INSULIN RESISTANCE AND COLON CANCER PROMOTION IN RATS ON DIETS DIFFERING IN FAT, ENERGY, AND STARCH

by

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ABSTRACT

McKeown-Eyssen and Giovannucci proposed that hyperinsulinemia associated with insulin resistance (IR) promotes colorectal cancer (CRC). In this thesis, first, it was hypothesized that, if IR led to CRC, IR should appear prior to evidence of CRC promotion. It was shown that a high saturated fat diet given to F344 rats increased IR prior to evidence of CRC promotion. Second, it was hypothesized that, if IR led to CRC, the degree of promotion/inhibition of CRC by different diets should be correlated with the degree of promotion/inhibition of IR. Effects of dietary energy, fat, n-3 fatty acids and starch were tested on IR and CRC promotion. The degree of CRC promotion was correlated with the degree of IR ($r=0.65$, $p<0.001$). In addition, energy intake and body weight were highly correlated with IR and CRC promotion. It was concluded that increased circulating energy (glucose, triglycerides) may lead to both IR and CRC.

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LIST OF ABBREVIATIONS

ACF	Aberrant crypt foci
AOM	Axozymethane
a.u.c.	Area under curve
CCT	Cafeteria control diet
CG	Corn grits diet
CHO	Carbohydrate
CR	Calorie restricted diet
CRC	Colorectal cancer
DAG	Diacyl glycerol
eng	Energy
FFA	Free fatty acids
GI	Glycemic index
HF	High fat diet
HF3	High fat - high-n-3 diet
IR	Insulin resistance
IVGTT	intravenous glucose tolerance test
LF	Low fat diet
LF3	Low fat - high n-3 diet
MUFA	Monounsaturated fatty acids
NEFA	Non estrified fatty acids
NIDDM	Non insulin dependent diabetes mellitus
OGTT	Oral glucose tolerance test
PKC	Protein kinase C
SCFA	Short chain fatty acids
SEM	Standard error mean
SFA	Saturated fatty acids
TG	Triglyceride
USDA	United State Department of Agriculture
VLDL	Very low density lipoprotein

CHAPTER 1

INTRODUCTION

Colorectal cancer is a major cause of morbidity and mortality in western countries (National Cancer Institute of Canada, 1995). While genetic predisposition plays an important part in causing colon cancer (Cannon-Allbright et al., 1988, Vogelstein et al., 1988, Kinzler and Vogelstein, 1996), environmental factors are believed to play a much larger role (Higginson, 1969, Doll and Peto, 1981, Armstrong and Doll, 1975). Evidence from epidemiological studies suggest that diet and life style factors are involved in the etiology of colon cancer (Potter et al., 1993). Diets high in fat, energy, and readily digested carbohydrates are associated with increased colon cancer risk, while diets high in fruits, vegetables and fiber are associated with reduced risk (Giovannucci and Willett, 1994, Rogers et al., 1993, Wynder et al., 1992). Several mechanisms have been proposed to link these risk factors and colorectal cancer, such as a role for bile acids, antioxidants, short chain fatty acids and calcium (Potter, 1996, 1995, MacLennan et al., 1995, McKeown-Eyssen et al., 1988, Greenberg et al., 1994, McKeown-Eyssen et al., 1994, Bianchini et al., 1992). However, none of these mechanisms has provided a complete explanation.

Recently, a new mechanism has been proposed by McKeown-Eyssen and Giovannucci (McKeown-Eyssen, 1994, Giovannucci, 1995). They noted that risk factors for colon cancer, such as diets high in fat, energy, readily digested carbohydrates, and low in fiber, and a sedentary life style, are similar to those for insulin resistance. They hypothesized that these risk factors lead to insulin resistance and hyperinsulinemia, and that hyperinsulinemia through the growth promoting effect of insulin, promotes colon cancer. McKeown-Eyssen also suggested that other factors associated with insulin resistance such as elevated levels of

triglycerides and glucose, might also be important (McKeown-Eyssen, 1994). Early results of a case-control study suggest that colon polyp and cancer patients have higher levels of insulin, triglycerides and abdominal obesity than control patients (McKeown-Eyssen and the Toronto Polyp Prevention Group, 1996).

Insulin resistance is a syndrome characterized by an impaired response to an oral glucose load, increased fasting insulin and VLDL triglycerides and it is commonly associated with obesity. Insulin resistance is caused by genetic and/or environmental (high risk diet and sedentary lifestyle) factors which results in a resistance of peripheral cells to insulin action, and a compensatory increase in insulin production in order to normalize glucose level (DeFronzo and Ferrannini, 1991). In some cases insulin resistance can lead to non-insulin-dependent diabetes mellitus (NIDDM) through pancreatic β-cell "fatigue" (Khan, 1994).

Experimental studies support the association of insulin resistance and colon cancer. Colorectal cancer and insulin resistance share common risk factors, not only in the epidemiological and case-control studies (McKeown-Eyssen, 1994, Giovannucci, 1995), but also in experimental animal studies. Development of both is promoted by diets high in energy, fat and rapidly digested carbohydrate and inhibited by caloric restriction and n-3 fatty acids (Rizkalla et a., 1987, Clinton et al., 1992, Kern et al., 1990, Bull et al., 1979, Byrnes et al., 1995, Caderni et al., 1991, Okauchi et al., 1995, Kumar et al., 1990, Storlien et al., 1991, Reddy et al., 1991). However, none of these experimental studies has investigated the effect of the risk factors on insulin resistance and colon cancer at the same time in the same animals. Direct evidence for the effect of hyperinsulinemia on colon cancer has recently been evaluated in animal model in which exogenous insulin was injected daily into rats after initiation with a colon carcinogen (Tran et al., 1996, Corpet et al., 1997). Exogenous insulin promoted the

colon carcinogenesis in these studies but animals had 10 times higher insulin levels compared to controls, levels higher than those seen in food-stimulated hyperinsulinemia.

This thesis is concerned with a further investigation of the McKeown-Eyssen and Giovannucci hypothesis. Chapter 2 reviews the literature on risk factors for colorectal cancer and insulin resistance. Chapter 3 briefly presents the problem formulation, hypothesis and objectives of the thesis. Chapter 4 and 5 are the research studies papers. Chapter 4 has been published in Nutrition and Cancer (Koohestani et al., 1997), and chapter 5 has been submitted for publication. Experiments carried out in this thesis were designed by me with Dr. Bruce's guidance. I conducted and analyzed these experiments, and also benefited from the help of others (see acknowledgements). Chapter 6 is a general discussion with suggestions for further studies.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The importance of environmental factors in the development of colon cancer was considered briefly in the introduction. Some of the vast epidemiological literature and human studies on the importance of dietary factors, and putative mechanisms by which these risk factors might affect colon carcinogenesis, are reviewed below. Since many of risk factors for colon cancer are similar to those of insulin resistance and there may be a close relationship between the processes of these two conditions, a review of risk factors for insulin resistance and of possible mechanisms involved are also provided. Some experimental studies linking dietary risk factors and colon cancer, and dietary factors and insulin resistance are then reviewed. Finally, some of the previous studies investigating the relationship between colon cancer and insulin resistance are noted. This review thus provides a perspective of the present knowledge relating insulin resistance and colon cancer and provides a basis for the questions asked in my research work.

2.2 Colorectal cancer and risk factors

Colorectal cancer is the second leading cause of cancer mortality in North America (National Cancer Institute of Canada, 1995). UK incidence data show that colon cancer is the second most common malignancy after lung cancer in men and breast cancer in women (Muir et al., 1987). While genetic predisposition plays an important role for causing colon cancer (Cannon-Allbright et al., 1988, Vogelstein et al., 1988, Kinzler and Vogelstein, 1996),

environmental factors are also believed to be more important. The fact that colon cancer rates increase among groups immigrating from low incidence areas to high incidence areas (Haenszel and Kurihara, 1968) and the fact that rate of incidence in genetically stable populations is influenced by environment (Boyle et al., 1985), provide the evidence for the importance of environmental factors. Colon cancer is strongly associated with dietary patterns. Positive correlations have been observed with total energy intake, and with energy containing nutrients (fat, protein, and carbohydrate) in case control and cohort studies (Potter et al., 1993, Willett et al., 1990, Giovannucci et al. 1992). Obesity is also associated with risk of colorectal cancer in some studies (Bostick et al., 1994, Lew and Garfinkel, 1979, LeMarchand et al, 1992). Negative correlations have been reported with consumption of vegetables, fruits, cereals and fibers, and also with exercise (Steinmetz and Potter, 1991).

2.3 Colon cancer development

Colon cancer is traditionally considered to develop in three stages as shown in figure 2.1. The first is initiation in which cells through mutation or other changes lose or acquire functions that lead to abnormal control of cell proliferation and differentiation. The second is promotion in which these abnormal cells have a proliferative advantage. The third is progression in which clones of abnormal cells acquire the characteristics seen in malignant tumors.

