, gender, and education. Blood was collected and stored during most years of the study. Participants were evaluated for the prevalence of specific cardiovascular outcomes at baseline, including myocardial infarction, angina pectoris and stroke. (Mittelmark *et al.*, 1993)
For this study, 493 Caucasian and 50 non-Caucasian participants that completed both the 1991/1992 (baseline) and 1999/2000 (year 8) clinic examinations were randomly selected from CHS participants that provided consent to use genetic material. Duplicate cases were eliminated leaving a total of 540 samples used for analysis.

3.2.2 Measurement of DNA methylation

The panel of genes selected were based on their reported role in cardiovascular disease, and a comprehensive report is being published separately. Therefore, the findings will be interpreted with regard to the inflammation-related genes that could plausibly be involved in CC, namely *TNF* and *IL10*. Genomic DNA was isolated from peripheral blood leukocytes collected from the participants at clinic examinations at baseline and year 8. Bisulphite conversion of DNA (500 ng) was performed using the EZ DNA methylation Gold kit (Zymo Research) following manufacturers' instructions. An adapted quantitative methylation-specific PCR (QMSP) assay

(Fackler *et al.*, 2004) was used to quantify the methylation status of LINE-1 and the genes encoding *TNF* (tumour necrosis factor), *SNCG* (synuclein gamma), *SOD3* (superoxide dismutase 3), *IL10* (interleukin 10), *SERPINA5* (serpin peptidase inhibitor A5), *NOD2* (nucleotide-binding oligomerization domain 2), *ABCA1* (ATP-binding cassette A1) and *UGT1A1* (UDP glucuronosyltransferase 1A1). The expression of each of these genes is negatively regulated by DNA methylation (Belanger *et al.*, 2010; Christensen *et al.*, 2009; Dokun *et al.*, 2008; Jiang *et al.*, 2008; Kerkel *et al.*, 2010; Larsson *et al.*, 2012; Lee *et al.*, 2013; Liang *et al.*, 2013; Sullivan *et al.*, 2007). They have also previously been shown in at least two studies to be subject to differential methylation in human leukocytes in response to age and/or environmental exposures and to play a role in cardiovascular health (Baccarelli *et al.*, 2010; Bruunsgaard *et al.*, 2000; Christensen *et al.*, 2009; Fukai and Ushio-Fukai, 2011; Galluzzo *et al.*, 2011; Gowers *et al.*, 2011; Guay *et al.*, 2012; Hoang *et al.*, 2008; Juul *et al.*, 2004; Kolbel *et al.*, 2006; Oram and Vaughan, 2006; Rau *et al.*, 2007). The QMSP assay had two steps. First, PCR was used to amplify the region of interest from bisulphite-modified DNA independent of methylation status using COBRA primers that do not contain CpGs (Table 2.3). Second, two separate quantitative real-time PCRs were performed using the products of the first PCR as template with methylation status-specific (U-unmethylated; M-methylated) primers. This determined the copy numbers of unmethylated and methylated PCR fragments, calculated from standard curves from plasmids containing cloned methylated and unmethylated PCR fragments, to give: % methylation = 100 × M copies / (U copies + M copies). All assays were performed in triplicate. Methylation-specific primer sequences are also shown in Table 2.3.

3.2.3 Physical activity

Leisure time PA levels of the entire CHS cohort (n = 5,888) were recorded using an interview-based questionnaire at baseline and year 8. Participants provided information on the type of activity they undertook (e.g. walking for exercise, callisthenics, gardening), how often the activity was completed in the past two weeks, the duration of each bout, and the number of months per year that it was undertaken. The total weekly PA energy expenditure (PAEE: kcal) was calculated for selected

participants using the method of van Stralen (van Stralen *et al.*, 2008) which uses the metabolic equivalent task (MET) score for the intensity of each activity.

Weekly energy expenditure (kcal) = (MET intensity score × minutes spent in each activity) × (number of sessions reported in last two weeks)/2 × (number of months per year each activity was undertaken)/12.

This calculation was performed for each activity to provide total leisure time PAEE/week for each participant at both time points. Change in PA (ΔPAEE) was calculated as the difference between the two time points and then stratified into the following three categories: participants who increased PAEE by >500 kcal/wk (INC), those who decreased PAEE by >500 kcal/wk (DEC), and the remaining cohort who showed a nominal overall change in PAEE of <500 kcal/wk (NOC). The 500 kcal cut-off represents approximately 2 hours of brisk walking according to the calculation of van Stralen (van Stralen *et al.*, 2008). Brisk walking equates to a MET score of 3.8 (Ainsworth *et al.*, 2000), which in a male of average fitness aged 70-79 is perceived as 'moderate' activity (O'Donovan *et al.*, 2010). Hence, a change in PAEE of >500 kcal/wk represents a tangible change in PA level between baseline and year 8. Participants reporting dubious PAEE values (i.e. zero or >15,000 kcal/week) or with missing anthropometric data at either interview were excluded from the analysis, leaving a sample of 390 Caucasian participants comprising 253 women and 137 men with the following mean (SD) characteristics at baseline: women were aged 70.7 (4.0) years, with waist circumference 91.2 (13.2) cm and leisure time PAEE 1313 (1335) kcal/wk; men were aged 71.4 (4.3) years with waist circumference 97.0 (10.0) cm and PAEE 2226 (2209) kcal/wk. A further 31 non-Caucasian participants comprising 20 women aged 70.3 (4.1) years, with waist circumference 93.0 (12.5) cm and PAEE 1118 (1391) kcal/wk, and 11 men aged 70.8 (4.7), with waist circumference 99.2 (8.6) and PAEE 1277 (660) were also included. A flow diagram of the cohort selection procedure is presented (Figure 3.1).

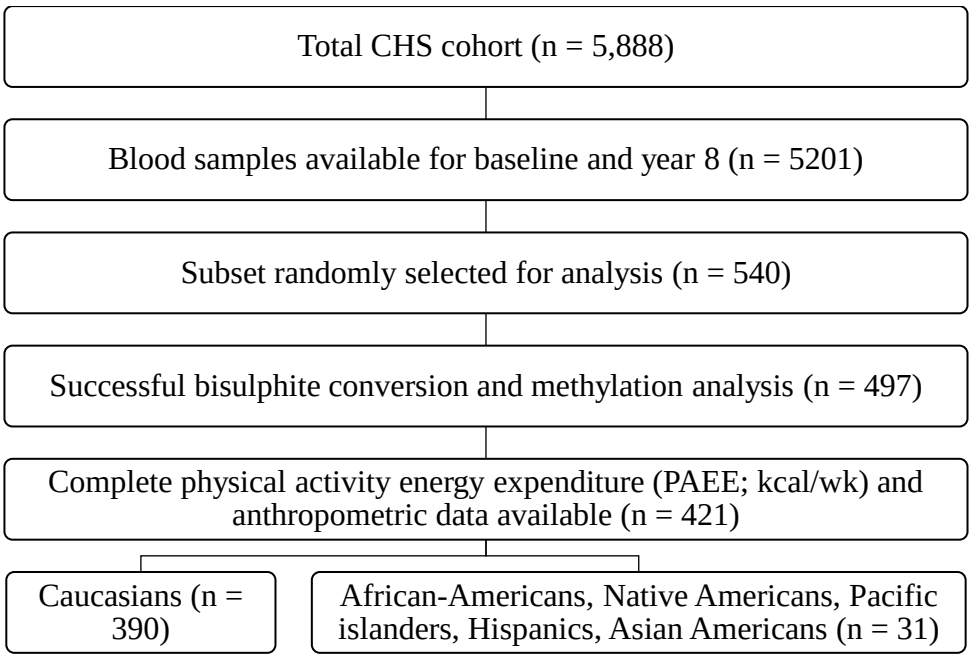


Figure 3.1 Flow diagram depicting the selection of participants used for analysis.

3.2.4 Statistical analysis

Exploratory investigations were performed using n-way analysis of covariance (ANCOVA) (SPSS v19, IBM, Armonk, IL) with methylation data that did not require transformation (i.e. *IL10* and *TNF*). ΔPAEE and gender were the independent variables, baseline age was the covariate, and change in methylation was the dependent variable. On the strength of these findings, further analysis was conducted to find i) whether there is an association between age, gender and the extent of DNA methylation, in line with previous reports ii) if ageing of the participants in the study impacted DNA methylation and iii) whether physical activity, or a change in physical activity, affected the extent of age-related DNA methylation independent of other confounding variables. To this end, gender differences in methylation at baseline were investigated using Wilcoxon-Mann-Whitney's rank sum test, and the change between baseline and year 8 using a Fisher sign test. Where evidence of association was found, Type I analysis of variance (ANOVA) was used to explore the explanatory ability of PAEE in the presence of the other variables ('age', 'sex', 'plate', 'log(Wbld)' and 'ΔWaist'), after applying logit and fourth-root transformations to the fractional methylation and PAEE variables, respectively.

Using all available data (irrespective of PAEE), associations between baseline methylation and age were investigated using Spearman rank correlation. The change in the proportion of methylation between baseline and year 8 for the eight genes and LINE-1 was treated as the response variable and analysed using analysis of variance, ANOVA. Associations between baseline methylation and age (Spearman correlation) were compared with longitudinal changes in methylation (Fisher's sign test) using standardised z-scores, which for age were calculated as $z_{SRC} = \sqrt{(n-3)/1.06}\,\mathrm{atanh}(\rho_{\mathrm{SRC}})$, where n and ρ_{SRC} are the sample size and Spearman rank correlation, respectively.

To determine any potential impact of a change (Δ) in PAEE on change in methylation, independent of some key variables with which it might be associated (age, gender, waist circumference and white blood cell count), three sets of modelling were conducted. First, models were constructed using the stratified ΔPAEE as a single explanatory categorical variable. Second, an additional five explanatory variables were introduced as main effects and the explanatory significance of ΔPAEE investigated in their presence (type I ANOVA). The five additional explanatory variables comprised two categorical variables and three continuous variables. The categorical variables were the 96-well plate batch identifier, which was treated as a block effect ('Plate'), and gender ('Sex'). The continuous variables were age at baseline ('Age'), logarithmically transformed white blood cell count collected at baseline ('log(Wbld)') and change in waist circumference between baseline and year 8 ('ΔWaist'). Third, backward elimination (using hierarchical type II ANOVA) was used to simplify an initial model comprising the five main effects; interactions between two categorical variables (ΔPAEE, gender); and interactions between these categorical variables and the three continuous variables. Power transformation of the response was applied where appropriate based on Box-Cox diagnostics. Summary ANOVA tables of simplified models containing ΔPAEE are provided as supplementary material.

Samples from the non-Caucasians were located on a single 96 well plate, and there was considerable heterogeneity of ethnicity therein (African-American, Hispanic, Native American, Pacific Islander, and Asian American). Given that considerable genomic variation occurs between populations (Haga, 2010) and that the effects of ethnicity (if any) could not be separated from the batch effects of sample

plates, this analysis was performed separately and provided as supplementary material. This highlights the need for targeted recruitment of these under-represented populations in future studies.

Approximately 10% of participants showed lower levels of *SERPINA5* methylation (< 10%) compared with the rest of the group (21 – 93%). The reason for this was unclear, especially considering that several of the 'low methylators' fell into this category at only one time point. That this was the case suggests a failure of the assay might have been responsible, although this could not be confirmed. To reduce the risk of a Type 1 error, the 'low methylators' were therefore excluded from all analyses of *SERPINA5* only. The size of the main study group and for *SERPINA5*, by gender and ΔPAEE, are summarised in Table 4.1. Statistical analysis was performed using Matlab (Mathworks Inc., Cambridge, UK). Significance was accepted where $P < 0.05$.

Table 3.1 Size of main study group and *SERPINA5* subgroup stratified by gender and ΔPAEE: DEC, decrease of >500 kcal/wk; NOC, change of <500 kcal/wk; INC, increase of >500 kcal/wk.

Subgroup sizes	**Main study group**			***SERPINA5* subgroup**		
	DEC (%)	NOC (%)	INC (%)	DEC (%)	NOC (%)	INC (%)
Females	92 (36)	122 (48)	39 (16)	79 (34)	114 (50)	37 (16)
Males	65 (47)	49 (36)	23 (17)	57 (47)	45 (37)	20 (16)
All	157 (40)	171 (44)	62 (16)	136 (39)	159 (45)	57 (16)

3.3 Results

3.3.1 Differences in DNA methylation by gender

The methylation status of the eight genes and LINE-1 at baseline and year 8 are summarised in Figure 3.2 and Table 3.2. Table 3.2 also shows the differences in methylation at baseline between genders, and the change in methylation between baseline and year 8. At baseline, methylation of *NOD2*, *SNCG* and *SOD3* was significantly greater in women, while LINE-1 was significantly less methylated.

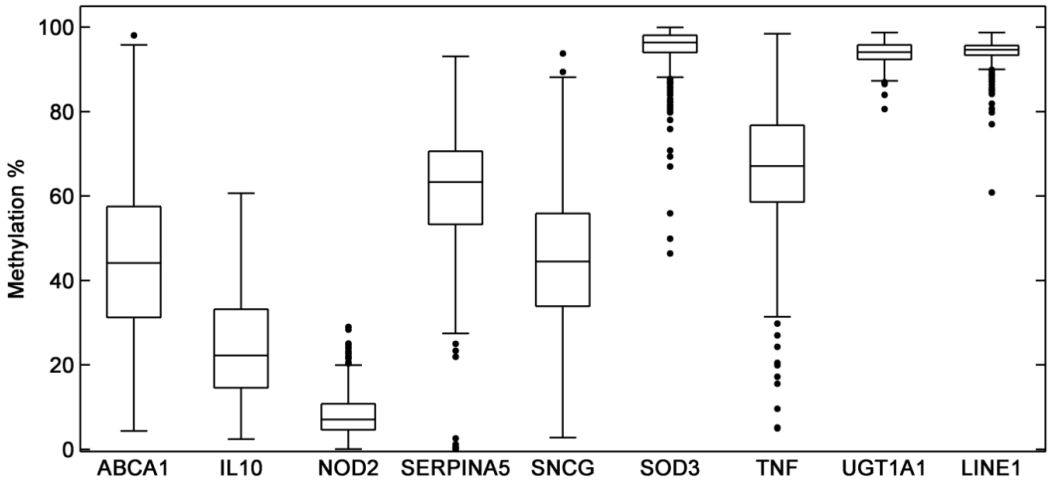


Figure 3.2 Summary descriptions of DNA methylation values for the eight genes and for LINE-1 at baseline

3.3.2 Associations between DNA methylation, baseline age and ageing

The associations between DNA methylation at baseline with age were compared with the longitudinal changes in methylation using all the available data (497 cases, or 444 for *SERPINA5*. Figure 3.3 provides strong evidence ($P < 0.01$) for a negative association between age and *NOD2* methylation and a positive association with *SERPINA5* methylation. Figure 3.3 also shows significant longitudinal changes between baseline and year 8 for reduced methylation of *IL10*, *NOD2* and *SNCG* and increased methylation of *TNF* and *SOD3*. Together these observations show that for several genes the relationship between age and methylation changed significantly during the course of the study.

Table 3.2 Methylation levels (%) at baseline, gender differences in methylation at baseline, and change in methylation between year 8 and baseline. The first and third quartiles are denoted as Q1 and Q3, respectively. The lower and upper 95% confidence intervals are denoted L95%CI and U95%CI, respectively, and were calculated using a large sample approximation. The difference (female – male) in baseline methylation is denoted ΔM_{F-M}, and was evaluated using the Wilcoxon-Mann-Whitney rank sum test. The change in methylation (year 8 – baseline) is denoted ΔM_{8-0}, and was evaluated using the Fisher sign test. †: Analysis of *SERPINA5* was conducted on a subgroup of 352 individuals.

DNA sequence	**Median**	**Q1**	**Q3**	**ΔM_{F-M}**	**L95%CI**	**U95%CI**	**p-value**	**ΔM_{8-0}**	**L95%CI**	**U95%CI**	**p-value**
ABCA1	44.36	30.84	57.22	-2.87	-6.98	1.43	0.211	0.174	-1.536	1.266	0.879
IL10	21.70	14.23	32.16	1.93	-0.52	4.40	0.122	-4.397	-6.067	-3.022	9.3E-13
NOD2	7.09	4.72	11.29	1.24	0.40	2.16	0.005	-1.938	-2.265	-1.660	1.3E-19
SERPINA5†	64.70	56.10	70.60	-1.83	-4.35	0.55	0.139	-0.398	-1.434	0.525	0.488
SNCG	44.05	33.64	55.53	5.69	2.39	8.92	8.19E-04	-2.074	-4.307	-0.899	0.004
SOD3	96.36	94.10	98.17	1.79	1.11	2.47	5.59E-08	0.256	0.002	0.414	0.038
TNF	67.96	58.94	77.27	-0.89	-3.62	1.85	0.506	2.538	0.515	3.854	0.004
UGT1A1	94.47	92.69	95.97	-0.24	-0.73	0.27	0.357	0.029	-0.112	0.180	0.578
LINE-1	94.68	93.39	95.60	-0.82	-1.14	-0.51	8.80E-07	-0.158	-0.286	0.046	0.244

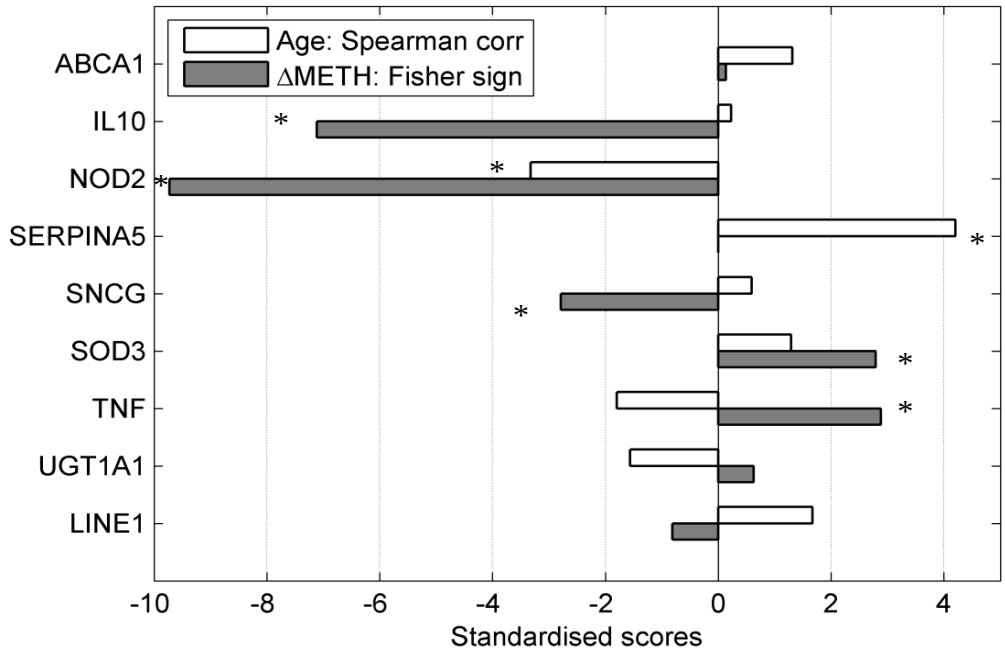


Figure 3.3 Standardised (z) scores for effect of baseline age on DNA methylation (open bars) and longitudinal changes during the study on change in methylation (ΔMETH: filled bars) using Spearman's correlation and Fisher's sign test, respectively. *denotes significance (P ≤ 0.05), accepted if standardised score >1.96 or <-1.96.

3.3.3 Baseline physical activity, changes in physical activity and associations with DNA methylation

Baseline PAEE was negatively correlated with the methylation of *SOD3* ($P < 0.01$) and *SERPINA5* ($P < 0.05$) but was not a significant explainer ($P > 0.05$) of either gene's methylation after adjusting for 'Plate' and 'Sex' (*SOD3*), or 'Plate' and 'Age' (*SERPINA5*). The investigation of an effect of physical activity on the change in *TNF* methylation between baseline and year 8 showed that ΔPAEE was a significant single explanatory variable ($P = 0.0004$); was significant after adjusting for the five additional explanatory variables ($P = 0.0006$); and was the most significant term ($P = 0.0007$) in a simplified ANOVA model that also contained 'Plate' and 'Age'; and 'Sex', ΔWaist and their interaction. Figure 3.4 shows a significant increase in *TNF* methylation in NOC and INC participants compared with DEC participants. ΔPAEE was not a significant single explanatory variable for the change in *IL10* methylation, but was significant after adjusting for the five additional explanatory variables ($P =$

0.0377), and was moderately significant in a simplified model that also contained 'Plate' (P = 0.0471).

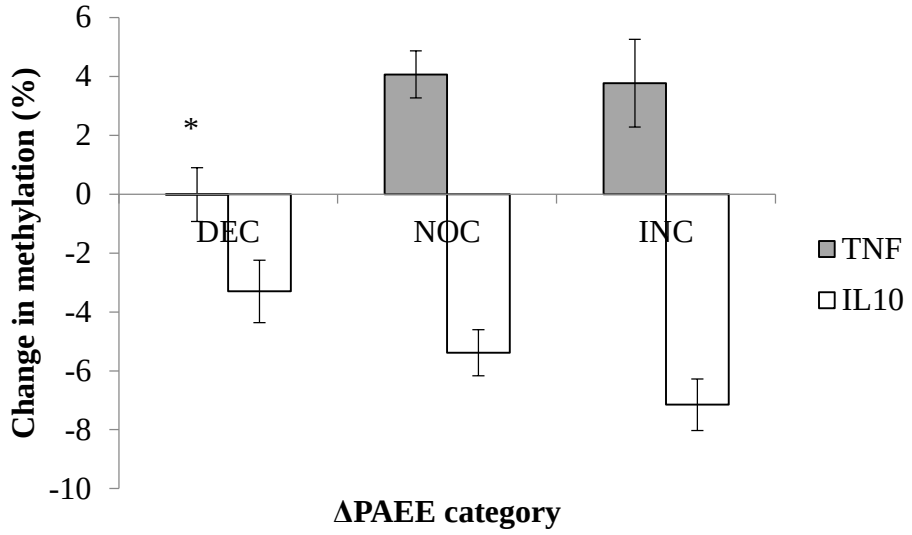
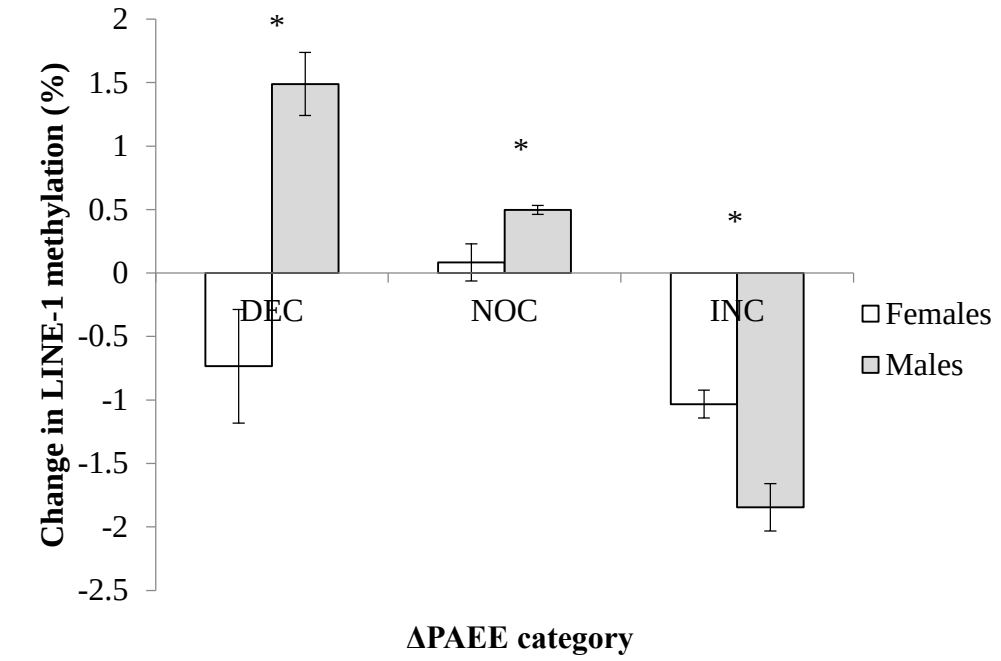
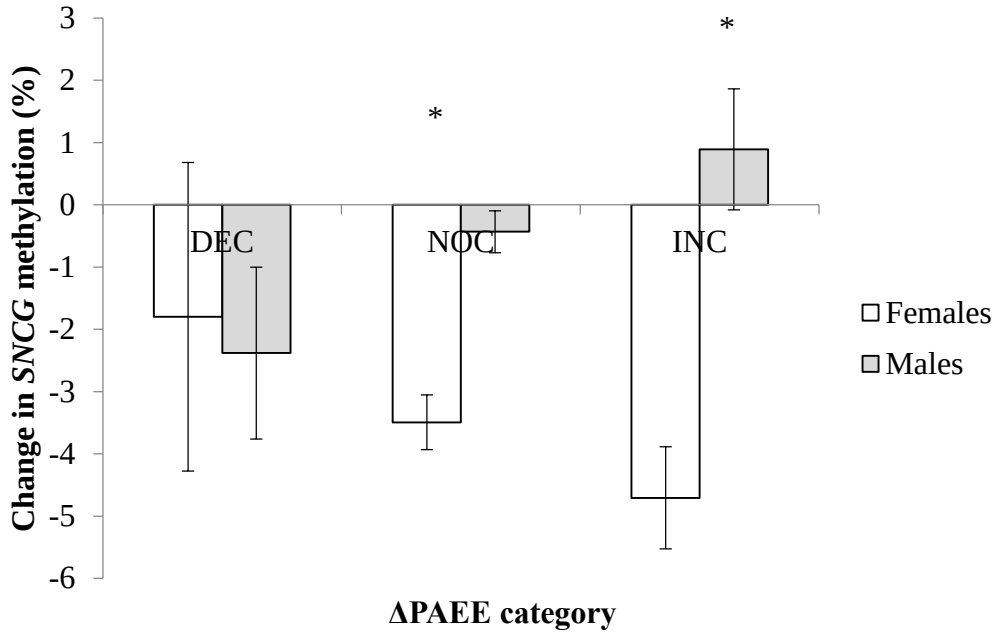


Figure 3.4. Variation in the change in DNA methylation (%) between year 8 and baseline for TNF and IL10 with ΔPAEE: DEC, decrease of >500 kcal/wk; NOC, change of <500kcal/wk; INC, increase of >500 kcal/wk. * significant difference (P < 0.05) between DEC and remaing PAEE categories.

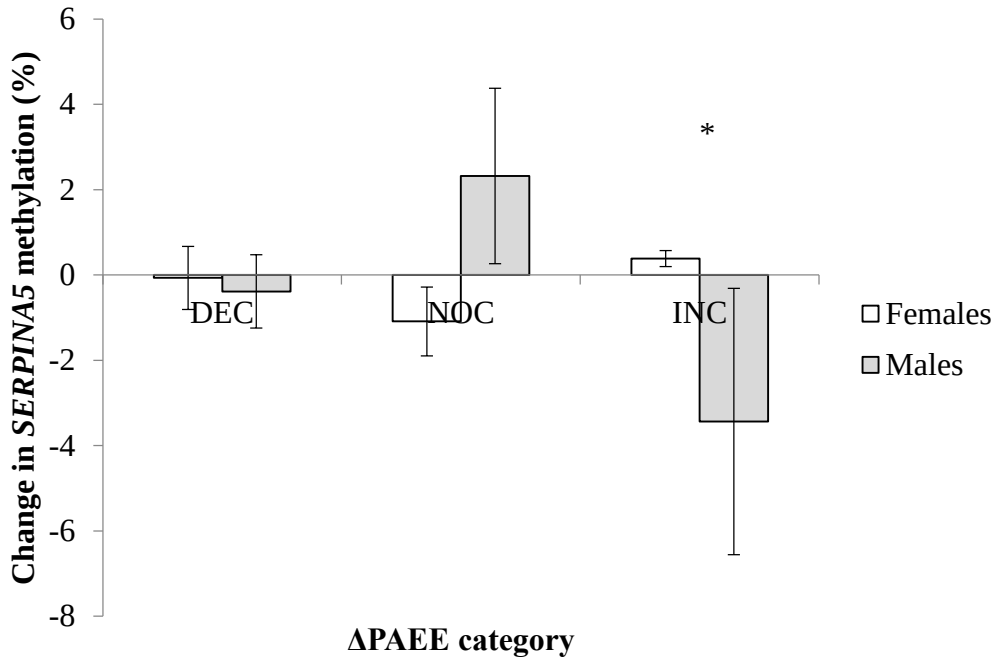
Figure 3.4 shows that changes in *IL10* methylation were negatively associated with increasing ΔPAEE, although the mean values for each stratum did not differ significantly. Figure 3.5a shows there was a positive association with the change in LINE-1 methylation and ΔPAEE in females, and a negative association in males, with the largest gender difference seen in subjects with increased ΔPAEE. An interaction between gender and ΔPAEE was also significant in a model for the change in LINE-1 methylation that also contained 'Plate'. Similarly for *SNCG* methylation, ΔPAEE was significant as an interaction with gender in a model that also contained 'Plate' (P = 0.0163). Figure 3.5b shows *SNCG* methylation decreased more in men with increased ΔPAEE. (P = 0.0302). ΔPAEE was a significant single explanatory variable for the change in *SERPINA5* methylation (P = 0.0275). It was significant after adjusting for the five additional explanatory variables (P = 0.0123), and was significant as an interaction with gender in a simplified model that also contained 'Plate' (P = 0.0104). Figure 3.5c shows that *SERPINA5* methylation decreased significantly in INC vs NOC men. ΔPAEE was not significantly associated with the changes in *ABCA1*, *NOD2*, *SOD3* and *UGT1A1* methylation.


2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
Change in LINE-1 methylation (%)
*
*
*
DEC
NOC
INC
Females
Males
ΔPAEE category

(a)


3
2
1
0
-1
-2
-3
-4
-5
-6
Change in SNCG methylation (%)
*
*
DEC
NOC
INC
Females
Males
ΔPAEE category

(b)



(c)

Figure 3.5 Gender-specific variation in the change in DNA methylation (%) between baseline and year 8 for (a) LINE-1 (b) SNCG and (c) SERPINA5 with ΔPAEE: DEC, decrease of >500 kcal/wk; NOC, change of <500kcal/wk; INC, increase of >500 kcal/wk. * denotes significant difference ($P < 0.05$) between gender at PAEE category.

3.4 Discussion

3.4.1 Differences in DNA methylation by gender

Methylation of LINE-1 repetitive elements has been shown to be a valid surrogate marker of the 5-methylcytosine content of the genome often referred to as global DNA methylation (Weisenberger *et al.*, 2005). Decreased LINE-1 methylation, which is suggested to induce genomic instability, has been observed during aging (Tapp *et al.*, 2013) and is associated with a number of age-related diseases including CC (Belshaw *et al.*, 2010), ischaemic heart disease and stroke (Baccarelli *et al.*, 2010) and also with CVD risk factors (Cash *et al.*, 2011). Therefore, the positive association between increasing PAEE and increased LINE-1 methylation identified here in women is consistent with a role for PA in reducing the risk of developing these

diseases and is concordant with observations from previous studies (White *et al.*, 2013; Zhang *et al.*, 2011).

Synuclein γ, encoded by *SNCG*, plays a role in lipid metabolism (Millership *et al.*, 2013) and shows increased expression in adipocytes from obese women (Oort *et al.*, 2008). Methylation of *SNCG* in peripheral blood was previously shown to decrease with age but increase in lung tissue with smoking (Christensen *et al.*, 2009). Therefore, the significance of the inverse association between increased PAEE and *SNCG* methylation in our male participants in relation to disease risk requires further investigation. The observed gender-specific differences for associations between changes in the methylation of *SNCG*, *SERPINA5* and LINE-1 and PA are consistent with a previous report that indicated a differential response to environmental factors on DNA methylation in the colorectal mucosa (Tapp *et al.*, 2013). The reasons for this are not known, but may reflect gender differences in age-related disease risk (Mosca *et al.*, 2011).

SERPINA5, also known as Protein C Inhibitor (PCI), plays a regulatory role in the coagulation system and has been suggested as a risk marker for acute coronary events (Carroll *et al.*, 1997). However, PCI has been shown to have both pro- and anticoagulant properties (Rau *et al.*, 2007), which means that its role in CVD pathogenesis is not clear. The functional consequence of reduced *SERPINA5* methylation in men with increased PAEE therefore requires further investigation. A previous study reported increased methylation of *SERPINA5* in peripheral blood from ever versus never alcohol drinkers and in lung tissue of smokers (Christensen *et al.*, 2009), suggesting that decreased *SERPINA5* is associated with healthier lifestyle behaviours.

3.4.2 Associations between DNA methylation, baseline age and ageing

In this study we investigated the relationship between DNA methylation in leukocytes and physical activity in the elderly. We showed that participant age was associated with DNA methylation, in agreement with previous reports (Christensen *et al.*, 2009; Xu *et al.*, 2013). Also, it was observed that DNA methylation patterns which correlated with baseline age were often dissimilar to those associated with longitudinal changes observed during the course of the study. For example, *TNF*

methylation at baseline was negatively (albeit not significantly) correlated with age, in agreement with a previous study that showed a significant negative correlation (Gowers *et al.*, 2011). The narrow age range (65-82 years) of the participants in this study may explain the weaker correlation. However, a significant increase in mean *TNF* methylation in our study population over the course of the study suggests a reversal in the age-related decline in the methylation status of this gene. Similar effects were also observed for the methylation of *SNCG*, *SERPINA5* and *IL10*, and to a lesser extent *UGT1A1* and LINE-1. We postulate that the mandatory fortification of foods with folic acid in the U.S. in 1996-7, which occurred after the baseline blood samples were collected but before the year 8 samples, might explain this effect. Folate is a major source of methyl groups and its availability has been shown to impact upon DNA methylation (Bae *et al.*, 2014; Charles *et al.*, 2012; Tapp *et al.*, 2013; Wallace *et al.*, 2010). Similar perturbations in leukocyte DNA methylation patterns were observed in the Women's Health Study, where erythrocyte folate status was correlated with increased leukocyte LINE-1 methylation prior to fortification, but with decreased LINE-1 methylation 1-2 years post-fortification (Bae *et al.*, 2014). Measurement of blood folate in the study population is therefore necessary to elucidate whether this contributed to our observations.

3.4.3 Changes in physical activity and DNA methylation

To explore the relationship between PA and DNA methylation we utilized the availability of participant blood samples together with physical activity data collected at baseline and 8 years later. This enabled an investigation of associations between changes in PAEE with changes in DNA methylation, while also accounting for the effects of age, gender, changes in waist circumference and the potential batch-related effects of sample plate. We identified ΔPAEE as a significant main effect in explaining changes in the methylation of *TNF* and *IL10*. A gender-specific interaction with ΔPAEE was also significant when modelling changes in *SERPINA5*, *SNCG* and LINE-1 methylation. These observations indicate that changes in PA are associated with changes in the methylation status of genes known to be associated with aging and inflammation, and therefore might constitute a mechanistic link between PA and reduced CC risk. Importantly, associations between changes in DNA methylation and

changes in PAEE were independent of any time-dependent changes in waist circumference. This suggests that PA and central adiposity affect DNA methylation via independent mechanisms.

DNA methylation plays a significant role in the regulation of gene expression; therefore, the association of PA with DNA methylation possibly suggests a role for the altered expression of the affected genes in attenuating CC risk. Potentially significant in this respect are the observed associations between ΔPAEE and changes in the methylation of *TNF* and *IL10*. There is evidence for a role of systemic inflammation in increasing adenoma and CC risk, and CC mortality (Comstock *et al.*, 2014a; Il'yasova *et al.*, 2005; Joshi *et al.*, 2014; Kim *et al.*, 2008; Zhou *et al.*, 2014). *TNF* encodes the pro-inflammatory cytokine tumour necrosis factor alpha (TNFα) and TNFα expression in peripheral blood is associated with atherosclerosis in the elderly (Bruunsgaard *et al.*, 2000). The cytokine interleukin 10, encoded by *IL10*, inhibits inflammatory cytokine production, including *TNF*α (Cassatella *et al.*, 1993).

Our results suggest, therefore, that an increase in PA in people of this age group may be associated with reduced risk of CC risk by decreasing systemic inflammation via the concomitant, methylation-induced down-regulation of *TNF* expression and the demethylation-induced up-regulation of *IL10* expression. A similar association with exercise and increased methylation of a pro-inflammatory gene (*ASC*) has previously been demonstrated in older Japanese persons after a 6-month brisk walking exercise intervention (Nakajima *et al.*, 2010). Aberrant methylation of this gene has been demonstrated to affect p53-mediated chemosensitivity in colon cancer (Ohtsuka *et al.*, 2006). Overall, this suggests that changes in the methylation status of inflammatory genes could underpin some of the reported anti-inflammatory effects of exercise training in the elderly (Woods *et al.*, 2012), which might consequently attenuate CC risk.

3.4.4 Limitations

One limitation of the current study is that CC precursor tissue (i.e. colon biopsies) was not available. Therefore, whilst there is good evidence that aberrant methylation in colon tissue increases risk of CC (Ahuja *et al*., 1998) there is limited evidence that altered methylation in blood impacts CC risk. Of particular relevance is the recent finding that age-related DNA methylation in blood is associated with earlier mortality

in later life (Marioni *et al*., 2015). It is yet to be determined whether this is also associated with CC risk and mortality.

Another potential limitation of the current study is that the methylation analyses were performed on leukocyte DNA, which is derived from a mixed population of cell types. It is possible, therefore, that the associations between DNA methylation and PA may simply reflect an impact on the relative proportion of cell types. For example, aerobic fitness has been associated with a decreased relative proportion of senescent T-cells (Spielmann *et al.*, 2011). Furthermore, differential patterns of age-related demethylation of *TNF* have previously been observed in peripheral blood leukocytes and monocyte-derived macrophages (Gowers *et al.*, 2011). Given the potential significance of the observed effects of PA on the methylation status of genes with plausible role in modulating CC risk, this warrants further investigation.

In addition, the amount of PA was self-reported, which potentially introduces recall bias. This might, in part, explain why no associations between DNA methylation and PAEE at baseline were observed. Since any participant-specific recall bias probably remained consistent between baseline and year 8, stratifying the participants by ΔPAEE will have negated this. Furthermore, stratification allowed a more sensitive appraisal of how a tangible change in PA is associated with DNA methylation patterns. In this respect, our observations are in agreement with previous work demonstrating that changes in physical activity level over the course of an exercise intervention was associated with altered DNA methylation patterns in a variety of tissues (Bryan *et al.*, 2013; Nakajima *et al.*, 2010; Nitert *et al.*, 2012; Ronn *et al.*, 2013).

It should be noted that, in this longitudinal multi-centre study, trial site was not included as a covariate in the model. This could plausibly introduce variation due to differences in demography, but perhaps more importantly this might have caused variation in standard operating procedures for data collection. For example, whilst other work in this population has suggested that measurement of waist circumference was performed about the umbilicus (Luchsinger *et al*., 2013) there is no intimation of standardisation between sites. This brings into question the reliability of 'ΔWaist' in the statistical models used.

Another possibility is that seasonal variation might have impacted on PA participation at the time of the clinic visits, confounding the PA data. Furthermore, one study has suggested that month of blood draw could impact on DNA methylation status of CC-related genes (*RASSF1A* and *MGMT*) and LINE-1 (Ricceri *et al.*, 2014). A separate analysis was conducted to determine whether this was the case, and it was found that only one participant in the cohort attended clinic visits in opposite seasons (i.e. summer and winter). In addition, a change in PA participation might have reflected reflect a change in health status. However, a separate χ^2 analysis showed there were no differences in categories of ΔPAEE by change in self-report health status, and indeed no impact of change in health status on change in methylation (see Appendix). Of course, this might be subject to recall bias. Equally, there was no difference in mean ΔPAEE between those who had an incident cardiovascular event over the course of the study and those who did not (see Appendix Aii). While it is not clear from the PAEE calculation whether the intensity of activity (i.e. moderate or vigorous) or time spent in these activities accounted for the associations with DNA methylation, it is clear that an increase in leisure time PAEE, irrespective of how this was achieved, was associated with apparently favourable changes to the methylation status of genes plausibly associated with CC risk.

3.4.5 Conclusion

In conclusion, a change in PAEE was associated with changes in the methylation status of several inflammation-related genes and LINE-1 in a manner that is generally consistent with the role of PA in modulating CC risk. The observed associations with PA were independent of changes in measured central adiposity, suggesting PA might influence the epigenome, and consequently health, by mechanisms that are independent of its effect on body fat. We showed that changes in PAEE of 500 kcal per week, equivalent to approximately 120 min of brisk walking in older people, (Ainsworth *et al.*, 2000; O'Donovan *et al.*, 2010) correlated with significant changes in DNA methylation. Future studies utilising the increasing availability of approaches for genome-wide DNA methylation analysis in other tissues will further our understanding of how PA modulates the epigenome and the consequential influence on health.