The second and third stage in the pathology of malignant tumors in the colon follows a sequence of events known as the adenoma-carcinoma sequence (Morson, 1974). According to this theory, the process of tumor development begins with a mutation event with a normal epithelial cell, leading to abnormal proliferation and the formation of a small, clonal polyp or

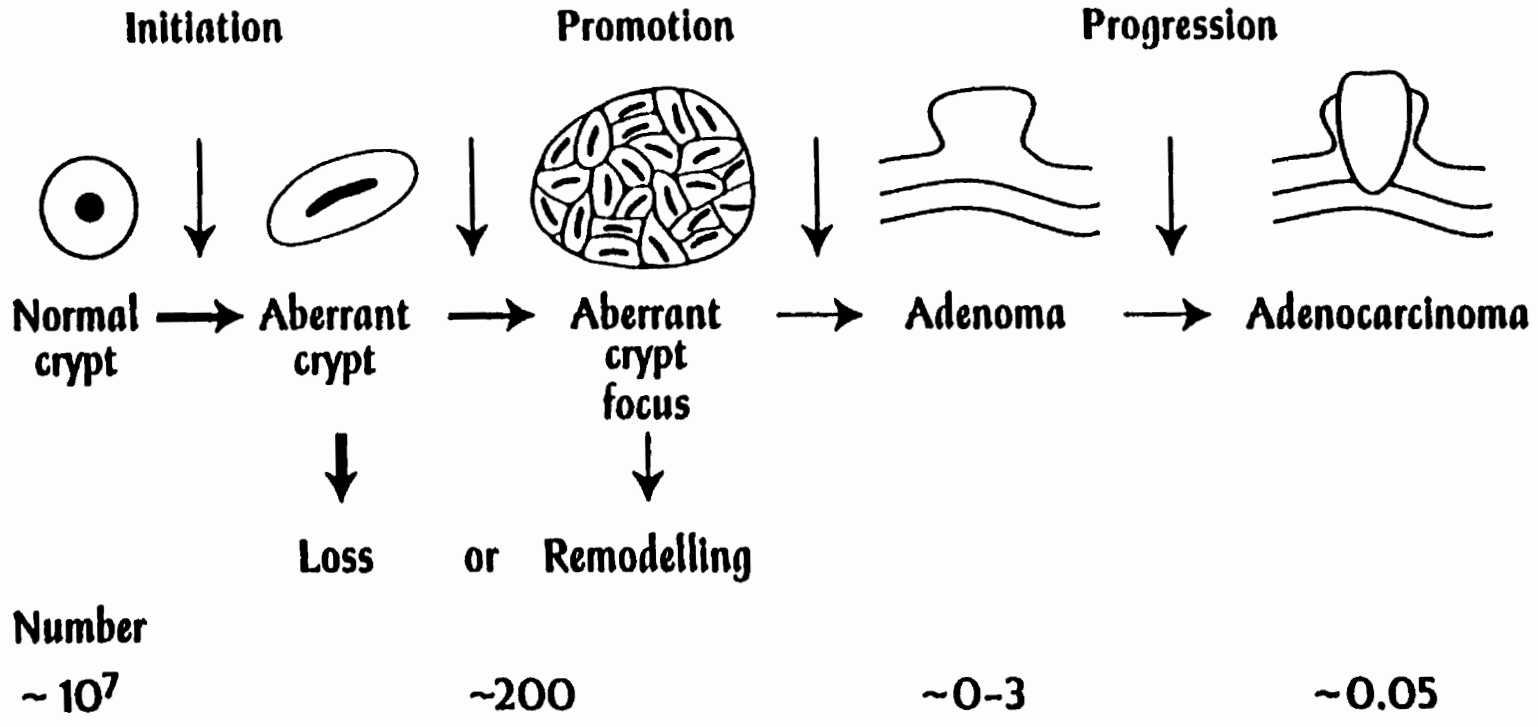


Fig 2.1. The multi-step process of colon carcinogenesis (Bruce et al., 1993).

adenoma (stage 1). Such adenomas, although benign, are widely believed to be precursor lesions to carcinomas. During the growth of adenomas (stage 2) a proportion of them will accumulate the genetic alterations necessary for progression (stage 3) to cancer. Several genetic alterations are known to occur in colon cancer (Fearon and Vogelstein, 1990, Vogelstein et al., 1988), which will not be discussed here. Generally, mutations in oncogenes stimulate cell division while loss of tumor suppressor genes leads to a loss of the control of normal cell division. Both result in inappropriate growth.

Diet may be important in all three stages of colon carcinogenesis. Initiation of tumorigenesis may occur through the action of chemicals such as heterocyclic amines or perhaps by free radicals generated in the metabolism of food which cause mutations in DNA. Although repair mechanisms are constantly operating, alteration of the DNA sequence once replicated can not be corrected, and the aberrant genetic format is propagated. Primitive dividing cells are, therefore, particularly susceptible to irreparable damage by carcinogenic or mutagenic agents, and factors that stimulate cell division tend to promote tumor development. Normal rates of proliferation in the colon are dependant on the presence of nutrients in the intestinal lumen. Cell turn over is inhibited during starvation or on feeding a liquid diet. Some dietary factors, such as fat, appear to increase and others, such as fiber, to inhibit proliferation.

2.3.1 Commonly used biomarkers of colon cancer risk

2.3.1.1. Proliferative activity of colon cells

Hyperproliferation of the colonic epithelium may be an early feature of the multi step process in the development of colon cancer (Fearon and Vogelstein et al., 1990). Proliferative

activity has been used as a measure of colon cancer risk in human and experimental animals (Paganelli et al., 1994, Steinbach et al., 1993). Proliferation is measured by several methods: bromodeoxyuridine labelling, [^{3}H]thymidine labelling, and PCNA. These methods are mostly used to measure the proliferative activity of differentiated colonic cells. Colonic epithelial stem cells are progenitor cells which can give rise to more of themselves as well as to other differentiated coloncytes in the crypt (Sato and Ahnen, 1992). Since these cells are long-lived, it is assumed that they are the cells that are at risk for accumulating sufficient mutations which may lead to the formation of tumors (Bjerknes, 1996).

2.3.1.2. ACF (aberrant crypt foci)

Aberrant crypt foci (ACF) are putative precursors of adenomas, colonic polyps and colon cancer. ACF may give rise to colon cancer through a multi-step process (Bruce et al., 1993). ACF can be viewed under light microscopy after methylene blue treatment. The crypts of ACF are characterized by their increased crypt size, elongated crypt opening, crowding of their nuclei, and thicker epithelial lining (McLellan and Bird, 1988). ACF were first described by Bird (Bird, 1987) in 1987 and since then have been studied as markers for colon cancer risk. ACF have been examined for dysplastic characteristics and molecular abnormalities associated with tumors (Roncucci et al., 1991, Stopera and Bird, 1992, Vivona et al., 1993). Although some ACF have dysplastic features, it is not clear whether all ACFs are preneoplastic. It is possible that some ACFs regress and disappear while others, which are exposed to promotional factors for a longer period of time, may lead to tumor formation.

2.4 Mechanisms linking dietary factors and colon cancer

The influence of fat on colon carcinogenesis is traditionally thought to be related to the stimulating effect of bile acids. Some studies (Aries et al., 1969, Hill et al., 1971) showed that feces from high risk Americans contained greater concentrations of biliary steroids than those from low risk Japanese. Other studies showed that patients with adenomatous polyps excrete greater quantities of bile acids than healthy subjects (Hill et al., 1975, Reddy and Wynder, 1977). Differences in the colon cancer incidence in different countries might be explained by differences in the ability of colonic bacteria to convert primary bile acids to carcinogenic metabolites (Aries et al., 1969, Hill et al., 1971). The stimulation of cell proliferation by bile acids may be mediated by the calcium dependent enzyme Protein Kinase C (PKC). Bile salts have been shown to stimulate membrane phospholipid turn over resulting in the release of diacyl glycerol (DAG) which activates PKC. Changes in PKC activity are in turn associated with increases in the activity of mucosal ornithine decarboxylase, a rate limiting enzyme in polyamine production (DeRubertis and Craven, 1987). It has also been suggested that DAG from malabsorbed fat acts as an intracellular mediator of cell proliferation through its effect on PKC.