3.5 Acknowledgements

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Chapter 4

Study 2: Physical Activity and Risk of Colon cancer (PARC)

4.1 Introduction

The role of PA in modulating DNA methylation patterns is being increasingly recognised (Denham *et al.*, 2014; Voisin *et al.*, 2015). The biological ageing process is predicted by DNA methylation (Horvath, 2013) and strongly influenced by environmental factors (Christensen *et al.*, 2009), even amongst monozygotic twins (Talens *et al.*, 2012). As aberrant DNA methylation is associated with adenomatous polyps and CC (Belshaw *et al.*, 2008; Esteller *et al.*, 2001; Issa *et al.*, 1994) it is of interest to determine whether PA in individuals at risk can alter DNA methylation patterns that are commonly linked to disease likelihood and progression. The presence of colon polyps suggests a perturbed epigenetic field (Belshaw *et al.*, 2008; Chan *et al.*, 2002) that might be malleable to unprompted changes in PA patterns (Figure 3.4) or a PA intervention (Bryan *et al.*, 2013; Nitert *et al.*, 2012). Previous reports have indicated that a six-month aerobic PA intervention is sufficient to induce changes in DNA methylation patterns in genes associated with metabolism and inflammation in blood, adipose tissue and muscle tissue (Nakajima *et al.*, 2010; Nitert *et al.*, 2012; Ronn *et al.*, 2013). Additionally, minutes of walking achieved after 12-month low-moderate intensity walking intervention amongst overweight premenopausal women was correlated with decreased methylation in genes associated with breast cancer risk in buccal cells (Bryan *et al.*, 2013). It is not known whether a similar intervention in colon adenoma patients, who are at elevated risk for CC (Citarda *et al.*, 2001; Winawer *et al.*, 1993), could alter methylation patterns commonly associated with gut ageing and inflammation and hence cancer risk (Issa *et al.*, 2001).

As part of the UK National Bowel Cancer screening programme, patients who present with colon polyps have them surgically removed upon initial colonoscopic investigation. Results from the National Polyp Study (NPS) suggested that this procedure, known as a polypectomy, reduced colon cancer mortality by 53% in US

adults who presented with an adenoma between the years of 1980-1990 (Zauber *et al*., 2012). Those with more than 6 small or more than 3 large (> 1 cm diameter) are invited for a follow-up colonoscopy 12 months afterward (Atkin and Saunders, 2002). This group of patients provide a unique opportunity to investigate any alterations in DNA methylation (amongst other measures) at the level the colon epithelia after a PA intervention, or indeed any lifestyle intervention. Since overall methylation of breast cancer-specific genes in buccal cells was modified by walking behaviour in premenopausal women (Bryan *et al.*, 2013), the DNA methylation of this epithelial cell has potential for use as a surrogate marker.

This population also provides an opportunity to investigate whether a PA intervention affects any serum factors that might be able to mitigate the progression of well-characterised *in vitro* models of colonic epithelia. This approach is especially usefully given the difficulty of obtaining colon biopsies, and can provide additional information as to the mechanisms by which PA could affect CC risk. Indeed, greater levels of PA are associated with apparently beneficial effects on serum factors that are linked with CC risk. For one, PA is correlated with reduced levels of pro-inflammatory cytokines in serum in older persons (Woods *et al.*, 2012), which is moderately associated with risk of CC and adenoma (Il'yasova *et al.*, 2005; Kim *et al.*, 2008). In addition, PA can reduce fasting glucose and insulin (Knowler *et al.*, 2002), which is also linked to CC risk (Giovannucci, 2007). There are a wide variety of CC cell lines for such an experiment (Ahmed *et al*., 2013), each with differing genetic and epigenetic characteristics (Table 1.2). It is of interest, therefore, to use these models should follow-up biopsies be unavailable.

4.2 Methods

4.2.1 Participants

Participants were patients who had attended the Norwich and Norfolk University Hospital (NNUH) Gastroenterology Unit for a screening colonoscopy as part of the NHS Bowel Cancer Screening Programme after a positive faecal occult blood test (FOBT), or after direct referral for gastrointestinal symptoms by their General Practitioner, within the last three years. Those who presented with colon polyps upon

investigation were eligible to take part in the study. Inclusion criteria were i) diagnosis adenomatous polyps as a result of the screening colonoscopy; ii) aged 50-80 years and iii) physically able to partake in regular exercise. Exclusion criteria included i) no adenomatous polyps or diagnosis of CRC following the colonoscopy; ii) PA levels that meet the most recent American Cancer Society (ACS) guidelines for maintenance of health for at least the past 6 months (150 min/wk MVPA, in bouts of ten minutes or more); iii) presence or history of other co-morbid conditions which might preclude patients from safely undertaking regular exercise iv) presence of other colorectal conditions (e.g. inflammatory bowel disease) or known familial CRC syndrome; v) previous diagnosis of CRC; vi) inability to adequately understand written and spoken English, and vii) current involvement in other ongoing research. Current health and demographic data were captured from consenting participants using a bespoke questionnaire designed by the researchers. Data captured included age, gender, ethnicity, medication profile (type of medications, dosage level and frequency), family history of CRC, co-morbidities, spouse present in the home, occupation, level of education, alcohol consumption, smoking status and number of GP visits in the past year. The questionnaire was administered again after 12 months to monitor any changes that occurred during the trial.

4.2.2 Recruitment

Patients attending the hospital for their pre-assessment were given a study invitation letter, a patient information leaflet and a consent form. The form requested their approval for the collection of research biopsies should any polyps be found during the colonoscopy. On the day of their screening colonoscopy, patients returned their signed consent form in the presence of the researchers. A letter was also sent to their GP outlining their interest in the study and providing contact details if they had any further questions

If the patient presented with any adenomatous polyps, a small research biopsy was taken from the sigmoid colon, placed in a 1.5 ml collection tube and frozen on dry ice. This was subsequently transferred to the Institute of Food Research and frozen at -80 °C until analysis. Once participants were informed of the outcome of their procedure by the clinical staff the researchers organised an appointment at the Exercise Science Laboratory at The University of East Anglia. At the appointment, the

researchers explained the study and gave the participant the opportunity to ask any questions before gaining full written informed consent.

5.2.2.1 Randomisation

After baseline measures were completed, participants were randomised into the control or intervention group and stratified by risk status. Risk status, in this instance, referred to the severity (number and size) of adenomas at investigation. Those with a more severe case were deemed 'high risk' by the clinical team and were invited to follow-up colonoscopy within 12 months; whereas those with fewer polyps and deemed 'moderate risk' were invited for follow-up after 36 months. Randomisation was performed using 'R' (R Core development team, http://www.R-project.org) by an independent biostatistician (Dr Jack Dainty) based at the Institute of Food Research. To ensure balancing between control and intervention groups, a covariate adaptive randomisation approach was used. The covariates were risk status, age, gender and BMI. These were chosen based on the probability that the severity of polyposis might indicate a greater epigenetic perturbation of the mucosal field, and that advancing age, being male and having a higher BMI have consistently been associated with increased risk of CC. Initially, participants were allocated randomly, and after the first five participants the balancing came into effect. Participants were assigned a unique code that blinded the researcher to their group allocation during analysis.

4.2.3 Outcomes

All biological outcome measures pertinent to this study were repeated after 6 and 12 months. To minimise bias due to perceived expectancy, all physiological samples collected were coded so as to blind the researcher conducting the analysis as to the group allocation.

4.2.3.1 Anthropometry

Patients arrived at the Clinical Exercise Science laboratory after an overnight fast and having refrained from moderate-vigorous PA for 72 h, and from consumption of alcohol for 24 h. Height (m) and mass (kg) was measured using a calibrated stadiometer and balance (Seca 711, Seca UK, Birmingham, UK). Waist and hip

measurements (cm) were taken about the umbilicus and the widest point of the hips with a tape measure (Seca) three times and the mean taken. When consecutive measurements deviated by greater then 2 mm, a further measurements was taken such that all three results were within 2 mm. Resting blood pressure (DINAMAP v1000, GE Healthcare, Bucks, UK) was recorded in a semi-supine position after two minutes of resting quietly, and after participants voided the bladder body composition was obtained via bioelectrical impedance analysis (AKERN BIA 101, SMT Medical GmbH & Co., KG, Germany) and integrated software (BodyGram Pro 3.0, SMT Medical GmbH & Co., KG, Germany).

4.2.3.2 Serum and buccal cell collection

Serum and buccal cells were collected according to the procedures detailed in General Methods 2.2.

4.2.3.3 Cardiorespiratory fitness

Height (m) and mass (kg) was measured using a calibrated stadiometer and balance (Seca 711, Seca UK, Birmingham, UK). Maximal aerobic capacity ($\dot{V}O_{2\,max}$; $ml \cdot kg \cdot min^{-1}$) was determined using a Medgraphics™ Ultima Cardio2 suite (Medical Graphics Corporation, St. Paul, MN, USA) using a 20-watt incremental ramp protocol on an electrically braked cycle ergometer (Lode Excalibur Sport, Lode B.V. Groningen, NL). Briefly, the test started with a 2 min 'freewheel' period against no resistance so that the participant was accustomed to the protocol. Thereafter, the resistance increased every minute by 20 watts until $\dot{V}O_{2\,max}$ (as indicated by a plateau in $\dot{V}O_2$ on the graphical display) or volitional exhaustion. During the test, a continuous 10 lead ECG trace (Mortara XScribe 5, Mortara Dolby UK Ltd, Stirling, UK) was monitored by a medical professional, and the test stopped immediately where any abnormalities arose during the exercise bout. Once the participant reached their $\dot{V}O_{2\,max}$ and was unable to continue, the test finished and the participant allowed to 'freewheel' against reduced resistance for as long as they deemed necessary. Participants then had the opportunity to shower and change and were allowed to leave after their resting heart rate and blood pressure has been checked. The test was repeated after six and twelve months, and prescribed exercise intensity in the intervention group adjusted accordingly.

4.2.3.4 Habitual physical activity

Objective free-living PA level was assessed over 7 days using accelerometry (ActiGraph® GT3X, ActiGraph Corps, Pensacola, FL, USA). The small unobtrusive accelerometer was worn on the hip and collected data including estimated activity counts, step counts, minutes of moderate-vigorous PA (MVPA) per day and per week, bouts of MVPA of ten minutes or more, and total exercise energy expenditure. Data was analysed using integrated software (ActiLife version 6.9.1, ActiGraph Corps, Pensacola, FL, USA). Self-reported PA was also assessed using the International PA Questionnaire (IPAQ) (Friedenreich *et al.*, 1998) and the Godin Leisure Time Exercise Questionnaire (Godin and Shephard, 1985). The Godin Leisure Time Exercise Questionnaire is a short four-item questionnaire that assesses the number of times that strenuous, moderate or mild exercise was performed for more than 15 min over the last 7 days.

4.2.3.5 Diet

Diet was monitored at baseline and after 6 and 12 months of the intervention with a diet diary, which formed part of the PARC workbook. Participants had to complete the diary for a minimum of four week days and one weekend day. Participants were instructed not to change their dietary habit during the study. Diet composition was analysed using COMPEAT5 software.

4.2.4 Intervention

4.2.4a Active Lifestyle Programme (ALP)

All participants in the Active Lifestyle Programme (ALP) attended a familiarisation session in the week before the trial started. They were introduced to the equipment available in the exercise facility (treadmill/rowing machine/cycle ergometer) and the various resistance/bodyweight exercises that the participants were required to perform. These included bicep curls, dumbbell flys, sit-ups and Theraband exercises. In the first 12 weeks of the study, participants attended the exercise facility on 2 d/wk and complete a supervised exercise session. This consisted of a ten minute warm up, 30 minutes of aerobic exercise at 65-80% $\dot{V}O_{2\,max}$ as determined by the $\dot{V}O_{2\,max}$ test

(some participants were unable to exercise at a heart rate corresponding to 65% $\dot{V}O_{2\,max}$ for 30 min at the onset of the trial, so intensity was adjusted accordingly to ensure a full 30 min bout was completed) and 30 min of resistance exercise using the exercises described above. Sessions followed the principles of progression and overload such that participants continued to improve their fitness. On ≥ 3 days per week, participants completed home-based exercise to complement these sessions. In the second 12 weeks of the study, supervised exercise at the exercise facility took place on 1 d/wk only, and home-based exercise took place on ≥ 4 d wk. For the remaining 24 weeks (for those participants recruited earlier in the study) participants were asked to complete ≥ 300 min of moderate to vigorous exercise per week, spread over ≥ 5 days. To help monitor non-supervised exercise activity, participants were provided with a physical activity diary with which to record the time spent, type and intensity of any occupational and leisure-time PA, with a copy of the 6-20 point Borg scale of perceived exertion (RPE; Borg, 1982) attached. Both the diary and Borg scale were introduced and explained to them at the initial familiarisation session. The research team advised that any leisure-time PA should ideally be completed at an RPE of 12-14, which equates to a 'somewhat hard' to 'hard' effort.

The protocol was an adapted, less vigorous version of one used in a previous study of polyp patients (Campbell *et al*., 2007), where participants were encouraged to engage in 60 min of aerobic exercise at 60-85% VO2 max on six days per week. It was adjusted based on the assumption that the patients in the present study would be significantly older than those in the previous study. Indeed, the mean age of participants in the work of Campbell and colleagues was 55 years; whereas the National Bowel Cancer Screening Programme is typically offered to individuals over the age of 60. Secondly, a resistance exercise component was added to increase the variety of exercise options available, thereby increasing the likelihood of continuing the intervention.

To encourage exercise participation and maintain adherence, ALP were provided with a bespoke PA workbook (the PARC workbook) designed by the researchers, which outlines suggestions for PA, and included PA logs, progress monitors and contact details of the researchers. The participant kept this workbook for the duration of the trial. Furthermore, ALP was provided with pedometers, which were used as a motivational tool to promote exercise (i.e. brisk walking) behaviour. To

promote adherence to the programme, theory-based workshops took place at the University of East Anglia every fortnight for the first 6 months of ALP and once a month for the remaining 6 months. The workshops were based upon the Self-Determination Theory (Ryan and Deci, 2000) and covered a range of topics including goal-setting, benefits of regular exercise and exercise barriers. The workshops were designed and delivered by Liane Lewis, a member of the research team. During the first 24 weeks of the intervention, ALP attended one workshop every two weeks after a supervised exercise session, which lasted for approximately 30-45 min**.** A schematic outline of the ALP intervention is presented in Figure 4.2.

4.2.4b Usual Care (UC) Group

The UC group did not receive an intervention or any other form of advice with regard to lifestyle behaviours. However, they had the opportunity to take part in a limited number of supervised exercise sessions and received an intervention workbook at the end of the study. They did not receive any lifestyle advice or supervised exercise sessions until the end of the 12 months study period. Participants in the UC group undertook the baseline measures and repeated these at the same time points as ALP. These included a fitness test, body composition, blood samples and all questionnaires at 6 and 12 months. A schematic outline of UC is presented in Figure 4.1.

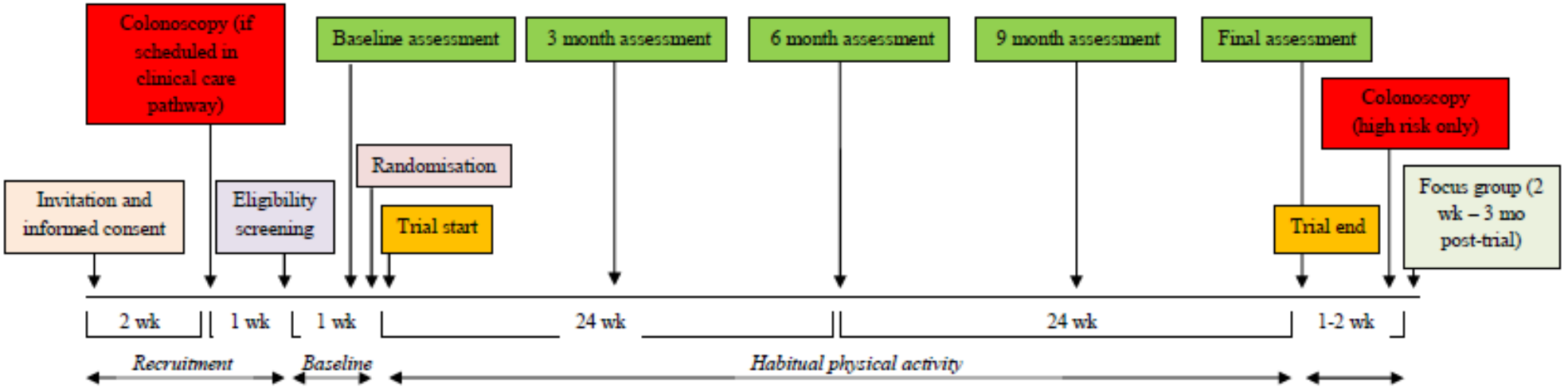

Figure 4.1. Schematic diagram of Usual Care (UC)

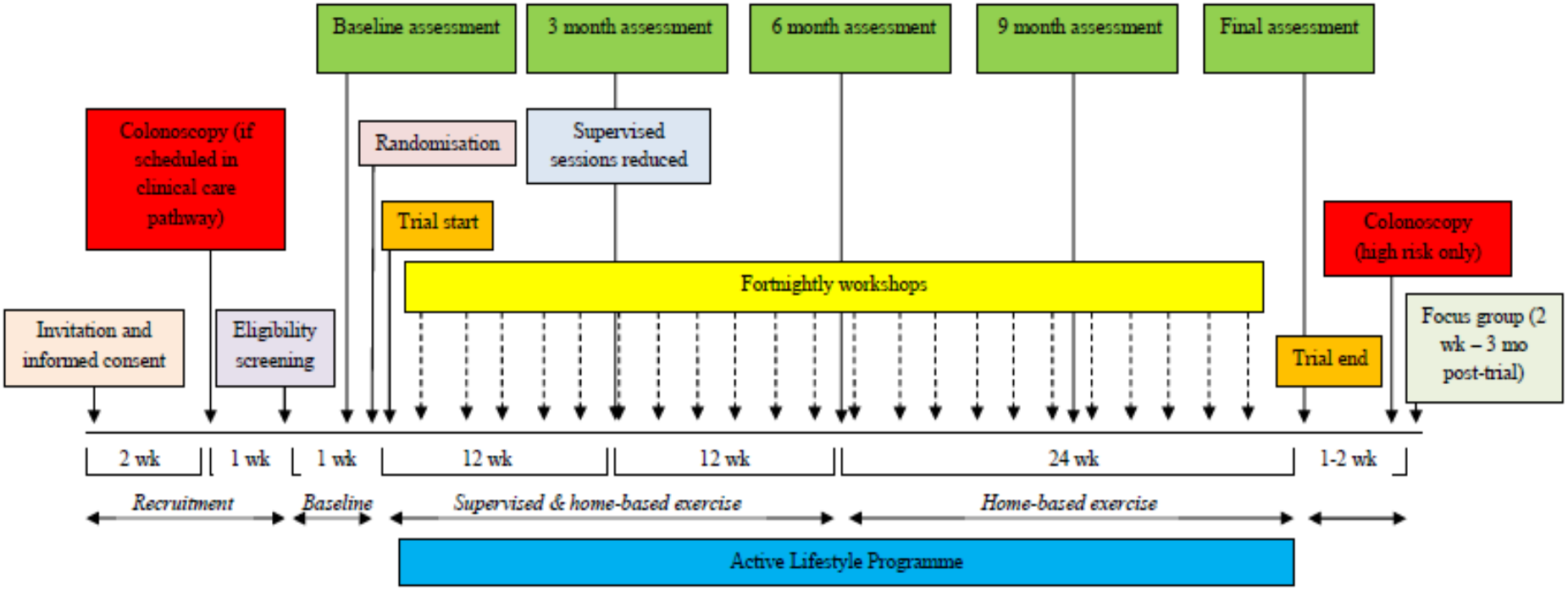

Figure 4.2 Schematic diagram of the Active Lifestyle Programme (ALP).

4.2.5. DNA methylation

Biopsies were collected at initial surveillance colonoscopy (baseline) and after 12 months at a follow-up visit where possible. Biopsies were analysed for global DNA methylation status by quantifying the methylation of the repetitive element LINE-1, previously demonstrated to be suitable surrogate index of global methylation, using an adapted qPCR assay (Iacopetta *et al.*, 2007). Gene-specific CGI methylation status of a panel of genes were also determined using combined bisulphite restriction analysis (COBRA) and 'mega gel' electrophoresis (Wang *et al.*, 2003; Xiong and Laird, 1997) (see 3.8.1 Combined bisulphite restriction analysis (COBRA) and gel electrophoresis). Genes (*ASC*, *IL6*, *IL10*, *TNF* and *TSLP*) were selected based on their involvement in chronic inflammation, colon inflammation and their apparent amenability to changes in PA based on previous investigations from our laboratory (Chapter 4) and others (Nakajima *et al.*, 2010). Other genes (*APC*, *SOX17*, *WIF1*, and *HPP1*) and LINE-1 were selected based on their involvement in age-related methylation of the colorectal mucosa (Belshaw *et al.*, 2008). Another gene, *BHMT2*, was analysed based on its role in methyl group donation, and its possible amenability to exercise training (Buehmeyer *et al.*, 2008).

Briefly, primers were designed to capture an area rich in CpGs after the colon biopsy and buccal DNA samples had undergone bisulphite modification (Zymo Research Corp, Irvine, CA, USA) and amplified by PCR (see General Methods 2.8). The product was then EtOH-precipitated and digested with a specific restriction enzyme designed to cut methylated DNA fragments (Table 2.2) at a concentration of 1% enzyme, 10% buffer and 89% RNAse free water (10 µl volume per sample) at the recommended temperature for ≥ 4 hours. The pool of cut and uncut fragments were then stained with 5 x loading dye and separated by gel electrophoresis in a high throughput 'mega gel' (C.B.S Scientific, San Diego, CA, USA). Gels were stained for > 1 hour with 10 µl neat SYBR Green and bands visualised with a PharosFX Molecular Imager (Applied Biosystems). The volume in each band was quantified using TotalLab software (Non-linear dynamics, Newcastle-upon-Tyne, UK) and percentage methylation calculated by using the formula 100 × M signal intensity/ (U signal intensity + M signal intensity).

4.2.6 Statistics

4.2.6.1 Sample size calculation

The sample size was based upon the numbers required to demonstrate a clinically important change in aberrant CGI methylation. Previous work has demonstrated that aberrant CGI methylation in key genes is inversely related to the progression of sporadic CC (Grady and Carethers, 2008; Kim *et al.*, 2010b). Aberrant activation of the *Wnt* signalling pathway is a common pathological feature of colon carcinogenesis. One reason for this is that the gene encoding the lipid binding protein *Wnt* inhibitory factor 1 (*WIF1*) that can inhibit this pathway is frequently methylated. Therefore, the statistical power for the present study was based on the assumption that PA will i) significantly reduce the proportion of participants in whom the *WIF1* gene is methylated in > 11% of alleles, and ii) reduce their *WIF1* methylation profile by the equivalent of ten years of ageing. The 11% threshold was based upon data collected from the Biomarkers of Risk of CRC (BORICC; Food Standards Agency) study which indicated that 11% of participants aged between 47 – 53 have > 11% of *WIF1* alleles methylated, compared with 33% in those aged from 57 – 63 (Tapp *et al*., 2013). The Cohen's *d* calculation for independent samples (R Core development team, http://www.R-project.org) suggested that to achieve a significant reduction ($P \leq 0.05$, 80% power) of *WIF1* gene methylation from 33% to 11%, in participants with > 11% of *WIF1* alleles methylated, it was calculated that n = 124 (i.e. 62 participants per group) was required.

4.2.6.2 Statistical methods

Differences in gene-specific colon and buccal CGI methylation, and LINE-1 methylation due to group allocation (ALP vs UC) after 6 and 12 months was analysed using a type 1 analysis of covariance (type 1 ANCOVA) with baseline methylation as a covariate. This method was also used to detect differences in anthropometric measures and accelerometer derived and self-report MVPA after 6 and 12 months, again using baseline values as covariates. Any possible associations between any of the participants' characteristics (e.g. age, mass, CRF) and cell metabolic activity were explored using Pearson correlations. All tests were performed, and figures created,

using the Statistical Package for Social Sciences (SPSS) v.20 (IBM, Armonk, NY, USA).

4.3 Results

4.3.1 Recruitment

In total, 736 patients were assessed for eligibility, and 31 randomised (see CONSORT Figure 4.3). Baseline characteristics of the participants are presented in Table 4.1. Of the participants, 22 completed ALP or UC for 6 months, and 13 completed 12 months.

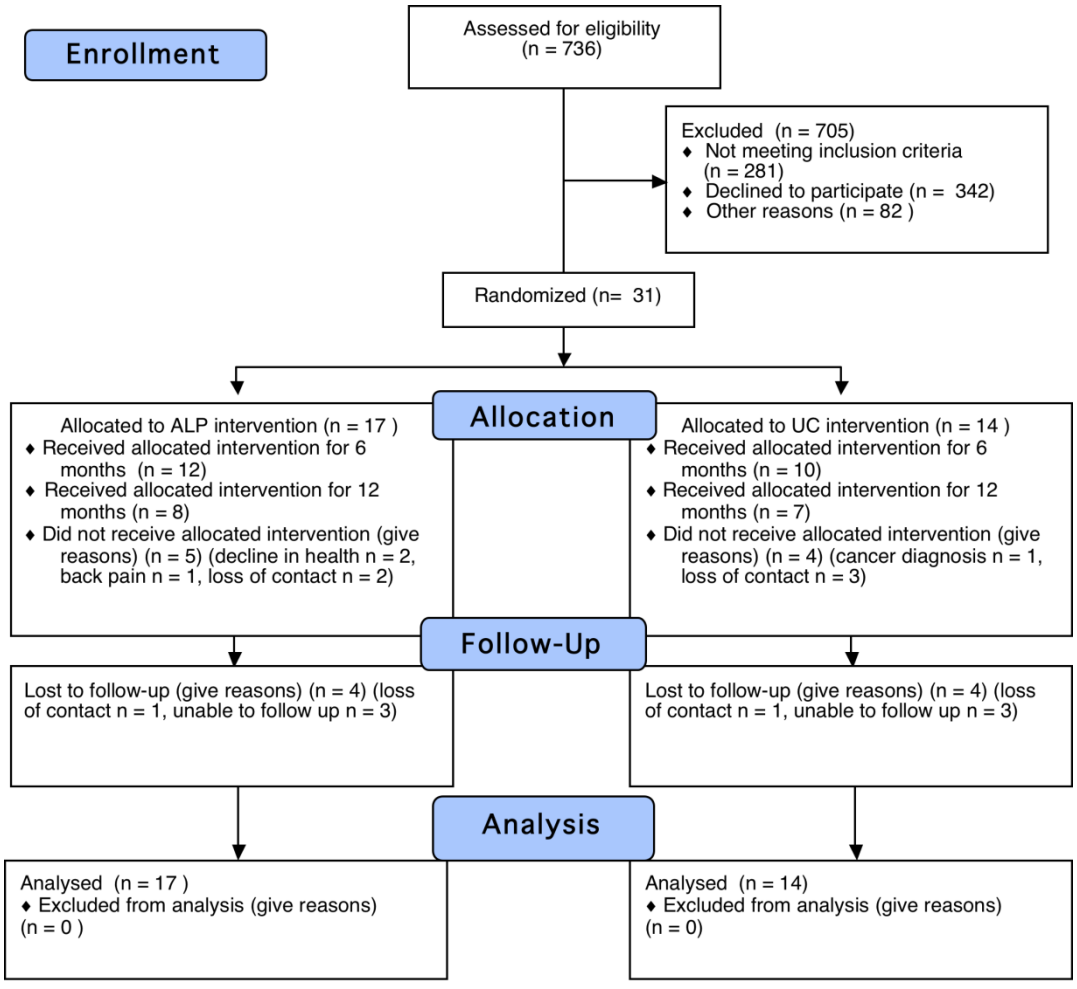

Figure 4.3 CONSORT diagram of PARC RCT

4.3.2 Compliance

Attendance at the supervised exercise sessions and workshops after randomisation was 62% and 53%, respectively. All participants gave advanced notice of non-

attendance. Principal reasons for non-attendance included illness, holidays and work commitments. However, when participants who dropped out of ALP shortly after randomisation were excluded, the remaining participants attended 82% of exercise sessions. The combined attrition rate across ALP and UC was 29%.

4.3.3 Anthropometry, cardiorespiratory fitness and physical activity

Body weight, BMI and body fat was unaffected by group allocation at all time points. CRF was significantly greater in UC at baseline (Table 4.1). ALP significantly increased CRF after 6 months ($P < 0.05$); whereas no change was observed in UC (Figure 4.4). However, there were no significant differences between groups after 12 months (Figure 4.5). There were group-related differences in self-report PA between UC and ALP after six and twelve months of the intervention. ALP significantly increased self-report leisure-time PA after 6 months ($P < 0.05$), with no change in UC (Figure 4.6). However, the increase in ALP was no longer significant after 12 months when corrected for baseline self-report leisure-time PA (Figure 4.7). According to accelerometry data at baseline, ALP completed significantly more moderate-vigorous PA (Table 4.1). The number of minutes spent in MVPA in of bouts of ten minutes or more did not change significantly in UC or ALP from baseline after 6 months (Figure 4.8) or 12 months (Figure 4.9).

Table 4.1 Baseline characteristics of patients in the PARC randomised controlled trial. All values in upper portion of table presented as mean (SD). Values in lower portion of table denote numbers of participants with each co-morbidity. * denotes significant difference between groups, $P < 0.05$.

Group	**UC**			**ALP**		
	Females	*Males*	*All*	*Females*	*Males*	*All*
N	5	9	14	6	11	17
Age	65.2 (2.7)	71.8 (1.8)	69.4 (6.3)	66.6 (1.6)	68.2 (2.7)	67.7 (3.0)
Mass (kg)	68.5 (7.3)	89.2 (3.8)	81.8 (16.3)	81.2 (3.8)	90.9 (20.7)	87.7(17.8)
BMI	25.6 (7.0)	28.9 (2.9)	27.7 (4.8)	30.3 (3.5)	29.8 (5.8)	30.0 (5.0)
Body fat (%)	31.0 (8.8)	23.8 (5.5)	26.4 (7.5)	37.7 (3.8)	25.7 (7.2)	29.7 (8.5)
Self-report leisure-time PA (MET h/wk)	6.0 (3.3)	7.0 (3.6)	6.7 (9.4)	4.7 (4.4)	10.3 (14)	8.5 (11.8)
Accelerometer-derived MVPA (min/wk)	132 (81)	101 (87)	112 (83)*	103 (108)	194 (128)	164 (126)
$\dot{V}O_{2\ max}$ $(ml \cdot kg \cdot min^{-1})$	23.7 (2.9)	25.1 (4.6)	24.6 (4.0)*	17 (3.6)	25.8 (5.8)	22.8 (6.6)
Obesity	2	2	4	4	6	10
Hypertension	1	2	3		3	3
Cardiovascular disease		2	2	2	2	4
Type II diabetes		4	4			
Pulmonary disease		1	1	2	2	4
Family history of colon cancer	2	2	4		1	1

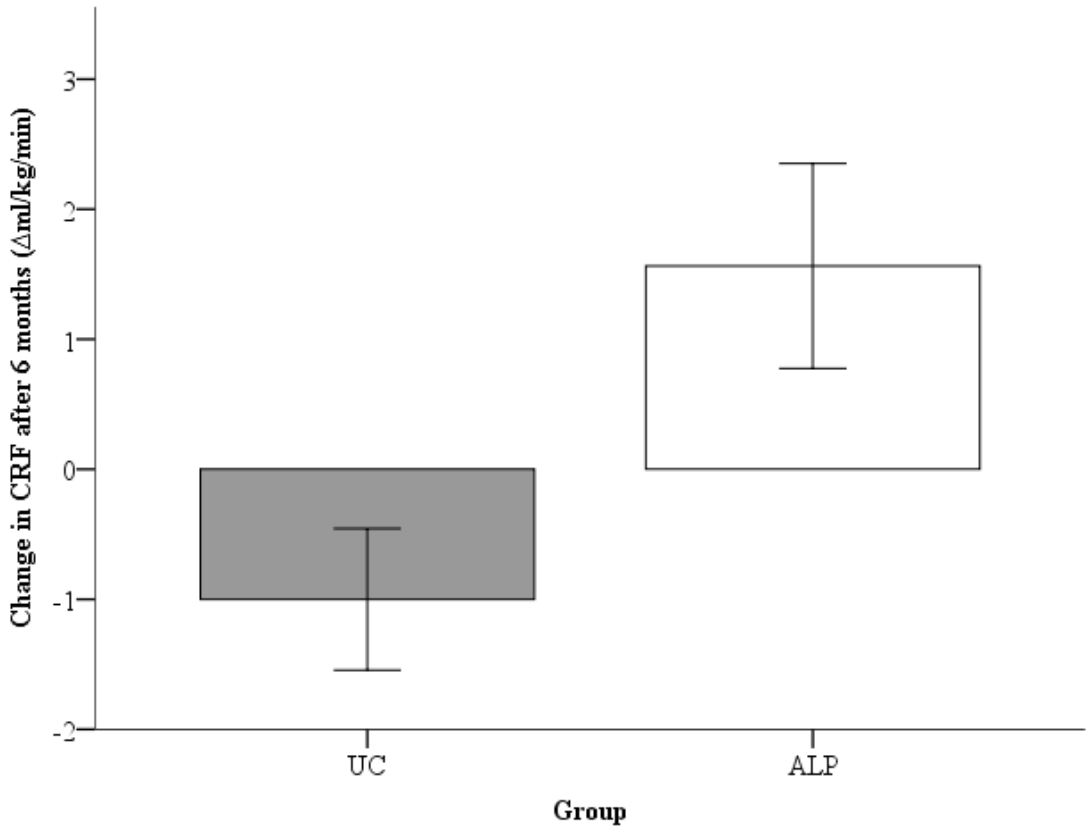

Figure 4.4 Significant (P = 0.015) difference between Usual Care (UC: filled bar) and Active Lifestyle Programme (ALP: open bar) in change in cardiorespiratory fitness (CRF, $\Delta ml \cdot kg \cdot min^{-1}$) from baseline to 6 months. Error bars ± 1 SE.

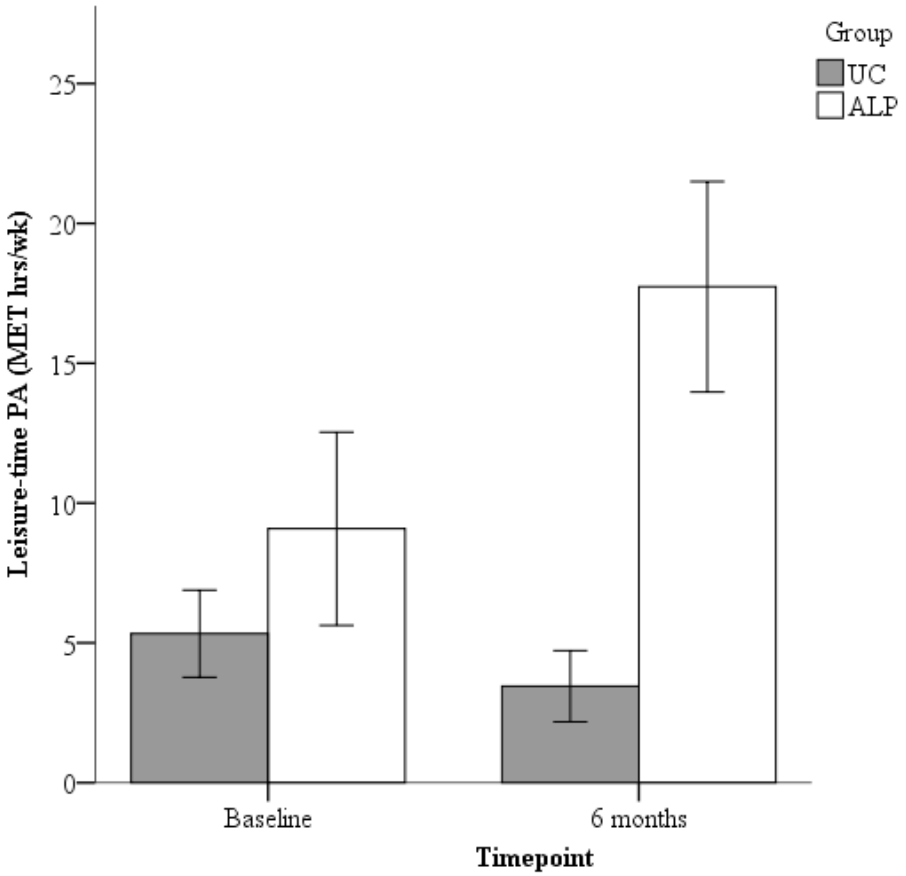

Figure 4.5 Significant (P = 0.002) difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) in self-report leisure-time physical activity (PA) in metabolic equivalent of task (MET) hours per week after six months when adjusted for baseline leisure-time PA . Error bars ± 1 SE.

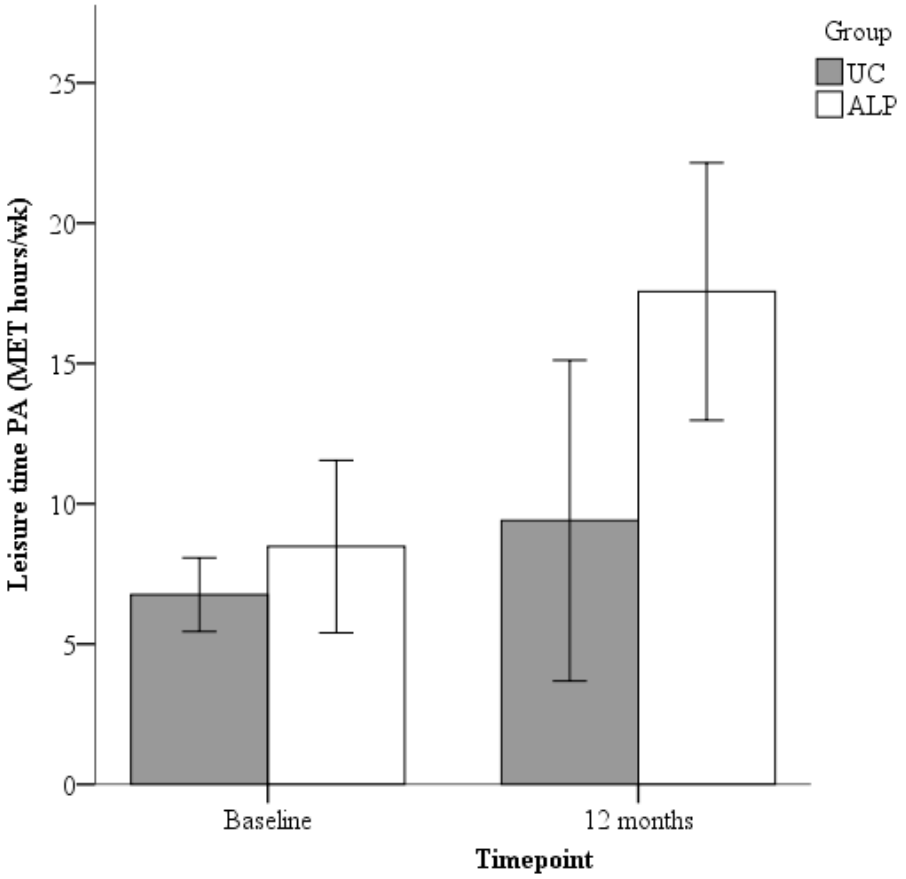

Figure 4.6 No significant difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) in self-report leisure-time physical activity (PA) in metabolic equivalent of task (MET) hours per week at baseline and after 12 months. No difference after 12 months by group allocation when adjusted for baseline leisure-time PA . P = 0.328, error bars ± 1 SE.

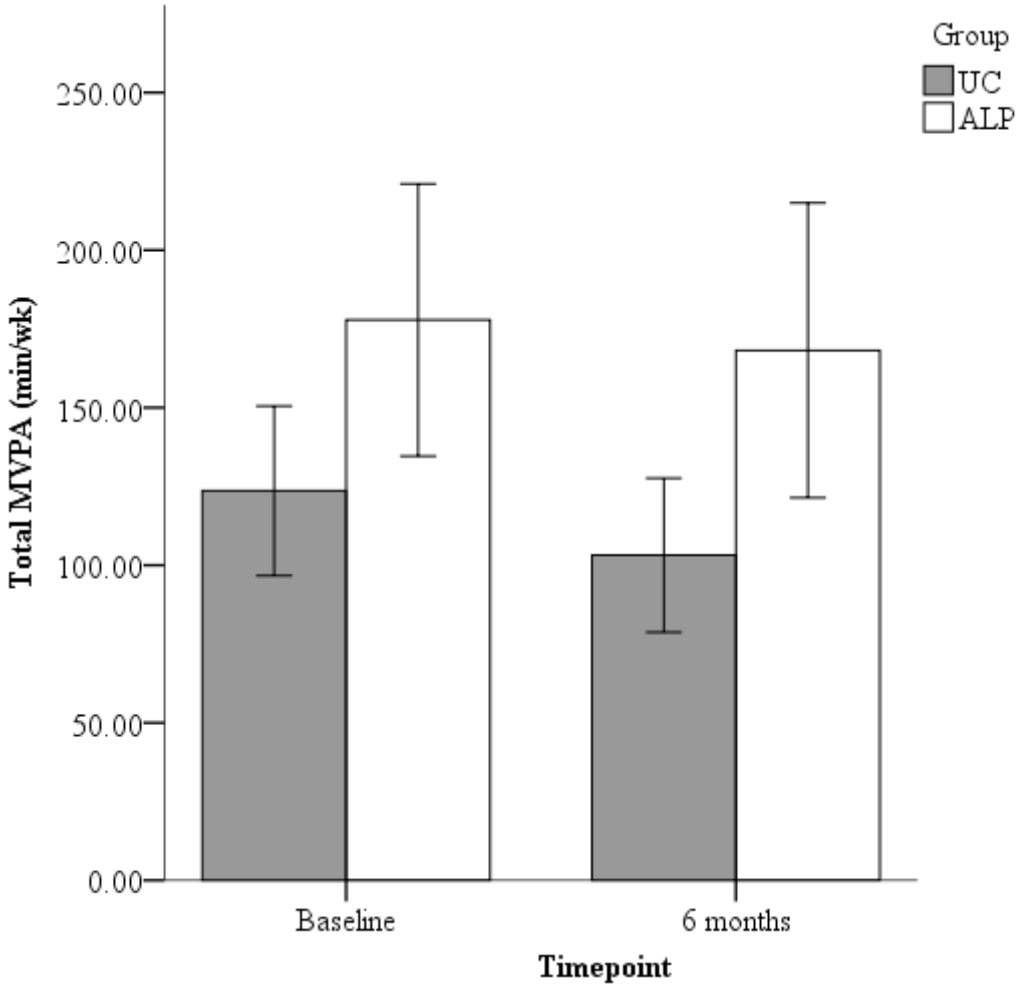

Figure 4.7 No significant difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) in minutes per week spent in moderate or vigorous physical activity (MVPA) in bouts of ten minutes or more according to accelerometer data at baseline and after 6 months. $P > 0.05$, error bars ± 1 SE.

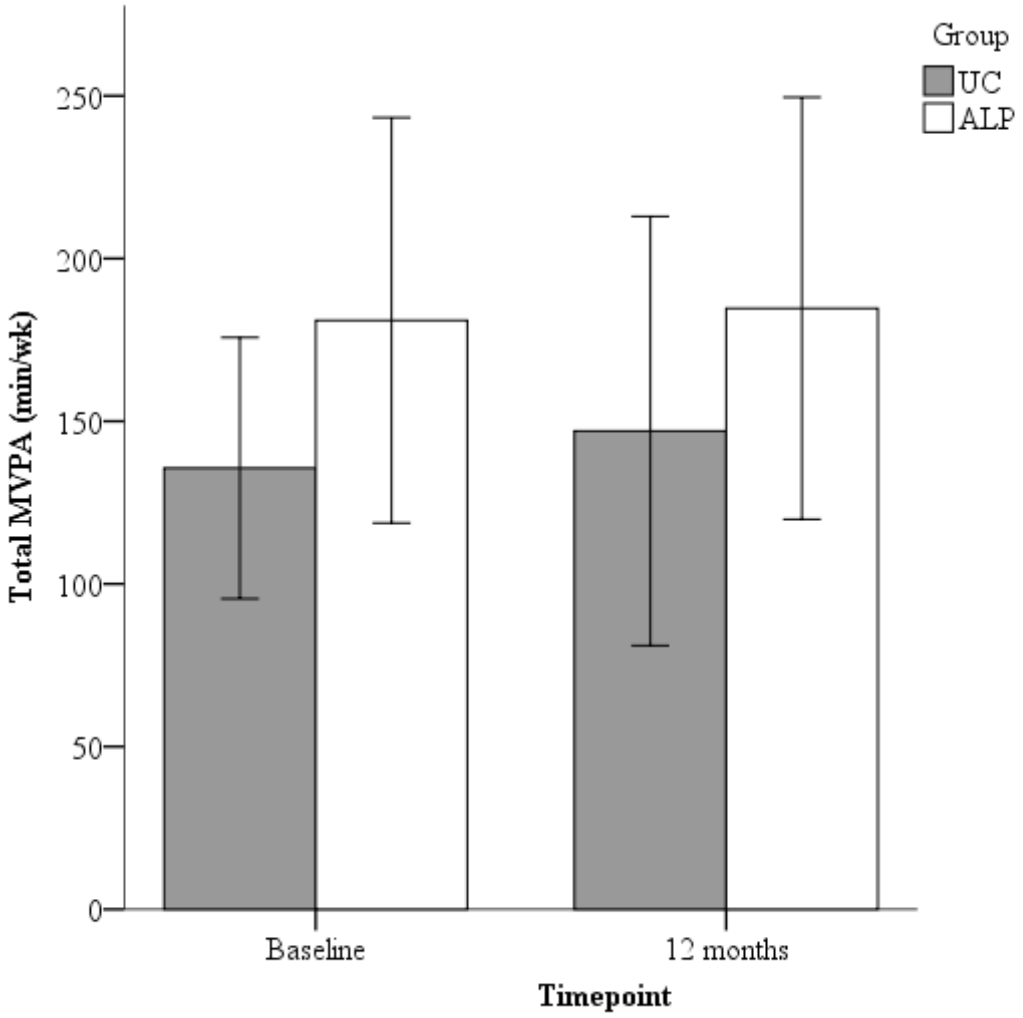

Figure 4.8 No significant difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) in minutes per week spent in moderate or vigorous physical activity (MVPA) in bouts of ten minutes or more according to accelerometer data at baseline and after 12 months. $P > 0.05$, error bars ± 1 SE.

4.3.4 Diet

Details of macronutrient and some micronutrient composition of the diet recorded by ALP and UC participants at baseline, 6- and 12-months are displayed in Table 4.2. Taking part in either ALP or UC did not affect diet composition over time, nor were there any inter-group differences in diet composition at any time point.

Table 4.2 Diet composition in PARC participants

Group	**UC**			**ALP**		
	Baseline	*6-mo*	*12-mo*	*Baseline*	*6-mo*	*12-mo*
N	14	10	7	17	12	8
Energy (kcal)	1697 (576)	2049 (676)	1648 (418)	1871 (823)	1584 (705)	1454 (546)
Protein (g)	65 (22)	76 (25)	63 (14)	80 (55)	66 (24)	71 (19)
Carbohydrate (g)	214 (89)	272 (100)	200 (69)	214 (93)	193 (145)	175 (98)
Fat (g)	64 (21)	76 (29)	62 (15)	73 (47)	53 (23)	53 (22)
Fibre	18 (10)	25 (14)	18 (11)	18 (8)	16 (9)	18 (11)
Selenium	36 (13)	39 (16)	27 (10)	53 (67)	39 (20)	45 (19)
Vitamin D	2.8 (4.0)	3.4 (2.0)	1.6 (0.7)	2.8 (3.1)	1.8 (1.4)	3.1 (2.5)
Folate	240 (134)	289 (115)	234 (134)	255 (94)	257 (237)	254 (121)

4.3.5 DNA methylation

Summary descriptions for the extent of DNA methylation for the eight genes for which there was sufficient DNA to analyse in both colon and buccal cells are displayed in Figure 4.10 and Figure 4.11, respectively. There was no correlation between buccal cell and colon DNA methylation in any of the genes studied or LINE-1. *APC ASC* and *HPP1* were predominantly unmethylated in colon biopsies (Figure 4.10) and *IL6, HPP1*, *APC* and *ASC* were found to be unmethylated in buccal cells (Figure 4.11). Of the remaining genes with normal distribution of methylation at baseline, none were associated with any of the variables in Table 5.1. There were no effects of sampling time i.e. baseline, 6 and 12 months on differences in DNA methylation. There was no difference between ALP and UC on the methylation status of *IL10* after 6 or 12 months, after correcting for baseline methylation levels (Figure 4.12 and Figure 4.13, respectively). There was also no difference between ALP and UC on the methylation status of *TNF* after 6 or 12 months, after correcting for baseline methylation levels (Figure 4.14 and Figure 4.15, respectively). Equally, there

was no difference in change in methylation between ALP and UC in the remaining genes and LINE-1. *WIF1* could not be analysed due to a lack of sufficient DNA, evidenced by multiple bands on the mega-gel.

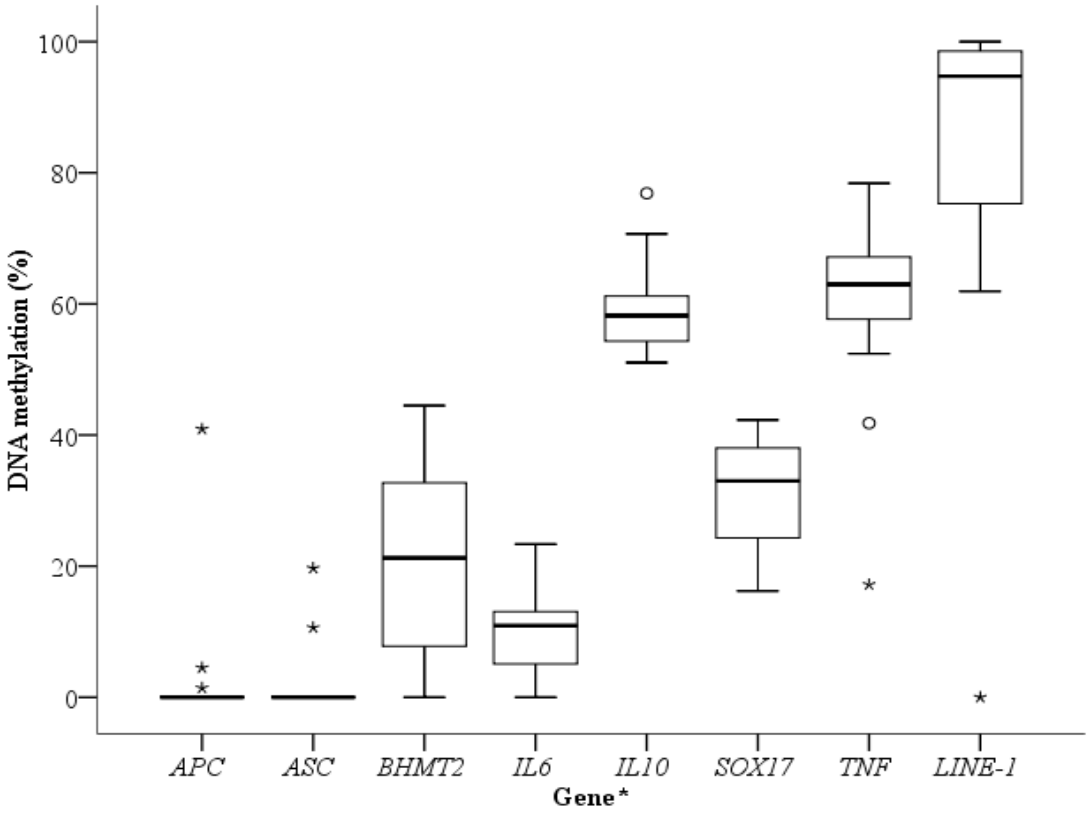

Figure 4.9. Summary descriptions of sigmoid colon biopsy DNA methylation values for seven genes and for LINE-1 at baseline.

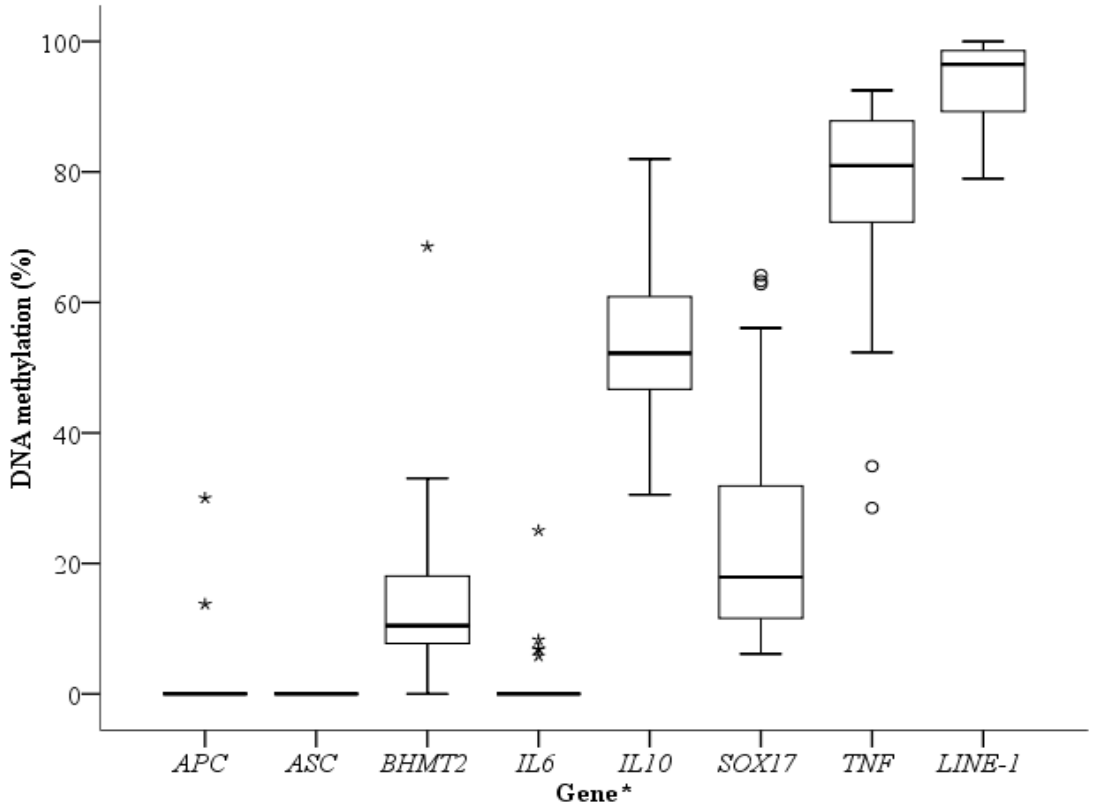

Figure 4.10. Summary descriptions of buccal cell DNA methylation values for seven genes and for LINE-1 at baseline.

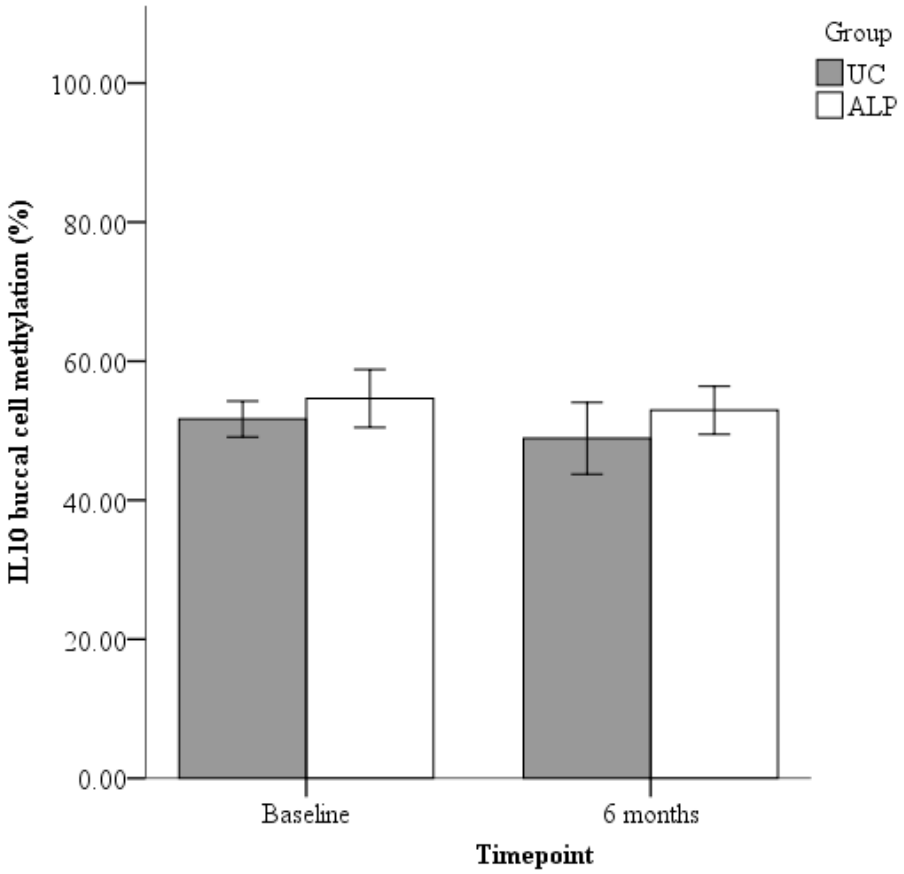

Figure 4.11 No significant difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) in IL10 buccal cell methylation (%) at baseline and after 6 months. Error bars ± 1 SE.

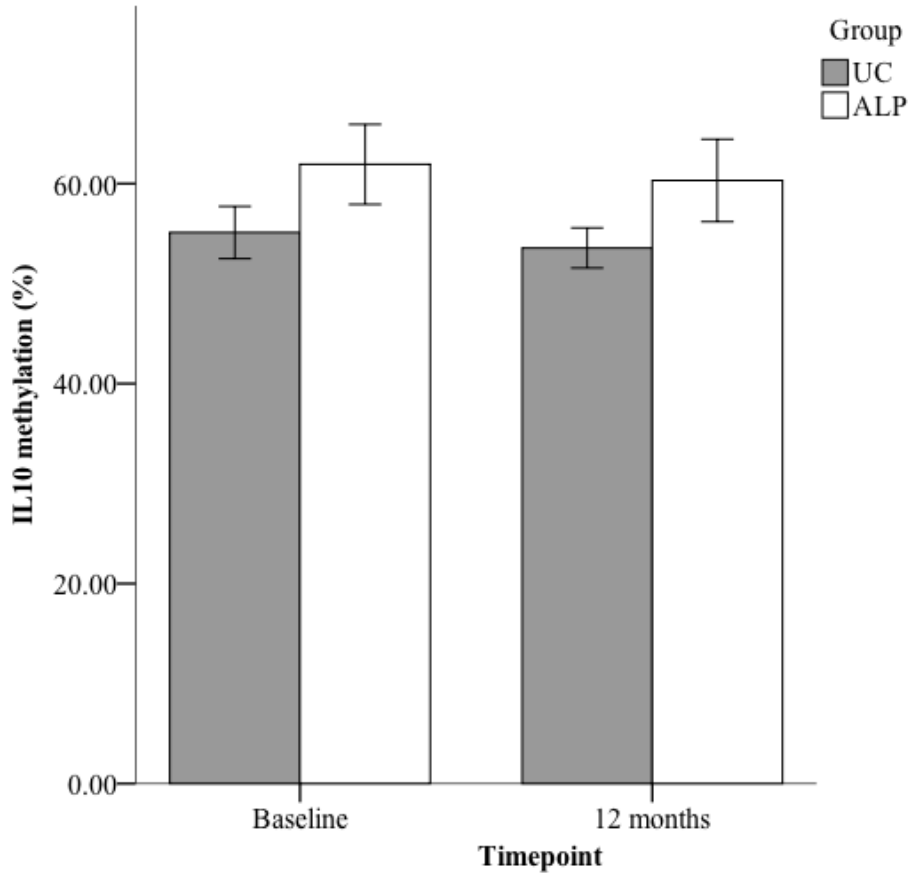

Figure 4.12 No significant difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) in IL10 buccal cell methylation (%) at baseline and after 12 months. $P > 0.05$, error bars ± 1 SE.

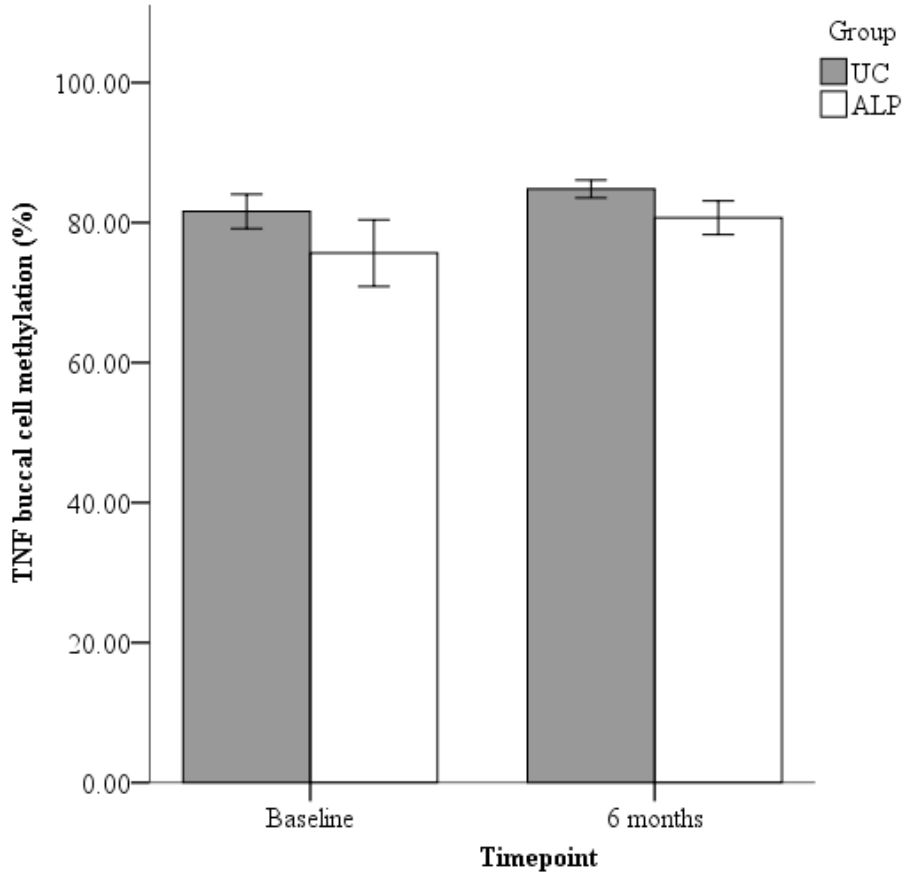

Figure 4.13 No significant difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) in TNF buccal cell methylation (%) at baseline and after 6 months. Error bars ± 1 SE.

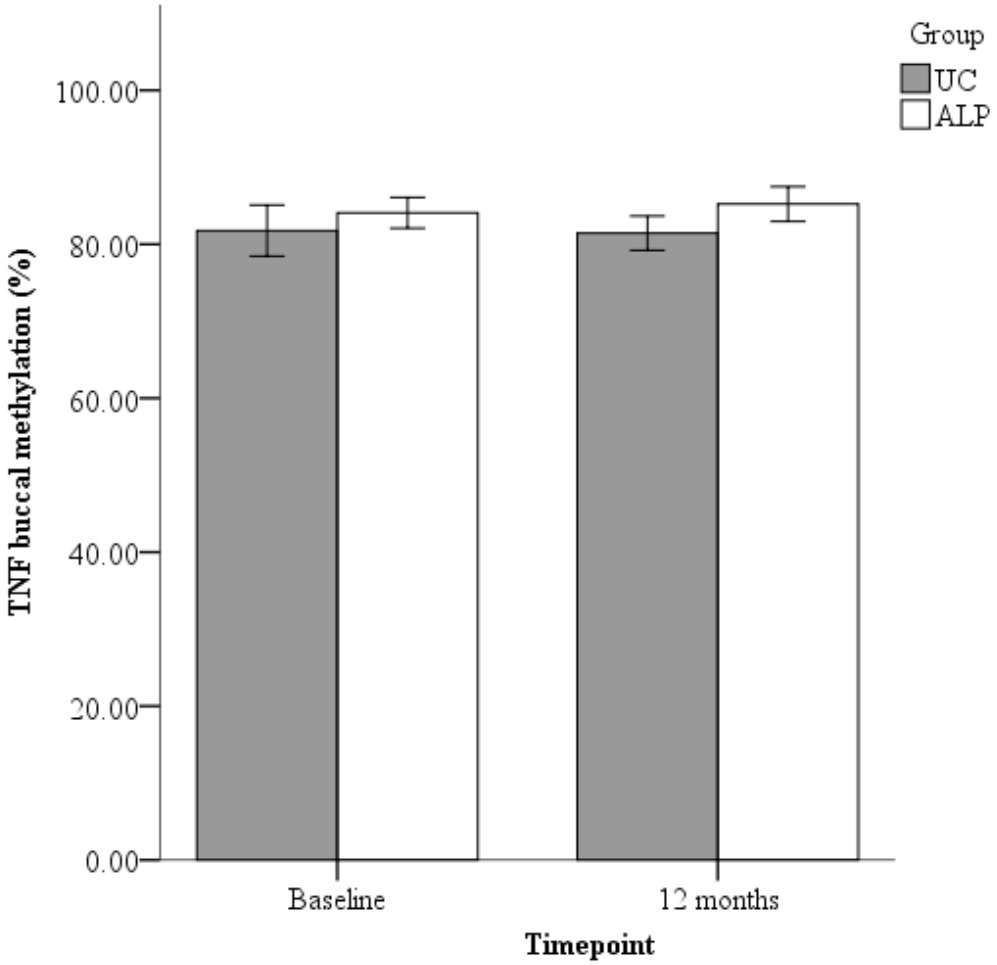

Figure 4.14 No significant difference between Usual Care (UC: filled bars) and Active Lifestyle Programme (ALP: open bars) TNF buccal cell methylation at baseline and after 12 months. Error bars ± 1 SE.

4.4 Discussion

4.4.1 Physical activity and cardiorespiratory fitness

According to the IPAQ and Godin Leisure Time Exercise Questionnaire, ALP successfully increased the amount of self-report leisure-time PA to a mean of 18 MET hrs per week after 6 months (Figure 4.4), which was maintained after 12 months (4.5), although there was no significant difference between ALP and UC after 12 months when baseline PA was taken into account (Figure 4.5). However, this ultimately fell short of the 300 min/wk target set by the research team. This was accompanied by increases in CRF in ALP (Figure 4.6), although this was not observed after 12 months

(Figure 4.7). This is possibly because i) the contact time with participants in ALP was reduced, and ii) fewer participants completed the study to 12 months (n = 15), which introduces the possibility that there was insufficient power to detect differences. Change in CRF was unrelated to body mass, which remained stable in both groups (Table 4.1). Surprisingly, it was also not associated with any changes in time spent in bouts of moderate-vigorous PA measured via accelerometer (Figure 4.8). There are several possible explanations for this finding. For one, it might be that the accelerometer was worn during a week where less moderate-vigorous PA was performed, despite more time being devoted to leisure-time PA. Another reason might be that the accelerometer was insufficiently sensitive in detecting lower intensity PA in our participants. Given that the majority of our participants had very poor fitness for their age at baseline according to previous work (Sui *et al.*, 2007), it is highly possible that very modest changes in PA behaviour resulted in substantial fitness gains.

4.4.2 DNA methylation

Buccal cell methylation and colon DNA methylation of all genes studied were not correlated at baseline. This is perhaps because age-related methylation of individual genes is tissue specific (Sliekerk *et al*., 2013; Lokk *et al.*, 2014) and this specificity is still apparent in response to environmental stimuli (Christensen *et al.*, 2009). Despite methylation patterns being similar across functionally similar tissues (Lokk *et al.*, 2014) this relationship might not extend to buccal and gut epithelia, which perform dissimilar roles. This heterogeneity might render surrogate gene-specific methylation markers unsuitable for accurately estimating methylation status of more inaccessible tissues such as the colon. As we could not obtain follow-up colon biopsies, we cannot be sure that beneficial adaptations not observed in buccal tissue also did not occur in the colorectal mucosa. Given the unpredictable nature of colonoscopy outcomes, and the considerable time between investigations after adenoma detection (1 – 3 years (Atkin and Saunders, 2002)) future investigations into colon DNA methylation and other biomarkers of risk from colon biopsies in a randomised trial setting might have to occur outside the auspices of the National Bowel Cancer Screening Programme.

In contrast to the present work, other exercise studies have succeeded in affecting DNA methylation patterns after six months in adipose tissue and muscle, in

men at elevated risk of type 2 diabetes (Nitert *et al.*, 2012; Ronn *et al.*, 2013). Interestingly, the majority of DNA methylation changes in those investigations did not occur in CpG islands but CpG island shores distant from the promoter. Similarly, widespread changes in CpG island shore methylation are abundant in cancerous colon tissue (Irizarry *et al.*, 2009). As the current study investigated the promoter region, there is a possibility that differentially methylated regions were not measured. Of the genes with altered CpG methylation after exercise training in adipose tissue, 39 were candidate genes for type 2 diabetes or obesity and involved in the metabolism of adipocytes, which is possibly related to the loss of fat as a result of the intervention (Ronn *et al.*, 2013). In addition, the genes that displayed differential methylation in skeletal muscle were principally involved in pathways including insulin signalling, metabolism of carbohydrate, and cell signalling (Nitert *et al.*, 2012). In both cases, the differences observed were plausibly linked with the morphological and functional changes that accompany exercise training, e.g. improved insulin sensitivity, fat metabolism and decreased reliance on carbohydrates for muscle fuel. Given these findings, it is perhaps unsurprising that we did not find any effect of ALP on buccal cell DNA methylation, as the dose of PA was much lower in intensity than the work of Ronn and colleagues, and the tissue had no direct involvement in exercise training. Indeed, it might be that such dramatic changes could not be achieved in the buccal cells or colon tissue in older persons at risk of CC given that i) it is unlikely that sedentary older adults will enrol on or independently maintain a programme of high-intensity aerobic exercise and ii) the tissues have no direct involvement in exercise training.

It is possible that had we measured the DNA methylation status of genes in peripheral blood we might have observed changes associated with exercise behaviour, as our group has demonstrated previously. Another example of this phenomenon was shown by Nakajima and colleagues, who found that compared with aged-matched controls, a six month moderate-intensity walking protocol in older Japanese persons was associated with ~ 1% increased methylation of *ASC* in peripheral blood leukocytes compared with the control group, which indicates a reversal of age-related methylation in the selected CpG sites of that gene (Nakajima *et al.*, 2010) and has been shown to increase with age in a separate report (Christensen *et al.*, 2009). However, the study was not a randomised controlled trial, nor was any information pertaining to balancing or matching the participants in the control and intervention

arms published. Therefore, it cannot be ruled out that the differences observed were not due to the exercise protocol. In the present study, it was found that *ASC* was typically unmethylated in both colon and buccal cells (Figure 4.10, Figure 4.11). In addition, the extent of DNA methylation of *IL10* and *TNF* observed in the colon biopsies obtained in this study (Figure 4.10) was dissimilar to those obtained from blood samples in the CHS, notably for *IL10*. Whilst we cannot infer that this represents a true difference between the methylation statuses of these genes in different tissues, it is worth noting that the baseline anthropometric characteristics of both study populations were similar. Again, this suggests that any effect of increasing PA on DNA methylation is tissue and gene-specific.

4.4.3 Limitations

Unfortunately, the study failed to recruit the number of participants deemed necessary at the outset. With hindsight, a smaller-scale feasibility study, or sham recruitment, would have been very useful in order to better estimate the likely uptake of participants into the study. Not performing this was a major limitation. As a consequence, this is reported as a pilot study. Previous work from our laboratory has indicated that *TNF* methylation might be susceptible to changes in PA (Chapter 3). A post-hoc estimate of achieved power with our small sample size was conducted using GPower software. Using the mean (SD) difference in *TNF* buccal cell methylation from baseline to 6 months by group, (n =10 in UC; n = 12 in ALP) the post-hoc effect size (*d*) was 0.154, which translated into an achieved power (1 – beta error probability) of 0.0636. An a priori calculation of required sample size based on obtaining 80% power was 1334 (Figure 4.14). It seems that the present study was highly underpowered, so it is perhaps unsurprising we did not find any significant differences in DNA methylation with PA. It is also possible that the original target of 124 participants would have been insufficient to detect whether PA had any effect.

The reasons for the low recruitment are multi-faceted and are probably related to the study design, location and patient group. Firstly, the FOBT has a specificity of ~50% for polyps and cancer in those who have a positive test (NHS, 2015). This is broadly in agreement with the number of patients who agreed to take part who were ineligible due to absence of adenomas at colonoscopy in the present study. n addition, a UK-based qualitative investigation, published after recruitment had begun, had

strongly suggested that most colon polyp patients did not associate polyps with CC, were unaware of lifestyle influences on the disease, and were not prepared to change behaviour to reduce risk (Dowswell *et al.*, 2012).

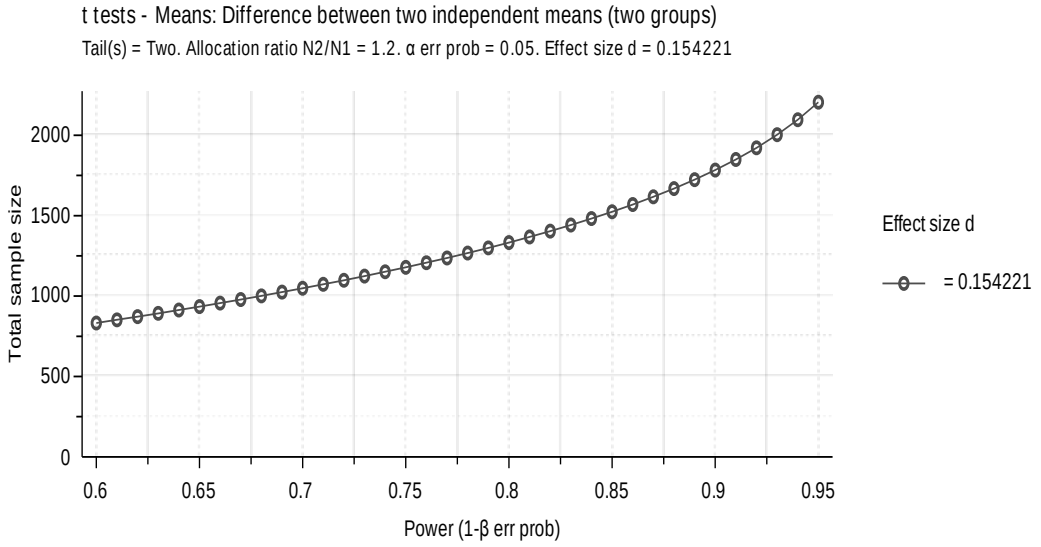

Figure 4.15 Estimated sample size required to demonstrate effect of PA on buccal cell *TNF* methylation based on achieved power in the present study

More recently, an investigation in New Zealand found that <13% of polyp patients with a family history of CRC who responded to a health questionnaire believed that the disease was related to lifestyle factors (Tarr *et al.*, 2014). This might in part explain the high number of refusals to take part in eligible patients contacted. This is an attitude that requires tackling, since the estimated conversion rate of advanced adenomas to carcinoma is 25 – 43% depending on age and gender (Brenner *et al.*, 2007b). Another potential explanation for the low recruitment rate is that the Norfolk and Norwich University Hospital, whilst providing hospital services for approximately 995,000 persons, does so for the largely rural Norfolk and Waveney catchment area, regions of which have some of the lowest population densities in England. As such, many participants were unable to commit to ALP due to travel difficulties.

Previous intensive lifestyle interventions have recruited larger numbers of participants from this patient group. For example, a 12-month exercise study based in Washington State, U.S.A, recruited 202 patients with a history of colon adenoma (McTiernan *et al.*, 2006). However, this was achieved by sending invitations to 9,828

potentially eligible participants and utilised four health centres, with complimentary gym memberships provided. Even then, the actual recruitment rate was 2.1%, which was less than that of the present study (4.2%). Another year-long study, which focused on weight loss of 7% body weight in overweight polyp patients, achieved a recruitment rate of 33% from an initial pool of 997 (Anderson *et al.*, 2014). Unlike the present study, this was achieved in a multicentre setting over 17 months. Also, the study required far less contact time with the research team (5.25 hrs per participant), which probably made taking part more palatable despite the demands of the intervention.

Furthermore, the attrition rate for this study was 29%. This is considerably greater than that seen in similar exercise intervention studies in cancer survivors, which typically report approximately 10% drop-out (Bisschop *et al*., 2015). This is possibly because cancer survivors might be more motivated to continue with such an intervention. Certainly, although the participants in the current study were polyp patients, with some suffering from other chronic disease, they were cancer-free. A comparable attrition rate (35%) was observed in cancer-free physically inactive Australians aged > 66 during a six-month randomised walking and resistance training intervention (Jancey *et al*., 2007). The only previous randomised exercise study in colon polyp patients failed to publish drop-out rates (McTiernan *et al*., 2006), so it remains unclear whether the present investigation experienced a greater or lesser extent of attrition than might be expected.

Another limitation was that the groups were not balanced in terms of CRF and baseline PA (Table 4.1). It was envisaged that the participants would follow similar physical activity patterns, in that they would all be sedentary. However, there was considerable heterogeneity in their PA behaviour patterns. This could plausibly have affected baseline DNA methylation, as minutes per week of PA and CRF have been associated with differential DNA methylation of buccal cell DNA in a previous report (Bryan *et al*., 2013). This was an oversight, and if the experiment were to be run again PA and CRF would form part of the randomisation strategy.

In addition, whilst participants recorded their diet and were asked not to alter their dietary habit, it was not controlled. This could have had a profound effect on DNA methylation patterns, as it has been demonstrated that age-related DNA methylation in the rectal mucosa is inversely correlated with Vitamin D and selenium intake, but positively correlated with folic acid intake (Tapp *et al*. 2013). Folate is a

methyl group donor and can profoundly affect DNA methylation (Bae *et al.*, 2014; Charles *et al.*, 2012; Tapp *et al.*, 2013; Wallace *et al.*, 2010). Whilst there were no significant differences in self-reported consumption of these micronutrients by group or over time (Table 4.2) it cannot be ruled out that they did not change.

Finally, peripheral blood leukocyte samples were not collected from the participants, which meant that we did not investigate whether the associations that were observed in our previous work in the CHS cohort could be replicated in a randomised trial. Whilst it is uncertain whether any changes in these cells are a proxy marker for colon DNA methylation patterns, this was perhaps a missed opportunity.

4.4.4 Conclusion

In conclusion, this study has demonstrated that the recruitment of adenoma patients to exercise intervention studies presents numerous challenges, which might render intensive exercise-based methods to decrease CC-specific risk in this group ineffectual. Secondly, there is little correlation between the methylation status of the inflammation and CC-risk specific genes in buccal and colon tissue analysed in this study, which suggests that buccal cell methylation might not be a suitable biomarker of CC-specific risk. Furthermore, there was no impact of a six-month to one-year aerobic physical activity programme on buccal cell methylation patterns. However, since the study was underpowered it is not possible to draw any definitive conclusions.

Chapter 5

Study 3: The Regular exercise and colon cell growth (ReGro) study: Does cardiorespiratory fitness and physical activity participation in older men affect growth of colon cancer cells *in vitro*?

5.1 Introduction

Obtaining colon biopsies from persons at risk of CC in the context of a randomised trial presents numerous challenges. An alternative to obtaining colon biopsies can be found in cell line work, where at least 24 distinct types of colorectal cancer cell are used for research purposes (Ahmed *et al*., 2013). Physical activity (PA) and cardiorespiratory fitness (CRF) have been inversely associated with risk of breast, prostate and colon cancer CC, and overall cancer mortality based on strong observational evidence (Evenson *et al.*, 2003), although there is no evidence from randomised trials. The mechanisms behind this phenomenon are not well understood, but are chiefly attributed to PA-induced alterations in chronic inflammation, immune function, hormonal concentrations and oxidative stress (Friedenreich *et al.*, 2010a; Gleeson *et al.*, 2011). These purported effects of PA on cancer risk and progression can be investigated through *in vitro* models. This method has demonstrated that growth of the prostate cancer cell line LNCaP is potentially susceptible to alterations in energy balance. For example, a two-week intensive Pritikin diet and exercise programme resulting in weight loss in obese men inhibited growth of LNCaP by 30% compared with baseline (Ngo *et al.*, 2002). Incubation in serum from long-term (14 y) adherents to the Pritikin programme reduced cell growth by a further 14% (Ngo *et al.*, 2002). Furthermore, it was found that serum from obese men also induced LNCaP proliferation compared with lean individuals who had not followed the Pritikin regimen (Leung *et al.*, 2004). The authors attributed this to a reduction in insulin-like growth factor-1 (IGF-1) and an increase in its

binding protein IGFBP-3, which was confirmed to affect LNCaP growth in a related study (Ngo *et al.*, 2003).

The findings from Ngo and colleagues must be viewed with caution since the most likely explanation for altered IGF-1 and IGFBP-3, resulting in reduced cell growth after two weeks was energy restriction rather than a change in diet and exercise patterns. Acute energy restriction has long been known to induce this effect (Thissen *et al.*, 1994), and importantly this does not translate to reductions in IGF-1 or IGFBP-3 in the long term in the absence of malnutrition (Belobrajdic *et al.*, 2010; Fontana *et al.*, 2008). Moreover, the long-term adherents to the Pritikin programme were of normal weight, which confounds any comparison with the obese men in that study. Indeed, obesity is associated with higher free concentrations of these hormones (Nam *et al.*, 1997) so any differences cannot be reliably attributed to diet and exercise.

More recent work has shown that pooled serum taken from the femoral artery in ten healthy young men after 60 min of cycling exercise at 50-65% $\dot{V}O_{2\,max}$ reduced LNCaP growth by 31% compared with incubation in serum taken prior to exercise (Rundqvist *et al.*, 2013). This might, in part, be related to the inflammatory response to exercise. An acute bout of exercise induces changes in some inflammatory biomarkers. Notably, IL-6 has been demonstrated to be elevated four-fold after thirty minutes of moderate intensity treadmill exercise in the serum of trained runners (Ostrowski *et al.*, 1998) and two-fold after 40 minutes of moderate cycling exercise in sedentary, middle aged men (Mendham *et al.*, 2011). his is also associated with an increase in the anti-inflammatory cytokine IL-1ra in lean men immediately after 60 min of moderate intensity running (Peake *et al.*, 2005; Scott *et al.*, 2011), which peaks approximately 1 hr after exercise (Ostrowski *et al.*, 1998; Peake *et al.*, 2005; Scott *et al.*, 2011). After high-intensity interval running in active males a transient increase in the pro-inflammatory mediator IL-8 is also observed (Croft *et al.*, 2009). In addition, a high-intensity continuous bout of running (60 min, 85% VO_{2max}) induces increases in the anti-inflammatory cytokines IL-1ra and IL-10 in endurance-trained men, which again peaks 1 hr after exercise (Peake *et al.*, 2005). Since inflammatory markers are modulated by PA, and chronic inflammation is associated with CC risk (Caruso *et al.*, 2004; Franceschi *et al.*, 2000a; Il'yasova *et al.*, 2005), this could be a mechanism by which a single bout of exercise mitigates cell growth *in vitro*.