Alternatively, the action of fat on cell proliferation may be a result of lipid oxidation products (Bull et al., 1984). Hydroperoxy and hydroxy fatty acids derived from linoleic and arachidonic acids may induce ornithine decarboxylase activity and DNA synthesis. Fish oil, rich in n-3 fatty acids, has been shown to inhibit cell proliferation in rectal mucosal cells of humans and animals (Anti et al., 1992, Rao and Reddy et al. 1993). The protective effect may relate to the replacement of arachidonic acid (20:4, n-6) in cell membranes by n-3 fatty acids, and its consequence in terms of reduced prostaglandin activity. Both arachidonic acid and

prostaglandins have been proposed to play a role in cell proliferation (Simopoulos, 1987, Dao and Hilf, 1992).

Increased risk of colon cancer with sugar consumption has been related to changes in bile acids and to bacterial metabolism in colon. High sucrose feeding increases fecal secretion of total and secondary bile acids in human (Kruis et al., 1991). It also increases pH in the colon compared to a starch based diet. pH may play a role in increased proliferation (Caderni et al., 1993). In addition, less digestible carbohydrates increase colonic fermentation which results in increased production of short chain fatty acids (SCFA), acetate, propionate and butyrate. Butyrate has been shown to inhibit cell proliferation (Bianchini et al., 1992, Young, 1992). The protective effect of dietary fiber may also be partly attributed to SCFA. The principal components of dietary fiber are non-starch polysaccharides which are not digested in the human small intestine, but are substrates for bacterial fermentation in the colon (Stephen and Cummings, 1980). In addition, fiber may inhibit carcinogenesis by increasing stool bulk and decreasing intestinal transit time therefore reducing the exposure of colon cells to carcinogens in the fecal stream (Cummings et al., 1992, 1982, Stephen et al., 1987, McPherson-Kay, 1987). The protective effect of vegetables and fruits may be partly attributed to their content of fiber. However, they also contain vitamins and other components that might scavenge free radicals or block carcinogen formation (Potter, 1992, Greenberg et al., 1994).

Most of the mechanisms that have been proposed to explain the relation between diet and colon cancer such as bile acids, assume that the risk factors associated with the diet reaches the colonic cells from the luminal surface. Only a few mechanisms such as those involving lipid oxidation products have proposed that risk factors could reach the colonic cells

by way of the general circulation. Almost all of the proposed mechanisms explain only some dietary risk factors. It was only recently that hypothesis proposed by McKeown-Eyssen and Giovannucci, linked dietary factors and colon cancer in a broader sense.

2.5 McKeown-Eyssen and Giovannucci hypothesis

This hypothesis is based on the striking similarity of the dietary and lifestyle factors for colon cancer with those for insulin resistance. Promotional effects of diets high in energy , fat, readily digested high glycemic index carbohydrates, and a sedentary lifestyle, and inhibitory effect of caloric restriction and n-3 fatty acids are common risk factors for both diseases. McKeown-Eyssen and Giovannucci hypothesized that these high risk diets and lifestyles lead to insulin resistance, a condition associated with elevated levels of insulin, glucose and triglycerides, and that insulin then promotes colon cancer. McKeown-Eyssen suggested further that an elevated level of energy as glucose and triglycerides may also be involved in the promotion of neoplastic cells and the appearance of colorectal cancer.

2.5.1 Evidence to support this hypothesis

Some epidemiological studies, have shown an association between non-insulin-dependent diabetes mellitus (a condition associated with insulin resistance) and colon cancer risk (Williams et al., 1984, Adami et al., 1991, Weiderpass et al., 1997). However, the association is not very strong.

Early results of a case-control study suggest that colon polyp and cancer patients have higher level of insulin, triglycerides and abdominal obesity than control patients (McKeown-Eyssen and the Toronto Polyp Prevention Group, 1996).

Direct evidence for the effects of hyperinsulinemia on colon cancer has been evaluated in animal models in which exogenous insulin was injected daily into rats after initiation with a colon carcinogen (Tran et al., 1996, Corpet et al., 1997). Exogenous insulin promoted the development of colon tumors and the growth of aberrant crypt foci (ACF), putative precursors of colon cancer (Bruce et al., 1993).

2.6 Insulin resistance and risk factors

Insulin resistance is characterized by an impaired response to an oral glucose load, increased fasting plasma insulin, increased VLDL triglycerides, increased abdominal obesity , and in some cases hyperglycemia (DeFronzo and Ferrannini, 1991, Hansen, 1995). Both genetic predisposition and environmental factors may lead to a resistance of peripheral cells to respond to insulin and to a decreased glucose transport. This in turn results in a compensatory increase in the production of insulin to reduce the level of plasma glucose. The most important environmental risk factors are likely energy intake and lack of physical activity because both are very important in terms of weight gain and obesity (Dowse et al., 1991). Positive association between obesity, serum triglycerides and blood glucose are well established (Ashley and Kannel, 1974, Committee on Diet and Health, 1989, Lovejoy and DiGirolamo, 1992, Hansen, 1995). Some studies have shown that exercise improves insulin sensitivity and lowers insulin and triglyceride levels (Koivisto et al., 1986, Regensteiner et al., 1991, Lindgarde and Saltin 1981, Huttunen et al., 1979). In addition diets high in fat and energy density and simple sugar carbohydrates are important in promoting (Coulston et al., 1983, Albrink and Ullrich, 1986), and diets high in fiber and n-3 fatty acids (Anderson et al., 1980, Marshall et al., 1991) in improving insulin resistance (World Health Organization,

1994).

2.7 Development of insulin resistance

Many investigators have shown that tissue sensitivity to insulin declines by ~30-40% when an individual is >35-40% over ideal weight (DeFronzo and Ferrannini, 1991). Resistance of peripheral cells to insulin action can be a result of obesity, of excessive energy intake or of a genetic defect in which a patient inherits a gene or set of genes that confer increased insulin resistance (DeFronzo and Ferrannini, 1991). Insulin resistance is associated with impaired glucose tolerance (World Health Organization, 1994), post-prandial and sometimes fasting hyperinsulinemia and hypertriglyceridemia (DeFronzo and Ferrannini, 1991). However, all of these effects may not be present at the same time. Insulin resistance can lead to non-insulin-dependent diabetes mellitus (NIDDM) in susceptible individuals, probably through pancreatic β-cell "fatigue" (Khan, 1994).

2.7.1 Commonly used measures for insulin resistance

These measures include the euglycemic clamp, glucose and insulin tolerance tests, and fasting and post-prandial insulin, triglycerides and glucose. In the euglycemic clamp method, subjects are given a continuous infusion of insulin and then an infusion of glucose sufficient to hold the plasma glucose in the normal range. As insulin resistance increases, peripheral cells are less efficient in removing glucose from the blood stream, and therefore less infused glucose is required to hold the plasma glucose in normal range. Insulin sensitivity is quantitavely calculated from the rate of glucose infusion. Oral and intravenous glucose tolerance tests (OGTT and IVGTT) are also used for indicating insulin resistance. In this test,

after a fasting period, a load of glucose is given to the subject either orally or through intravenous injection. Blood glucose and/or insulin are measured in the fasting state and at intervals after the glucose load for about two hours. Insulin resistant subjects have higher and more prolonged rises of plasma glucose and insulin compared to normal controls. Areas under these curves provide a measure of insulin resistance.

The differences between the methods are sensitivity of the detection and the degree of precision for abnormal insulin conditions. The euglycemic method is the standard measure for insulin resistance and has a high sensitivity and precision, but it requires catheterization of subject and technical skills. OGTT and IVGTT provide a reasonable sensitivity and precision (Bergman et al., 1985).

2.8 Mechanisms linking dietary risk factors and insulin resistance

Most studies show a strong correlation between obesity and insulin resistance (DeFronzo and Ferrannini, 1991). However, it is not clear whether obesity initiates the resistance of peripheral tissues to insulin and if it does, by what mechanism this occurs. It has been suggested that an intracellular glucose-fatty acid cycle in muscle is involved in determining insulin sensitivity. A high fat diet may induce insulin resistance by causing an accumulation of triglycerides in skeletal muscle. This accumulation affects the glucose-fatty acid cycle and results in less glucose uptake by muscle. Consequently, insulin sensitivity decreases (Storlien et al, 1991). An over supply of non-esterified fatty acids (NEFA) may play a role in this glucose-fatty acid cycle (Boden et al, 1991). Inhibitory effects of n-3 fatty acids on insulin resistance has been related to changes in cell membranes. It has been suggested that n-3 fatty acids increase membrane permeability to ion flux, and it increases

energy requirements of cells by ion pumping to maintain appropriate compartmental gradients (Else and Hulbert, 1987). It has been suggested that alterations in the energy requirement of cells increase insulin sensitivity (Storlien et al., 1993). A role for non- enzymatic, free radical-mediated oxidation of biological molecules or oxidative stress has also been suggested. Oxidative stress is a function of the balance between prooxidant factors and those scavenging them. Oxidative stress might impair insulin action by i) changing the physical state of the plasma membrane (permeability and fluidity) ii) increasing in the intracellular calcium content. Membrane protein mobility and lipid fluidity decrease when plasma free radicals increase. Changes in membrane may affect signalling pathways for insulin. Increased oxidative stress could be a result of over supply of fuel such as glucose and NEFA. Oxidative stress indicators are significantly correlated with NEFA concentration (Paolisso and Giugliano, 1996). There is a strong negative correlation between calcium content of cells and insulin sensitivity in vitro but the mechanism of action and importance is unknown at the present (Paolisso and Giugliano, 1996).