Combined, the results of these studies lend partial support to the hypothesis that PA participation could induce biologically relevant changes in serum that affects the proliferative

capacity of one type of malignant cell. To the author's knowledge, this hypothesis is yet to be tested in CC cell lines using serum from participants who have partaken in regular moderate-vigorous PA (MVPA) over many years and who have excellent CRF, those who have a sedentary lifestyle, and those who have recently taken up a programme of MVPA. It is also not known whether a single bout of exercise can affect the capacity of serum to retard CC cell line growth.

Therefore, to examine whether serum-induced growth of CC cells is modulated by long-term MVPA, CRF or a single bout of exercise, we sought to recruit older men free of metabolic or cardiovascular disease with varying CRF. We also aimed to investigate whether a single bout of exercise could affect growth of these cells, as has been demonstrated in the LNCaP cell line (Rundqvist *et al.*, 2013). Therefore, given the negative association between MVPA and CRF on CC risk and mortality (Wolin *et al.*, 2009), the aim of this pilot study (the Regular Exercise and colon cell GROwth study: ReGro) was to investigate whether serum from long-term MVPA participants and with excellent CRF affects growth of CC cell lines, in the fasted state and after a single bout of exercise, compared with disease-free controls. To test the hypothesis that recently changing exercise behaviour might influence serum composition that could also meaningfully affect cell growth, a population previously recruited to a randomised exercise trial in our laboratory was used. Twenty male participants from the PARC RCT (Chapter 4) provided serum at baseline, and after 6 months of taking part in the supervised exercise portion of the study. These samples were used to treat CC cell lines to determine whether the ALP intervention influenced the ability of serum to inhibit cell growth.

5.2 Methods

5.2.1 Study design

This investigation was split into two components. The first of which was a repeated measures, counterbalanced, crossover design in older men free of metabolic and cardiovascular disease (the ReGro study, Figure 5.1). Participants were randomly allocated to either exercise followed by rest group (Group 1: EX-REST) or vice-versa (Group 2: REST-EX). Randomisation was completed by a member of the Biostatistics team at the Institute of Food Research (Dr Jack Dainty). Participants were assigned a unique code which blinded the researcher as to their group allocation and time of sampling during analysis. The second was

a randomised controlled trial of MVPA participation in older colon adenoma patients (Figure 4.1, 4.2). A detailed description of the PARC intervention study can be found in Chapter 4.2 Methods. Briefly, adenoma patients were randomly assigned to either the Active Lifestyle Programme (ALP; a six-month predominantly aerobic exercise intervention) or UC (Usual Care).

5.2.2 Participants

Twenty male volunteers free of known cardiovascular or metabolic disease and with no history of malignancy were recruited via paper and online flyers placed on the Norwich Research Park, the Norfolk and Norwich University Hospital, and in the Norwich Evening News. The baseline characteristics of the participants can be found in Table 5.1. Recruitment of the PARC participants was completed via the Norfolk and Norwich University Hospital (see 4.4.1 Participants and 4.2.2 Recruitment). The baseline characteristics of these participants can be viewed in Table 5.2.

5.2.3 Cardiorespiratory fitness

Maximal aerobic capacity ($\dot{V}O_{2\,max}$; $ml \cdot kg \cdot min^{-1}$) in the ReGro participants was determined using a Medgraphics™ Ultima Cardio2 suite (Medical Graphics Corporation, St. Paul, MN, USA) with a Balke graded treadmill protocol. A comfortable running/brisk walking speed was selected for each participant (5.5 - 9 kph) and gradient increased by 1% every minute until volitional exhaustion or $\dot{V}O_{2\,max}$ was achieved. This was considered to have occurred if a plateau in $\dot{V}O_2$ was evident on the graphical display, respiratory exchange ratio (RER) was at least >1.15, and age-predicted maximum heart rate was attained. A real-time 10 lead ECG trace was monitored by a medical professional to ensure safety both during and after completion of the test. Owing to the reduced physical capacity of the PARC participants, a cycling protocol was used instead (see 4.2.3.3 Cardiorespiratory fitness).

5.2.4 Current physical activity

Current PA for both sets of participants was assessed using a self-report diary on which participants were instructed to list all occupational and recreational PA for a minimum of 7 days. In addition, objective free-living PA level was assessed over this period using an accelerometer worn about the right hip (ActiGraph® GT3X, ActiGraph Corps, Pensacola, FL, USA). The small unobtrusive accelerometer was worn on the hip and collected data including estimated activity counts, step counts, minutes of MVPA per day and per week, bouts of MVPA of ten minutes or more, and total estimated exercise energy expenditure. Data was analysed using integrated software (ActiLife version 6.9.1, ActiGraph Corps, Pensacola, FL, USA). Data for both ReGro and PARC participants is displayed in Table 5.1 and 5.2, respectively. The activity count cut off points used are displayed in Table 2.1.

5.2.5 Historical physical activity in ReGro participants

For ReGro participants, historical physical activity was assessed using the Historical Adulthood Physical Activity Questionnaire (HAPAQ) (Besson *et al.*, 2010). Participants completed the 'Life Calendar' section (which was not analysed by the researcher) followed by the formal HAPAQ questionnaire in line with the original authors' instructions. A PA 'prompt card' was also used to identify the specific types of occupational or leisure time PA performed by the participants. The number of years the participants spent with the present physical activity pattern, according to accelerometer and PA diary, against which the HAPAQ has been validated (Besson *et al.*, 2010), can be viewed in Table 5.1.

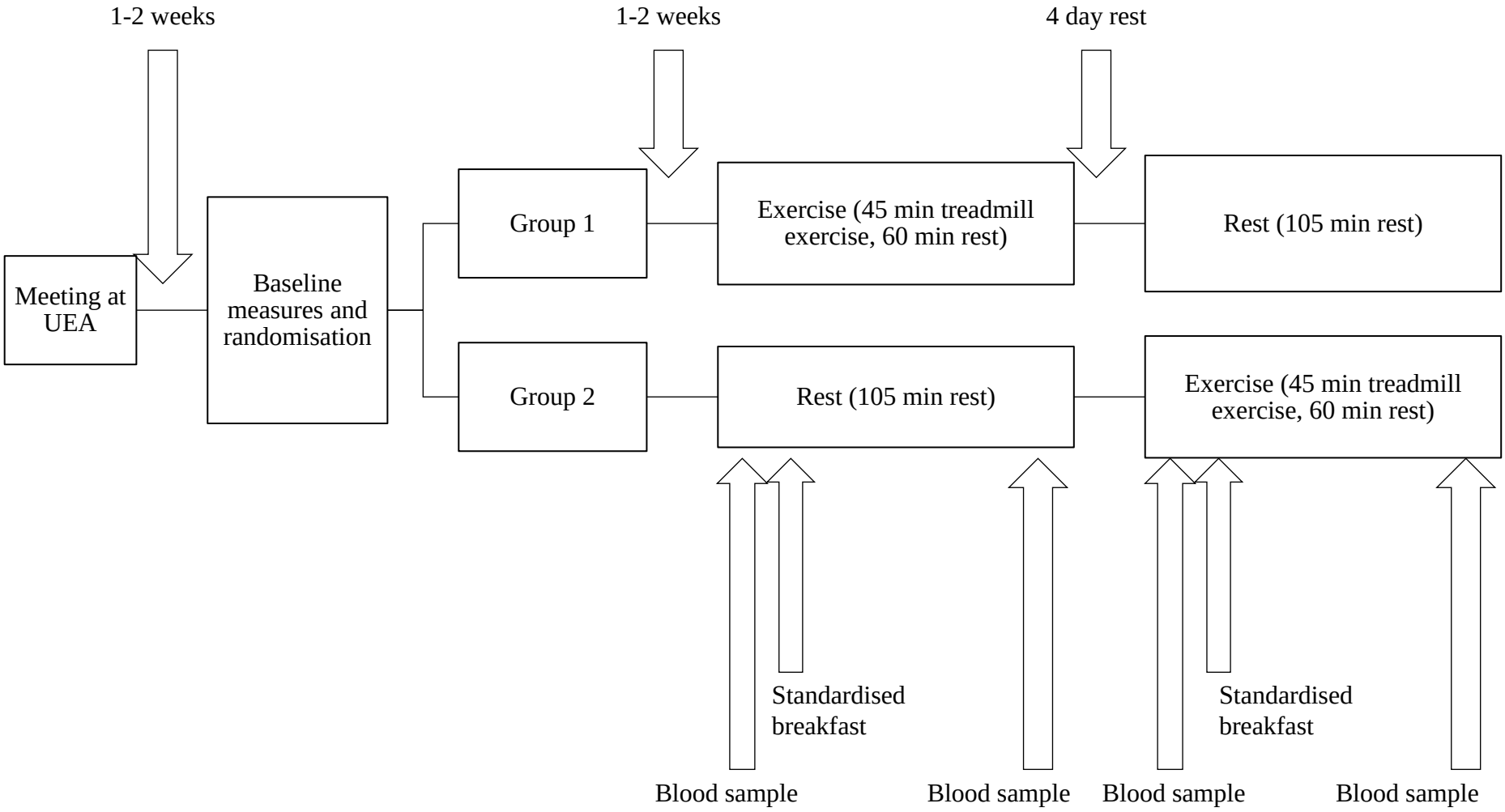

Figure 5.1 Study design for ReGro

5.2.6 Study days

Participants arrived at the Clinical Exercise Science laboratory after an overnight fast and having refrained from moderate-vigorous physical activity for 72 h, and from consumption of alcohol for 24 h. On one of the visits, participants had resting blood pressure (DINAMAP v1000, GE Healthcare, Bucks, UK), height, mass, and body composition recorded via bioelectrical impedance analysis (AKERN BIA 101, SMT Medical GmbH & Co., KG, Germany) and integrated software (BodyGram Pro 3.0, SMT Medical GmbH & Co., KG, Germany). 20 ml of venous blood was taken from a vein about the cubital fossa using the Vacutainer® technique in serum collection tubes (Beckton, Dickinson and Company, Franklin Lakes, NJ, USA). The tubes containing whole blood were inverted gently several times and allowed to clot for 30 min at ambient temperature. They were then centrifuged at 3600 rpm at 4 °C for 15 min (Thermo Fisher Scientific 1-SR Multifuge, Thermo Fisher Scientific, Waltham, MA, USA). Serum was extracted, pipetted into 1.5 ml Eppendorf tubes in 500 µl aliquots and frozen on dry ice. Each tube was given a 4 digit code which blinded the researcher to the participant identity and time of sampling. Serum samples were then stored at -75 °C at the Institute of Food Research.

Immediately afterwards the first blood sample had been taken, ReGro participants consumed a standardised breakfast. The rationale for providing a standardised breakfast was based on initial ethical review, where it was suggested that refreshments should be provided to the participants after the exercise bout. Secondly, it was envisaged that several of the participants would be unaccustomed to sustained endurance exercise, and might experience discomfort if asked to do so in the fasting state. To minimise the impact of this on the blood samples post-exercise, the researcher decided to provide a standardised breakfast before exercise began. The breakfast consisted of a plain bagel (New York Bakery Company, Rotherham, UK) with a sachet of Robertson's strawberry jam (Premier Foods, St Albans, UK) and Lurpak butter (Arla Foods, Viby, Denmark) a 250ml orange juice drink (Tesco PLC, Cheshunt, Herts, UK), and water ad libitum, within ten minutes. The total energy content was 353 kcal, with 66% coming from carbohydrate, 9% from protein, and 25% from fat. The standardised breakfast was modelled on previous work into breakfast consumption and exercise metabolism (Farah and Gill, 2013).

After a further 20 min, ReGro participants either rested for 105 min or completed a treadmill run/walk for 45 min at 65% $\dot{V}O_{2\,max}$ followed by 60 min of rest. Previous work has indicated that 120 min of rest after 65 min of cycling exercise corresponding to approximately 60% VO2 max was sufficient to retard LNCaP growth (Rundqvist *et al*., 2013). However, the lack of control group meant that the effects might have occurred in the absence of exercise, and/or were related to the long time-frame after exercise. To ensure that that any effects were truly due to the exercise bout it was decided to reduce the time-frame post-exercise to 60 min and perform the study in a crossover fashion. Furthermore, his timeframe was chosen to coincide with a potential increase in anti-inflammatory mediators such as IL-1ra and IL-10 (Fig 5.1). An additional 20 ml of venous blood was then taken. Heart rate was monitored (Polar FT1, Polar Electro, Kempele, Finland) during the exercise bout to ensure that it corresponded to expected levels as determined by the maximal exercise test.

5.2.7 Cell experiments

5.2.7.1 Incubation protocol

In order to gain a more comprehensive understanding of how serum factors might affect a wide variety of molecular phenotypes of CC, four different cell lines were chosen, namely, LoVo, Caco-2, HCT-116 and HT-29 (ATCC, Vanassas, MA, USA). A description of their phenotype can be found in Table 1.2. Detailed methods of cell handing and seeding procedures can be found in General Methods section 2.9. For the ReGro participants, the outer 36 wells of a 96-well cell culture plates (Nunc™; Thermo Fisher Scientific) were filled with 100 µl sterile PBS with a multi-channel pipette to limit evaporation. The remaining 60 wells were divided into four quadrants of 12 wells each, which were seeded with 1 x 10^4 cells, except in the case of HCT-116 which was seeded with 5 x 10^3 cells. Each quadrant represented one participant, and three wells were designated for serum obtained from each timepoint (i.e. Pre-Exercise, Post-Exercise, Pre-Rest, Post-Rest: Figure 5.1). For PARC participants, the remaining 60 wells were instead divided into eight lanes of six wells each. Each lane represented one participant at each timepoint (i.e. baseline, 6 months, 12 months).

Six of the remaining 12 wells were used for the foetal bovine serum (FBS) control, and the final six were left empty. One of the empty wells was used as a negative control on the day of the assay. The position of the twenty PARC and ReGro participants across the five plates was distributed such that there was heterogeneity of CRF on each plate. Serum was

prepared on the day of seeding by thawing on ice for ~ 30 min. 200 µl was extracted after mixing and added to 1.8 ml of DMEM/F12 free of FBS, but containing PS, in a 2 ml Eppendorf. The serum concentration was therefore 10% of the total fluid volume. Tubes were labelled and refrigerated at 4 °C. All tubes were incubated at 37 °C for 20 min prior to use. After 24 h of seeding, the media containing FBS was removed by multi-channel pipette and replaced with media supplemented with participants' serum or control serum containing FBS, and returned to the incubator. The procedure was repeated 48 hours later, except in the case of HCT-116 cells which were assayed after 48 hours. For all other cell lines, the assay was performed 48 hours after the second media replacement, when cells had reached 60-80% confluence.

5.2.7.2 WST-1 Assay

Culture media was removed from cells and replaced with 100 µl cell proliferation reagent WST-1 (Roche Diagnostics Corporation, Indianapolis, IN, USA) and fresh DMEM/F12 in a 1/10 dilution. An empty well was also filled to serve as a background measurement, whose absorbance was subtracted from experimental wells. Plates were then incubated for three hours. The colorimetric assay was performed with a microplate reader using a single read Endpoint protocol (Bio-Rad Benchmark Plus, Bio-Rad Laboratories, Hercules, CA, USA). Dual absorbance was set between 450nm and 690nm as per manufacturers' instructions, with a five second mix time at medium speed prior to plate read. Mean absorbance of six replicate wells and the six control wells treated with FBS on the same plate was calculated. The absorbance of the negative control was subtracted, and the results expressed as a percentage of the FBS control.

5.2.7.3 Gene expression from cultured cells

Where a significant result was returned from the WST-1 assay, gene expression was investigated to determine which pathways might have been responsible. 12 well cell culture plates (Nunc™; Thermo Fisher Scientific) were seeded with 3 x 10^4 cells in 300 µl media. Cells were incubated for 24 hours and media replaced with 300 µl 10% serum-supplemented media, which was replaced after 48 hours as above. After the 96-hour incubation, cells were lysed *in situ* and RNA extracted using an Isolate II RNA Mini Kit (Bioline) according to manufacturers' instructions. Purified RNA was quantified by Nanodrop Spectrophotometer (Thermo Fisher Scientific). 4 µl of eluted RNA was reverse-transcribed using qScript™

cDNA Supermix (Quanta Biosciences, Gaithersburg, MD, USA) as per the recommended protocol. 1.5 µl of triplicate samples were RT-PCR amplified in a Viia™ 7 Real-Time PCR System (Applied Biosystems) in a 5 µl reaction volume using the methods described previously (see 3.9.4 Gene expression from cultured cells) Primer sequences for the selected genes can be found in Table 2.4. Three genes (*Ki67*, *c-Myc*, *MUC2*) were selected due to their known associations with cell proliferation and oncogenesis (Gerdes *et al.*, 1984; He *et al.*, 1998; Ho *et al.*, 1993; Leoni *et al.*, 2012), *CDH1 (E-Cad)* was selected based on its association with tumour suppression (Esteller *et al.*, 2001; Visintin *et al.*, 1997), *LGR5* to assess presence of stem cells (Barker *et al.*, 2007), *COX*-2 for its involvement in inflammation and metastasis (Tsujii *et al.*, 1997) and *DNMT3b* for de novo methylation (Miranda and Jones, 2007) The ribosomal DNA sequence *18S* was used as a reference gene (Table 2.4). Fold changes in gene expression relative to *18S* reference gene in both sample and control wells treated with FBS was calculated using the ΔΔCT method described previously (Schmittgen and Livak, 2008).

5.2.8 Statistical analysis

In the ReGro study, a repeated measures analysis of variance (type 1 ANOVA) was conducted to determine any differences in cell metabolic activity due to treatment (EX *vs.* REST) or time (Pre *vs.* Post). Where significant results were returned, Mauchley's Test of Sphericity was conducted to check if sphericity assumptions had been violated. In addition, where any differences were returned, post hoc t-tests with the Bonferroni correction were used to locate them. For the PARC participants, differences in cell metabolic activity due to group allocation (ALP vs UC) after 6 and 12 months was analysed using a type 1 analysis of covariance (type 1 ANCOVA) with baseline metabolic activity as a covariate. Any possible associations between any of the participants' characteristics (e.g. age, mass, CRF) and cell metabolic activity were explored using Pearson correlations. All tests were performed, and figures created, using the Statistical Package for Social Sciences (SPSS) v.20 (IBM, Armonk, NY, USA).

5.3 Results

5.3.1 Anthropometry, physical activity and cardiorespiratory fitness

Baseline characteristics of all participants from ReGro and PARC studies are presented in Table 5.1 and 5.2, respectively. According to the Compendium of Physical Activity MET Intensities (Ainsworth *et al.*, 2000) ten ReGro participants were taking part in regular (≥ 3 times per week) vigorous intensity, predominantly aerobic exercise training (e.g. running, interval training, lap swimming, circuit training, fast cycling or spinning), and had maintained this pattern of activity for ≥ 10 years. Their baseline characteristics can be viewed in Table 5.3. The remaining ten were currently not performing any vigorous exercise training (all recorded activities being < 6 METS and/or being performed for < 10 min according to PA diary and accelerometer readout), and had maintained this pattern for at least 6 years. Their characteristics are displayed in Table 5.4. Minutes spent in bouts of MVPA lasting ten minutes or more was moderately correlated with CRF (Figure 5.2) and body fat percentage was strongly negatively correlated with CRF (Figure 5.3). There was no correlation between CRF and age.

5.3.2 Associations between cell metabolic activity and ReGro participants' baseline characteristics

In ReGro participants, there were no associations between any of the participants' characteristics, including age, body fat, MVPA or CRF on cell metabolic activity in any cell lines after being incubated in serum taken in the fasted state. The lack of correlation between cell metabolic activity and CRF is displayed in Figure 5.4. After grouping the participants by training status, there was a significant reduction ($P = 0.046$) in cell metabolic activity of Caco-2 cells following incubation in serum taken in the fasted state in the 'Rest' condition in those who did not perform any vigorous exercise training compared with those who did (Table 5.6). However, this was not observed in fasted samples taken in the 'Exercise' condition.

Table 5.1 Baseline characteristics of participants in the ReGro study (N = 20). All values mean (SD).

Age	**Mass (kg)**	**BMI**	**Body fat (%)**	$\dot{V}O_{2\,max}$	**MVPA (min/wk)**	**MVPA pattern (yrs)**
59 (6)	78.6 (14.3)	25.1 (4.6)	21.8 (7)	48 (11)	216 (128)	24 (15)

Table 5.2 Baseline characteristics of participants from PARC study. All values mean (SD) where appropriate.

Group	***ALP***	***UC***
N	9	11
Age	71.8 (1.8)	68.2 (2.7)
Mass (kg)	89.2 (3.8)	90.9 (20.7)
BMI	28.9 (2.9)	29.8 (5.8)
Body fat (%)	23.8 (5.5)	25.7 (7.2)
$\dot{V}O_{2\,max}$ **(ml · kg · min^{-1})**	25.1 (4.6)	25.8 (5.8)
Accelerometer-derived MVPA (min/wk)	101 (87)	194 (128)
Obesity	2	6
Hypertension	2	3
Cardiovascular disease	2	2
Type II diabetes	4	
Pulmonary disease	1	2
Family history of colon cancer	2	1

Table 5.3 Characteristics of the ten males in ReGro study who were undertaking at least three sessions of vigorous intensity exercise training per week at time of recruitment. All values mean (SD)

Age	**Mass (kg)**	**BMI**	**Body fat (%)**	$\dot{V}O_{2\,max}$	**MVPA (min/wk)**	**MVPA pattern (yrs)**
58 (4)	69.6 (11.9)	23.5 (4.0)	19 (8)	57 (7)	278 (112)	26 (12)

Table 5.4 Characteristics of the ten males in ReGro study who were not engaged in any vigorous intensity exercise training at time of recruitment. All values mean (SD)

Age	**Height**	**Mass (kg)**	**Body fat (%)**	$\dot{V}O_{2\,max}$	**MVPA (min/wk)**	**MVPA pattern (yrs)**
61 (7)	1.80 (0.08)	87.5 (10.4)	25 (6)	39 (7)	153 (116)	25 (6)

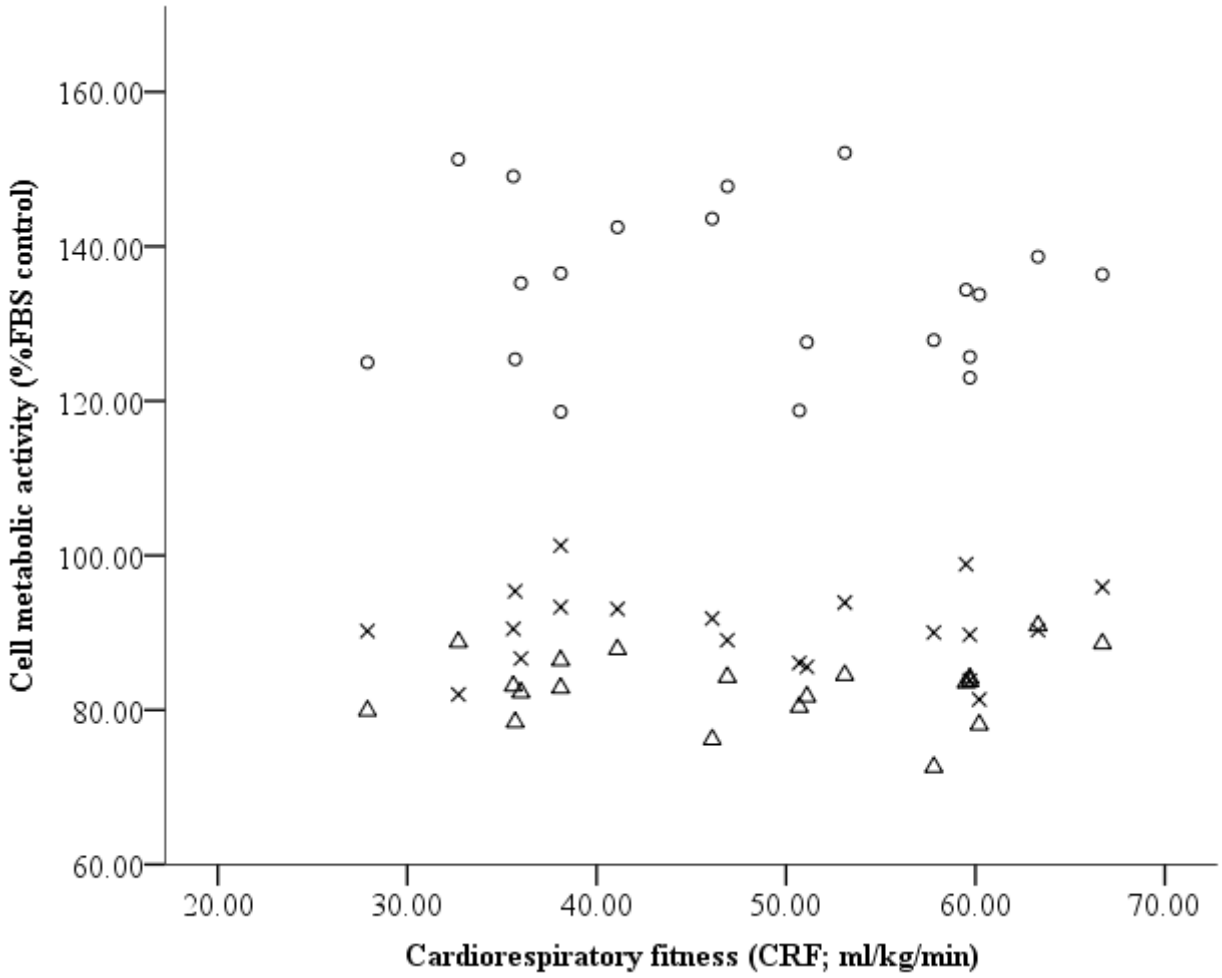

Figure 5.2 No correlation between HCT 116, Caco-2 or HT-29 cell metabolic activity (y axis) after incubation in serum from older men free of metabolic and cardiovascular disease in the postabsorptive state, and their cardiorespiratory fitness (x-axis). All results expressed as percentage foetal bovine serum (FBS) control. Key: Open circles = HCT-116, Crosses = Caco-2, Open triangles = HT-29.

Table 5.5 Cell metabolic activity after incubation in serum from ReGro participants who undertook regular vigorous exercise training versus no vigorous exercise training (% FBS control). All values displayed as mean (SD). * Significant difference between conditions ($P < 0.05$)

Cell line	Condition	Cell metabolic activity (% FBS control)	
		Vigorous exercise training	No vigorous exercise training
Caco-2	Pre-Exercise	90.31 (5.43)	90.58 (5.40)
	Post-Exercise	89.80 (7.34)	88.67 (8.92)
	Pre-Rest	92.62 (4.55)*	86.65 (7.52)
	Post-Rest	90.88 (7.76)	91.24 (7.00)
HCT-116	Pre-Exercise	158.00 (10.49)	157.69 (5.28)
	Post-Exercise	161.35 (6.27)	157.95 5.48
	Pre-Rest	160.10 (10.74)	153.37 (7.89)
	Post-Rest	160.47 (5.55)	158.77 (7.58)
HT-29	Pre-Exercise	83.44 (5.36)	82.40 (3.69)
	Post-Exercise	85.80 (5.50)	82.01 (3.87)
	Pre-Rest	85.28 (4.69)	81.73 (3.41)
	Post-Rest	85.98 (5.15)	82.16 (4.25)

5.3.3 Cell metabolic activity in response to Exercise or Rest

LoVo cells failed to adhere to the surface of the cell culture wells, and so the results were not included. There was no significant effect of treatment (EX vs REST) or time (Pre vs Post) on the metabolic activity of HCT-116 cells (Figure 5.5). Incubation in 10% human serum stimulated cell metabolic activity to a greater extent (15-55%) than FBS (Figure 5.5). Similarly, there was no effect of treatment or time on metabolic activity of HT-29 and Caco-2 cells (Figure 5.6, Figure 5.7). In contrast, participants' serum typically stimulated cell metabolic activity to a lesser extent than FBS in these cell lines (Figure 5.6, Figure 5.7). There was consistent agreement between results within participants irrespective of treatment or time of sampling, notably in the HCT-116 cell line (Figure 5.5). There were also no significant differences related to whether participants were currently undertaking regular vigorous exercise training.

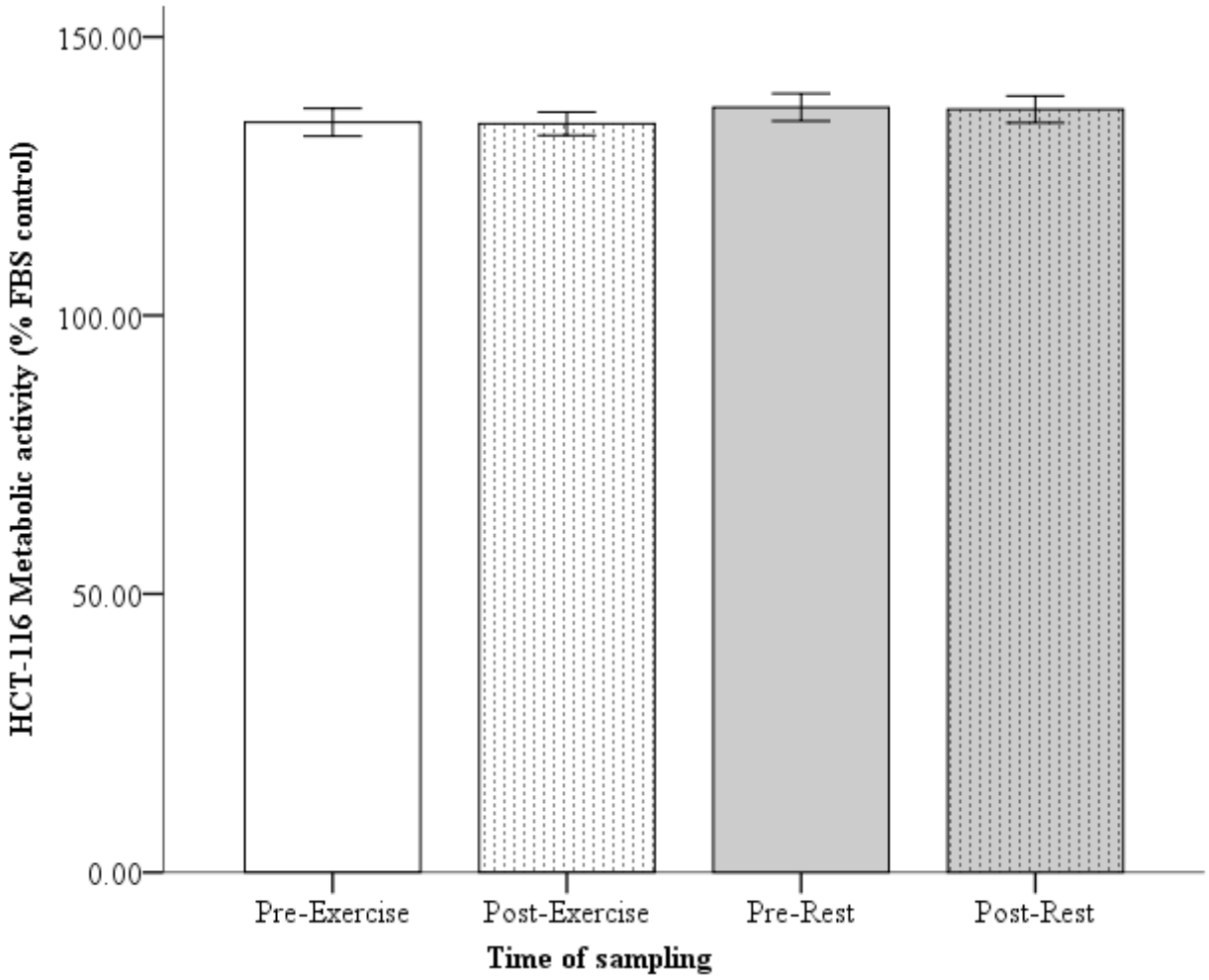

Figure 5.3. HCT-116 cell metabolic activity after 48 hours incubation in serum taken when in the post-absorptive state (Pre-Exercise and Pre-Rest, solid white and grey bars, respectively), one hour after completion of 45 min treadmill exercise at 65% VO_{2max} in the fed state (Post-Exercise, white dotted bar) and after 105 min of rest in the fed state (Post-Rest, grey dotted bar) from older men free of metabolic and cardiovascular disease. All results expressed as percentage foetal bovine serum (FBS) control. No significant differences between treatments. Error bars ± 1 SE.

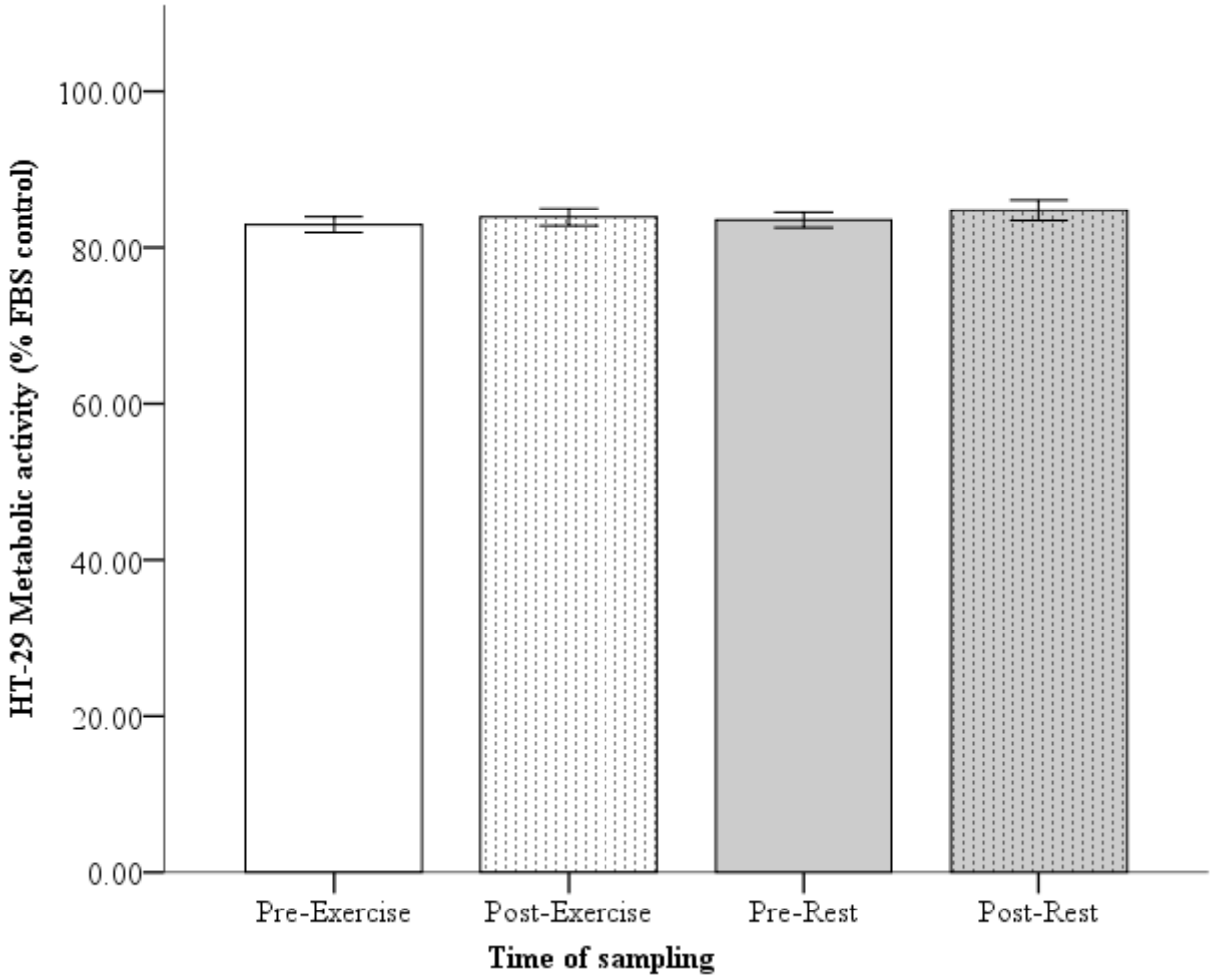

Figure 5.4. HT-29 cell metabolic activity after 96 hours incubation in serum taken when in the post-absorptive state (Pre-Exercise and Pre-Rest, solid white and grey bars, respectively), one hour after completion of 45 min treadmill exercise at 65% VO_{2max} max in the fed state (Post-Exercise, white dotted bar) and after 105 min of rest in the fed state (Post-Rest, grey dotted bar) from older men free of metabolic and cardiovascular disease. All results expressed as percentage foetal bovine serum (FBS) control. No significant differences between treatments. Error bars ± 1 SE.

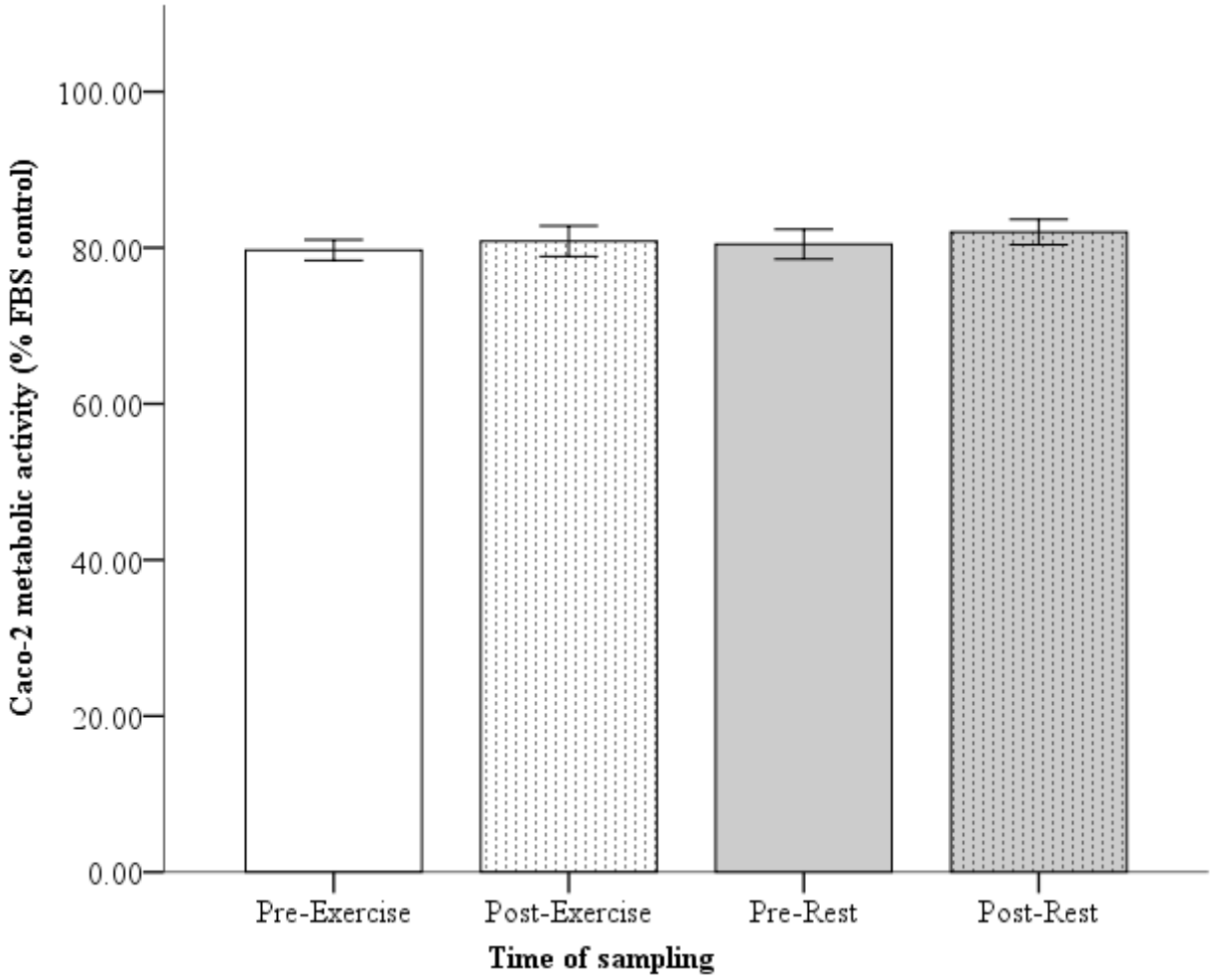

Figure 5.5. Caco-2 cell metabolic activity after 96 hours incubation in serum taken when in the post-absorptive state (Pre-Exercise and Pre-Rest, solid white and grey bars, respectively), one hour after completion of 45 min treadmill exercise at 65% VO_{2max} max in the fed state (Post-Exercise, white dotted bar) and after 105 min of rest in the fed state (Post-Rest, grey dotted bar) from older men free of metabolic and cardiovascular disease. All results expressed as percentage foetal bovine serum (FBS) control. No significant differences between treatments. Error bars ± 1 SE.