2.9 Experimental animal studies and risk factors

2.9.1 Energy intake

Animal studies manipulating dietary energy have used either a cafeteria diet or a caloric restricted diet to provide higher or lower than normal energy intake levels.

Excess energy intake increases colon cancer promotion as well as insulin resistance. In cancer studies excess energy has been provided by very high fat diets though the effects of fat and energy are not really separated (Narisawa et al., 1991, Bird and Lafave, 1995). Although

a clear association between excess energy intake and increased risk of colon cancer has been established in human studies (Bostick et al., 1994), the relationship between excess energy intake and colon cancer has not been modeled in animal studies. One animal study using a 3 x 3 factorial design showed that *ad libitum* energy intake was significantly associated with intestinal carcinogenesis in Sprague-Dawley rats (Clinton et al., 1992). The study was originally designed to examine the effect of different amounts of protein and fat on intestinal carcinogenesis. However, different diets resulted in different energy intakes and the effect of energy intake was greater than the effect of protein or fat. Excess energy intake results in increased body weight, and it has been shown that exercise and reduced body weight reduce tumor incidence (Kritchevsky, 1993, Thorling et al., 1993).

In studies related to insulin resistance, excess energy intake has been provided by cafeteria feeding. It is a common feeding regimen used in studies of obesity syndromes in animals, it effectively induces the same conditions that give rise to obesity in humans (Louis-Sylvestre et al., 1984, Mandenoff et al., 1982, Rogers and Blundell., 1984, Rizkalla et al., 1987, Rolls et al., 1980, Sclafani and Springer, 1976, Llado et al., 1995). The essentials of a cafeteria diet are palatability and variety. Palatability refers to the preference value for a particular food and variety refers to the availability of different types of foods. Both can independently increase food intake (Rogers and Blundell, 1984). The cafeteria diet is characterized by the presentation of highly palatable "supermarket" foods in a combination of 3-4 foods each day in addition to an animal standard diet (Rizkalla et al., 1987, Mandenoff et al., 1982). Greatest energy intake is observed with high fat food choices (Harris, 1993) and simultaneous presentation of foods (Rolls et al., 1980). Insulin sensitivity of peripheral tissues decreases in a relatively short period of time, about 3 weeks, in animals on cafeteria diet

(Rizkalla et al., 1987).

Restricted energy intake has been well defined and commonly used in both cancer and insulin resistance studies. A calorie restricted diet is a straightforward restriction on the *ad libitum* calorie intake of a reference diet with controlled intake of protein, fat, fiber and macronutrients (Klurfeld et al., 1989). Caloric restriction of 30% - 40% has been shown to have a protective effect on colon carcinogenesis (Kumar et al., 1990). In a study by Reddy and co-workers four dietary groups were compared: low fat *ad libitum*, 20% restricted from low fat, high fat (3 times more than low fat) *ad libitum*, and 30% restricted from high fat. The high fat group had significantly higher colonic tumor incidence compare to all other groups, while the restricted high fat group had a tumor incidence as low as the low fat and restricted low fat groups (Steinbach et al., 1993).

Caloric restriction also improves insulin sensitivity and glucose uptake, and it lowers insulin levels (Okauchi et al., 1995, Bodkin et al., 1995, Escriva et al., 1992).

Exercise also improves insulin resistance (Nagasawa et al., 1995).

2.9.2 Fat intake

Animal studies suggest that the amount and type of dietary fat are important factors in both colon cancer and insulin resistance. Increased tumor incidence in rats fed high saturated fat or high n-6 fatty acid diets have been reported many times (Reddy et al., 1977, Reddy and Sugie, 1988). Bird and co-workers showed that a high fat diet increased the number of aberrant crypt per focus (Lafave et al., 1994). The promoting effect of a high fat diet on insulin resistance has been very well documented in animal studies as well. High fat diets increase plasma insulin, reduces insulin sensitivity and impair glucose tolerance (Barnard et al.,

1993, Harris and Jones, 1991, Kraegen et al., 1991). Fish oil, perilla oil and linseed oil have been used as a source of n-3 fatty acids in colon cancer as well as insulin resistance studies. n-3 fatty acids have been known to have a protective effect on both colon carcinogenesis and insulin resistance (Reddy et al., 1991, Narisawa et al.,1991, Storlien et al., 1991).

2.9.3 Carbohydrate intake

Dietary glucose, either in the form of simple sugars or starch, eventually is absorbed into the blood, producing an increased blood glucose level and a compensatory increase of insulin. However, the rise in blood glucose is directly related to the rate of absorption of dietary glucose. The rate of digestibility of carbohydrates is a determining factor in their promoting or protective effects. Diets high in readily digested carbohydrates promote colon cancer and also impair glucose tolerance (Stamp et al., 1993, Grimditch et al., 1988, Pagliassotti et al.,1994). High starch diets with a lower glycemic index reduce colon carcinogenesis as well as improve insulin resistance (Caderni et al.,1991, 1993, Bianchini et al., 1992, Thorup et al., 1995, Lerer-Metzger et al., 1996, Byrnes, et al., 1995).

2.10 Introduction to experimental work

The effect of dietary factors on colon cancer and insulin resistance has been investigated previously only in separate experiments. To investigate the McKeown-Eyssen and Giovannucci hypothesis, first step was to demonstrate the effect of some of the dietary risk factors on measures of insulin resistance and colon cancer promotion in one experiment in the same animals. The questions asked were: **i)** Does evidence of insulin resistance appear before evidence of colon cancer promotion? **ii)** Do different dietary risk factors affect insulin

resistance and colon cancer promotion to the same degree? Research work presented in the following chapters aims to answer these questions.

CHAPTER 3

HYPOTHESIS AND OBJECTIVES

3.1 Introduction

McKeown-Eyssen and Giovannucci noted that insulin resistance and colorectal cancer have similar risk factors. They hypothesized that high risk diets lead to insulin resistance, and factors associated with insulin resistance then lead to the promotion of colon cancer. They suggested that the hyperinsulinemia associated with insulin resistance leads to promotion through the growth promoting effect of insulin. This thesis tests the relationships between diet, insulin resistance and colon cancer promotion based on logical inferences drawn from McKeown-Eyssen and Giovannucci hypothesis.

3.2 Hypothesis

If insulin resistance leads to colon cancer promotion, at least two conditions must be fulfilled. These conditions are necessary but not sufficient to prove causality. They are:

i) Evidence of insulin resistance must appear before the evidence of colon cancer promotion.

ii) The degree of promotion or inhibition of colon cancer by different diets must be correlated with the degree of promotion or inhibition of insulin resistance by those diets.

Insulin resistance can be assessed by the oral glucose tolerance test (OGTT) and colon cancer promotion can be assessed by the average size of aberrant crypt foci (ACF) 100 days after initiation with carcinogen.

Therefore, two questions are asked in this thesis:

i) Does evidence of insulin resistance (impaired OGTT) appear before evidence of colon

cancer promotion (increased ACF size at 100 days) ?

ii) Do different dietary risk factors affect insulin resistance (OGTT) and colon cancer promotion (ACF) to the same degree?

3.3 Objectives

i) To determine if there is evidence of insulin resistance (assessed by OGTT) prior to the evidence of colon cancer promotion (assessed by size of ACF) in F344 rats on a high fat diet.

ii) To determine if there are correlations between measures of insulin resistance and colon cancer promotion (ACF) in animals fed diets with different potential risk for both conditions.

CHAPTER 4

INSULIN RESISTANCE AND PROMOTION OF ABERRANT CRYPT FOCI IN THE COLONS OF RATS ON A HIGH-FAT DIET

4.1 Introduction

As mentioned before, McKeown-Eyssen and Giovannucci (McKeown-Eyssen, 1994, Giovannucci, 1995) noted that risk factors for colorectal cancer (CRC) are similar to those for insulin resistance (IR). Thus, they proposed that insulin resistance and hyperinsulinemia associated with insulin resistance lead to colon cancer promotion.