5.3.4 Associations between cell metabolic activity and PARC participants' baseline characteristics

At all timepoints, serum-stimulated growth was typically greater than FBS control, and was most marked in the HCT-116 cell line (Figure 5.7). There were no differences with respect to treatment (ALP vs UC) or time (Baseline, 6 months) on serum-stimulated growth of the Caco-2 cell line (Figure 5.6). Similarly, there were no differences with respect to treatment (ALP vs UC) or time (Baseline, 6 months) on serum-stimulated growth of the HCT-116 cell line (Figure 5.7). There was a strong, statistically significant inverse correlation ($P < 0.01$)

between CRF and baseline Caco-2 metabolic activity in the male participants (Figure 5.8). There were no associations between serum-stimulated growth and any other variables displayed in Table 5.1. The subsequent investigation into the expression of genes in the selected panel (*DNMT3b*, *E-Cad*, *Ki67*, *LGR-5*, *C-Myc, COX-2 and MUC2*) demonstrated no associations with CRF, and indeed there were negligible fold changes compared with respective control wells (Table 5.7). Mean (SD) percentage methylation of LINE-1 in sample wells was 35.6 (5). There were no associations between LINE-1 methylation and CRF.

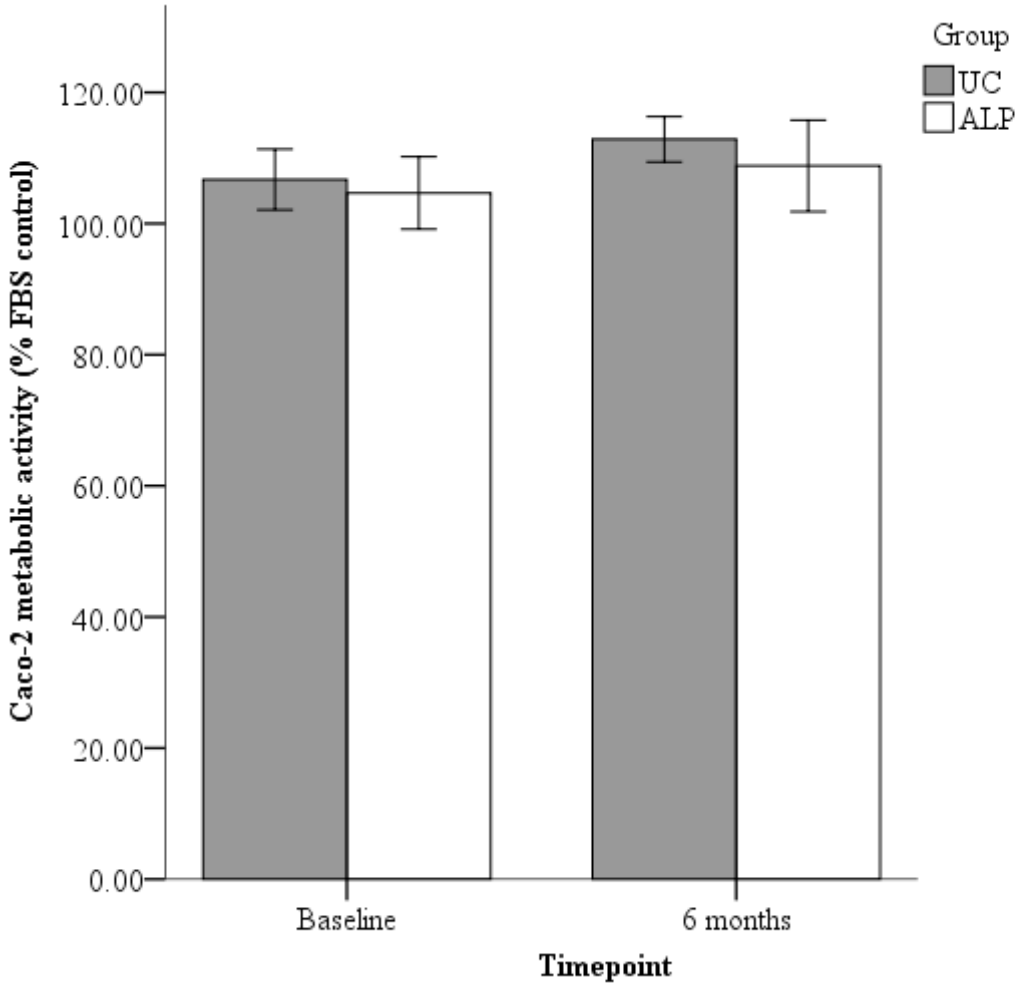

Figure 5.6 No significant difference between Usual Care (UC: filled bar) and Active Lifestyle Programme (ALP: open bar) in Caco-2 metabolic activity (expressed as % Foetal Bovine Serum control) when incubated in serum taken at baseline or after 6 months. Error bars ± 1 SE.

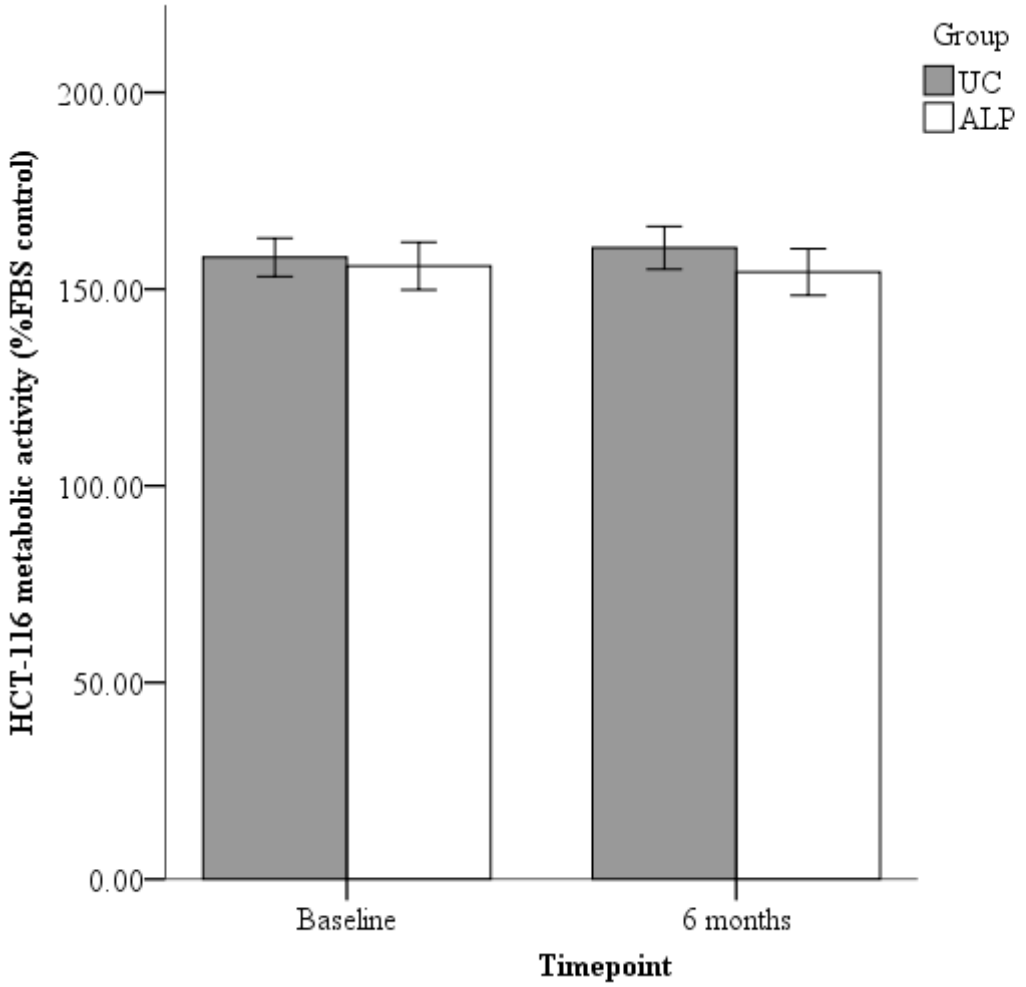

Figure 5.7 No significant difference between Usual Care (UC: filled bar) and Active Lifestyle Programme (ALP: open bar) in HCT-116 metabolic activity (expressed as % Foetal Bovine Serum control) when incubated in serum taken at baseline or after 6 months. Error bars ± 1 SE.

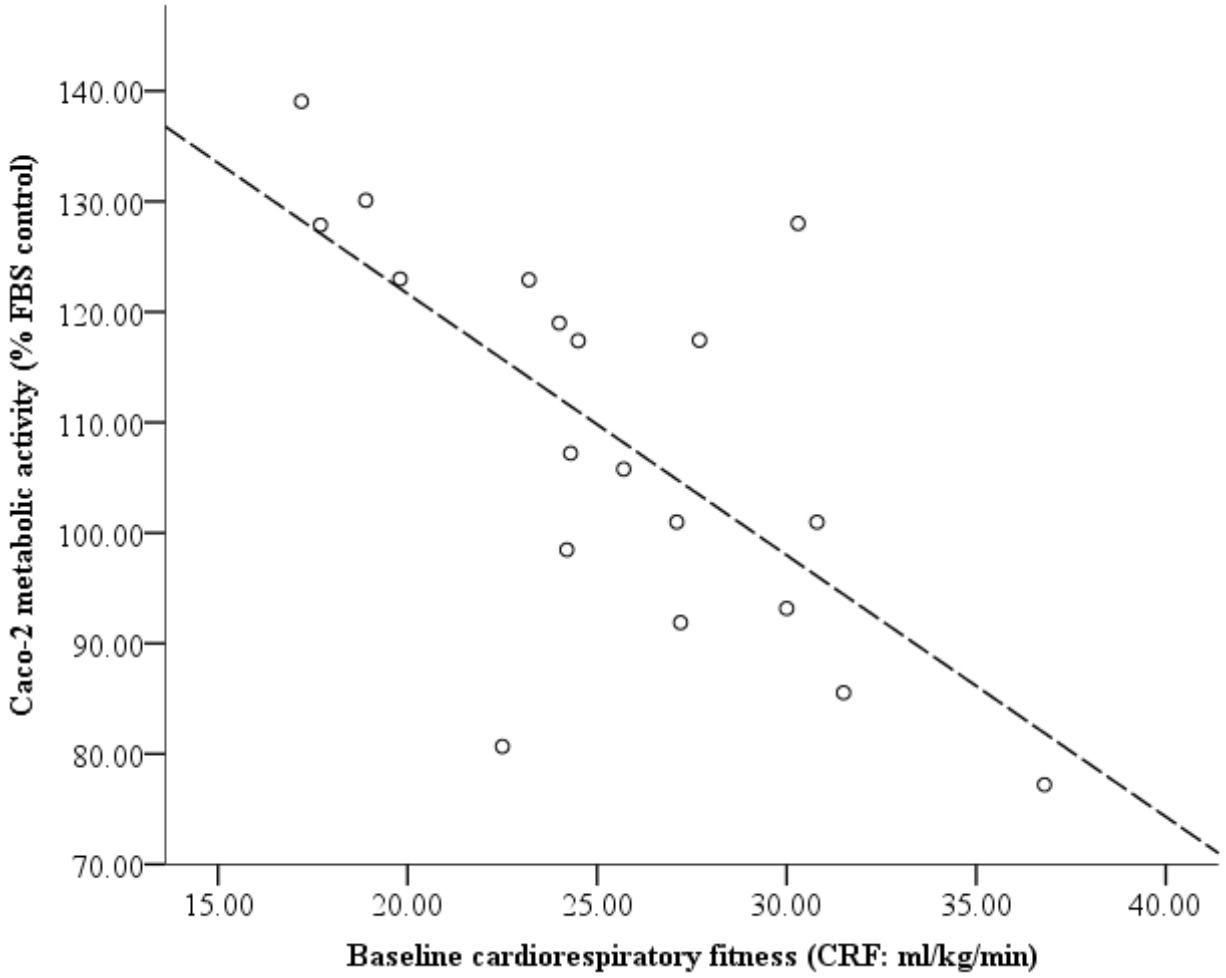

Figure 5.8 Significant (Pearson's R = - 0.672, P = 0.002) negative correlation between baseline cardiorespiratory fitness (CRF:$ml \cdot kg \cdot min^{-1}$) and Caco-2 metabolic activity when incubated in serum from male polyp patients enrolled in the PARC study (expressed as % of Foetal Bovine Serum control).

Table 5.6 Gene expression relative to internal control (*18S*) in samples after normalising for expression in untreated wells (FBS control)

Gene	Mean fold change (SD)	Range
C-Myc	1.31 (1.05)	3.25
E-Cad	2.11 (0.98)	3.28
COX-2	2.03 (0.76)	2.97
LGR5	1.81 (0.83)	2.72
Ki67	2.00 (1.04)	3.72
DNMT3b	1.88 (0.75)	2.30
MUC2	1.68 (1.80)	7.04

5.4 Discussion

5.4.1 Effect of cardiorespiratory fitness and physical activity on serum-stimulated growth of colon cancer cells

5.4.1.1 PARC participants

There was a significant correlation between Caco-2 proliferation and baseline CRF in male PARC participants. The present result is broadly in agreement with previous studies which found that LNCaP prostate cancer cell line growth using an identical incubation protocol was increased in serum from obese men compared with lean counterparts (Leung *et al.*, 2004; Ngo *et al.*, 2002). This was explained by differences in serum IGF-1, and subsequent work demonstrated that an intensive two-week diet and lifestyle intervention that reduced IGF-1 also reduced LNCaP growth (Ngo *et al.*, 2002); whereas re-introducing IGF-1 to pre-intervention concentrations restored growth (Ngo *et al.*, 2003).

The similarity of findings between LNCaP and Caco-2 in the current study, where there has been a lack of effect on other cell types, might be due to the sensitivity of both lines to IGF-1 (Leung *et al.*, 2004; Ngo *et al.*, 2003; Ngo *et al.*, 2002; Singh *et al.*, 1994). This could explain why no effects were observed in the HCT-116 cell line in the current investigation, as these are unresponsive to fluctuations in IGF-1 despite expressing its receptor (Guo *et al.*, 1992; Lahm *et al.*, 1992; Singh *et al.*, 1994). However, unlike the present study, the work of Ngo and colleagues used two well-defined groups i.e. lean vs obese (Ngo *et al.*, 2002) and the obese state is known to heighten circulating IGF-1 (Coe *et al.*, 2014; Nam *et al.*, 1997). In addition, to the author's knowledge, no study has found any associations between CRF and IGF-1. If anything, aerobic exercise training in the elderly that raises CRF induces greater production of IGF-1 in serum (Poehlman *et al.*, 1994). Therefore, it is unlikely that IGF-1 was responsible for our results.

On the contrary, there were no associations with CRF in the HCT-116 cell line. This might be because the rapid proliferative capacity of these cells, observed by the researcher and other groups (Ahmed *et al.*, 2013) did not allow for a 4-day incubation period before confluence. This could be of importance, as work into the LNCaP cell line also found that a 48 hr treatment was insufficient to induce any changes in cell growth after a high fibre diet and aerobic exercise intervention (Soliman *et al.*, 2011). Alternatively, it might be that HCT-

116 cells are resistant to any variations in serum that are associated with CRF or disease state. It is also possible that the findings are peculiar to the Caco-2 cell line. It is wild type for several mutations involved in carcinogenesis, including *KRAS*, *PI3KC*, *BRAF*, *PTEN*, *TGFBR2* and *B-Cat* (Ahmed *et al.*, 2013). Obesity and physical inactivity has been linked with risk of wild-type *B-Cat* colorectal tumours, but not when it is mutated (Morikawa *et al.*, 2013). Although this is highly speculative, these tumour subtype-specific effects might extend to the *in vitro* setting, and potentially explain the lack of consistency in the cell line work in the current and previous investigations (Ngo *et al.*, 2002).

The significant correlation between CRF and Caco-2 proliferation was not related to any differences in gene expression of *COX-2*, *DNMT3b*, *E-Cad*, *Ki67*, *LGR-5*, *C-Myc* or *MUC-2*, or global DNA methylation as measured by LINE-1. One possible explanation for a lack of findings is that these pathways are unaffected by serum factors associated with poor CRF, and the differences in growth patterns might have been related to genes in pathways other than those analysed. Additionally, post-translational modifications might have been partially responsible for the differences in cell growth. Finally, it might have been that an 'edge effect' in the cell wells confounded the expression of proliferation-associated genes. Indeed, there appeared to be pockets within the wells where cells had become confluent, which might explain why there were no associations between CRF and the genetic markers of cell proliferation, particularly *Ki67*.

5.4.1.2 ReGro participants

There were no significant effects of participants' baseline characteristics, including CRF, on any of the cell lines when incubated in serum obtained after an overnight fast (Figure 5.2). This is in contrast with the results from the PARC participants that has shown a clear, consistent inverse correlation between CRF and growth of the Caco-2 cell line (Figure 5.8). One explanation for this difference could be that the mean age of the ReGro participants in was ten years younger. In addition, 70% of the participants had a level of CRF that is considered 'excellent' or above for their age, with none attaining a score that would be considered 'poor', based on the age-adjusted criteria defined by the Aerobics Center Longitudinal Study group (Sui *et al.*, 2007). Indeed, according to self-report physical activity, accelerometry, and recent exercise behaviour recorded in the HAPAQ, most participants were also achieving an amount of moderate to vigorous PA (>150 min/wk; Table 5.1) deemed sufficient for maintenance of health (O'Donovan *et al.*, 2010). The author postulates that the

participants were of sufficient fitness to introduce a 'ceiling effect' on the ability of increased CRF to inhibit serum-stimulated cell growth. Conversely, the CRF of the male polyp patients in the PARC investigation was much lower (25.1 (5.2) vs. 48 (11.7) $ml \cdot kg \cdot min^{-1}$). Moreover, several of the PARC participants had a presence or history of obesity, and metabolic or cardiovascular disease, which might have meant that CRF was associated with differential cell growth patterns by happenstance. Certainly, obesity was associated with increased serum-induced proliferation of the LNCaP cell line compared with lean men (Ngo *et al.*, 2002). This could explain why no such findings were evident in this study, since the participants were disease-free in this respect. This raises the possibility that the exclusion criteria in this study for ReGro participants i.e. absence of a known metabolic or cardiovascular disease, obesity or previous malignancy prevented participants with sufficiently poor fitness to demonstrate an effect from taking part.

Although it might be argued that greater CRF brings with it alterations to hormone concentrations, enhanced immunity, reduced inflammation and antioxidative capacity that protected against Caco-2 cell proliferation in the present work, it is very likely that the correlation with CRF amongst the male polyp patients was confounded by health status, which is known to influence serum composition. Cardiovascular and metabolic diseases are associated with a host of changes to the serum metabolome (Du *et al.*, 2013). Within the male adenoma patients who enrolled in the PARC study were individuals with obesity, morbid obesity, diabetes, history of stroke, and cardiovascular disease (Table 5.2) Given the heterogeneity of disease states coupled with small sample size, it would be unwise to speculate on the potential growth-inhibiting mechanisms afforded by increased CRF, especially since it cannot be ruled out that the correlation was merely circumstantial. In support of this interpretation, neither increasing CRF in ALP participants, nor CRF in ReGro participants, affected Caco-2 cell growth (Figure 5.2, Figure 5.6, Figure 5.7).

5.4.2 Effect of a single bout of exercise on serum-stimulated growth of colon cancer cells in ReGro participants

A single bout of exercise did not affect cell proliferation, in contrast with previous work in the LNCaP cell line that found that moderate-intensity cycle ergometer exercise reduced proliferation by 31%, which was associated with increased serum IGFBP-3 and reduced endothelial growth factor (EGF) (Rundqvist *et al.*, 2013). This might be because the work of

Rundqvist and colleagues employed young healthy active men, retrieved serum from the femoral artery and did so two hours after the exercise bout had finished. It is possible that similar results might have been achieved in the present investigation had a serum sample been obtained two hours after cessation of the treadmill bout, as opposed to one hour. Unfortunately, Rundqvist and colleagues neglected to perform a crossover study as per the current study design (Figure 5.1), so it is not known whether the same results would have been achieved with an equivalent period of rest. It might reasonably be argued that 3 hours of rest in the postabsorptive state (whether or not the participants arrived in this state was not specified) might have induced changes to serum which could meaningfully have altered serum-stimulated growth of LNCaP cells. In the current study, a crossover design was used to negate this, and all participants exercised in the fed state. However, there were no consistent differences related to treatment (exercise vs rest) or time (pre vs post) (Figure 5.3, Figure 5.4, Figure 5.5). Based on the results of the present work, it might be suggested that the effects of moderate-intensity treadmill exercise on serum in the fed state was insufficient to significantly alter the growth of colon cancer cell lines *in vitro*.

5.4.3 Limitations

A major limitation of the current study was the failure to recruit individuals to the ReGro study who were sufficiently physically inactive, despite a prolonged recruitment campaign in several media channels, which specifically targeted highly active or inactive men. This could imply that the physically inactive, eligible men who viewed the advertisement material either felt they did not meet the inclusion criteria, or were disinterested in taking part in a study which involved a commitment to exercise. Indeed, it appears that studies into PA behaviour tend to attract individuals who are probably already sufficiently active for maintenance of health. One notable example was performed by Thompson and colleagues, who recruited 90 middle-aged men for an analysis of different PA recommendations from health authorities versus actual PA via combined accelerometry and heart rate monitoring. Of these men, 98% were achieving at least 150 min/week of ‘moderate’ PA (> 3 METS) in bouts of at least ten minutes or more, despite level of PA not being part of any inclusion criteria (Thompson *et al.*, 2009).

A further limitation is that the CC cell lines used are a limited model to use in studies of CC risk, since the cells are already cancerous and display a highly perturbed molecular phenotype (Ahmed *et al*., 2013). Whilst these models are certainly useful, and often match

the phenotype of actual cases (Ahmed *et al*., 2013) they have limited scope in the early stages of cancer development, prior to any mutations taking place. Recently, a reliable technique for maintaining populations of 'mini-guts' derived from biopsies of non-cancerous human colon has been developed, which could provide more insight into the origins of carcinogenesis (Sato *et al*., 2011). This could have served as a model of epigenetically 'vulnerable' mucosa, which might have responded to changes in serum factors that were altered as a result of any intervention. This could have included changes to gene expression and DNA methylation patterns, and may have been a more informative model than the CC lines used.

Another potential limitation was that as this investigation was a pilot study, there was a possibility that it was underpowered to detect the differences that might have occurred after a single bout of exercise. Furthermore, the contents of the serum collected were not analysed, largely due to financial constraints and the impending cessation of the period of study. It would have been of interest to screen samples for evidence of altered cytokine expression. Chronic inflammation is elevated in metabolic and cardiovascular disease states (Hotamisligil, 2006), so there might be a case for inflammatory markers being an explanatory variable for the differences in Caco-2 cell growth. This is not without precedent, as treatment with 50 ng/ml IL-6 has been shown to increase growth of HCT-116 colonies after 72 h of incubation (Foran *et al.*, 2010). However, even in heightened inflammatory states such as morbid obesity, individual cytokine concentrations of IL-6 and TNFα in serum are three orders of magnitude lower (Fontana *et al.*, 2007). The large dose of IL-6 used by Foran and colleagues is probably of greater relevance to colitis associated cancer (Terzic *et al.*, 2010). This necessitates further investigations using a metabolomics approach to understand the components of serum that could promote colon cancer cell growth.

Indeed, the timeframe for the study days in the ReGro investigation were largely based on the assumption that differences in inflammatory cytokines might be responsible for exercise-induce effects on cell growth. Another possibility from the realm of epigenetics is that miRNA expression might have also changed with exercise. Indeed, high-intensity interval training and vigorous continuous exercise in young men has been demonstrated to increase the plasma appearance of 12 miRNAs (Cui *et al*., 2016), which could plausibly have interfered with gene expression of CC cells *in vitro*, perhaps to a greater extent than cytokines. For example, miRNA-7 Some of these appear immediately after cessation of exercise (Cui *et al*., 2016) so it is possible that the timing of blood sampling missed the critical window where miRNAS could have impacted on cell growth, but this is speculative. Another issue was that despite the adenoma patients generally displaying increased growth of

cells compared with the ReGro participants, the experiments were conducted on separate plates. Therefore, batch effects might have played a role. It would be of interest to repeat such an experiment with adenoma patients and matched controls free of adenoma on the same plate to determine if this pattern was repeated. Also, a direct comparison between the groups is unwise given that it was not confirmed whether the ReGro participants were free of colon polyps.

5.4.4 Conclusion

In conclusion, cardiorespiratory fitness, minutes of MVPA per week, age, and body composition did not affect growth of HT-29, Caco-2 or HCT-116 cells when incubated in serum from an older non-obese Caucasian male population free of metabolic or cardiovascular disease. Furthermore, a single bout of moderate-intensity treadmill exercise in the fed state did not affect cell growth compared with an equivalent period of rest. Whilst this study suggests that in a disease-free state, CRF, long-term physical activity patterns nor a single bout of exercise in the fed state does not instigate any alterations in serum that consistently affect the growth of colorectal cancer cells *in vitro*, its pilot nature means that this conclusion cannot be drawn definitively. Equally, whilst CRF was inversely correlated with reduced Caco-2 proliferation in male adenoma patients, it is very possible that this association was confounded by health status.

Chapter 6
General Discussion

6.1 Summary

Study 1 has demonstrated that changes in self-report physical activity patterns over eight years in an aged Caucasian population are associated with changes in DNA methylation in peripheral blood, most notably for the genes *IL10* and *TNF*. Gender-specific associations were also observed for *SNCG*, *SERPINA5* and LINE-1. The results might have been confounded by differences in measurement techniques used at different study sites, the introduction of folic acid fortification to foods, and that PA was self-reported and might not have accurately reflected behaviour. Study 2 indicated buccal cell methylation patterns of the CC risk genes investigated in adenoma patients are not significantly affected by taking part in a 6 to 12-month randomised exercise intervention. However, owing to the paucity of participants it is likely that the study was underpowered. It also indicated that the recruitment of colon adenoma patients to a long-term randomised exercise intervention study, and that reliably obtaining follow-up colon biopsies for such an investigation presents numerous challenges. Study 3 inferred that whilst a 6-month exercise intervention in male adenoma patients does not affected serum stimulated growth of the HCT-116 and Caco-2 cell lines, cardiorespiratory fitness in this population was inversely related to growth of the Caco-2 cell line *in vitro*. This relationship was not observed in older men free of metabolic or cardiovascular disease, but the reasons for this are not clear. Furthermore, in the latter population, a single bout of moderate-intensity treadmill exercise in the fed state was not associated with differences in growth of the HT-29, HCT-116 or Caco-2 cell line. However, given the pilot nature of the investigation and study design it cannot be drawn conclusively that a single bout of exercise has no effect on serum factors that could modulate CC cell growth.

6.2 PA and DNA methylation of genes in peripheral blood

This series of investigations have lent some support to the notion that changes in physical activity patterns are associated with changes to DNA methylation patterns in the promoter regions in genes associated with risk or presence of chronic disease, particularly *IL10* and *TNF*. This was apparent in peripheral blood leukocytes from older participants in the Cardiovascular Health Study in samples spaced eight years apart. This is broadly in agreement with epidemiological studies that have suggested that greater PA in later life is associated with reduced serum markers of inflammation (Brinkley *et al.*, 2009; Cesari *et al.*, 2004; Colbert *et al.*, 2004; de Gonzalo-Calvo *et al.*, 2012b; Geffken *et al.*, 2001; Jankord and Jemiolo, 2004; King *et al.*, 2003; McFarlin *et al.*, 2006; Reuben *et al.*, 2003; Verdaet *et al.*, 2004), and that maintaining or increasing PA in later life is associated with improved inflammatory profile in peripheral blood (Hamer *et al.*, 2012), improved physical function and disease-free survival (Hamer *et al.*, 2014) and reduced cancer mortality (Zhang *et al.*, 2014). Beneficial alterations in DNA methylation status might therefore plausibly underpin some of the associations between PA and healthy ageing. This is the first study to demonstrate that changes in long-term PA patterns are associated with differential age-related DNA methylation patterns. Given the rapid turnover of peripheral blood leukocytes and that the samples were taken eight years apart, we can be confident that the changes in the methylation status of *TNF* and *IL10* reflected a genuine long-term adaptation.

Nonetheless, it cannot be ruled out that the apparently beneficial changes observed in the present study were linked to folic acid fortification. This has been observed in the Womens' Health Initiative Observational Study, where folic acid fortification resulted in increased global DNA methylation in those who were deficient; and conversely decreased methylation in those who were sufficient (Bae *et al.*, 2014). We observed that LINE-1 methylation decreased, albeit not significantly, over the course of eight years in the CHS cohort (Figure 4.2), and we cannot be confident that this was related to a change in dietary folic acid intake. In addition, in those individuals who maintained or substantially increased PAEE there was a *reversal* of age-related DNA methylation in both *TNF* and *IL10* (Figure 4.3), despite both genes following the 'expected' association with age observed in previous work (Figure 4.2) (Christensen *et al.*, 2009), which is without precedent. Given that this occurred in those who did not substantially change their PA behaviour over the course of eight years, it seems unlikely that PA was wholly responsible for these changes.

What is arguably more of note is that the apparently beneficial changes to *TNF* and *IL10* did not occur in those persons who substantially decreased PAEE by >500 kcal/wk (DEC; Figure 4.3). This might plausibly be associated with age-related functional decline, possibly as a result of disease, which has been shown to be linked with elevated markers of inflammation such as IL-6 (Reuben *et al.*, 2002). This could certainly have confounded the associations observed. However, the reduction in self-report PA was not associated with any change in self-assessment of health or objective measures of functional capacity (see Appendix Aii). On balance, this points to an independent role of reducing PA in shifting the methylome towards a more 'pro-inflammatory' state. This tallies with the available evidence from the Whitehall cohort linking a reduction in PA in a longitudinal setting being associated with increased peripheral markers of inflammation including CRP and IL-6 over ten years of follow-up (Hamer *et al.*, 2012). It could also play a role in the association between decreased fitness and an increase in all-cause, CVD and cancer mortality in the Aerobics Center Longitudinal Study cohort (Lee *et al.*, 2011b; Zhang *et al.*, 2014) although this is speculative. Based on this evidence, it might be advised that maintenance of habitual PA, and interventions to allow older persons to increase or conserve habitual PA, should be prioritised. However, it should be made clear that due to the observational nature of this investigation, we cannot confirm that the observed associations between PA and change in methylation are causally linked.

6.3 PA and DNA methylation of genes in epithelial cells

Previous studies have shown that six-month exercise programmes, and even a single bout of exercise, affects methylation status of genes involved in metabolic regulation in skeletal muscle and adipose tissue (Barres *et al.*, 2012; Nitert *et al.*, 2012; Ronn *et al.*, 2013). Nonetheless, it might be argued that the training effects on DNA methylation were merely effects of the last bout of exercise, which was performed a minimum of 48 hours previously (Nitert *et al.*, 2012; Ronn *et al.*, 2013). This is plausible, since the metabolic effects of vigorous exercise persist for several days following cessation of a bout (Goodyear and Kahn, 1998) and there could be a role for altered DNA methylation in this process. However, unpublished data from a separate group demonstrated that gene-specific DNA methylation at multiple loci in skeletal

muscle after a three-week exercise programme was unchanged 48 hours after cessation of exercise (Barres *et al.*, 2012). This suggests that the changes to the methylome observed by Nitert and colleagues was a genuine effect of prolonged training (Nitert *et al.*, 2012; Ronn *et al.*, 2013) as opposed to acute exercise, which has also been observed (Barres *et al.*, 2012). Therefore, the chronic and acute responses of the methylome to exercise training appear to be separate phenomena (Barres *et al.*, 2012).

Despite the apparently beneficial modulations in peripheral blood, adipose tissue and muscle tissue as observed by the present work and previous studies, it appears to be the case that the response of the methylome to changes in physical activity is time-dependent and tissue specific. This is evidenced by the finding that buccal cell DNA methylation was unaffected by increasing leisure-time physical activity and/or CRF in colon adenoma patients over the course of a single-blind, randomised six-month to one year exercise intervention, despite alterations being observed over the course of eight years with a change in self-report PA in the inflammation-related genes *IL10* and *TNF*. The tissue-specific effects on DNA methylation extend to short-term bouts of exercise. For example, overall CpG methylation in gene promoters is decreased in vastus lateralis muscle biopsies in the hours following isocaloric (400 kcal) moderate (40% $\dot{V}O_{2\,max}$) or high-intensity (80% $\dot{V}O_{2\,max}$) cycling exercise (Barres *et al.*, 2012), but unchanged in peripheral blood after 2 hours of treadmill running followed by a 5 km time trial in trained runners (Robson-Ansley *et al.*, 2014). Once more, this shows that the response of the methylome to exercise is tissue-specific.

Furthermore, there was no correlation between buccal or sigmoid colon DNA methylation at the regions investigated. This introduces the possibility that any beneficial epigenetic alterations induced by modestly increasing exercise behaviour might not be realised in buccal tissue or colorectal mucosa in the context of a short-term, well-controlled randomised trial, if at all. Indeed, whilst an apparent reversal of age-related methylation of inflammatory genes was observed in peripheral blood in older persons with increasing PA (Figure 3.4) it is possible that such findings do not extend to buccal or perhaps colon tissue. The paucity of participants in the PARC intervention meant that the investigation was likely underpowered, and precludes any definite conclusions.

In contrast to the findings of the current investigations, the COSTRIDE study found that methylation of 45 CpG sites in genes associated with breast cancer risk in buccal cells was negatively correlated with increasing time spent in PA after a 12 month exercise intervention (Bryan *et al.*, 2013). A potential explanation for this was that the gene panel from that investigation differed from the present study. Secondly, that study was conducted on premenopausal overweight women, and it might be that fluctuations in buccal cell DNA methylation are more malleable to changes in exercise behaviour in younger persons, although this is not known. Moreover, the study did not report whether the participants underwent any weight loss, which has previously been demonstrated to be associated with altered DNA methylation patterns in peripheral blood and adipose tissue (Milagro *et al.*, 2011), although the effects of weight loss on buccal cell methylation has not been investigated. Also, samples underwent whole-genome amplification, which is known to affect accurate detection of DNA methylation as amplification occurs in a non-linear fashion (Bundo *et al.*, 2012; Robinson *et al.*, 2010). In addition, the findings of Bryan and colleagues were only significant when the entire gene panel was included in their analysis, which they incorrectly stated as being representative of 'global methylation' (Bryan *et al.*, 2013). This introduces the strong possibility of a Type 1 error, and confounds any insight into the gene-specific effects (if any) of exercise behaviour on the buccal epigenome.

The author concedes that the tissue-specific nature of DNA methylation might be overcome with the advent of the 'DNA methylation clock', which is a ubiquitous predictor of tissue age based on robust DNA methylation marks (Horvath, 2013). As accelerated age-related DNA methylation is present in diseases that increase CC risk (Issa *et al.*, 2001) and in pre-cancerous and cancerous tissue (Ahuja *et al.*, 1998; Belshaw *et al.*, 2008; Chan *et al.*, 2002; Horvath, 2013) it would have been of particular interest in the present investigation to determine whether ALP affected the methylation status of clock genes in the buccal cell epigenome. Unfortunately, the less-than-expected yield of DNA from buccal swabs, and the explicit statement in our information materials that the research team would not sequence the entire genome of the participants, precluded this analysis.

Future work could be modelled on the BORICC study, an observational investigation which found associations between dietary components, age, gender, waist circumference, and colon DNA methylation in disease-free participants (Tapp *et al.*, 2013). The National Bowel Cancer Screening Programme provides opportunities

for research biopsy collection, providing an ideal platform for the analysis of colon DNA methylation using epigenome-wide techniques, which are becoming more cost-effective. This can be combined with increasingly accurate measures of current PA, CRF and historical physical activity levels (Besson *et al.*, 2010) from polyp patients and individuals free of polyps, and determine whether any of these indices are associated with risk of polyps and/or aberrant DNA methylation. As an adjunct outcome, such an approach might also be able to inform clinical decisions in patient care, since the least fit individuals are at the greatest risk of CC mortality (Peel *et al.*, 2009).

6.4 Potential role of CRF in mediating serum-stimulated cancer cell growth

In serum from male adenoma patients, poor CRF was associated with increased proliferation of the CC cell line, Caco-2 (Figure 5.8). This might point to a role of compromised metabolic and cardiovascular health, indicated by poor CRF, in promoting Caco-2 cell proliferation. Compromised metabolic and cardiovascular health is possibly a more credible explanation than CRF in explaining variation in Caco-2 cell proliferation. This was evidenced by the finding that in men free of known metabolic, cardiovascular, malignant or pre-malignant disease, CRF was not associated with growth of Caco-2, HT-29 and HCT-116 cells (Figure 5.2). Furthermore, improving CRF through taking part in the ALP arm of the PARC intervention was not associated with any differences in Caco-2 or HCT-116 proliferation (Figure 5.6, Figure 5.7). However, the variation might also be related to differing exercise protocols, a lack of statistical power and the timing of blood collection, so a direct comparison between the results obtained in these studies should be approached with caution.

There may be more applicability of the present results for cancer prognosis and survival. Indeed, low CRF (Peel *et al.*, 2009) and comorbidities such as diabetes have been repeatedly documented to increase risk of CC-specific mortality (Chen *et al.*, 2010; Dehal *et al.*, 2012; Huang *et al.*, 2011; Meyerhardt *et al.*, 2003; Mills *et al.*, 2013; Stein *et al.*, 2010; van de Poll-Franse *et al.*, 2012) as has obesity (Campbell *et al.*, 2012; Fedirko *et al.*, 2014; Gibson *et al.*, 2014). Importantly, obesity and PA have

been shown to differentially affect prognosis of survivors depending on CC phenotype, specifically nuclear beta-catenin and COX-2 status (Morikawa *et al.*, 2011; Yamauchi *et al.*, 2013). In support of these findings, the Caco-2 cell line is *COX-2* positive (Tsujii *et al.*, 1997); whereas HCT-116 does not express *COX-2* (Sheng *et al.*, 1998). Though speculative, if those survivors who undertook the greatest amount of PA were also most likely to have higher CRF in the study by Yamauchi and colleagues (Yamauchi *et al.*, 2013), this might in part explain why a strong correlation between proliferation and CRF was found with Caco-2 but not the HCT-116 cell line.

In a disease-free state, it might be argued that increasing fitness has no direct effect upon CC risk. Rather, it might be that a low CRF is indicative of associated pathology which increases risk of GI cancers. In support of this argument, higher CRF was associated with reduced risk of gastrointestinal cancers compared with lower CRF in older Finnish males aged 42-60 (Laukkanen *et al.*, 2010). However, the minimum cut-off point for the highest tertile of CRF was 33.2 $ml \cdot kg \cdot min^{-1}$, a score which is considered 'poor' in men of this age (Sui *et al.*, 2007). The maximum cut-off for the lowest tertile of fitness was 26.9 $ml \cdot kg \cdot min^{-1}$, a score which is considered 'very poor' according to findings from the Aerobics Center Longitudinal Study (Sui *et al.*, 2007). Furthermore, men in the lowest tertile of fitness had significantly increased BMI, waist-hip ratio, blood glucose, serum insulin, serum fatty acids, cigarette smoking in pack years, and alcohol consumption. Many of these are independently associated with increased CC risk (Watson and Collins, 2011), but puzzlingly none of these factors except CRF was found to be associated with reduced risk (Laukkanen *et al.*, 2010). This could suggest that Laukkanen and colleagues did not use an appropriate model to determine risk posed by these factors, which in isolation might not have a significant association with risk. As CRF was associated with all of these risk factors combined, it is perhaps unsurprising that CRF was significantly associated with future risk. In this respect, CRF was most likely a proxy marker for overall health.

Interestingly, a gain in age-adjusted fitness in older men is no more protective against cancer mortality that maintaining fitness (Zhang *et al.*, 2014), but a loss of fitness was associated with markedly increased risk by 26% (Zhang *et al.*, 2014). However, it should be noted that in a subgroup analysis, risk was reduced in those men who increased fitness sufficiently such that they were no longer in the lowest

quintile, compared with those who remained (Hazard Ratio 0.59, CI 0.37–0.94) (Zhang *et al.*, 2014). This finding hints at a 'threshold' of fitness below which risk of cancer mortality (as with digestive cancer mortality and all-cause mortality) is increased (Farrell *et al.*, 2007; Peel *et al.*, 2009; Sawada *et al.*, 2014; Sui *et al.*, 2007; Zhang *et al.*, 2014). Although this hypothesis requires more investigation, this might explain why poor CRF in male polyp patients was negatively associated with serum-stimulated Caco-2 cell growth (Figure 5.8), but was not associated with further increases in CRF in a disease-free population (Figure 5.2).

The results from the current series of experiments point to a possible role of cardiovascular and metabolic health (which could be indicated by low CRF) in mediating cancer cell proliferation *in vitro*. Future *in vitro* studies could focus upon recruiting a larger number of age-matched participants whose health status is clearly defined, e.g. obese vs lean, high-fitness vs low fitness and in more colorectal cancer cell lines to explore the effects of CRF on different phenotypes. Furthermore, it would be of great interest to utilise a metabolomics approach to understand the components of serum which might be associated with increased risk of cell proliferation. For example, recent metabolomics work in a cohort of 50 men and women aged 56 ± 4 years and with poor estimated CRF for their age ($\dot{V}O_{2\,max}$ = 29 ± 3 $ml \cdot kg \cdot min^{-1}$) demonstrated that higher fitness was associated with greater concentrations of phosphatidylcholines in serum, which are anti-inflammatory mediators (Wientzek *et al.*, 2014). This could present new avenues of treatment and prevention for CC, in particular of COX-2 positive tumours.

6.5 PA as a means to reduce risk of future polyps or colon cancer in adenoma patients

Some improvements in subjective colon crypt cell proliferation markers (i.e. Ki67 positive nuclei) have been observed in male polyp patients (McTiernan *et al.*, 2006), as has colon crypt expression of pro and anti-apoptotic proteins (Bax and Bcl-2) in both genders (Campbell *et al.*, 2007) after a one year PA intervention. This was achieved with a minimum of 250 min/week of MVPA participation, and no data has been published on whether this level of activity was maintained post-intervention. The same study showed no changes in intestinal PGE2 expression, which is known to be a

driver of CC risk and progression, particularly in the transition from adenoma to carcinoma (Burn *et al.*, 2011; Pugh and Thomas, 1994). Notably, this was after a well-controlled 12 month exercise programme where participants in the experimental arm engaged in an average of 330 min/week of moderate-vigorous PA (Abrahamson *et al.*, 2007). On the other hand aspirin, rapid weight loss in obese women, and dietary folate depletion reduces colorectal inflammation within months (Abrahamson *et al.*, 2007; Krishnan *et al.*, 2001; Pendyala *et al.*, 2011; Protiva *et al.*, 2011), so it is unlikely that modestly increasing PA alone in the absence of impacting these factors will have any effect on colon inflammation.

Despite the plentiful epidemiological evidence for an association between CC risk and PA, particularly over the life course (Boyle *et al.*, 2011; Wolin *et al.*, 2010; Wolin *et al.*, 2009) there is no longitudinal evidence that a change in self-report PA in older, sedentary persons affects future adenoma or CC risk. Indeed, the clinical outcomes of previous work suggest that a PA-induced reduction in polyp or cancer risk does not occur. Follow-up data from the Polyp Prevention Trial demonstrated no associations between changes in diet and or recent (i.e. three year) PA levels and risk of adenoma recurrence (Colbert *et al.*, 2002; Lanza *et al.*, 2007). Furthermore, based on a meta-analysis of studies which investigated associations between leisure-time PA and CC risk, those in the 95th percentile had a only a 14% risk reduction compared with those in the 20th percentile or below (Harriss *et al.*, 2009a). In light of this, the authors concluded that interventions aimed solely at increasing leisure-time PA are unlikely to meaningfully affect risk in older sedentary persons (Harriss *et al.*, 2009a). The conclusions of Harriss and colleagues (Harriss *et al.*, 2009a) are also supported by the available epidemiological evidence from the Cancer Prevention Study II cohort, which has suggested that an increase in frequency and intensity of PA over 10-15 years has no bearing on modifying CC risk, despite consistently higher levels of PA (>30 MET hours/week) being associated with reduced risk versus low (< 17.5 MET hours/week) (Wolin *et al.*, 2010). In agreement with this finding, total or moderate self-report PA after the age of 51 in an Australian cohort had no effect on future CC risk, but vigorous PA across the adult lifespan substantially reduced risk (Boyle *et al.*, 2011). One limitation of these findings is that they are based on self-report questionnaires, which are notoriously subject to recall bias and might not truly reflect PA behaviour, especially when compared with more objectively measured PA (Boyle *et al.*, 2015; Helmerhorst *et al.*, 2012). Another possible reason for a lack of

association is that older, sedentary persons seldom increase PA, particularly vigorous PA, into old age, which means that any associations could be missed without a sufficiently large sample size. A further explanation is that the relationships detected in these studies are present because an increase in PA in later life has a negligible effect on CC risk.

Whilst an intensive year-long MVPA programme has been successfully implemented in adenoma patients, the mean age was ~55 years, and the age range of the participants was 35 years (ages 40 to 75) (McTiernan *et al.*, 2006). Plus, those with co-morbid conditions (e.g. heart disease, diabetes, COPD, previous cancer) were excluded from the intervention (McTiernan *et al.*, 2006). It is highly likely that the group studied by McTiernan and colleagues was particularly physically capable compared with the typical UK adenoma patient, given that bowel cancer screening in the UK is currently offered to persons over 60, including the patients studied in Chapter 5 of this thesis (mean age 68.5 yrs). Furthermore, a cross-sectional study of health service users in Scotland found that in persons aged over 65, 64.9% were living with more than one co-morbidity (Barnett *et al.*, 2012). It is perhaps unrealistic to expect an older sedentary population such as this to embark on a programme of vigorous-intensity exercise. Also the available evidence strongly suggests that these populations, if anything, prefer 'moderate' activities. Certainly, in the ALP arm of the PARC intervention study, increases in self-report and accelerometer derived MVPA were predominantly achieved through doing 'moderate' activities such as brisk walking. In addition, the most preferable exercise for CC survivors considering taking part in a PA programme was walking or hiking (McGowan *et al.*, 2013), and walking was the most popular exercise amongst previously sedentary post-menopausal women in the ALPHA trial (Aparicio-Ting *et al.*, 2015).

Pertinently, the recruitment of patients for the PARC intervention fell short of the target number. Also, the low uptake of eligible participants for 'real world' exercise intervention studies aimed at reducing future disease risk is a frequent occurrence. The low (< 10%) recruitment rate of potentially eligible patients experienced by the PARC intervention study is reflective of other lifestyle interventions for chronic disease risk, including CC and polyp prevention (Jackson *et al.*, 2015; McTiernan *et al.*, 2006; Schatzkin *et al.*, 1996). One notable exception was the Alberta Physical Activity and Breast Cancer Prevention (ALPHA) trial, which recruited 19% of eligible patients contacted into a large (n = 320) RCT (Friedenreich

et al., 2010b). Due to the low uptake and long-term compliance experienced by the present and previous work (Jackson *et al.*, 2015; Vallance *et al.*, 2008), the usefulness of PA interventions alone as a public health strategy, specifically to combat CC risk in persons with polyps, is unclear. The outcomes of these studies bring into question the worth of continued large-scale studies into time-consuming, intensive exercise-only protocols in reducing polyp or CC risk in adenoma patients. Indeed, the available evidence strongly suggests that most individuals in this patient group are either unable or unwilling to dedicate such a large amount of time to change exercise behaviour in a trial setting due to a combination of a lack of knowledge and reluctance to change behaviour (Dowswell *et al.*, 2012; Tarr *et al.*, 2014). Therefore, it is highly unlikely that such an intensive approach as utilised by the present study will encounter much success in a population setting for reduction of CC or polyp risk at the present time.

Further evidence for this conclusion can be found in recent findings from the 'MOVE!' lifestyle intervention study to prevent diabetes in U.S. veterans, where from an eligible pool of 1.8 million just 1% adequately completed the programme (Jackson *et al.*, 2015). In addition, it is questionable that activity levels would be adequately maintained. This is supported by the results of the PARC intervention study where fitness gains and increased leisure-time PA after 6 months were not maintained after 12 months in ALP (see 5.5 Tables and figures: Figure 5.7, Figure 5.8, Figure 5.9, Figure 5.10). A previous larger RCT (n = 377) in breast cancer survivors demonstrated that higher levels of PA were also not maintained six months after a PA intervention (Vallance *et al.*, 2008), so there is also the possibility that fitness or PA increases might not be sustained after cessation of a trial. On the other hand, recent results from the ALPHA trial have shown that 62% of women who completed a one-year exercise intervention maintained an adequate (>150 min MVPA per week) level of activity 12 months later (Aparicio-Ting *et al.*, 2015). It should be noted that in that study 58% of the *control* group also achieved this target, the activity measurement was in the form of a self-report questionnaire (Friedenreich *et al.*, 2006) which was poorly correlated with accelerometer-derived PA (Friedenreich *et al.*, 2006; Helmerhorst *et al.*, 2012), and > 20% of the participants did not complete the questionnaire. More objective follow-up PA data is lacking from the extant literature.

6.6 From epidemiology to physiology: the future role of exercise science research in CC prevention

This series of investigations has focused principally on the role of PA in regulating the methylome and its relevance to colon cancer risk. However, this could ultimately be a moot point, since there is little likelihood that a sedentary older adenoma patient at risk of CC will increase PA sufficiently to induce a meaningful, clinically important change to future risk (Dowswell *et al.*, 2012; Tarr *et al.*, 2014). In the author's view, it is therefore of interest to pursue alternative avenues for reduction of CC-specific risk in high-risk populations. Examples include prescription of anti-inflammatory medications and Vitamin D and calcium supplements, where recruitment rates are high (> 30% eligible participants contacted) as is long-term compliance with medication (> 85% for > 2 years). Plus, the clinical trial data for effective reduction of future advanced adenoma or cancer risk is convincing (Baron *et al.*, 2003; Burn *et al.*, 2011; Flossmann *et al.*, 2007; Grau *et al.*, 2003; Sandler *et al.*, 2003). This is not to diminish the role of modestly increasing PA, for example walking behaviour, in improving other outcomes in chronic age-related disease and all-cause mortality (Blair *et al.*, 1996; Stofan *et al.*, 1998; Sui *et al.*, 2007; Swift *et al.*, 2013). It is also evident that rapid weight loss might induce similar benefits in obese persons (Pendyala *et al.*, 2011), although more clinical trial evidence is necessary to determine if this is the case. This is currently being pursued in the BeWEL study (Anderson *et al.*, 2014), and it is to be verified whether the modest weight loss (3.5 kg, or 3.9% of body weight) achieved in that study affects future polyp risk.

There is a need for a more mechanistic, targeted approach toward CC prevention via known biomarkers of risk, particularly with respect to exercise. To date, the PARC intervention included (Chapter 4), exercise prescription has been based on epidemiological associations over the life course (McTiernan *et al.*, 2006). This approach is not suitable for tackling CC risk in older, previously sedentary persons, for two reasons. First, there is likely to be considerable resistance to behaviour change (Dowswell *et al.*, 2012; Tarr *et al.*, 2014). Second, the effects of such prescriptions on risk biomarkers purportedly linked with PA are highly inconsistent, including CRF (Bouchard *et al.*, 2011; McTiernan *et al.*, 2006), insulin sensitivity (Boule *et al.*, 2005) chronic inflammation (Woods *et al.*, 2012) and

oxidative stress (Campbell *et al.*, 2010b), and might render untailored interventions ineffective. Future intervention studies should be based on the work of Pendyala and colleagues, who showed that a standardised weight loss protocol resulted in reduced colorectal inflammation, one of the most important risk markers for CC (Pendyala *et al.*, 2011). This was based on the evidence that obesity induces chronic inflammation (Fontana *et al.*, 2007), ergo weight (fat) loss combats gut inflammation, which was confirmed after the participants underwent rapid fat loss (Pendyala *et al.*, 2011).

Perhaps the most plausible risk biomarker that is linked with risk of CC and a lack of PA is hyperinsulinaemia (Giovannucci, 2007). There are convincing and consistent associations between metabolic syndrome and CC and adenoma risk, notably in relation to hyperinsulinaemia and particularly in men (Campbell *et al.*, 2010a; Chen *et al.*, 2013; Comstock *et al.*, 2014b; Giovannucci, 2007; Jenab *et al.*, 2007; Ollberding *et al.*, 2012; Ortiz *et al.*, 2012; Vidal *et al.*, 2012). There is strong clinical trial evidence that this is amenable to exercise training (Knowler *et al.*, 2002) and accumulating evidence that low-volume, high-intensity interval training (HIIT) can improve insulin sensitivity in a matter of weeks, in both non-clinical and clinical populations with metabolic disease (Babraj *et al.*, 2009; Gibala and McGee, 2008; Gillen *et al.*, 2014; Little *et al.*, 2011; Trapp *et al.*, 2008). The author tentatively suggests that some of the findings observed by McTiernan and colleagues with respect to crypt proliferation (Campbell *et al.*, 2007; McTiernan *et al.*, 2006), whilst they did not measure metabolic parameters such as fasting blood glucose and serum insulin, could have plausibly occurred due to improved insulin sensitivity that can accompany exercise training in previously sedentary individuals (Goodyear and Kahn, 1998). This could have impacted upon crypt cell growth patterns, and indeed insulin administration has been consistently demonstrated to invoke proliferation and tumorigenesis in human cell lines and rodent models (Corpet *et al.*, 1997; Hvid *et al.*, 2013; Sun and Jin, 2008; Tran *et al.*, 2006).

The author suggests that future investigations into PA and adenoma or CC risk using exercise as an intervention could utilise a HIIT approach in younger individuals with metabolic syndrome, given that this method appears to show the greatest effect in improving metabolic health, which is strongly linked with CC risk (Chen *et al.*, 2013; Giovannucci, 2007) and mortality (Wolpin *et al.*, 2009) and presents far fewer time constraints for study participants, and, indeed, the general population. A prior investigation in men with metabolic syndrome demonstrated beneficially altered DNA

methylation in muscle and adipose tissue after high-intensity exercise training (Nitert *et al.*, 2012; Ronn *et al.*, 2013). Colon biopsies could be obtained before and after a similar intervention and markers of risk, including crypt proliferation and CGI methylation, can then be analysed. Given the increase in incidence of metabolic syndrome in the UK, this could prove a fruitful direction of investigation.

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i. Physical activity report form for Cardiovascular Health Study
ii. Summary of ANOVA models and descriptions of Cardiovascular Health Study participants by PAEE group

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ii. Consent form for contact details
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Appendices

Appendix A

i. Physical activity report form for Cardiovascular Health Study
ii. Summary of ANOVA models and descriptions of Cardiovascular Health Study participants by PAEE group

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OMB #0925-0334 Exp: 3/95

Cardiovascular Health Study

THIRD FOLLOW-UP PHYSICAL ACTIVITY

Name
ID#: IDNO

1A I am going to read a list of activities. Please tell me which activities you have done in the past two weeks:

If the activity was done during the past two weeks, ask the following questions:

	no	yes	refuse	B How often have you *name of activity* in the last two weeks?	C What is the average amount of time that you spent per session?	D How many months per year do you *name of activity*?
walking for exercise?	WALK04 0	1	9	WALKFR04 # times:	WALKTM04 #hours: #min:	WALKMO04 # months:
Moderately strenuous household chores, Example, scrubbing or vacuuming?	CHOR04 0	1	9	CHORFR04 # times:	CHORTM04 #hours: #min:	CHORMO04 # months:
mowing the lawn?	MOW04 0	1	9	MOWFR04 # times:	MOWTM04 #hours: #min:	MOWMO04 # months:
raking the lawn?	RAKE04 0	1	9	RAKEFR04 # times:	RAKETM04 #hours: #min:	RAKEMO04 # months:
gardening?	GRDN04 0	1	9	GRDNFR04 # times:	GRDNTM04 #hours: #min:	GRDNMO04 # months:
hiking?	HIKE04 0	1	9	HIKEFR04 # times:	HIKETM04 #hours: #min:	HIKEMO04 # months:
jogging?	JOG04 0	1	9	JOGFR04 # times:	JOGTM04 #hours: #min:	JOGMO04 # months:
biking?	BIKE04 0	1	9	BIKEFR04 # times:	BIKETM04 #hours: #min:	BIKEMO04 # months:
exercise cycle?	EXCY04 0	1	9	EXCYFR04 # times:	EXCYTM04 #hours: #min:	EXCYMO04 # months:
dancing?	DANC04 0	1	9	DANCFR04 # times:	DANCTM04 #hours: #min:	DANCMO04 # months:
aerobics/aerobic dance?	AERO04 0	1	9	AEROFR04 # times:	AEROTM04 #hours: #min:	AEROMO04 # months:
bowling?	BOWL04 0	1	9	BOWLFR04 # times:	BOWLTM04 #hours: #min:	BOWLMO04 # months:
golf?	GOLF04 0	1	9	GOLFFR04 # times:	GOLFTM04 #hours: #min:	GOLFMO04 # months:
calisthenics/ general exercise?	EXER04 0	1	9	EXERFR04 # times:	EXERTM04 #hours: #min:	EXERMO04 # months:
swimming?	SWIM04 0	1	9	SWIMFR04 # times:	SWIMTM04 #hours: #min:	SWIMMO04 # months:

Have you done any other physical activities during the past two weeks in addition to those listed above?

(*If yes:*) Please tell me what they were:

other: NAME104	OTH104 0	1	9	OTH1FR04 # times:	OTH1TM04 #hours: #min:	OTH1MO04 # months:
other: NAME204	OTH204 0	1	9	OTH2FR04 # times:	OTH2TM04 #hours: #min:	OTH2MO04 # months:

Name
ID#:

2 Think about the walking you do outside your home. During the last week, about how many city blocks or miles did you walk? BLOCKØ4

blocks 1
or
miles 2
BLMILEØ4

6 In a usual 24 hour period, how many hours do you spend seated or lying down? Include all time spent sleeping, resting, and lying down, and also include all time spent watching TV, eating, reading, and any other time sitting down.

# of hours SEATØ4

3 Look at this card and tell me, when you walk outside your home, what is your usual pace?

PACEØ4 card #9

no walking at all	1
casual strolling (greater than 0 to 2.0 mph)	2
average or normal (greater than 2.0 to 3.0 mph)	3
fairly briskly (greater than 3.0 to 4.0 mph)	4
brisk or striding (greater than 4 mph)	5
	9

Think about how often you use stairs. Include stairs outside your home, and stairs at other ...e last week, about how many flights of ...airs did you climb up? (Ten steps = one flight of stairs)

# of flights of stairs FLIGHTØ4

5 Look at this card and tell me, how would you describe your level of activity since we saw you last year?

ACTLEVØ4 card #10

a lot less active	1
a little less active	2
about as active	3
a little more active	4
a lot more active	5
unknown	9

Interview					First Edit		Second Edit			
INTIRØ4										
interviewer	month	day	year		hour : min		month	day	hour : min	

Supplementary material: Analysis of variance in change in methylation with change in physical activity, ΔPAEE

1. Summary of ANOVA models: 390 Caucasian participants

Study group of 390 Caucasian participants comprising 253 women and 137 men with the following mean (SD) characteristics at baseline: women were aged 70.7 (4.0) years, with waist circumference 91.2 (13.2) and leisure time PAEE 1313 (1355) kcal/wk; men were aged 71.4 (4.3) years with waist circumference 97.0 (10.0) cm and PAEE 2226 (2209) kcal/wk

1.1 TNF

No power transformation

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.244	10	0.0244	1.97	0.0352
Sex	0.009	1	0.0091	0.74	0.3913
ΔPAEE	0.184	2	0.0922	7.45	0.0007
Age	0.050	1	0.0498	4.02	0.0456
ΔWaist	0.000	1	0.0003	0.02	0.8846
Sex*ΔWaist	0.058	1	0.0581	4.69	0.0310
Error	4.620	373	0.0124		
Total	5.220	389			

ANOVA table

1.2 IL10

No power transformation

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.480	10	0.0480	3.57	0.0002
ΔPAEE	0.083	2	0.0413	3.08	0.0471
Error	5.061	377	0.0134		
Total	5.644	389			

ANOVA table

1.3 SERPINA5

No power transformation

Study group of 352 individuals (230 women and 122 men)

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.089	10	0.0089	1.66	0.0887
Sex	0.003	1	0.0031	0.59	0.4447
ΔPAEE	0.046	2	0.0228	4.27	0.0148
Sex*ΔPAEE	0.050	2	0.0248	4.63	0.0104
Error	1.798	336	0.0054		
Total	1.971	351			

ANOVA table

1.4 SNCG

Transformation: $(y + 0.65)^{1.4}$

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.537	10	0.0537	2.17	0.0190
Sex	0.011	1	0.0112	0.45	0.5012
ΔPAEE	0.001	2	0.0004	0.01	0.9853
Sex*ΔPAEE	0.206	2	0.1031	4.16	0.0163
Error	9.258	374	0.0248		
Total	9.946	389			

ANOVA table

1.5 LINE1

Transformation: $(y+0.25)^{1.5}$

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.008	10	0.00082	1.64	0.0941
Sex	0.000	1	0.00038	0.75	0.3862
ΔPAEE	0.001	2	0.00069	1.38	0.2520
Sex* ΔPAEE	0.004	2	0.00176	3.53	0.0302
Error	0.187	374	0.00050		
Total	0.201	389			

ANOVA table

2. Summary of ANOVA models: 390 white and 31 non-white participants

Study group of 31 non-white participants comprising 19 women and 11 men with the following mean (SD) characteristics at baseline: women were aged 70.3(4.1) years, with waist circumference 93.0 (12.5) cm and leisure time PAEE 1118 (1391) kcal/wk; men were aged 70.8 (4.7) years with waist circumference 99.2 (8.9) cm and PAEE 1277 (1290) kcal/wk. The 31 individuals belonged to the following racial groups: 9 White Hispanics, 26 Black Americans; 1 Native American / Alaskan Native, 1 Asian / Pacific Islander, 2 Other, and 1 Hispanic Other. The ANOVA tables correspond to model terms derived from the sub-group of 390 whites and applied to the combined white and non-white participants.

2.1 TNF

No power transformation

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.253	11	0.0230	1.77	0.0570
Sex	0.012	1	0.0116	0.89	0.3451
ΔPAEE	0.152	2	0.0758	5.85	0.0031
Age	0.028	1	0.0282	2.17	0.1411
ΔWaist	0.000	1	0.0000	0.00	0.9641
Sex*ΔWaist	0.062	1	0.0616	4.75	0.0299
Error	5.225	403	0.0130		
Total	5.770	420			

ANOVA table

2.2 IL10

No power transformation

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.484	11	0.0440	3.22	0.0003
ΔPAEE	0.081	2	0.0404	2.96	0.0532
Error	5.566	407	0.0137		
Total	6.149	420			

ANOVA table

2.3 SERPINA5

No power transformation

Study group of 382 individuals (249 women and 133 men)

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.091	11	0.0082	1.47	0.1413
Sex	0.004	1	0.0042	0.75	0.3884
ΔPAEE	0.054	2	0.0271	4.83	0.0085
Sex*ΔPAEE	0.050	2	0.0248	4.42	0.0127
Error	2.049	365	0.0056		
Total	2.241	381			

ANOVA table

2.4 SNCG

Transformation: $(y + 0.65)^{1.4}$

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.644	11	0.0585	2.22	0.0128
Sex	0.032	1	0.0319	1.21	0.2715
ΔPAEE	0.006	2	0.0028	0.11	0.8995
Sex*ΔPAEE	0.146	2	0.0728	2.76	0.0643
Error	10.643	404	0.0263		
Total	11.420	420			

ANOVA table

2.5 LINE1

Transformation: $(y+0.25)^{1.5}$

Source	*Sum Sq.*	*d.f.*	*Mean Sq.*	*F*	*Prob>F*
Plate	0.009	11	0.00080	1.54	0.1156
Sex	0.001	1	0.00070	1.34	0.2481
ΔPAEE	0.001	2	0.00053	1.01	0.3634
Sex* ΔPAEE	0.003	2	0.00132	2.53	0.0809
Error	0.210	404	0.00052		
Total	0.224	420			

ANOVA table

3. Contingency Tables for change in DNA methylation (%) between baseline and year 8

Leukocyte DNA sequence	ΔPAEE group	390 Caucasians: 253 women and 137 men										31 non-whites: 20 women and 11 men					
		Women					Men					Women			Men		
		Down	Up	Mean	U95%CI	L95%CI	Down	Up	Mean	U95%CI	L95%CI	Down	Up	Mean	Down	Up	Mean
ABCA1	INC	20	19	-.38	3.0	-3.7	14	9	-4.79	0.5	-10.1	1	1	-.68	1	0	-1.23
	NOC	61	61	-.85	1.4	-3.1	23	26	-1.25	2.7	-5.2	7	5	-.18	2	5	1.51
	DEC	44	48	.72	3.5	-2.0	31	34	1.30	4.5	-1.9	1	5	7.08	2	1	5.80
IL10	INC	26	13	-6.19	-2.0	-10.4	19	4	-7.94	-3.3	-12.6	1	1	-3.18	1	0	-6.14
	NOC	87	35	-5.69	-3.7	-7.7	38	11	-6.09	-3.5	-8.6	9	3	-3.63	5	2	-5.16
	DEC	57	35	-3.49	-0.6	-6.4	39	26	-1.95	1.1	-5.0	5	1	-4.81	2	1	-.14
NOD2	INC	28	11	-2.55	-1.3	-3.8	16	7	-1.76	-0.3	-3.3	1	1	1.23	1	0	-2.65
	NOC	91	31	-2.44	-1.5	-3.3	38	11	-2.65	-1.4	-3.9	10	2	-2.34	5	2	-2.25
	DEC	67	25	-2.26	-1.4	-3.1	45	20	-1.57	-0.7	-2.4	6	0	-5.36	3	0	-1.49
SERPINA5	INC	23	16	-1.19	1.7	-4.1	17	6	-4.90	-1.5	-8.3	1	1	-2.21	1	0	-11.22
	NOC	66	56	-1.12	0.5	-2.8	17	32	1.71	3.7	-0.3	9	3	-.63	3	4	.23
	DEC	42	50	-.22	1.1	-1.5	32	33	.30	1.8	-1.2	1	5	3.77	0	3	13.43
SNCG	INC	18	21	-.69	3.4	-4.8	17	6	-6.47	-0.7	-12.3	0	2	8.81	1	0	-10.74
	NOC	70	52	-4.05	-1.4	-6.7	27	22	-.33	3.9	-4.6	4	8	9.38	3	4	-2.91
	DEC	50	42	-1.99	1.2	-5.2	42	23	-3.79	-0.9	-6.7	3	3	-2.79	2	1	-3.11
SOD3	INC	16	23	.00	0.9	-0.9	8	15	.07	1.5	-1.4	0	2	2.51	0	1	.60
	NOC	57	65	-.56	0.4	-1.5	20	29	.23	0.9	-0.5	8	4	.61	2	5	1.27
	DEC	43	49	-.48	0.5	-1.5	30	35	-.90	1.2	-3.0	1	5	9.14	0	3	3.16
TNF	INC	17	22	3.06	6.9	-0.8	7	16	5.02	10.7	-0.7	1	1	1.02	0	1	3.20
	NOC	48	74	3.82	5.8	1.8	17	32	5.91	8.8	3.0	6	6	-1.07	2	5	5.89
	DEC	44	48	-.68	2.0	-3.4	33	32	-.09	2.2	-2.4	1	5	8.08	1	2	-1.02
UGT1A1	INC	17	22	-.25	0.5	-1.0	12	11	.20	1.1	-0.7	1	1	-.72	0	1	.26
	NOC	59	63	.09	0.4	-0.3	23	26	-.25	0.5	-1.0	8	4	.29	4	3	-.25
	DEC	48	44	-.16	0.3	-0.6	30	35	-.83	-0.1	-1.6	3	3	.52	1	2	-.08
LINE1	INC	17	22	.94	2.2	-0.3	13	10	-.87	1.8	-3.5	0	2	.87	1	0	-1.05
	NOC	56	66	.24	0.7	-0.2	27	22	-.62	0.2	-1.5	5	7	.01	3	4	.15
	DEC	58	34	-.76	-0.2	-1.4	36	29	.00	0.6	-0.6	2	4	2.18	2	1	-1.45

Numbers of subjects whose leukocyte DNA methylation increased or decreased between baseline and year 8, stratified by racial group, gender and ΔPAEE: DEC, decrease of more than 500 kcal/wk; NOC, no overall change; INC, increase of more than 500 kcal/wk. These data refer to 421 subjects comprising 273 women and 148 men. 95% CI not provided for non-whites owing to small numbers of individuals in each PAEE change category.

Leukocyte DNA sequence	ΔPAEE group	352 whites: 230 women and 122 men										30 non-whites: 19 women and 11 men					
		Women			95% CI		Men			95% CI		Women			Men		
		Down	Up	Mean	Upper	Lower	Down	Up	Mean	Upper	Lower	Down	Up	Mean	Down	Up	Mean
SERPINA5	INC	22	15	-1.26	1.8	-4.3	17	3	-5.67	-1.9	-9.5	1	0	-4.68	1	0	-11.22
	NOC	63	51	-.49	0.9	-1.9	15	30	1.85	4.0	-0.4	9	3	-.63	3	4	.23
	DEC	37	42	-.26	1.3	-1.8	29	28	.33	2.0	-1.4	1	5	3.77	0	3	13.43

Numbers of subjects whose leukocyte *SERPINA5* methylation increased or decreased between baseline and year 8, and with year 8 methylation proportions greater than 20%, stratified by racial group, gender and ΔPAEE: DEC, decrease of more than 500 kcal/wk; NOC, no overall change; INC, increase of more than 500 kcal/wk. These data refer to 382 subjects comprising 249 women and 133 men. 95% CI not provided for non-whites owing to small numbers of individuals in each PAEE change category.

ΔPAEE group	Change in self-report health status (Baseline – year 8)			Any cardio-vascular disease		Have metabolic syndrome		Have Type 2 diabetes		Have pulmonary disease		Ever diagnosed with cancer		Have cancer		Any arthritis (wrist, hips, knee)		Any skeletal breaks/ fractures		Time taken to walk 15 feet (s)	Number of chair stands in 30 s
	Worse	Same	Better	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Mean (SD)	Mean (SD)
INC	12.9	46.8	40.3	51.6	48.4	45.2	54.8	8.1	91.9	16.1	83.9	14.5	85.5	1.6	98.4	51.6	48.4	6.5	93.5	5.38 (2.18)	14.49 (3.64)
NOC	12.9	32.9	54.1	56.1	43.9	52.6	47.4	17	83	12.3	87.7	12.9	87.1	5.3	94.7	46.2	53.8	3.5	96.5	5.65 (2.14)	15.48 (4.76)
DEC	10.3	46.2	43.6	56.7	43.3	40.1	59.9	14	86	8.9	91.1	13.4	86.6	5.7	94.3	49.7	50.3	5.1	94.9	5.54 (2.70)	15.32 (4.92)

Summary descriptions of general health status of white participants with available PAEE data (n = 390) by ΔPAEE Group (%). Includes change in subjective appraisal of health (rated as ‘Excellent’, ‘Very good’, ‘Good’, ‘Fair’ or ‘Poor’) from Baseline to year 8, and presence or history of cardiovascular, metabolic, malignant, pulmonary or musculoskeletal ailment by year 8. Also includes measures of functional capacity, presented as mean (SD).

Appendix Bi

University of East Anglia

Norfolk and Norwich University Hospitals NHS Foundation Trust

Bowel Cancer

Is the second most common cancer diagnosed in the UK…

Our Research

Is aimed at finding ways to reduce your risk…

The Active Lifestyle Programme

By taking part in a free 1-year physical activity programme with lifestyle workshops and free fitness tests…

If you are taking part in the *NHS National Bowel Cancer Screening Programme* then you may be eligible for our study. If you are interested, then please contact a member of the research team, or ask the screening nurse at NNUH about the 'Active Lifestyle Programme'.

Thank you.

Miss Kelly Semper:
To be determined
k.semper@uea.ac.uk

Mr Barnabas Shaw:
To be determined
b.shaw@uea.ac.uk

Miss Liane Thomas
To be determined
liane.thomas@uea.ac.uk

Alternatively, please call the
University of East Anglia Research Office: 01603 593098

Version 2 13/04/2012

Appendix Bii

The Effects of a 12 month Active Lifestyle Programme on patients diagnosed as being at increased risk of developing further polyps as determined by colonoscopy.

Dear

Re: Invitation to participate in an Active Lifestyle Study: What effect does physical activity have on exercise participation and bowel health?

I am delighted to let you know about a new research study for people who are undergoing a colonoscopy. Consultants from the Gastroenterology Unit and health researchers from the University of East Anglia are working together to investigate how a structured exercise and educational programme affects exercise participation and bowel health.

This study will be asking some eligible participants to undertake a programme of exercise and attend exercise-related educational workshops. We hope to gain a better understanding of how active lifestyle programmes like this impact upon physiological markers of bowel health and health behaviours.

Please find enclosed a patient information sheet, which describes the study in more detail and answers the most frequently asked questions.

If you are interested in the study please bring the tear off slip in the bottom of this letter with you to your appointment. A member of the study team (Mr Barnabas Shaw, Miss Kelly Semper or Mrs Liane Lewis) will be present at the NNUH on the day of your colonoscopy to talk to you about the study should you be interested. This is entirely voluntarily and your treatment will not be effected should you decide not to meet one of the researchers.

Yours sincerely,

Mr. James Hernon
Consultant Surgeon, Norfolk and Norwich University Hospital.

……

The Effects of a 12 month Active Lifestyle Programme on patients diagnosed as being at increased risk for developing further polyps as determined by colonoscopy.

☐ Yes. I am interested in the above named study.

☐ No, I am not interested in taking part in the study

Name: _____

Telephone Number: _____

Version 1 07/01/2013

University of East Anglia

Norfolk and Norwich University Hospitals NHS Foundation Trust

Consent Form – Initial Biopsies

The Effects of a 12 month Active Lifestyle Programme on patients diagnosed as being at increased risk of developing further polyps as determined by colonoscopy.

Thank you for your initial interest to take part in our study. You will soon be undergoing your colonoscopy at the Norwich and Norfolk University Hospital.

Depending on the outcome of your colonoscopy, you may be eligible to take part in this research study. With your consent the surgeon will take five further small biopsy samples from your colon for research purposes. These samples will be anonymised and transferred to the Institute of Food Research by the research team.

With your consent, we might store any leftover samples at a NHS approved tissue bank for future studies of bowel health after the study has finished for a period of 5 years. You can still take part in this study even if you do not consent to this tissue being stored, in which case the tissue will be destroyed after the study.

As the additional research biopsies to be taken are small in size (2-3mm across), there is only a small risk that you will experience any adverse health effects from this procedure. Simple biopsies such as this carry a very small risk of perforation (tearing) of the bowel, and this occurs in less than 1 in 1000 cases.

Please initial box

I confirm that I have read and understood the above information ☐

I consent for further tissue samples to be extracted during my colonoscopy for analysis purposes. ☐

I consent for these samples to be transferred to the Institute of Food Research by the research team. ☐

I consent for any leftover tissue extracted to be anonymously stored at a NHS approved tissue bank for future studies of bowel health after the study has finished for a period of five (5) years. ☐

Name of Participant	Date	Signature
Name of person taking informed consent	Date	Signature

Version 1 07/01/2013

Appendix Biv

University of East Anglia

Norfolk and Norwich University Hospitals NHS Foundation Trust

Consent Form – Initial Biopsies

The Effects of a 12 month Active Lifestyle Programme on patients diagnosed as being at increased risk of developing further polyps as determined by colonoscopy.

You will soon be undergoing your follow-up colonoscopy at the Norwich and Norfolk University Hospital.

With your consent the surgeon will take five further small biopsy samples from your colon for research purposes. These samples will be anonymised and transferred to the Institute of Food Research by the research team.

With your consent, we might store any leftover samples at a NHS approved tissue bank for future studies of bowel health after the study has finished for a period of 5 years. You can still take part in this study even if you do not consent to this tissue being stored, in which case the tissue will be destroyed after the study.

As the additional research biopsies to be taken are small in size (2-3mm across), there is only a small risk that you will experience any adverse health effects from this procedure. Simple biopsies such as this carry a very small risk of perforation (tearing) of the bowel, and this occurs in less than 1 in 1000 cases.

Please initial box

I confirm that I have read and understood the above information ☐

I consent for further tissue samples to be extracted during my colonoscopy for analysis purposes. ☐

I consent for these samples to be transferred to the Institute of Food Research by the research team. ☐

I consent for any leftover tissue extracted to be anonymously stored at a NHS approved tissue bank for future studies of bowel health after the study has finished for a period of five (5) years. ☐

Name of Participant	Date	Signature
Name of person taking informed consent	Date	Signature

Version 1 16/01/13

Norfolk and Norwich University Hospitals NHS Foundation Trust

University of East Anglia

Dear

Re: Invitation to participate in an Active Lifestyle Study: What effect does physical activity have on exercise participation and bowel health?

I am delighted to let you know about a new research study for people who have undergone a screening colonoscopy. Consultants from the Gastroenterology Unit and health researchers from the University of East Anglia are working together to investigate how a structured exercise and educational programme affects exercise participation and bowel health.

This study will be asking some eligible participants to undertake a programme of exercise and attend exercise-related educational workshops. We hope to gain a better understanding of how active lifestyle programmes like this impact upon physiological markers of bowel health and health behaviours.

Please find enclosed a patient information sheet, which describes the study in more detail and answers the most frequently asked questions.

If you are interested in participating in this study, please contact a member of the research team, and we can arrange a formal consultation at the University of East Anglia:

Mr Barnabas Shaw BSc. MSc.
Email: B.Shaw@uea.ac.uk
Tel: 07933090196

Mrs Liane Lewis BSc.
Email: Liane.Thomas@uea.ac.uk
Tel: 07933090197

Miss Kelly Semper BSc.
Email: K.Semper@uea.ac.uk

Yours sincerely,

Mr. James Hernon

Consultant Surgeon, Norfolk and Norwich University Hospital.

Appendix Bvi

PATIENT CONSENT FORM

The University of East Anglia

The Effects of a 12 month Active Lifestyle Programme on patients diagnosed as being at increased risk of developing further polyps as determined by colonoscopy.

Patient Identification Number for this study:

Investigators: Consultant, Professor John Saxton, Students

Name of patient:

Please initial box

1. **I confirm that I have read and understood the Patient Information Sheet Version ____ dated ____/____/____ for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.** ☐
2. **I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.** ☐
3. **I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals of the research team, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.** ☐
4. **I consent for tissue collected during the study (i.e. venous blood, cheek swab and colon tissue) to be transferred to the Institute of Food Research for analysis purposes** ☐
5. **I agree to my G.P. being informed of my participation in the study.** ☐
6. **I agree to take part in the above study.** ☐
7. **I am aware that I may be contacted to be interviewed at 1 and 12 months.** ☐

Name of Participant	Date	Signature
Name of individual taking consent (if not researcher)	Date	Signature
Researcher	Date	Signature

University of East Anglia

Norfolk and Norwich University Hospitals NHS Foundation Trust

Dear Dr Cooper

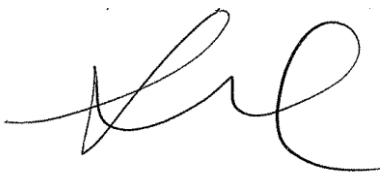
Re: patient name (xx/xx/19xx)

I am writing to inform you that xxxxx has consented to be contacted regarding his involvement in an exercise intervention based at the University of East Anglia, supported by the Norfolk and Norwich University Hospital.

The new project is aiming to identify the biological and psychological effects of a 12 month Active Lifestyle Programme on patients diagnosed as being at increased risk for developing further colon polyps at their screening colonoscopy. Alongside the 12 month exercise intervention, questionnaires to assess behavioural changes with regards to lifestyle factors will also be administered to each participant. As well as these procedures some participants may be asked to participate in interviews at the start and end of the intervention, and focus groups after trial completion to gain a more detailed account of personal experiences with both physical activity and the trial itself.

If you have any concerns or questions regarding your patient participating in this study please do not hesitate to contact a member of the study team on 01603 593098.

Yours Sincerely,



Professor John Saxton

Patient Information Sheet

The Effects of a 12 month Active Lifestyle Programme on patients diagnosed as being at increased risk for developing further polyps by their screening colonoscopy.

We are inviting you to take part in our research study. Before you decide whether or not to take part we want you to understand why we are doing this research and what it will involve for you. This information sheet provides an overview of the study, and it should take about fifteen minutes to read. Please feel free to discuss the study with family and friends. If there is anything you are not clear about, the contact details of the researchers are provided at the end. We will happily go through the information sheet with you and answer any questions you have.

We have compiled a list of Frequently Asked Questions (FAQs) which cover the main aspects of the research:

What is the purpose of the study?

Recently, some evidence has accumulated which suggests that people who have exercised regularly throughout their life might be at reduced risk of developing certain types of cancer, in particular colon (bowel) cancer. However, at the present time, we do not know whether a physically active lifestyle can have a positive effect on biological markers associated with colon cancer risk. Also, we do not know how taking part in a programme like this affects exercise behaviour and attitudes towards exercising after colonoscopy screening.

Therefore, we are aiming to find out whether an active lifestyle programme, incorporating supervised exercise sessions and healthy living workshops over a 12-month period has a positive impact on bowel health and exercise behaviour in people diagnosed with benign polyps or adenomas as a result of their screening colonoscopy. We also want to investigate if changes in exercise habits can affect physical function and feelings of well-being.

Why have I been invited?

You have been selected as being a potentially suitable participant as you have presented to the Norfolk and Norwich University Hospital on the National Bowel Cancer Screening Programme. We are looking to recruit participants from this population subject to the outcome of your test.