Animal studies show that dietary factors that lead to increased IR also seem to lead to increased CRC. IR and CRC appear to be promoted by dietary saturated fat (Kern et al., 1990, Bull et al., 1979) and readily digested carbohydrate (Byrnes et al., 1995, Caderni et al., 1991) and to be inhibited by calorie restriction (Okauchi et al., 1995, Kumar et al., 1990), some dietary fibers (Wakabayashi et al., 1995, Jacob, 1987, Madar et al., 1993), and n-3 fatty acids (Reddy et al., 1991, Storlien et al., 1991). Support for a specific relationship between insulin and colon carcinogenesis has recently been reported. Repeated injections of insulin promote tumor development in the colon of the rat (Tran et al., 1996). In addition, Corpet and co-worker (Corpet et al., 1997) recently showed that insulin promotes the growth of aberrant crypt foci (ACF), putative precursors of colon cancer (Bird, 1987, Bruce et al., 1993, Kinzler and Vogelstein, 1996).

The results of a further test of the IR hypothesis are reported here. It was reasoned that if diets affect colon carcinogenesis through their effects on hyperinsulinemia and IR, evidence of colon cancer promotion would necessarily be preceded by IR and increased levels

of plasma insulin. This will be the answer to question 1 in chapter 3. To test this expectation, the effects of a diet high in animal fat and a low-fat diet were compared on measures of IR and tumor promotion in F344 rats, a strain and species frequently used in carcinogenesis studies. Promotion of CRC was assessed with the growth of ACF, which permits the unambiguous assessment of promotion as early as 100 days after initiation (Bird, 1987, Bruce et al., 1993). IR was assessed indirectly with oral glucose tolerance tests (OGTT) and with fasting insulin and triglyceride, inasmuch as differences in these measures have been observed in cancer patients (Marks and Bishop, 1957) and in a colonic polyp and cancer case-control study (McKeown-Eyssen and Toronto Polyp Prevention Group, 1996). Thus the expectation was that initiated rats fed the high-fat diet would show promotion with increased ACF size at 100 days, and the question was: Is there evidence of IR before 100 days?

4.2 Materials and methods

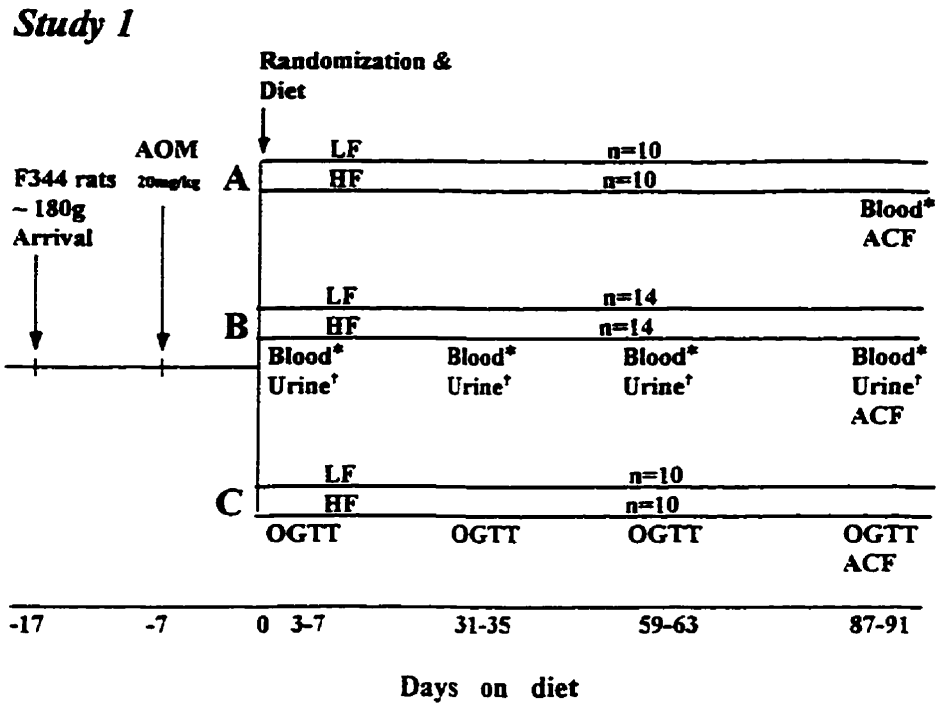
4.2.1 Animals

Male F344 rats were obtained from Harlan Sprague Dawley (Indianapolis, IN) at approximately eight weeks of age. The 68 animals in Study 1 weighed about 180 g and were housed in plastic cages with sterile wood chip bedding. The 60 animals in Study 2 weighed about 165 g and were housed in wire-bottomed cages. Animal weight was the same at the time of carcinogen injection in both studies. Humidity and temperature were maintained at approximately 50% and 22°C, respectively, in a controlled room with a 12:12-hour light-dark cycle, with the dark cycle extending from 7 PM to 7 AM. Tap water from an automated system was provided ad libitum. Animals were treated in compliance with the guidelines of

the Canadian Council on Animal Care, and the protocol was approved by the University of Toronto Animal Care Committee.

4.2.2 Design

Study 1 (Figure 4.1, top) compared the effect of the high-fat (HF) and the low-fat (LF) diet on the development of IR and ACF promotion. The 68 animals were acclimatized for 10 days and were initiated with the colon carcinogen azoxymethane (AOM; Sigma Chemical, St. Louis, MO) at 20 mg/kg body wt between 9 and 11 AM. One week later they were randomized to the HF and LF diets and further randomized into three experimental comparisons of the diets on the development of IR and ACF promotion. Three independent comparisons were made to determine the effect of handling of animals, inasmuch as it was possible that the stress of blood collection, metabolic caging, and gavaging used to assess the development of IR would affect IR and also tumor promotion (Gartner et al., 1980). In Experiment A, the animals were not handled until just before they were killed, when blood was collected by cardiac puncture under halothane anesthesia (Sigma Chemical). In Experiment B the animals were placed in metabolic cages for one week on the day the diets were started and every 4 weeks thereafter (with collections on Days 3-7, 31-35, 59-63, and 88-91). During the last two days of the week in the metabolic cages, urine samples were collected over two 24-hour periods, and blood samples were collected from the orbital sinus under light anesthesia (halothane) at the end of the week after a seven-hour fast (7 AM-2 PM). In Experiment C, the animals were fasted for seven hours every four weeks before they were given an OGTT. Tail vein blood glucose was determined by glucometer (Medisense Canada, Toronto, ON, Canada) before and 30, 60, and 120 minutes after an oral glucose



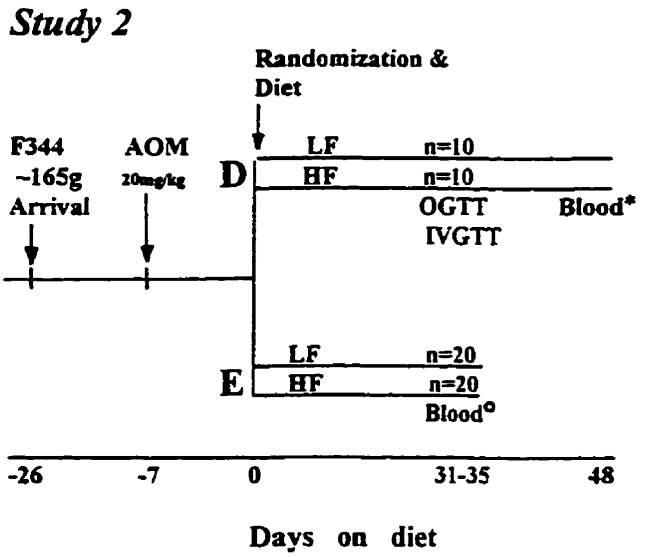


Fig 4.1. Diagrammatic representation of experimental protocols of Studies 1 and 2. All animals received azoxymethane (AOM, 20 mg/kg) 7 days before randomization and assignment to study groups low-fat (LF) or high-fat (HF) diet. *, Blood (fasting, 7 AM-2 PM) was obtained for serum insulin, free fatty acids, triglycerides, and glucose; ✪, blood was obtained 1 hr after a glucose gavage (4 mg/g body wt); †, urine was obtained at 24 hrs for C-peptide. ACF, aberrant crypt foci; OGTT, oral glucose tolerance test; IVGTT, intravenous glucose tolerance test.

gavage (1.2 mg/g anhydrous glucose). At 100 days all the animals in the three experimental comparisons were sacrificed for the assessment of ACF promotion.

Study 2 (Figure 4.1, bottom) was carried out to further investigate the development of IR after 32-48 days on the diet. The 60 animals were acclimatized (19 days) and treated with AOM in the same manner as in Study 1 but were placed in wire bottomed cages to reduce coprophagia and the consumption of bedding during fasting periods. They were randomized to the HF and LF diets and further randomized for two comparisons of the development of IR (Also see appendices A & B). In Experiment D an OGTT was performed as described above (Experiment C) after 32 days on the diet, and an intravenous glucose tolerance test (IVGTT) was performed after 40 days on the diet. The animals were sacrificed at 48 days, when blood was collected by cardiac puncture under anesthesia (halothane) after a seven-hour fast. In Experiment E the animals were fasted for seven hours on Days 39-41 and were then given an oral gavage of anhydrous glucose (4 mg/g body wt). One hour later blood was collected by cardiac puncture under anesthesia (halothane).

In both studies, coded blood samples collected by cardiac puncture and orbital sinus were centrifuged after the addition of Trasylol-EDTA (Miles Canada, Etobicoke, ON, Canada) at 1,800 rpm for 20 minutes at 4°C. Glucose was measured by the glucose oxidase method (kit from Synermod, Quebec, PQ, Canada), insulin by radioimmunoassay (RIA; kit from Diagnostic Products), and triglyceride using an enzymatic method [triglycerides GPO-PAP (TRIG) kit, Boehringer, Mannheim] by Vita-tech (Markham, ON, Canada). Insulin measurements were repeated for samples from Study 2 by the Banting and Best Diabetes Centre Laboratory (Toronto, ON, Canada) by RIA using rat antibody (rat insulin RIA kit, Linco Research, St. Louis, MO). Results for insulin obtained by the latter kit were generally

higher. However, insulin results from the two kits were highly correlated (r=0.93), and ratios of insulin values for the HF to the LF diet were the same for the two kits. Twenty-four-hour urinary C-peptide was measured by RIA using rat antibody (rat C-peptide RIA kit, Linco Research) by the Banting and Best Diabetes Centre Laboratory and is reported as concentration. Free fatty acids (FFAs) were measured enzymatically [non esterified fatty acid (NEFA) C test kit, Wako Chemicals, Richmond, VA].

4.2.3 Diets

All the rats were fed rodent chow (Ralston Purina, Strathroy, ON, Canada) on their arrival. After the acclimatization period and another week after the carcinogen, the animals were fed the experimental diets (Dyets, Bethlehem, PA). The LF diet was based on AIN-76 D-65, a high-starch control diet providing 11% of energy as fat in the form of corn oil (Table 4.1). The HF diet provided 59% of energy as fat, with 40% of those calories provided by beef tallow, a value chosen to correspond to the presumed high-risk extreme for a population at risk for colon cancer (Rizek et al., 1983). Protein, micronutrients, and fiber (1% cellulose) were adjusted in the

HF diet so that they were similar to the LF diet on a caloric density basis.

4.2.4 Measure of ACF promotion

ACF were assayed as previously described (Bird, 1987, Bruce et al., 1993). Briefly, the colons were removed, rinsed in Krebs-Ringer bicarbonate buffer, cut longitudinally, and fixed flat between filter papers in 10% buffered Formalin. They were then stained briefly with 0.2% methylene blue and examined mucosal surface up under a light microscope at ×40

Table 4.1. Composition of experimental diets[a]

	Diet	
	LF	HF
Casein	16	22
DL-Methionine	0.3	0.4
Corn starch	68	26.2
Sucrose	5	6.8
Corn oil	5	6.8
Beef tallow	0	30
Cellulose	1	1.4
AIN-76 mineral mix	3.5	4.8
AIN-76 vitamin mix	1	1.3
Choline bitartrate	0.2	0.3
TBHQ	0.001	0.007
Total	100	100
% Calories		
Protein	16	16
Fat	11	59
CHO		
Complex	67	19
Simple	6	6
Total kcal/100 g	410	563

a: Values are g/100 g diet unless otherwise noted. HF, high fat; LF, low fat diet; CHO, carbohydrate; TBHQ, *tert*-butylhydroquinone.

magnification for ACF. ACF were identified on the basis of their darkly staining and enlarged crypts, which appeared to bulge from the mucosal surface. The number of ACF per colon and the number of crypts comprising each focus were recorded to provide the mean number of ACF and average number of aberrant crypts per ACF reported.

4.2.5 Statistical measures

Two-tailed Student's *t*-test was used to evaluate the differences in weight gain between dietary groups. For Experiments A, D, and E, one-way analysis of variance (ANOVA) and *t*-tests were used. For Experiment C, ANOVA was used with a model that had the following fixed effects: group, minutes (after gavage), days (on diet), the three two-level interactions, and the triple interaction. The random effects were animal within group, the interaction of animals and minutes, and the interaction of animals and days. For Experiment B, a procedure similar to Experiment C was used. In this case, fixed effects were group, days, and their interaction, and the only random effect was animal within group. ACF results were processed as previously described (Minkin, 1994).

4.3 Results

4.3.1 Study 1

The weight gain of groups of animals was significantly higher on the HF than on the LF diet, with slight differences in the separate experiments over the intervals shown (Figure 4.2). Animals in Experiment B, however, lost approximately 1-2% of their body weight when

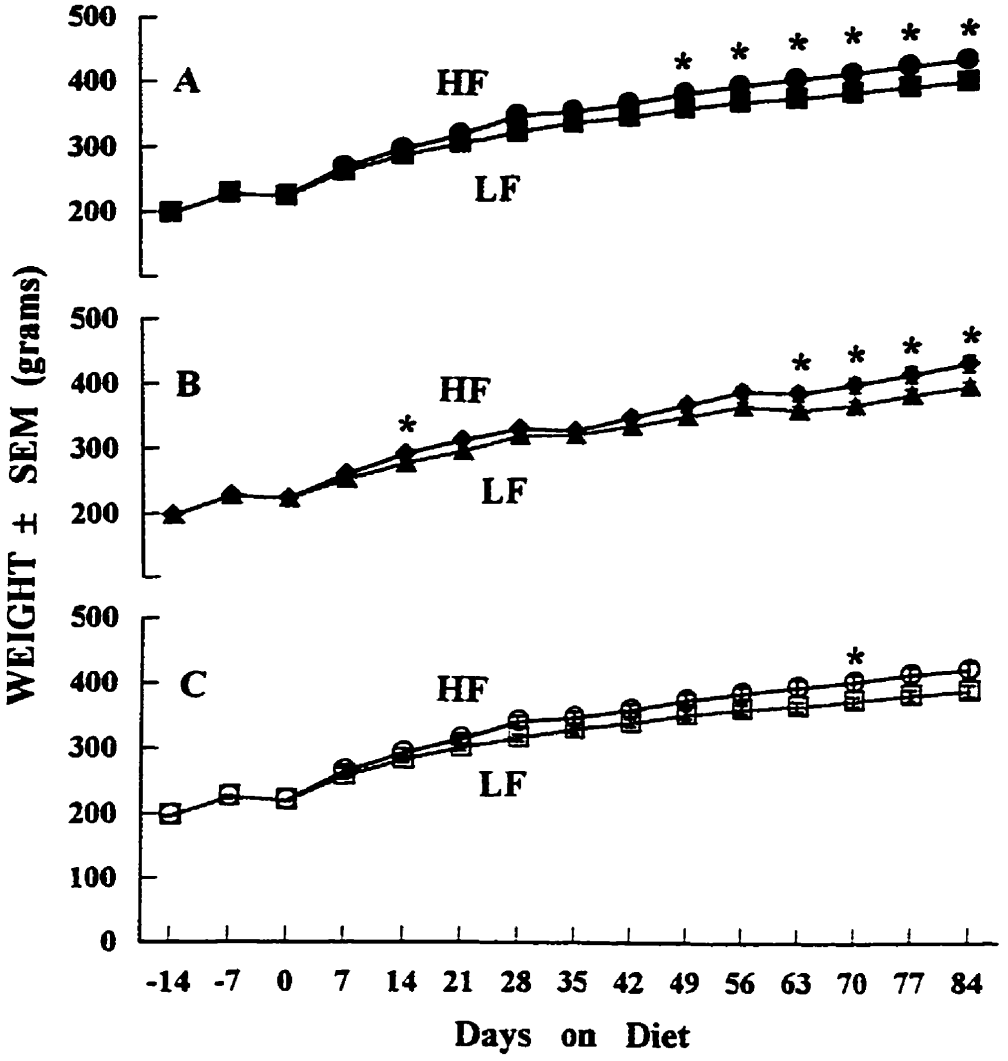


Fig 4.2. Body weight of F344 rats during acclimatization (-14 to -7 days), after carcinogen injection (-7 to 0 days), and after assignment to diets (0-84 days). A, B, and C, results for Experiments A, B, and C; LF, group fed low-fat diet; HF, group fed high-fat diet. Values are means ± SEM. *, Significantly different from corresponding LF group ($p<0.05$) as tested with 2-tailed Student's *t*-test as modified for unequal variance.

they were placed in metabolic cages over the seven-day period.