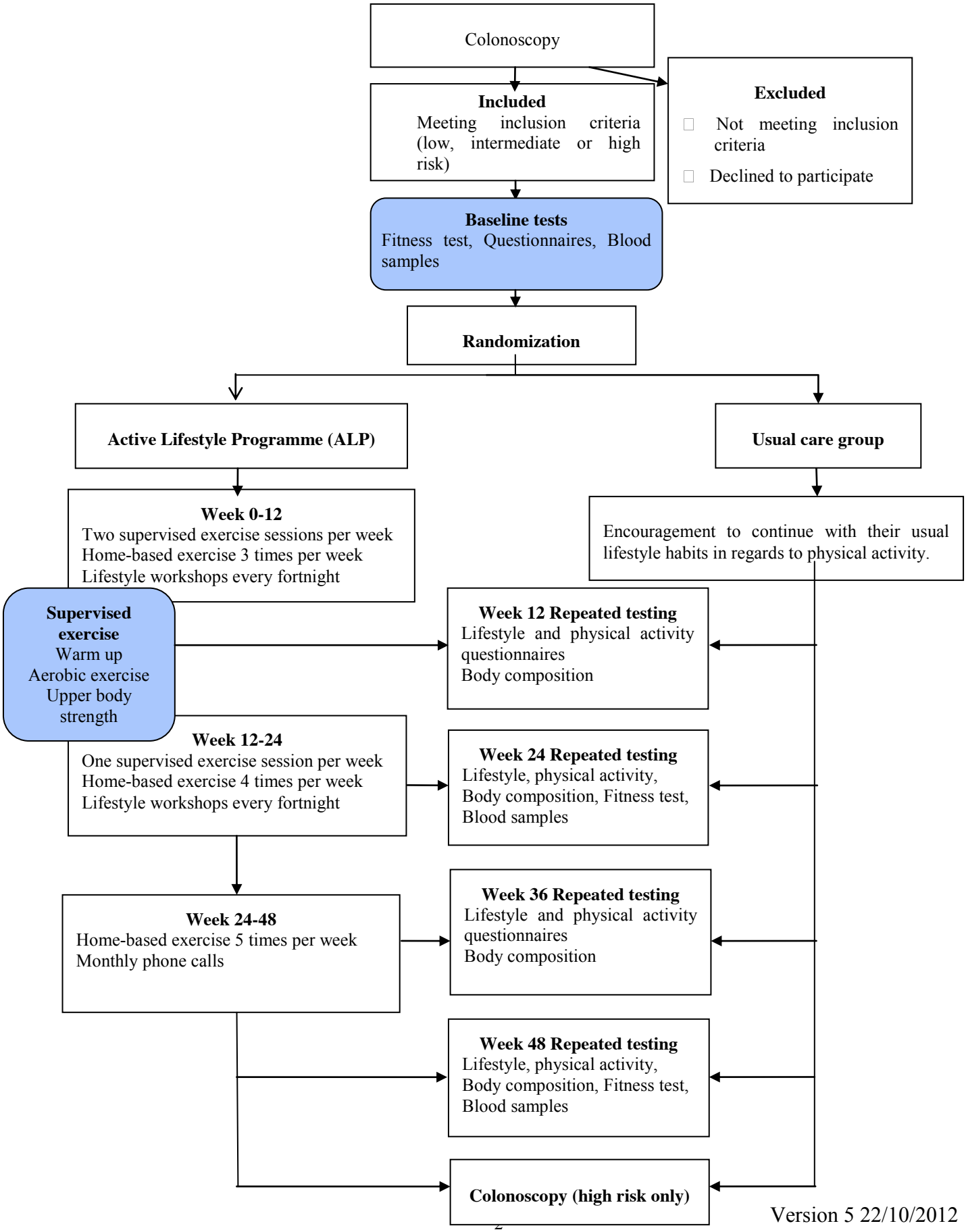
Do I have to take part?

Your participation is entirely voluntary. If you decide not to take part, this will not affect the standard of care you receive from the hospital or any other health professional. You are also free to withdraw from the study at any time without giving reason.

What will happen to me if I take part/what do I have to do?

If you decide to take part and you meet our inclusion criteria (i.e. the surgeon identifies you as being as either 'low', 'intermediate' or 'high' risk for developing further polyps during the routine colonoscopy) you will have five small pinch biopsies taken from your colon, as well as any abnormal tissue that would be routinely removed. Once you have the results of your colonoscopy we will contact you to arrange a formal meeting with the research team at the University of East Anglia. If after the meeting you are still happy to take part, we will invite you to complete baseline tests. Afterwards, you will be randomly assigned to one of two groups; namely the Active Lifestyle Programme (ALP) or Usual Care (UC). You have an equal chance of being in either group. This is known as a randomised controlled trial, and we are running the study this way because we do not know which treatment is best.

You will be involved in the study for 12 months, and the total length of the research will be 2 years. The figure below outlines all of the procedures involved:



The second colonoscopy at the end of the study only applies to those individuals diagnosed as 'high' risk at the first colonoscopy, which is routine. As before, the surgeon will take five further pinch biopsies as well as any abnormal tissue. We will monitor your physical activity levels, body composition, and diet, and ask both groups to complete questionnaires every 3 months. We will take venous blood samples from you and ask you to complete a fitness test every 6 months. The principal difference will be that the ALP group will aim to achieve 300 min per week of moderate to vigorous physical activity for the duration of the study; whereas UC will maintain their normal lifestyle habits. To help achieve this goal, ALP will receive 36 personal training sessions at the University of East Anglia over 6 months. This will be complemented by 12 lifestyle workshops at the University. You *may* also be asked to participate in a face to face interview at the start and end of the study, or a focus group after 12 months, subject to your consent.

Can I expect any payment/reimbursement of costs?

Unfortunately, we cannot offer any financial reward or cover any personal expenses. However, ALP will receive free personal training and lifestyle workshops, and we will make data pertaining to health such as body composition, cardiorespiratory fitness and diet analysis available to both groups at the end of the study.

What are the treatment alternatives?

Currently, there are no treatment guidelines for individuals diagnosed as being intermediate or high risk for developing further polyps, other than further screening colonoscopies.

What are the possible disadvantages/risks of taking part?

The potential for risks to occur will be minimised. We will make sure that you can safely complete the exercise sessions before you take part, so that the likelihood of anything untoward happening during the exercise will be minimal. Exercise protocols will be tailored to your needs and your heart rate will be monitored during the exercise. In the event that something does go wrong and you are harmed during the research study, there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you might have grounds for legal action for compensation, but you could have to pay your legal costs.

Are there any side-effects of taking part?

If you haven't exercised for a while, and are part of the ALP group, physical activity might initially make you feel tired, and you could feel slightly breathless, but as you do it more regularly you will feel increasingly better.

What are the possible benefits of taking part in this study?

We cannot guarantee that you will benefit personally, but you will receive free fitness tests. The information which we will obtain might help improve medical care for patients at elevated risk like yourself.

What happens when the research study stops?

When the study finishes, we plan to publish the findings in a peer-reviewed scientific journal. We will not monitor you after your involvement in the study has finished. We will make data pertaining to health such as body composition, cardiorespiratory fitness and diet analysis available to both groups at the end of the study. If you are randomised to UC, you will be given the materials provided to ALP should you request them.

What if there is a problem?

In the event that something does go wrong and you are harmed during the research study, there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you might have grounds for legal action for compensation, but you could have to pay your legal costs.

Will my taking part in the study be kept confidential?

The confidentiality of our patients and the data which this study will generate is of utmost importance. All data from this study will be anonymised with a unique code during the study so the researcher analysing your data will be blinded as to your identity, which group you are in and to information collected during the study. This is one of the clauses, which you will sign in agreement on the official consent form. Our procedures for handling, processing and storage of and destruction of data are compliant with the Data Protection Act 1998.

What if relevant new information becomes available?

We will inform you if relevant new information becomes available which might affect the way we treat you. We will also discuss whether we need to make any amendments with the trial steering committee, which is responsible for the conduct of the research.

What will happen if I do not want to carry on with the study?

You are free to withdraw from the study at any time without giving reason, and this will not affect the standard of care you receive from the hospital or any other health professional. Should you wish, we can also destroy any identifiable data/tissue samples that we have collected from you.

What if there is a problem?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, the Patient Advice and Liaison Service is available to you. In order to use this service you can choose one of the following options:

Phone: 01603 289036
Email: PALS@nnuh.nhs.uk
Website: http://www.pals.nhs.uk/

Will my GP be notified?

With your consent, we will write and inform your family doctor that you are taking part in this study.

What will happen to any samples I give?

Any tissue samples that you provide will be transferred to the Institute of Food Research, where we will analyse them for indicators of bowel health. They will be stored there for the duration of the study. Responses to questionnaires, physical activity, diet and fitness data will be stored and analysed at the University of East Anglia for the duration of the study.

Will any genetic tests be done?

There are several genes which are known to be involved with the development of bowel cancer. These genes can be affected by ageing, which may in turn affect the risk of developing the disease. We want to understand whether exercise can reverse the gene ageing process, and we will look for signs of this in the colon biopsies we obtain. We also would like to see whether any changes are reflected in other areas of the body, which is why we would like to analyse these genes in your blood and cheek cells. We will only analyse genes that are known to be implicated in bowel cancer, and we will *not* sequence your entire genome.

What will happen to the results of the research study?

We plan to publish the results of this study in a peer-reviewed scientific journal. However, you will not be personally identifiable from these results. In addition, the results from initial fitness testing and overall conclusions of the study will be available to you. Any further information will be available upon request. With your consent, we will anonymously store any leftover blood and tissue samples we collected from you at a NHS approved tissue

bank for a period of 5 years. These samples might be used for future research into bowel health. You can still take part in the study even if you do not wish to have any leftover tissue stored in this way, in which case they shall be destroyed once the study has finished. All of these procedures will be compliant with the Human Tissue Act 2004.

Who is organising and funding the research?

The research forms part of a PhD programme funded by the University of East Anglia. The research is being conducted in collaboration with the Norfolk and Norwich University Hospital and the Institute of Food Research.

Who has reviewed the study?

All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Norfolk Research Ethics Committee.

Further information and contact details

If you have any specific questions about the study, we will be more than happy to answer them for you.

In the event of further questions please contact:

Mr Barnabas Shaw BSc. MSc.
Email: B.Shaw@uea.ac.uk
07933090196

Miss Liane Lewis BSc.
Email: Liane.Thomas@uea.ac.uk
07933090197

Miss Kelly Semper BSc.
Email: K.Semper@uea.ac.uk

Thank you for taking the time to consider participating in this study

Prof John Saxton (Project co-ordinator), Tel: 01603 593098, Email: john.saxton@uea.ac.uk

University of East Anglia

Norfolk and Norwich University Hospitals NHS Foundation Trust

Health Questionnaire

Preventative Action against risk of Colon Cancer (PARC)

Title: **Name:**	**Gender** M ☐ F ☐	**DOB (dd/mm/yyyy) :** __ __/__ __/ __ __ __ __

Address: **Postcode:**	**Telephone number:** **Mobile number:** **Email:**

Medical information

1. Please tick if you suffer from, or have suffered from, any of the following:

☐ Heart disease (e.g. angina, palpitations) ☐ High blood pressure ☐ Vascular disease (e.g. peripheral arterial disease, hypercholesterolaemia) ☐ Stroke ☐ Type 2 Diabetes ☐ Pulmonary disease (e.g. asthma, COPD) ☐ Inflammatory bowel disease (e.g. Crohn's disease, ulcerative colitis) ☐ Familial adenomatous polyposis (FAP) ☐ Cancer ☐ Seizures ☐ Joint problems (e.g. arthritis) ☐ Back pain ☐ Other _____

If you have ticked any of the above, please give details, including any treatments:

2. Is there a history of colon cancer in your family ☐ Yes ☐ No

If yes, please give details:

3. Has your doctor advised you against taking regular exercise? ☐ Yes ☐ No

If yes, please give any reasons why:

4. Do you take any laxatives (e.g. bulking agents, stool softeners, irritants)?
☐ Yes ☐ No

If yes, please give details as to the type of medication, the amount, and how often it is taken:

Medication	Amount	Frequency (per week)

5. Do you take any non-steroidal anti-inflammatory drugs (NSAIDS) (e.g. aspirin, ibuprofen)? ☐ Yes ☐ No

If yes, please give details as to the type of medication, the amount, and how often it is taken:

Medication	Amount	Frequency (per week)

6. Do you regularly take any other prescribed or over the counter medication?
☐ Yes ☐ No

If yes, please give details:

Medication	Amount	Frequency (per week)

7. Do you smoke cigarettes? ☐ Yes ☐ No

If yes, how many per day?: _____

8. Do you drink alcoholic beverages? ☐ Yes ☐ No

If yes, how often per week?

☐ Less than once ☐ Once ☐ Twice ☐ Three to six times ☐ Daily

Approximately how many units of alcohol do you drink per week (*for reference, 1 pint of standard beer, lager or cider = ~ 3 units; a standard 175 ml glass of wine = ~ 2 units; and a single measure of a spirit = ~ 1 unit)*?: _____

9. What is your marital status?

☐ Single ☐ Partnered
☐ Married ☐ Separated
☐ Divorced ☐ Widowed

10. How many visits have you made to your GP in the past 12 months?

Please state: ____

Thank you.

Appendix Bx

Health Questionnaire

PARC- Physical Activity and Risk of Colon Cancer

Participant ID: _____ Date: _____
Time point: _____

University of East Anglia

Norfolk and Norwich University Hospitals NHS Foundation Trust

Version 1 14/02/2012

Your Health and Well-Being

The following survey asks questions about your general health, lifestyle behaviour in regards to exercise and diet and assesses your weekly physical activity habits. It is important that you answer EACH question of the following survey.

1. In general, would you say your health is:

Excellent	Very good	Good	Fair	Poor
▼	▼	▼	▼	▼
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

2. <u>Compared to one week ago</u>, how would you rate your health in general <u>now</u>?

Much better now than one week ago	Somewhat better now than one week ago	About the same as one week ago	Somewhat worse now than one week ago	Much worse now than one week ago
▼	▼	▼	▼	▼
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

3. The following questions are about activities you might do during a typical day. Does <u>your health now limit you</u> in these activities? If so, how much?

	Yes, limited a lot	Yes, limited a little	No, not limited at all
a <u>Vigorous activities</u>, such as running, lifting heavy objects, participating in strenuous sports	☐ 1	☐ 2	☐ 3
b <u>Moderate activities</u>, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf	☐ 1	☐ 2	☐ 3
c Lifting or carrying groceries	☐ 1	☐ 2	☐ 3
d Climbing <u>several</u> flights of stairs	☐ 1	☐ 2	☐ 3
e Climbing <u>one</u> flight of stairs	☐ 1	☐ 2	☐ 3
f Bending, kneeling, or stooping	☐ 1	☐ 2	☐ 3
g Walking <u>more than a mile</u>	☐ 1	☐ 2	☐ 3
h Walking <u>several hundred yards</u>	☐ 1	☐ 2	☐ 3
i Walking <u>one hundred yards</u>	☐ 1	☐ 2	☐ 3
j Bathing or dressing yourself	☐ 1	☐ 2	☐ 3

4. During the past week, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of your physical health?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Cut down on the amount of time you spent on work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b Accomplished less than you would like	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c Were limited in the kind of work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d Had difficulty performing the work or other activities (for example, it took extra effort)	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

5. During the past week, how much of the time have you had any of the following problems with your work or other regular daily activities as a result of any emotional problems (such as feeling depressed or a nxious)?

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Cut down on the amount of time you spent on work or other activities	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b Accomplished less than you would like	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c Did work or other activities less carefully than usual	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

6. During the past week, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbours, or groups?

Not at all	Slightly	Moderately	Quite a bit	Extremely
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

7. How much <u>bodily</u> pain have you had during the <u>past week</u>?

None	Very mild	Mild	Moderate	Severe	Very severe
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5	☐ 6

8. During the <u>past week</u>, how much did <u>pain</u> interfere with your normal work (including both work outside the home and housework)?

Not at all	A little bit	Moderately	Quite a bit	Extremely
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

9. These questions are about how you feel and how things have been with you <u>during the past week</u>. For each question, please give the one answer that comes closest to the way you have been feeling. How much of the time during the <u>past week</u>…

	All of the time	Most of the time	Some of the time	A little of the time	None of the time
a Did you feel full of life?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b Have you been very nervous?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c Have you felt so down in the dumps that nothing could cheer you up?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d Have you felt calm and peaceful?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
e Did you have a lot of energy?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
f Have you felt downhearted and low?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
g Did you feel worn out?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
h Have you been happy?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
i Did you feel tired?	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

10. During the <u>past week</u>, how much of the time has your <u>physical health or emotional problems</u> interfered with your social activities (like visiting with friends, relatives, etc.)?

All of the time	Most of the time	Some of the time	A little of the time	None of the time
☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

11. How TRUE or FALSE is <u>each</u> of the following statements for you?

	Definitely true	Mostly true	Don't know	Mostly false	Definitely false
a I seem to get ill more easily than other people	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
b I am as healthy as anybody I know	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
c I expect my health to get worse	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5
d My health is excellent	☐ 1	☐ 2	☐ 3	☐ 4	☐ 5

2. EXERCISE BEHAVIOUR

WHY DO YOU ENGAGE IN EXERCISE?

We are interested in the reasons underlying peoples' decisions to engage, or not engage in physical exercise. Using the scale below, please indicate to what extent each of the following items is true for you. Please note that there are no right or wrong answers and no trick questions. We simply want to know how you personally feel about exercise.

(1-19) Please circle one number on each line indicating how true each statement is for you.

		Not true for me		Sometimes true for me		Very true for me
1	I exercise because other people say I should	0	1	2	3	4
2	I feel guilty when I don't exercise	0	1	2	3	4
3	I value the benefits of exercise	0	1	2	3	4
4	I exercise because it's fun	0	1	2	3	4
5	I don't see why I should have to exercise	0	1	2	3	4
6	I take part in exercise because my friends/family/partner say I should	0	1	2	3	4
7	I feel ashamed when I miss an exercise sessions	0	1	2	3	4
8	It's important to me to exercise regularly	0	1	2	3	4
9	I can't see why I should bother exercising	0	1	2	3	4
10	I enjoy my exercise sessions	0	1	2	3	4
11	I exercise because others will not be pleased with me if I don't	0	1	2	3	4
12	I don't see the point in exercising	0	1	2	3	4
13	I feel like a failure when I haven't exercise in a while	0	1	2	3	4
14	I think it is important to make the effort to exercise regularly	0	1	2	3	4
15	I find exercise a pleasurable activity	0	1	2	3	4
16	I feel under pressure from my friends/family to exercise	0	1	2	3	4
17	I get restless if I don't exercise regularly	0	1	2	3	4
18	I get pleasure and satisfaction from participating in exercise	0	1	2	3	4
19	I think exercising is a waste of time	0	1	2	3	4

(20-29) With the next questions we want to know how confident you are right now that you could exercise 30min on most days of the week?

		Not very confident										Very confident
20	The weather was bothering you	0	1	2	3	4	5	6	7	8	9	10
21	You were bored by the programme or activity	0	1	2	3	4	5	6	7	8	9	10
22	You were not exactly sure what exercise to do	0	1	2	3	4	5	6	7	8	9	10
23	You felt pain when exercising	0	1	2	3	4	5	6	7	8	9	10
24	You had to exercise alone	0	1	2	3	4	5	6	7	8	9	10
25	You did not enjoy it	0	1	2	3	4	5	6	7	8	9	10
26	You were too busy with other activities	0	1	2	3	4	5	6	7	8	9	10
27	You felt tired	0	1	2	3	4	5	6	7	8	9	10
28	You felt stressed	0	1	2	3	4	5	6	7	8	9	10
29	You felt depressed	0	1	2	3	4	5	6	7	8	9	10

		Do not agree at all						Completely agree
33	I intend to exercise regularly over the next month	1	2	3	4	5	6	7
34	I intend to exercise regularly over the next 6 months							

3. How much PHYSICAL ACTIVITY do you do?

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport. Think about all the vigorous and moderate activities that you did in the last 7 days. Vigorous physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Moderate activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, and any other unpaid work that you did outside your home. **Do not** include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

☐ Yes

☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the last 7 days as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, digging, heavy construction, or climbing up stairs as part of your work? Think about only those physical activities that you did for at least 10 minutes at a time.

_____ **days per week**

☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing vigorous physical activities as part of your work?

_____ **hours per day**

_____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like carrying light loads as part of your work? Please do not include walking.

_____ **days per week**

☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing moderate physical activities as part of your work?

_____ **hours per day**

_____ **minutes per day**

6. During the last 7 days, on how many days did you walk for at least 10 minutes at a time as part of your work? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days walking as part of your work?

_____ **hours per day**

_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the last 7 days, on how many days did you travel in a motor vehicle like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days traveling in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**

_____ **minutes per day**

Now think only about the bicycling and walking you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the last 7 days, on how many days did you bicycle for at least 10 minutes at a time to go from place to place?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to bicycle from place to place?

_____ **hours per day**

_____ **minutes per day**

12. During the last 7 days, on how many days did you walk for at least 10 minutes at a time to go from place to place?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days walking from place to place?

_____ **hours per day**

_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the last 7 days in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like heavy lifting, chopping wood, shoveling snow, or digging in the garden or yard?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing vigorous physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, sweeping, washing windows, and raking in the garden or yard?

_____ days per week

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing moderate physical activities in the garden or yard?

_____ hours per day

_____ minutes per day

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate activities like carrying light loads, washing windows, scrubbing floors and sweeping inside your home?

_____ days per week

☐ No moderate activity inside home ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing moderate physical activities inside your home?

_____ hours per day

_____ minutes per day

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the last 7 days solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the last 7 days, on how many days did you walk for at least 10 minutes at a time in your leisure time?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days walking in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do vigorous physical activities like aerobics, running, fast bicycling, or fast swimming in your leisure time?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing vigorous physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do moderate physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis in your leisure time?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing moderate physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the last 7 days, how much time did you usually spend sitting on a weekday?

_____ **hours per day**
_____ **minutes per day**

27. During the last 7 days, how much time did you usually spend sitting on a weekend day?

_____ **hours per day**
_____ **minutes per day**

28. During a typical 7-Day period (a week), how many times on the average do you do the following kinds of exercise for more than 15 minutes during your free time (write on each line the appropriate number).

	Times Per Week
a) STRENUOUS EXERCISE (HEART BEATS RAPIDLY) (e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)	_____
b) MODERATE EXERCISE (NOT EXHAUSTING) (e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)	_____
c) MILD EXERCISE (MINIMAL EFFORT) (e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)	_____

2. During a typical **7-Day period** (a week), in your leisure time, how often do you engage in any regular activity **long enough to work up a sweat** (heart beats rapidly)?

OFTEN	SOMETIMES	NEVER/RARELY
1. ☐	2. ☐	3. ☐

Food diary

Record all foods and portion sizes you eat on four days of the week in the table below. The record should include at least one day of the weekend and three week days. Try to give as much detail as possible.

Week day	Type of food eaten	Time eaten	Portion size (in grams, cups, table spoons)
e.g. Monday	*Semi-skimmed Milk*	*8:53am*	*250ml glass*
	Toast- white bread	*8:55am*	*3 slices*
	Butter	*8:55am*	*1 tsp*
	Cheddar cheese	*8:55*	*30g*
Week day	**Type of food eaten**	**Time eaten**	**Portion size (in**

			grams, cups, table spoons)

Week day	Type of food eaten	Time eaten	Portion size (in grams, cups, table spoons)

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P A R C

Physical Activity and Risk of Colon Cancer

Active Lifestyle Booklet

University of East Anglia

What you should know...

Colorectal cancer is the second most common cancer in the UK, but is also the **most preventable**.

As with all cancers, the risk of developing colon cancer depends on a number of factors and varies from person to person.
Many of the factors that increase our chances of developing the disease are linked to our way of life.
This means that there are changes we can make to reduce our risk:
- Healthy/fibre rich diet
- Physical Activity
- Maintain Healthy Weight
- Limit Alcohol Intake and Cigarette Smoking.

Some important Facts...

- regular physical can reduce colon cancer risk by 24%
- being overweight or carrying extra weight around your waist increases the risk of developing colon cancer
- physical activity can help you to maintain a healthy body weight and therefore decrease your risk of developing colon cancer

Understanding the science behind the benefits of exercise....

The immune system also benefits from exercise. Regular physical activity can boost immune function to help fight off disease.

Elevated levels of insulin are thought to be related to colon cancer risk. Physical activity has been shown to lower insulin levels and could therefore protect against colon cancer.

Physical activity can help to move food through the digestive system more efficiently. This can reduce exposure of the colon to potentially cancerous substances.

Did you know..?

Being physically active for at least 30 minutes a day helps you feel and look better. It can even improve your mood and decrease stress levels.

Getting started...

- Wear comfortable clothing
- Bring suitable shoes (trainers)
- Don't exercise on an empty stomach but also leave time after a large meal (at least 2 hours)
- Make sure you have plenty of fluids with you
- Always handy to carry a small snack in case it is needed

Don't worry if...

- You have not exercised in the past- we are here to help you to settle in and familiarise you with the exercise equipment. As well as this we will tailor the exercise programme to your individual capabilities.
- You feel breathless- this is normal, especially if you are not used to exercising. During the supervised exercise, a qualified exercise instructor will be present to monitor your condition.
- Your muscles hurt after the exercise- this is also quite normal, frequent exercise will make your muscles adapt, which will result in increased in strength.

How much is enough?

- Research shows that risk of colon cancer decreases with increasing amounts of physical activity
- The **minimum** amount for health benefits is 30 minutes of moderate to vigorous activity per day on most days of the week.
- However, to maximise the health benefits of exercise, an attainable target of 300 minutes of moderate to vigorous physical activity per week is recommended.
- Examples of moderate and vigorous physical activities are shown in the table below.
- Physical activity does not have to happen in a gym. There are many alternative ways to be physically active.

Moderate Intensity	Vigorous Intensity
Cycling = Sweeping	Aerobics = Mow Lawn
Swimming = Hoovering	Tennis = Climb Stairs
Brisk Walk = Gardening	Jogging = Heavy Lifting

Structure of an Exercise Session

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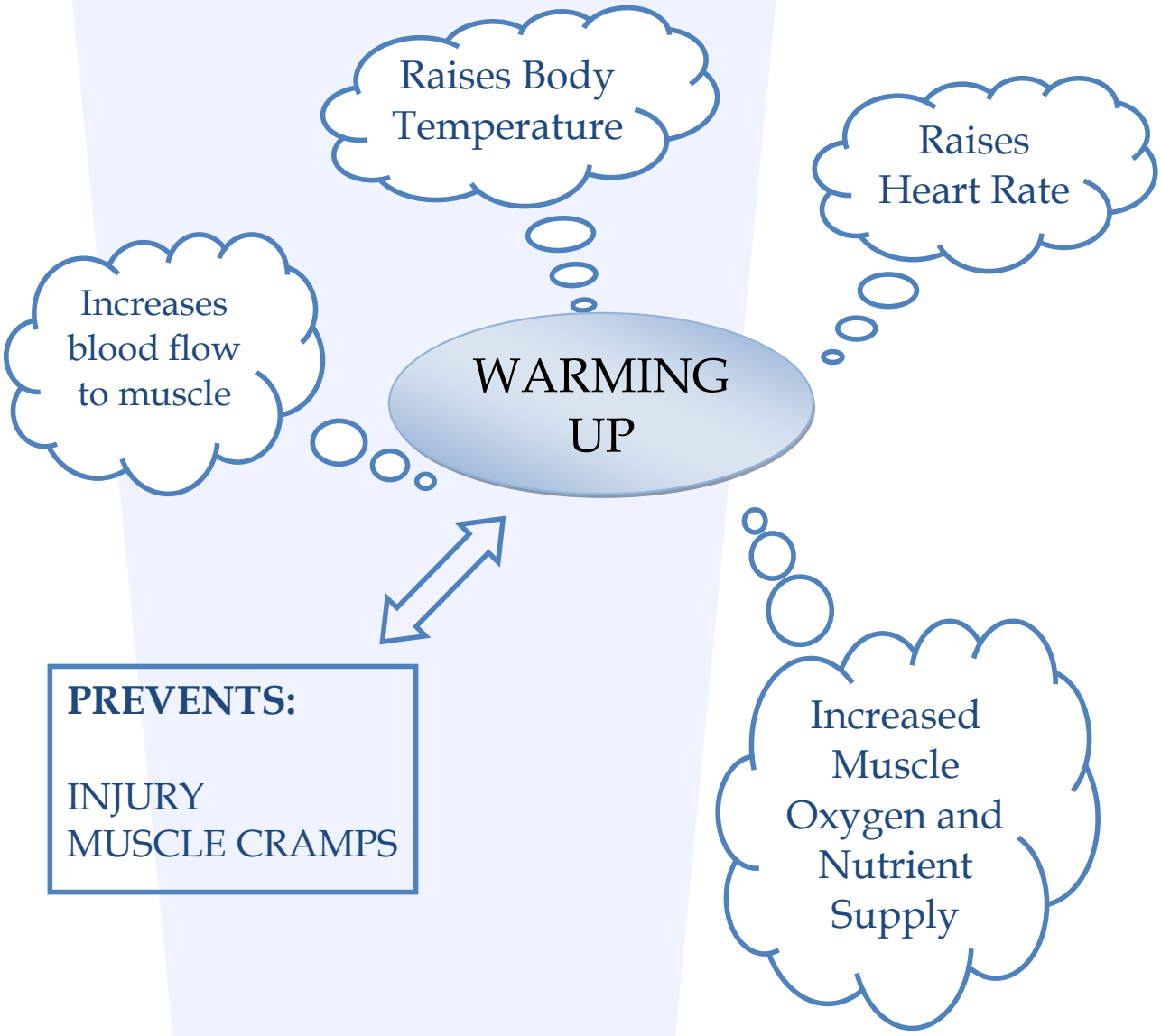


Use the RPE Scale below to monitor how hard you exercise.
Rate your exercise intensity on a scale of 6 (at rest) to 20 (extremely hard).
Try to aim for moderate to hard intensity throughout exercise.
Use this scale to record your level of activity later on in the booklet.

Rate of Perceived Exertion (RPE) Scale

6	8- 9	10- 11	12 - 13	14- 15	16- 18	20
Rest	Very Light	Light	Moderate	Hard	Very Hard	Extremely Hard

Warm Up

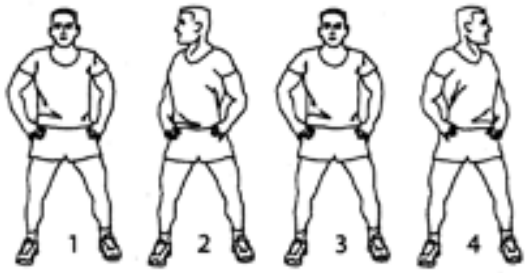
5 – 10 minutes to gradually elevate heart rate.
Mobilise your joints (see exercises below)

HEAD

Head Tilt (side to side)

Side Bends

TORSO

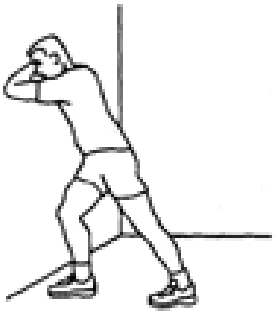


Twist

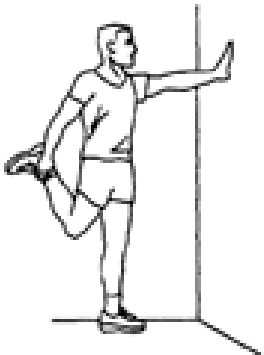
SHOULDERS

Side Reach

LEGS



Calf Stretch
(hold 10 seconds)



Quadriceps
(hold 10 seconds)

Main exercises

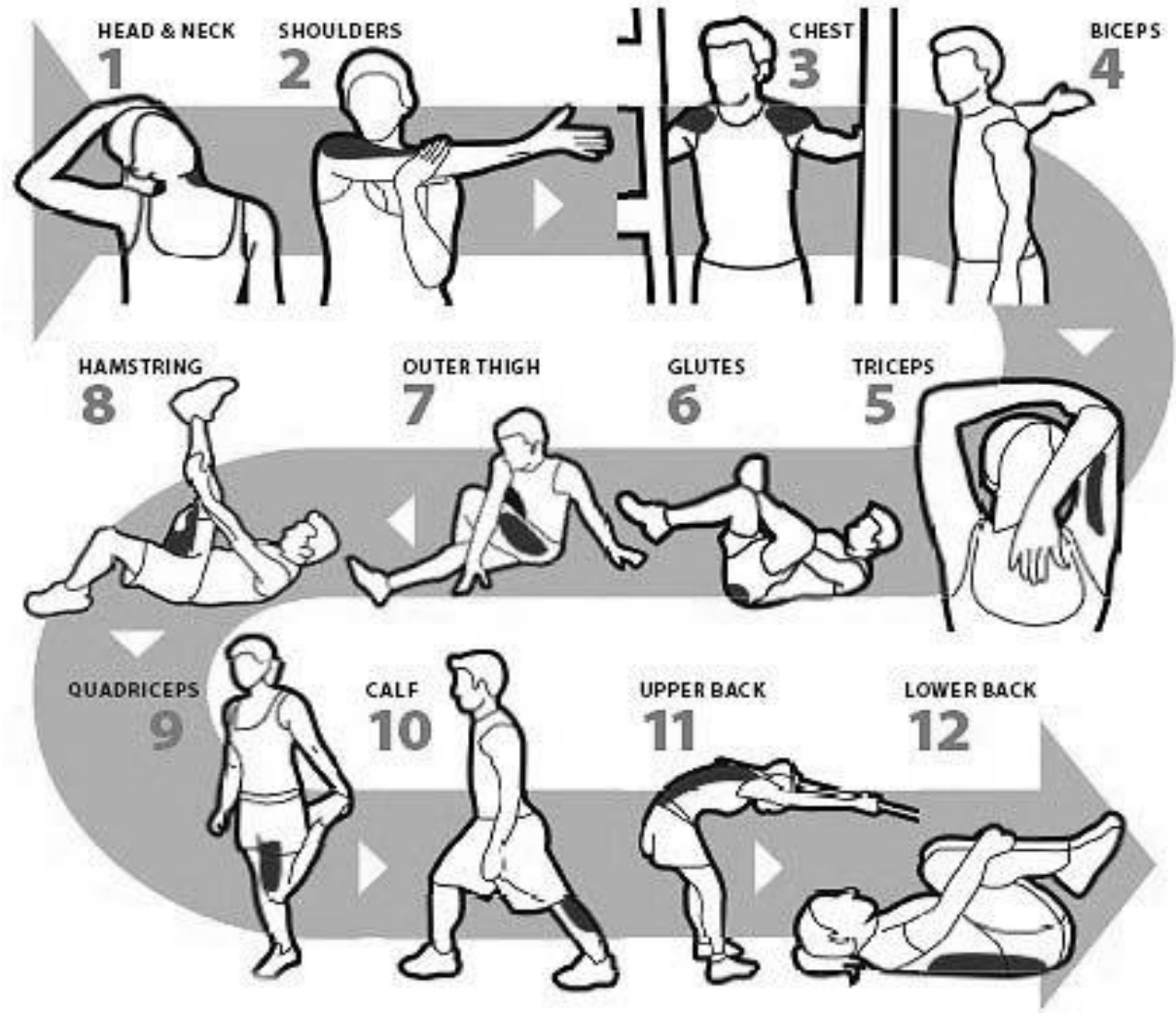
Start your exercise at a low intensity (RPE 9-10) and slowly build up over a period of 10minutes until you reach RPE 13-15

- Brisk walking
- Slow cycling
- Light jogging

Keep a moderate to hard intensity for a minimum of 20-30 minutes, depending on how comfortable you feel.

Cool down

Stretch your muscles to avoid stiffness.



Strategies for success...

- Try to build as much activity into your daily routine as possible.
- Plan a minimum of 30-minutes per day where you can be active...and stick to it, e.g. a brisk walk
- Get friends and family involved in your daily activities.
- Make sure you incorporate a variety of activities to avoid

How to include exercise into my daily routine...

- Try to take the stairs instead of the lift
- Walk to the corner shop instead of driving.
- Take the dog for long walks in the park.
- Get off the bus one stop earlier.
- Don't use the remote control- get up and change the channel.

How will I incorporate exercise into MY daily routine...?

Is there anyone I could exercise with on a regular basis?

My Goals

Goal setting should be S.M.A.R.T.

- ✔ Specific
- ✔ Measurable
- ✔ Attainable
- ✔ Realistic
- ✔ Timely

I aim to increase my step count by 3000 steps each day for the next month.

My goals for the future

Short term goals (by the end of the month)

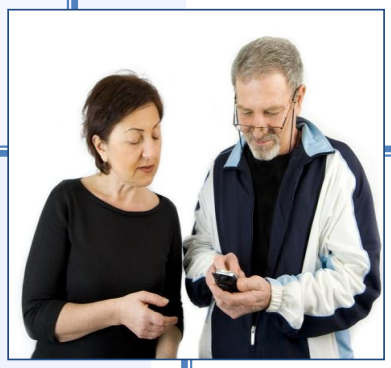
Long term (by the end of the intervention), i.e. how am I going to make sure that I remain physically active?

"Failing to prepare, is preparing to fail" – John Wooden.

Overcoming Barriers...

Support from Family and Friends

- A bicycle ride with your family
- Play with your grand children
- Sunday afternoon walks



Planning for success

- Make specific activity diaries for each week
- Adjust for unexpected circumstances
- Check timetable of activities in your area and plan around these

Make housework fun

- Do your housework to music you like
- Gardening can be as strenuous as cycling

Ideas to get you started at home...

GP Referral Scheme

Ask your GP for details.
The scheme offers a variety of activities in several centres in and around Norwich. You can choose from circuit training and aqua classes.
Cost: £2.50 per class
http://www.exercise-referral.co.uk/

Active Norfolk

FREE lead group walks
Exercise classes for over 50s
Where: several locations around Norfolk
http://www.activenorfolk.org/
Tel: 01603- 732 333

Fitness in later life (FILL)

Badminton
Aerobic classes
Walking, stretching and strengthening
Where: Sportspark at University of East Anglia
Costs: £3.20 per class
http://sportspark.co.uk/
Tel: 01603- 592 398

Go4less

This is a FREE discount card by Norwich City Council
Up to 50% discount
Can be used at a variety of fitness centres.

- Riverside Leisure Centre
- Norman Centre

Available at Tourist Information in the Forum

Guided walks around Mousehold Heath

Organised by Norwich City Council

ATP Health and Fitness

A mixture of exercise classes, from tailored programmes to structured sessions.
Where: locations throughout Norwich
Costs: £5 per class- special offers are available

Eaton Community Centre

Circuit Classes, Line Dancing
Cost: £5
Where: Eaton Park Community Centre
www.friendsofeatonpark.co.uk/community.html
Tel: 01603- 504 420

Ask one of the instructors if you want any further details.

Physical Activity Diary

My Start Date.....

Week 1 Date Commencing……

Day	Activity, Duration (minutes) and RPE	Step count
Monday		
Tuesday		
Wednesday		
Thursday		
Friday		
Saturday		
Sunday		

Week 2 Date Commencing……

Day	Activity, Duration (minutes) and RPE	Step count
Monday		
Tuesday		
Wednesday		
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Saturday		
Sunday		

Week 3 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
Monday		
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Sunday		

Week 4 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 5 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 6 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 7 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 8 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 9 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 10 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 11 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Saturday		
Sunday		

Week 12 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 13 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 14 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 15 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 16 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 17 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 18 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 19 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 20 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 21 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 22 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 23 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 24 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 25 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 26 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 27 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 28 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 29 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 30 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 31 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 32 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 33 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 34 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 35 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 36 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 37 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 38 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 39 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 40 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 41 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 42 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 43 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Sunday		

Week 44 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 45 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Sunday		

Week 46 **Date Commencing……**

Day	Activity, Duration (minutes) and RPE	Step count
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Week 47 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
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Saturday		

Week 48 **Date Commencing……..**

Day	Activity, Duration (minutes) and RPE	Step count
Monday		
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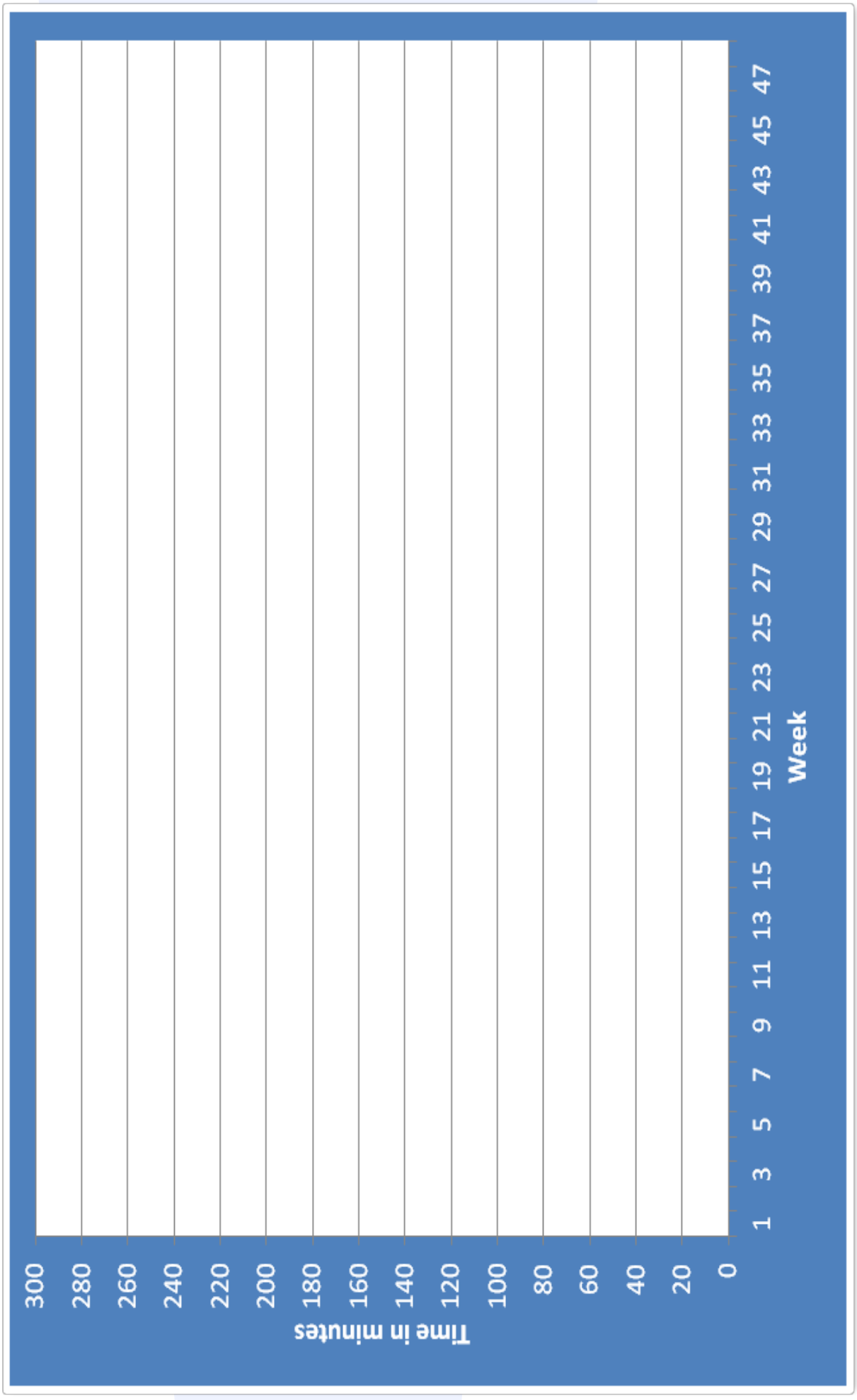
Sunday		

Plot all your physical activities here...