Promotion, which is usually assessed as size of ACF (aberrant crypts per ACF), was significantly higher in the HF than in the LF group. In Experiment A, in which there was no manipulation over the promotional period of 100 days, the HF diet promoted the growth of ACF during the period on the diet (Table 4.2; size ratio = 1.43, 95% confidence interval = 1.30-1.58, $p<0.001$). There was no effect of the diets on the number of ACF per colon (Table 4.2). Similar results were obtained in Experiments B and C (size ratios = 1.42 and 1.36, each $p<0.001$), although the number of ACF per colon was significantly higher in the HF group in Experiment B, the group that showed weight losses and gains with caging changes.

In Experiment A, at 100 days, the concentrations of fasting insulin, triglycerides, and glucose were higher in the HF group [by 15% (18.6 $\pm$ 3.4 vs. 16.2 $\pm$ 1.7 μIU/ml), 20% (1.88 $\pm$ 0.23 vs. 1.56 $\pm$ 0.16 mmol/l), and 10% (7.9 $\pm$ 0.6 and 7.2 $\pm$ 0.5 mmol/l), respectively], although none of these differences reached statistical significance. The fasting FFAs were lower in the HF group (0.43 $\pm$ 0.02 and 0.53 $\pm$ 0.02 me/l, $p<0.02$).

In Experiment B, in which repeated measures were made over the 100-day period (Figure 4.3), the results for C-peptide showed a similar pattern for all days, with no difference between the HF and LF groups, and this was also true for insulin (no significant interaction between dietary group and days on the diet and no significant group effect). For triglycerides (TG) and FFA the differences between the HF and LF groups depended on which time point was chosen to measure these variables (significant interaction between dietary group and days on diet, $p<0.04$ and $p<0.006$, respectively). Because of this interaction, estimating a group effect for these two variables (TG & FFA) was not meaningful. For glucose the patterns were significantly different (no significant interaction between group and days, a significant group

Table 4.2. Effect of dietary treatment on ACF in F344 rats 98 days after a single AOM injection[a,b]

		Average Size of ACF	
	ACF/Colon[c]	AC/ACF	Ratio[d]
Expt A			
LF	112 ± 5	2.49	1.43 (1.30-1.58)
HF	122 ± 3	3.57*	
Expt B			
LF	107 ± 3	2.41	1.42 (1.33-1.52)
HF	132 ± 5*	3.79*	
Expt C			
LF	113 ± 5	2.66	1.36 (1.25-1.48)
HF	127 ± 3	3.28*	

a: Azoxymethane (AOM) was injected at 20 mg/kg. ACF, aberrant crypt foci; AC, aberrant crypts; HF, high fat; LF, low fat; Expt A, fasting insulin; Expt B, repeated fasting insulin + C-peptide; Expt C, repeated oral glucose tolerance test.

b: Statistical significance is as follows: *, significantly different from LF, $p<0.001$.

c: Values are means ± SEM.

d: Values in parentheses are confidence intervals.

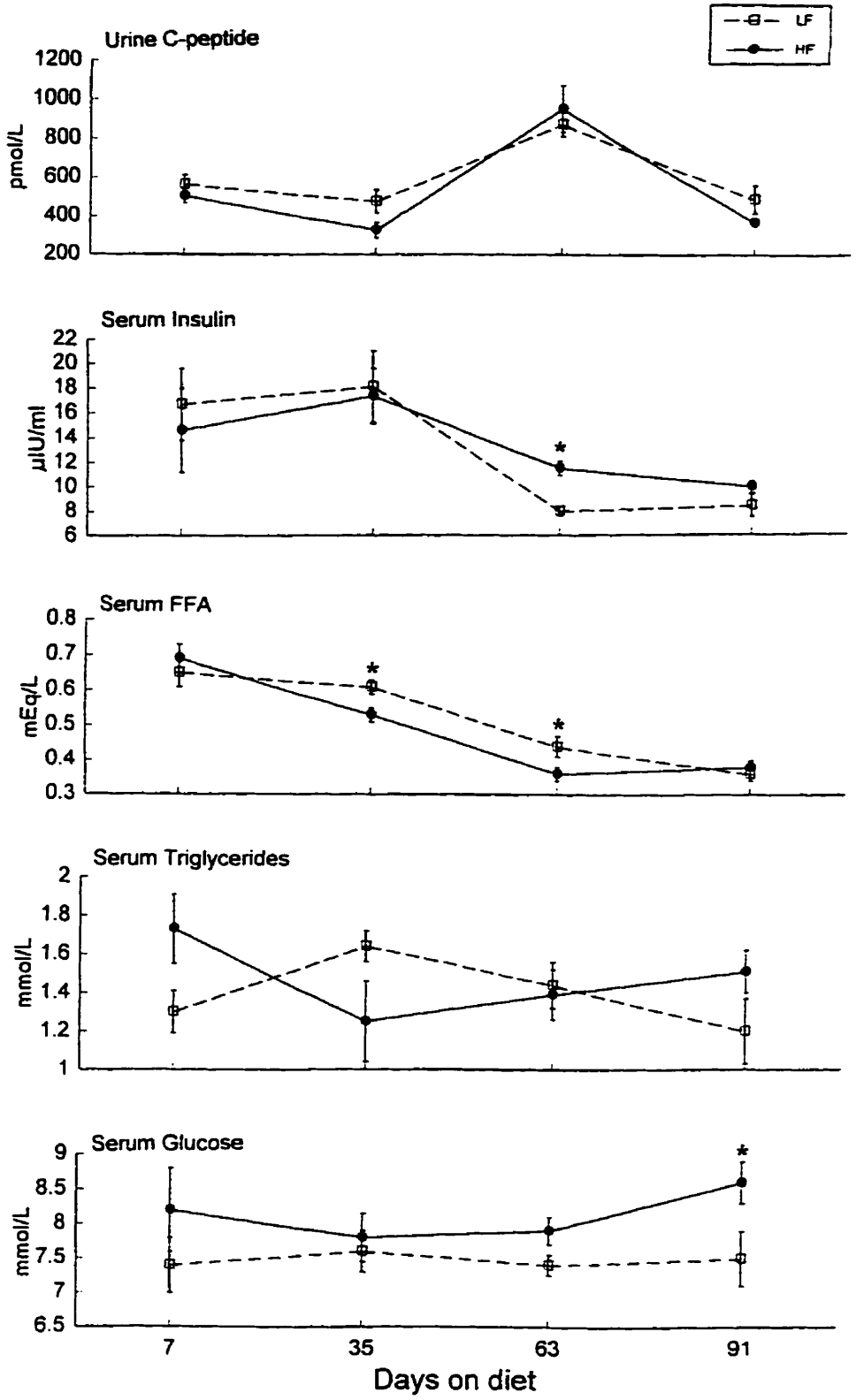


Fig 4.3. Results of Experiment B. Values are means ± SEM. C-peptide showed a similar pattern for all days with no difference between HF and LF. This was also the case for insulin. Differences for triglycerides and free fatty acids (FFA) between HF and LF groups depended on time point. For glucose, patterns were significantly different for HF and LF groups (significant group effect, $p<0.04$). *, Significantly different from corresponding LF group ($p<0.05$) as tested with 2-tailed Student's *t*-test as modified for unequal variance.

effect, $p<0.04$). To obtain an estimation of the group effect, mean of all fasting glucose measures throughout the experiment was calculated for HF as well as for LF group. The HF diet glucose level was higher than the LF diet glucose level (8.1 ± 0.20 vs. 7.5 ± 0.14, mmol/l, $p<0.01$) as tested by a one-way ANOVA.

In Experiment C, the OGTT was measured four times throughout the 100-day period on the two diets (Figure 4.4). Differences were observed for all glucose measures for each OGTT and for the different days the tests were performed (no significant interaction between dietary group and minutes after gavage or between dietary group and days on diet, a significant group effect, $p<0.001$). To obtain an estimation of the group effect, mean of all glucose measures throughout the experiment was calculated for HF as well as for LF group. Glucose levels were higher in the HF than in the LF group (6.9 ± 0.10 vs. 6.2 ± 0.10, mmol/l, $p<0.001$) as tested by a one-way ANOVA.

4.3.2 Study 2

Study 2 was undertaken to confirm the early effect of the HF diet on glucose tolerance and to determine whether this test, when a glucose dose approximating the level of carbohydrate consumed per day by the animals on their diet was utilized, led to an increased level of circulating

insulin and triglyceride.