Tell us what you think about the programme:

What did you like?

What would change?

Any other comments?

Thank you for taking part in our study…

If you have any queries, comments or suggestion please contact:

Barnabas Shaw: B.Shaw@uea.ac.uk,
Tel:
Liane Thomas: Liane.Thomas@uea.ac.uk
Tel:
Kelly Semper: K.Semper@uea.ac.uk
Tel:
Project Supervisor: Prof John Saxton
John.Saxton@uea.ac.uk
Tel:

Appendix Ci

Volunteers needed!

Bowel Cancer

Is the third most common cancer diagnosed in the UK…

Our Research

Is aimed at understanding how physical activity reduces the risk of the disease

You can help

By taking part in the ReGro (Regular exercise and colon cell Growth) study. Your involvement will be less than three weeks in total

If you are:

- **male**
- **aged 50-75**
- **non-smoking**
- **free of any current or previous bowel conditions (e.g. bowel cancer, polyps or inflammatory bowel disease)**
- **able to attend two morning, and two afternoon or evening visits (can be weekend days) at the University of East Anglia**
- **prepared to do one fitness test and one exercise session**
- **prepared to have 4 blood samples taken (4 teaspoons worth per occasion, on the morning visits)**

then you might be eligible. Please contact Mr Barney Shaw if you are interested, and we can post/email an invitation letter and a Participant Information Sheet.

Barney Shaw:
07933090196
b.shaw@uea.ac.uk

Version 3 26/04/2013

Appendix Cii

RE: Does Regular and acute exercise affect Growth of colon cells (ReGro)?

Thank you for your interest in the above study. Our research is investigating the links between exercise and bowel cancer risk.

Please find enclosed a Patient Information Sheet, which explains the study in more detail and answers some of the most frequently asked questions.

Please feel free to discuss this information with family and friends before making any decisions, however if you still have any further questions, please feel free to contact me on the addresses/ telephone numbers provided below.

Yours sincerely,

Barnabas Shaw

Mr Barnabas Shaw, BSc., MSc.
ENV 0.58
School of Allied Health Professions
Faculty of Medicine & Health Sciences
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Norwich
Norfolk
NR4 7TJ
Email: B.Shaw@uea.ac.uk
Research telephone: 07933090196

Participant Information Sheet

Does Regular and acute exercise affect Growth of colon cells (ReGro)?

We are inviting you to take part in our research study (the ReGro study). Before you decide whether or not to take part we want you to understand why we are doing this research and what it will involve for you. This information sheet provides an overview of the study, and it should take about fifteen minutes to read. Please feel free to discuss this document with family and friends. If there is anything you are not clear about, the contact details of the researchers are provided at the end. We will happily go through the information sheet with you and answer any questions you have.

We have compiled a list of Frequently Asked Questions (FAQs) which cover the main aspects of the research:

What is the purpose of the study?

Evidence suggests that people who have exercised regularly throughout their life might be at reduced risk of developing certain types of cancer, in particular colon (bowel) cancer. Secondly, bowel cancer survivors who exercise regularly are less likely to contract it again, and more likely to live longer. This might be related to changes seen in blood with physical exercise.

We think that these changes make colon cancer less likely to develop and spread. We want to test this by treating colon cells with serum from blood obtained from people who exercise regularly and people who do not exercise regularly.

Why have I been invited?

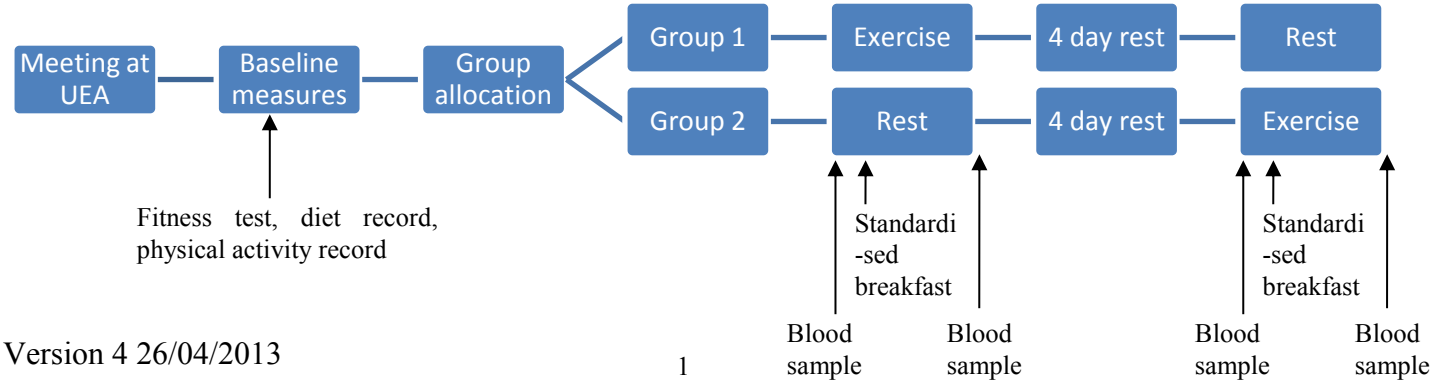
You have been selected as being a potentially suitable participant as you have responded to an advertisement for the study and you fit the study inclusion criteria.

Do I have to take part?

Your participation is entirely voluntary. You are also free to withdraw from the study at any time without giving reason.

What will happen to me if I take part/what do I have to do?

If you decide to take part and you meet our inclusion criteria we will invite you for a meeting at the Exercise Science Laboratory at the University of East Anglia, answer any questions you might have, and take written informed consent (see below for a flowchart showing what will happen if you take part).
After we take your consent we will ask you to complete two questionnaires: one about your general health which will take about 10 minutes to complete, and another about the amount of physical activity you have done throughout your life, which will take about one hour. Both will be completed at your informed consent meeting.



At the end of your informed consent meeting, you will be given a 4 day food record (to record your normal diet, including alcohol and other liquids) and a physical activity record to complete, and a small device, called an accelerometer, to wear on your hip for one week. This is the size of a small matchbox, about the same weight, and records any movements you do. When you return (at least one week later later) we will take your

- height
- weight
- blood pressure
- body fat percentage
- and perform an exercise stress test, which involves walking/running on a treadmill until you reach your exercise limit.

This will be conducted in the presence of a medical professional who will ensure that it is safe for you to complete the test. These will be your 'baseline measures'. You will also sign another consent form countersigned by the medical professional, which will permit us to take your blood during the study. We will make sure all of this can be completed at a time that suits you.

After the tests, you will be randomly allocated into either Group 1 or Group 2 (as shown in the flowchart)

At least 4 days later, you will arrive in the morning at the laboratory having not eaten for at least 10 hours. After you are rested and feel comfortable, the researcher (Mr Barnabas Shaw) will take a 16 ml (4 x 4 ml tubes) blood sample from your right or left arm (just above the crease of the elbow).

Afterwards, you will consume a standardised breakfast consisting of a plain bagel, a sachet of butter and strawberry jam, an orange juice drink, and as much water as you like. You will then complete either an 'Exercise' session on the treadmill, which will last for 45 min at continuous speed, or a 'Rest' session which will last for the same length of time depending on whether you are in Group 1 or 2.

After the session has finished, you will be given the opportunity to shower and change. Exactly 1 hour after finishing the session, the researcher will take another 16 ml blood sample from the other arm.

This process will be repeated at least 4 days later, with the only difference being that you will complete the session that you did not undergo first time round.

Between the sessions, you will be asked not to do any other exercise for 4 days prior to the second session (other than that you would normally do with work). We will also ask you to consume the same foods you have already recorded on your 4 day record.

Each time blood is taken, the yellow fluid in the blood (the 'serum') will be extracted, frozen and later used for treating the colon cells. We are planning to add the serum we collected from you to human colon cells (some of which are cancerous, others which are healthy). We will measure the growth of the cells and some of the factors produced by the cells which are associated with cancer risk and development after we add the serum. We think that changes in the serum caused by physical exercise (over a long period of training or after just one session) might affect growth and some of these factors.

Can I expect any payment/reimbursement of costs?

We will cover the cost of the standardised breakfast, travel costs and any parking charges you might incur.

What are the treatment alternatives?

We are not testing the effectiveness of exercise as a treatment for any disease in this study. This is an investigative study of the biological links between exercise and bowel cancer risk.

What are the possible disadvantages/risks of taking part?

The potential for risks to occur will be minimised. We will make sure that you can safely complete the exercise sessions before you take part, so that the likelihood of anything untoward happening during the exercise will be minimal. The medical professional will not be present for the exercise session, but the intensity of the session is less than that of the exercise stress test, which is designed to find out whether you can safely complete the exercise session There will be at least two individuals who are immediate life support trained in the vicinity of the laboratory whilst you do the exercise session. This session is tailored based on your capability and your heart rate will be monitored during the exercise.

In the event that something does go wrong and you are harmed during the research study, there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you might have grounds for legal action for compensation, but you could have to pay your legal costs.

Are there any side-effects of taking part?

If you haven't exercised for a while, you might find the fitness test and exercise sessions demanding. However, we will ensure that you are physically capable of doing the exercise before taking part.

What are the possible benefits of taking part in this study?

We cannot guarantee that you will benefit personally, but the information which we will obtain might help improve our understanding of the link between exercise and bowel cancer.

What happens when the research study stops?

We will not monitor you after your involvement in the study has finished. We will make data pertaining to health such as body composition, cardiorespiratory fitness and diet analysis available at the end of the study.

Will my taking part in the study be kept confidential?

The confidentiality of our patients and the data which this study will generate is of utmost importance. All data from this study will be anonymised with a unique code during the study so the researcher analysing your data will be unaware of your identity, which group you are in and to information collected during the study. This is one of the clauses, which you will sign in agreement on the official consent form. As soon as your involvement in the study has finished (i.e. after your final laboratory visit) any personally identifiable information such as your name, address and telephone number will be destroyed. We plan to store non-identifiable data such as height, weight and fitness level, together with any serum samples at the Institute of Food Research, until the project has been completed, after which these will be destroyed. Our procedures for handling, processing and storage of and destruction of data are compliant with the Data Protection Act 1998.

What if relevant new information becomes available?

We will inform you if relevant new information becomes available which might affect how the study is run. We will also discuss whether we need to make any amendments with the trial steering committee, which is responsible for the conduct of the research.

What will happen if I do not want to carry on with the study?

You are free to withdraw from the study at any time without giving reason. Should you wish, we can also destroy any identifiable samples that we have collected from you.

What if there is a problem?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of this study, then please contact the researcher (Mr Barnabas Shaw) in the first instance. Alternatively you can contact project co-ordinator (Professor John Saxton), or the Associate Dean for Postgraduate Research (Dr Christina Jerosch-Herold). All contact details are presented at the end of this document.

Will my GP be notified?

We will write and inform your family doctor that you are taking part in this study should you so wish, but if you are in otherwise good health this will not be necessary. However, if as a result of the exercise stress test an abnormality in your heart function is detected, we will inform your GP and recommend you visit them at the earliest possible opportunity.

What will happen to any samples I give?

The blood samples that you provide will be processed at the University of East Anglia Exercise Science Laboratory, and serum from this blood transferred to the Institute of Food Research, where we will use it for cell experiments and analyse it for indicators of bowel health. They will be stored there for the duration of the study. Responses to questionnaires, physical activity, diet and fitness data will be securely stored and analysed at the University of East Anglia for the duration of the study. Any leftover samples will be disposed of in accordance with the codes of practice at the University of East Anglia and Institute of Food Research.

Will any genetic tests be done?

No genetic data will be collected from you.

What will happen to the results of the research study?

We plan to publish the results of this study in a peer-reviewed scientific journal. However, you will not be personally identifiable from these results. In addition, the results from initial fitness testing and overall conclusions of the study will be available to you. Any further information will be available upon request.

Who is organising and funding the research?

The research forms part of a PhD programme funded by the University of East Anglia. The research is being conducted in collaboration with the Institute of Food Research.

Who has reviewed the study?

This study has been reviewed and given favourable opinion by University of East Anglia Faculty of Medicine and Health Sciences Ethics Committee.

Further information and contact details

If you have any specific questions about the study, we will be more than happy to answer them for you.

In the event of further questions please contact:

Mr Barnabas Shaw BSc. MSc. Email: B.Shaw@uea.ac.uk

Research Tel: 07933090196

Thank you for taking the time to consider participating in this study

Prof John Saxton (Project co-ordinator), Tel: 01603 593098, Email: john.saxton@uea.ac.uk
Dr Christina Jerosch-Herold (Associate Dean for Postgraduate Research), Tel: 01603 593316, Email: C.Jerosch-herold@uea.ac.uk

Appendix Civ

PARTICIPANT CONSENT FORM

The University of East Anglia

Does Regular and acute exercise affect Growth of colon cells (ReGro)?

Participant Identification Number for this study:

Investigators: Mr Barnabas Shaw, Professor John Saxton, Dr Nigel Belshaw

Name of participant:

Please initial box

1. **I confirm that I have read and understood the Participant Information Sheet Version ____ dated ____/____/____ for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.** ☐
2. **I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.** ☐
3. **I consent for samples collected during the study (i.e. serum from venous blood) to be transferred to the Institute of Food Research for analysis purposes** ☐
4. **I agree to take part in the above study.** ☐

Name of Participant	Date	Signature
Name of individual taking consent (if not researcher)	Date	Signature
Researcher	Date	Signature

2 copies to be kept; 1 for site file, 1 for participant.

Appendix Cv

Physical Activity Diary **Participant:** **Date:**

Day:

Time in minutes	Activity

Physical Activity Diary **Participant:** **Date:**

Day:	
Time in minutes	Activity

Physical Activity Diary **Participant:** **Date:**

Day:	
Time in minutes	Activity

Physical Activity Diary **Participant:** **Date:**

Day:	
Time in minutes	Activity

Physical Activity Diary **Participant:** **Date:**

Day:	
Time in minutes	Activity

Physical Activity Diary **Participant:** **Date:**

Day:	
Time in minutes	Activity

Physical Activity Diary **Participant:** **Date:**

Day:	
Time in minutes	Activity

Life Calendar

Similar to timelines, life calendars are used to document events in your life as they occurred in time. Research has shown that when questions are asked about events and activities from the past, life calendars can be very useful in helping you to remember these events more accurately.

Our questionnaire will ask you about physical activity performed from the age of 21 years onwards. Therefore, to help you recall events, we will construct your own life calendar. To do this we would like you to try and remember the year of certain important life events, both happy and sad. We will ask you a set of questions to guide you, but you do not have to answer them if you feel uncomfortable.

We will then use the calendar to refer back to whilst filling in the questionnaire. However, the details you give here will not be used as part of the research project and at the end of the interview the calendar will be yours to keep.

All information you provide will be treated as confidential information.

To know when to start your life calendar, we need to know:-

1. What is your date of birth?.....
2. What was the year was when you were 21 years old?.....

Life Calendar questions

Housing

1. What year did you buy your first house?
2. Do you remember any other house moves or changes in location as particularly memorable?

Education and Work

3. Have you been a student at any time from the age of 21 years old?
4. Do you have any memorable job or career changes from the age of 21 years old?

Life Events

5. If you are married, what year did you get married?
6. If you have children, what year were they born?
7. If you have grandchildren, what year were they born?
8. Do you remember any significant deaths in your family or close friends?
9. Have you ever been divorced?

Health

10. Do you remember any particular time when you were admitted to hospital or had a long period of time off work due to illness?
11. Have you had any significant accidents or injuries that you remember?

Holidays and Travel

12. Are there any holidays you consider particularly memorable?
13. Have you ever spent a longer period of time travelling abroad or around the country?

Others

14. Can you recall any other memorable events in your life, such as passing a test/exam or winning the lottery?

Life calendar: Years 1959 – 2013

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
1959	Fidel Castro becomes Cuban Premier First sections of M1 motorway opened						
1960	Ben Hur wins Oscar for Best Picture Nigeria gains independence from UK Farthing removed from UK currency						
1961	John F. Kennedy becomes President, Spurs do League and Cup double						
1962	Telstar launched Marilyn Monroe found dead Pete Best replaced by Ringo Starr Cuban Missile crisis The 'Big Freeze' in the UK, 1962-63						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
1963	Beeching report published First Bond film (Dr No) released Martin Luther King 'I have a dream' speech JFK assassinated						
1964	BBC2 starts broadcasting Daily Herald replaced by The Sun Harold Wilson becomes Prime Minister						
1965	Winston Churchill dies						
1966	Hosts England win football World Cup						
1967	Beatles release Sgt Pepper's Lonely Hearts Club Band (Summer of Love) Che Guevara captured in Bolivia						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
1968	Enoch Powell delivers 'Rivers of Blood' speech Robert F. Kennedy assassinated Black Power Salute from John Carlos and Tommie Smith at Mexico Olympics						
1969	Apollo 11 Moon landing						
1970	Apollo 13 mission Beatles disband Brazil '70 World Cup						
1971	Miners' strike The Godfather released in cinemas UK currency goes decimal						
1972	Bobby Fischer becomes world chess champion Munich Olympic massacre						
1973	UK enters EEC Watergate scandal emerges						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
1974	Muhammad Ali defeats George Foreman in the 'Rumble in the Jungle'						
1975	Birmingham Six arrested Fall of Saigon signifies end of Vietnam War						
1976	Raid on Entebbe, Agatha Christie dies, UK summer heatwave						
1977	Red Rum wins 3rd Grand National Elvis Presley dies						
1978	John Paul II becomes Pope Hosts Argentina win World Cup						
1979	Margaret Thatcher becomes Prime Minister Lord Mountbatten assassinated						
1980	John Lennon shot Ronald Reagan becomes president						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
1981	Diana and Charles wedding, Anwar Sadat assassinated						
1982	Falklands War						
1983	USA invades Grenada						
1984	World Aid following drought in Ethiopia						
1985	Live Aid Concert at Wembley						
1986	Space Shuttle 'Challenger' disaster Maradona 'Hand of God'						
1987	Great storm of 1987 Black Monday						
1988	Lockerbie Airline bomb						
1989	Berlin wall destroyed						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
1990	Nelson Mandela released Margaret Thatcher resigns Gazza's tears at Italia '90						
1991	Gulf War						
1992	European Union formed. Summer Olympics in Barcelona						
1993	Waco massacre						
1994	Nelson Mandela elected President of South Africa Channel Tunnel opens						
1995	Kobe earthquake South Africa win Rugby World Cup						
1996	Euro '96 in UK, Summer Olympics in Atlanta						
1997	Tony Blair elected Prime Minister, Princess Diana dies						
1998	France win football World Cup						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
1999	Euro currency established War in Kosovo Manchester United win Treble Millennium celebrations						
2000	Concorde crash in Paris George Bush elected President						
2001	September 11th attacks Foot and Mouth outbreak						
2002	Queen Elizabeth II Golden Jubilee War in Afghanistan begins						
2003	Iraq War begins						
2004	Indian Ocean tsunami						
2005	Pope John Paul II dies Labour wins third successive General Election						
2006	Italy win World Cup in Germany						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
2007	iPhone released Benazir Bhutto assassinated						
2008	Beijing Olympics Global Financial Crisis begins Barack Obama elected President						
2009	Swine flu pandemic Michael Jackson dies						
2010	Iceland volcano ash cloud Aung San Suu Kyi released Tory/Lib Dem coalition government formed						
2011	Hosni Mubarak resigns, Colonel Gaddafi, Osama bin Laden killed Prince William and Kate Middleton married						
2012	London Olympics Manchester City win football Premiership						

Yr	World Events	Housing	Education/Work	Life Events	Health	Travel	Others
2013	Pope Benedict XVI resigns Hugo Chavez dies Sir Alex Ferguson retires						

Historical Adulthood Physical Activity Questionnaire (HAPAQ)

Period between 21 and 29 years old

Activity in and around the house

Between the ages of 21 and 29 years old, how many hours on average per day did you spend **sitting at home**? This includes activities such as eating, drinking, reading, doing needlework, listening to radio, watching TV. You should give a separate answer for working and non-working days.

Hours per working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Hours per non-working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Did you do any **regular housework**? This includes preparing food, cooking, washing up, cleaning the house, doing the laundry and ironing. This should be on average at least one hour per day for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular DIY or house and car maintenance** (excluding gardening)? This should be on average at least one hour per week for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular gardening**? This includes mowing, watering, weeding, pruning, digging, chopping wood. This should be on average at least one hour per week for at least one growing season.

Yes	○	***No***	○

For how many growing seasons?

Years	

Excluding travel to and from work, what **form of transportation** did you use most often **to get about**? You can give more than one form of transport if you think you used them equally as much.

Usual mode of travel			
Car / motor vehicle	***Walk***	***Public transport***	***Cycle***
☐	☐	☐	☐

Activity at work

Between the ages of 21 and 29 years old, how many years were you in each of the following occupations?

	Years
Student	
Employed *(Paid and unpaid: voluntary work)*	
Housewife/husband	
Retired	
Professional sport person	

Guide: The sum of years should not be over 10, unless the participant has been carrying 2 occupations the same time.

I am now going to show you some pictures which demonstrate four different types of work classified depending on the intensity of physical activity involved.

During this decade try to remember the type of activity your work involved, excluding housework. Try to classify each job into one of these four categories, which ever is most representative of what you did.

For each category, how many years of the decade did you hold such a job and how many hours per week on average did you work?

	Years	***Months***	***Hours per week***
Sedentary occupation			
Standing occupation			
Manual work			
Heavy manual work			

Guide: The sum of years should not be over the number of years previously indicated unless some works have been held concomitantly.

For how many years did you work as a **professional sports person**? On average how many hours per week did you do this job? What sport was it?

	Years	***Months***	***Hours per week***
Sport:			

Commuting to work

Between the ages of 21 and 29 years old, regarding travel to and from your place of work or study, did you ever **regularly** travel **by bike**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Years	

On average, how many journeys per week did you cycle, counting return journeys only?

Number of journeys	

On average, how many miles was that return journey?

Mileage	

Did you ever **regularly** travel to your place of work or study **by foot**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Number of journeys	

On average, how many journeys per week did you walk, counting return journeys only? On average, how many miles was that return journey?

Mileage	

Sporting and Recreational Activity

From the ages of 21 to 29 years old, did you do any **regular sports in a competitive or strenuous nature**? By this we mean it made you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 60 and 69 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Swimming			
Cycling			
Running			
Football, rugby or hockey			
Cricket			
Volleyball, basketball or netball			
Racket sports			
Aerobics			
Fighting sports			
Other sport:			
Other sport:			

From the ages of 60 to 69 years old, did you undertake any **regular sport or recreational exercise on a casual basis**? This means it was not competitive and didn't make you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 60 and 69 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Walking for pleasure			
Swimming for pleasure			
Jogging			
Conditioning exercises *(Yoga, weights…)*			
Golf			
Bowling			
Fishing or hunting			
Other exercise undertaken on a casual basis:			
Other exercise undertaken on a casual basis:			

Period between 30 and 39 years old

Activity in and around the house

Between the ages of 30 and 39 years old, how many hours on average per day did you spend **sitting at home**? This includes activities such as eating, drinking, reading, doing needlework, listening to radio, watching TV. You should give a separate answer for working and non-working days.

Hours per working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Hours per non-working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Did you do any **regular housework**? This includes preparing food, cooking, washing up, cleaning the house, doing the laundry and ironing. This should be on average at least one hour per day for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular DIY or house and car maintenance** (excluding gardening)? This should be on average at least one hour per week for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular gardening**? This includes mowing, watering, weeding, pruning, digging, chopping wood. This should be on average at least one hour per week for at least one growing season.

Yes	○	***No***	○

For how many growing seasons?

Years	

Excluding travel to and from work, what **form of transportation** did you use most often **to get about**? You can give more than one form of transport if you think you used them equally as much.

Usual mode of travel			
Car / motor vehicle	***Walk***	***Public transport***	***Cycle***
☐	☐	☐	☐

Activity at work

Between the ages of 30 and 39 years old, how many years were you in each of the following occupations?

	Years
Student	
Employed *(Paid and unpaid: voluntary work)*	
Housewife/husband	
Retired	
Professional sport person	

Guide: The sum of years should not be over 10, unless the participant has been carrying 2 occupations the same time.

I am now going to show you some pictures which demonstrate four different types of work classified depending on the intensity of physical activity involved.

During this decade try to remember the type of activity your work involved, excluding housework. Try to classify each job into one of these four categories, which ever is most representative of what you did.

For each category, how many years of the decade did you hold such a job and how many hours per week on average did you work?

	Years	***Months***	***Hours per week***
Sedentary occupation			
Standing occupation			
Manual work			
Heavy manual work			

Guide: The sum of years should not be over the number of years previously indicated unless some works have been held concomitantly.

For how many years did you work as a **professional sports person**? On average how many hours per week did you do this job? What sport was it?

	Years	***Months***	***Hours per week***
Sport:			

Commuting to work

Between the ages of 30 and 39 years old, regarding travel to and from your place of work or study, did you ever **regularly** travel **by bike**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Years	

On average, how many journeys per week did you cycle, counting return journeys only?

Number of journeys	

On average, how many miles was that return journey?

Mileage	

Did you ever **regularly** travel to your place of work or study **by foot**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Number of journeys	

On average, how many journeys per week did you walk, counting return journeys only? On average, how many miles was that return journey?

Mileage	

Sporting and Recreational Activity

From the ages of 30 to 39 years old, did you do any **regular sports in a competitive or strenuous nature**? By this we mean it made you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 30 and 39 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Swimming			
Cycling			
Running			
Football, rugby or hockey			
Cricket			
Volleyball, basketball or netball			
Racket sports			
Aerobics			
Fighting sports			
Other sport:			
Other sport:			

From the ages of 30 to 39 years old, did you undertake any **regular sport or recreational exercise on a casual basis**? This means it was not competitive and didn't make you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 30 and 39 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Walking for pleasure			
Swimming for pleasure			
Jogging			
Conditioning exercises *(Yoga, weights…)*			
Golf			
Bowling			
Fishing or hunting			
Other exercise undertaken on a casual basis:			
Other exercise undertaken on a casual basis:			

Period between 40 and 49 years old

Activity in and around the house

Between the ages of 40 and 49 years old, how many hours on average per day did you spend **sitting at home**? This includes activities such as eating, drinking, reading, doing needlework, listening to radio, watching TV. You should give a separate answer for working and non-working days.

Hours per working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Hours per non-working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Did you do any **regular housework**? This includes preparing food, cooking, washing up, cleaning the house, doing the laundry and ironing. This should be on average at least one hour per day for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular DIY or house and car maintenance** (excluding gardening)? This should be on average at least one hour per week for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular gardening**? This includes mowing, watering, weeding, pruning, digging, chopping wood. This should be on average at least one hour per week for at least one growing season.

Yes	○	***No***	○

For how many growing seasons?

Years	

Excluding travel to and from work, what **form of transportation** did you use most often **to get about**? You can give more than one form of transport if you think you used them equally as much.

Usual mode of travel			
Car / motor vehicle	***Walk***	***Public transport***	***Cycle***
☐	☐	☐	☐

Activity at work

Between the ages of 40 and 49 years old, how many years were you in each of the following occupations?

	Years
Student	
Employed *(Paid and unpaid: voluntary work)*	
Housewife/husband	
Retired	
Professional sport person	

Guide: The sum of years should not be over 10, unless the participant has been carrying 2 occupations the same time.

I am now going to show you some pictures which demonstrate four different types of work classified depending on the intensity of physical activity involved.

During this decade try to remember the type of activity your work involved, excluding housework. Try to classify each job into one of these four categories, which ever is most representative of what you did.

For each category, how many years of the decade did you hold such a job and how many hours per week on average did you work?

	Years	***Months***	***Hours per week***
Sedentary occupation			
Standing occupation			
Manual work			
Heavy manual work			

Guide: The sum of years should not be over the number of years previously indicated unless some works have been held concomitantly.

For how many years did you work as a **professional sports person**? On average how many hours per week did you do this job? What sport was it?

	Years	***Months***	***Hours per week***
Sport:			

Commuting to work

Between the ages of 40 and 49 years old, regarding travel to and from your place of work or study, did you ever **regularly** travel **by bike**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Years	

On average, how many journeys per week did you cycle, counting return journeys only?

Number of journeys	

On average, how many miles was that return journey?

Mileage	

Did you ever **regularly** travel to your place of work or study **by foot**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Number of journeys	

On average, how many journeys per week did you walk, counting return journeys only? On average, how many miles was that return journey?

Mileage	

Sporting and Recreational Activity

From the ages of 40 to 49 years old, did you do any **regular sports in a competitive or strenuous nature**? By this we mean it made you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes		***No***	

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 40 and 49 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Swimming			
Cycling			
Running			
Football, rugby or hockey			
Cricket			
Volleyball, basketball or netball			
Racket sports			
Aerobics			
Fighting sports			
Other sport:			
Other sport:			

From the ages of 40 to 49 years old, did you undertake any **regular sport or recreational exercise on a casual basis**? This means it was not competitive and didn't make you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes		***No***	

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 40 and 49 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Walking for pleasure			
Swimming for pleasure			
Jogging			
Conditioning exercises *(Yoga, weights…)*			
Golf			
Bowling			
Fishing or hunting			
Other exercise undertaken on a casual basis:			
Other exercise undertaken on a casual basis:			

Period between 50 and 59 years old

Activity in and around the house

Between the ages of 50 and 59 years old, how many hours on average per day did you spend **sitting at home**? This includes activities such as eating, drinking, reading, doing needlework, listening to radio, watching TV. You should give a separate answer for working and non-working days.

Hours per working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Hours per non-working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Did you do any **regular housework**? This includes preparing food, cooking, washing up, cleaning the house, doing the laundry and ironing. This should be on average at least one hour per day for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular DIY or house and car maintenance** (excluding gardening)? This should be on average at least one hour per week for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular gardening**? This includes mowing, watering, weeding, pruning, digging, chopping wood. This should be on average at least one hour per week for at least one growing season.

Yes	○	***No***	○

For how many growing seasons?

Years	

Excluding travel to and from work, what **form of transportation** did you use most often **to get about**? You can give more than one form of transport if you think you used them equally as much.

Usual mode of travel			
Car / motor vehicle	***Walk***	***Public transport***	***Cycle***
☐	☐	☐	☐

Activity at work

Between the ages of 50 and 59 years old, how many years were you in each of the following occupations?

	Years
Student	
Employed *(Paid and unpaid: voluntary work)*	
Housewife/husband	
Retired	
Professional sport person	

Guide: The sum of years should not be over 10, unless the participant has been carrying 2 occupations the same time.

I am now going to show you some pictures which demonstrate four different types of work classified depending on the intensity of physical activity involved.

During this decade try to remember the type of activity your work involved, excluding housework. Try to classify each job into one of these four categories, which ever is most representative of what you did.

For each category, how many years of the decade did you hold such a job and how many hours per week on average did you work?

	Years	***Months***	***Hours per week***
Sedentary occupation			
Standing occupation			
Manual work			
Heavy manual work			

Guide: The sum of years should not be over the number of years previously indicated unless some works have been held concomitantly.

For how many years did you work as a **professional sports person**? On average how many hours per week did you do this job? What sport was it?

	Years	***Months***	***Hours per week***
Sport:			

Commuting to work

Between the ages of 50 and 59 years old, regarding travel to and from your place of work or study, did you ever **regularly** travel **by bike**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Years	

On average, how many journeys per week did you cycle, counting return journeys only?

Number of journeys	

On average, how many miles was that return journey?

Mileage	

Did you ever **regularly** travel to your place of work or study **by foot**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Number of journeys	

On average, how many journeys per week did you walk, counting return journeys only? On average, how many miles was that return journey?

Mileage	

Sporting and Recreational Activity

From the ages of 50 to 59 years old, did you do any **regular sports in a competitive or strenuous nature**? By this we mean it made you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 50 and 59 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Swimming			
Cycling			
Running			
Football, rugby or hockey			
Cricket			
Volleyball, basketball or netball			
Racket sports			
Aerobics			
Fighting sports			
Other sport:			
Other sport:			

From the ages of 50 to 59 years old, did you undertake any **regular sport or recreational exercise on a casual basis**? This means it was not competitive and didn't make you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 50 and 59 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Walking for pleasure			
Swimming for pleasure			
Jogging			
Conditioning exercises *(Yoga, weights…)*			
Golf			
Bowling			
Fishing or hunting			
Other exercise undertaken on a casual basis:			
Other exercise undertaken on a casual basis:			

Period between 60 and 69 years old

Activity in and around the house

Between the ages of 60 and 69 years old, how many hours on average per day did you spend **sitting at home**? This includes activities such as eating, drinking, reading, doing needlework, listening to radio, watching TV. You should give a separate answer for working and non-working days.

Hours per working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Hours per non-working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Did you do any **regular housework**? This includes preparing food, cooking, washing up, cleaning the house, doing the laundry and ironing. This should be on average at least one hour per day for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular DIY or house and car maintenance** (excluding gardening)? This should be on average at least one hour per week for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular gardening**? This includes mowing, watering, weeding, pruning, digging, chopping wood. This should be on average at least one hour per week for at least one growing season.

Yes	○	***No***	○

For how many growing seasons?

Years	

Excluding travel to and from work, what **form of transportation** did you use most often **to get about**? You can give more than one form of transport if you think you used them equally as much.

Usual mode of travel			
Car / motor vehicle	***Walk***	***Public transport***	***Cycle***
☐	☐	☐	☐

Activity at work

Between the ages of 60 and 69 years old, how many years were you in each of the following occupations?

	Years
Student	
Employed *(Paid and unpaid: voluntary work)*	
Housewife/husband	
Retired	
Professional sport person	

Guide: The sum of years should not be over 10, unless the participant has been carrying 2 occupations the same time.

I am now going to show you some pictures which demonstrate four different types of work classified depending on the intensity of physical activity involved.

During this decade try to remember the type of activity your work involved, excluding housework. Try to classify each job into one of these four categories, which ever is most representative of what you did.

For each category, how many years of the decade did you hold such a job and how many hours per week on average did you work?

	Years	***Months***	***Hours per week***
Sedentary occupation			
Standing occupation			
Manual work			
Heavy manual work			

Guide: The sum of years should not be over the number of years previously indicated unless some works have been held concomitantly.

For how many years did you work as a **professional sports person**? On average how many hours per week did you do this job? What sport was it?

	Years	***Months***	***Hours per week***
Sport:			

Commuting to work

Between the ages of 60 and 69 years old, regarding travel to and from your place of work or study, did you ever **regularly** travel **by bike**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Years	

On average, how many journeys per week did you cycle, counting return journeys only?

Number of journeys	

On average, how many miles was that return journey?

Mileage	

Did you ever **regularly** travel to your place of work or study **by foot**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Number of journeys	

On average, how many journeys per week did you walk, counting return journeys only? On average, how many miles was that return journey?

Mileage	

Sporting and Recreational Activity

From the ages of 60 to 69 years old, did you do any **regular sports in a competitive or strenuous nature**? By this we mean it made you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 60 and 69 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Swimming			
Cycling			
Running			
Football, rugby or hockey			
Cricket			
Volleyball, basketball or netball			
Racket sports			
Aerobics			
Fighting sports			
Other sport:			
Other sport:			

From the ages of 60 to 69 years old, did you undertake any **regular sport or recreational exercise on a casual basis**? This means it was not competitive and didn't make you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 60 and 69 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Walking for pleasure			
Swimming for pleasure			
Jogging			
Conditioning exercises *(Yoga, weights…)*			
Golf			
Bowling			
Fishing or hunting			
Other exercise undertaken on a casual basis:			
Other exercise undertaken on a casual basis:			

Period between 70 and 75 years old

Activity in and around the house

Between the ages of 70 and 75 years old, how many hours on average per day did you spend **sitting at home**? This includes activities such as eating, drinking, reading, doing needlework, listening to radio, watching TV. You should give a separate answer for working and non-working days.

Hours per working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Hours per non-working day					
Up to 1	***Up to 2***	***Up to 3***	***Up to 4***	***Up to 6***	***Over 6***
○	○	○	○	○	○

Did you do any **regular housework**? This includes preparing food, cooking, washing up, cleaning the house, doing the laundry and ironing. This should be on average at least one hour per day for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular DIY or house and car maintenance** (excluding gardening)? This should be on average at least one hour per week for at least one year.

Yes	○	***No***	○

For how many years?

Years	

Did you do any **regular gardening**? This includes mowing, watering, weeding, pruning, digging, chopping wood. This should be on average at least one hour per week for at least one growing season.

Yes	○	***No***	○

For how many growing seasons?

Years	

Excluding travel to and from work, what **form of transportation** did you use most often **to get about**? You can give more than one form of transport if you think you used them equally as much.

Usual mode of travel			
Car / motor vehicle	***Walk***	***Public transport***	***Cycle***
☐	☐	☐	☐

Activity at work

Between the ages of 70 and 75 years old, how many years were you in each of the following occupations?

	Years
Student	
Employed *(Paid and unpaid: voluntary work)*	
Housewife/husband	
Retired	
Professional sport person	

Guide: The sum of years should not be over 10, unless the participant has been carrying 2 occupations the same time.

I am now going to show you some pictures which demonstrate four different types of work classified depending on the intensity of physical activity involved.

During this decade try to remember the type of activity your work involved, excluding housework. Try to classify each job into one of these four categories, which ever is most representative of what you did.

For each category, how many years of the decade did you hold such a job and how many hours per week on average did you work?

	Years	***Months***	***Hours per week***
Sedentary occupation			
Standing occupation			
Manual work			
Heavy manual work			

Guide: The sum of years should not be over the number of years previously indicated unless some works have been held concomitantly.

For how many years did you work as a **professional sports person**? On average how many hours per week did you do this job? What sport was it?

	Years	***Months***	***Hours per week***
Sport:			

Commuting to work

Between the ages of 70 and 75 years old, regarding travel to and from your place of work or study, did you ever **regularly** travel **by bike**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Years	

On average, how many journeys per week did you cycle, counting return journeys only?

Number of journeys	

On average, how many miles was that return journey?

Mileage	

Did you ever **regularly** travel to your place of work or study **by foot**, at least once a week for at least one year?

Yes	○	***No***	○

For how many years did you do this?

Number of journeys	

On average, how many journeys per week did you walk, counting return journeys only? On average, how many miles was that return journey?

Mileage	

Sporting and Recreational Activity

From the ages of 70 to 75 years old, did you do any **regular sports in a competitive or strenuous nature**? By this we mean it made you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 70 and 75 years old?
For how many years did you do this sport and on average how many hours per week?

	Years	***Hours per week***	***Minutes per week***
Swimming			
Cycling			
Running			
Football, rugby or hockey			
Cricket			
Volleyball, basketball or netball			
Racket sports			
Aerobics			
Fighting sports			
Other sport:			
Other sport:			

From the ages of 60 to 69 years old, did you undertake any **regular sport or recreational exercise on a casual basis**? This means it was not competitive and didn't make you out of breath and/or sweat a lot. It must have been undertaken for at least one hour per week for at least one year.

Yes	○	***No***	○

I am now going to show you some illustrations of different sports which may be undertaken on a competitive level.

Can you identify your sport amongst these? If not what other sports did you do between the ages of 70 and 75 years old?
For how many years did you do this sport and on average how many hours per week?

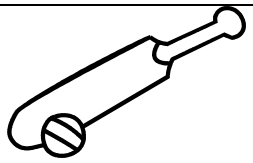
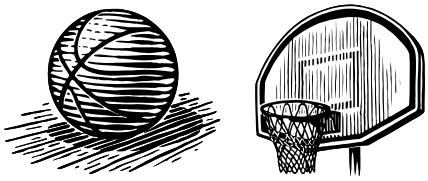
	Years	***Hours per week***	***Minutes per week***
Walking for pleasure			
Swimming for pleasure			
Jogging			
Conditioning exercises *(Yoga, weights…)*			
Golf			
Bowling			
Fishing or hunting			
Other exercise undertaken on a casual basis:			
Other exercise undertaken on a casual basis:			

Prompt cards

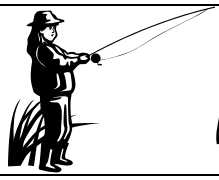
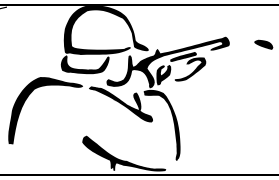
OCCUPATIONS

1. Sedentary occupation You spent most of your time sitting (such as in an office).	
2. Standing occupation You spent most of your time standing or walking. However, your work does not require intense physical effort (e.g. shop assistant, hairdresser, guard).	
3. Manual work This involved some physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter).	
4. Heavy manual work This implied very vigorous physical activity including handling of very heavy objects (e.g. dock worker, miner, bricklayer, construction worker).	

SPORTS

1. Swimming	
2. Cycling	
3. Running	
4.Football, rugby or hockey	
5. Cricket	
6. Volleyball, basketball or netball	
7. Racket sports	
8. Aerobics	
9. Fighting sports	
10. Other sports	

EXERCISES

1. Walking for pleasure	
2. Swimming for Pleasure	
3. Jogging	
4. Conditioning exercises (e.g. yoga, weights etc)	 
5. Golf	
6. Bowling	
7. Fishing or Hunting	 
8. Other exercises	