In Experiment D, the OGTT at 32 days and the IVGTT at 40 days confirmed an impairment in glucose tolerance in the HF group, as observed in Experiment C (Figure 4.5). At 48 days the fasting, terminal values for the HF and LF groups also showed differences in insulin (18.7 ± 3.3 vs. 9.3 ± 1.2 μIU/ml, $p<0.03$) and triglyceride (2.1 ± 0.28 vs. 1.21 ± 0.18

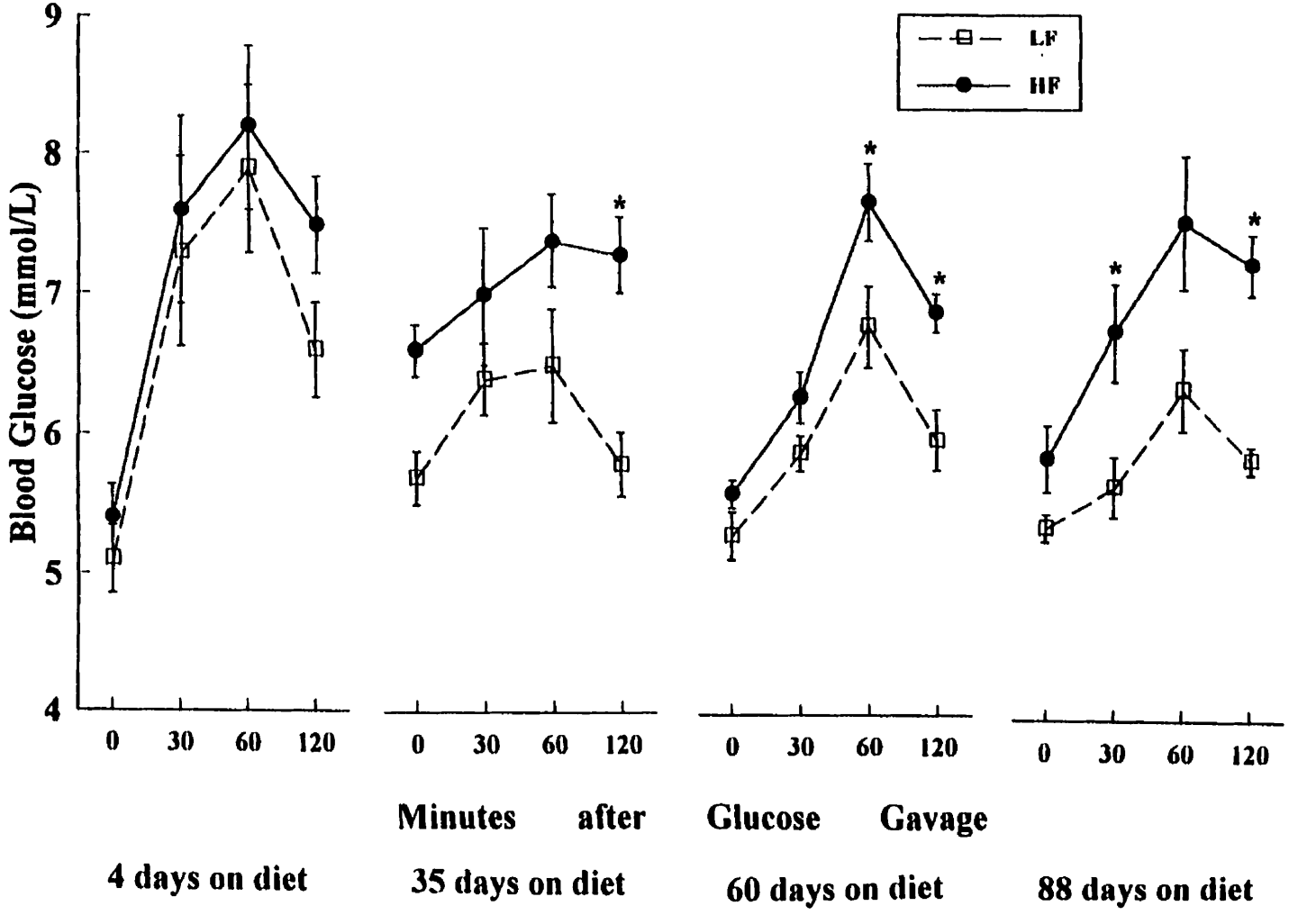


Fig 4.4. Results of Experiment C. Values are means ± SEM. Blood glucose was measured before and 30, 60, and 120 minutes after a glucose gavage at 4, 32, 60, and 88 days on diet. Dietary group had a significant effect ($p<0.001$). *, Significantly different from corresponding LF group ($p<0.05$) as tested with 2-tailed Student's *t*-test as modified for unequal variance.

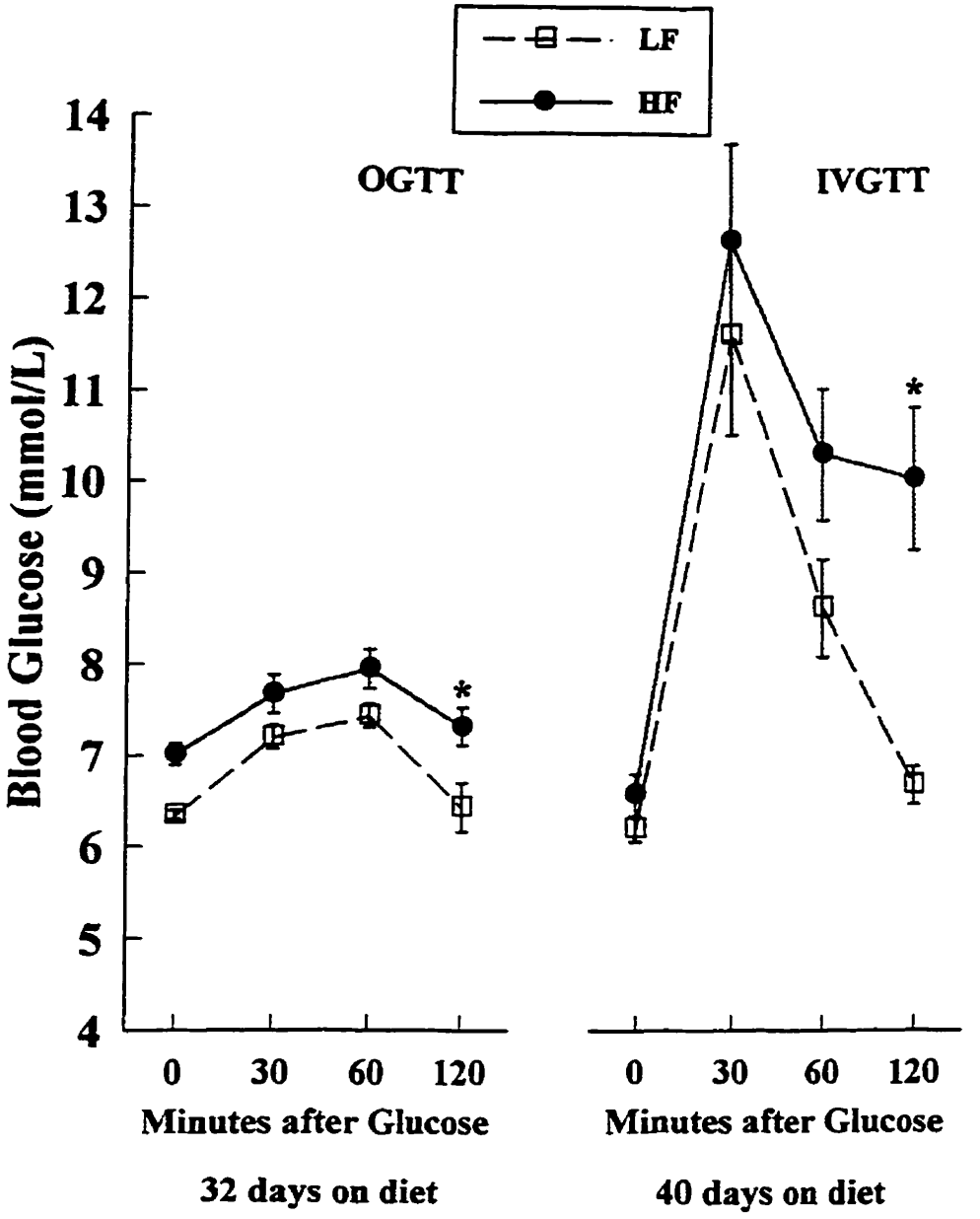


Fig 4.5. Results of Experiment D. OGTT at 32 days and IVGTT at 40 days confirmed differences measured in Experiment C. *, Significantly different from corresponding LF group ($p<0.05$) as tested with 2-tailed Student's *t*-test as modified for unequal variance.

mmol/l, $p<0.03$), but not glucose (10.9 ± 0.4 vs. 10.4 ± 0.4 mmol/l).

In Experiment E, the HF and LF groups were given glucose at a higher concentration, i.e., 4 mg/g, and blood was collected for insulin, glucose, and triglyceride measurements one hour later. The results showed that the concentration of insulin was significantly elevated in the animals receiving the HF diet (19.5 ± 1.8 vs. 14.1 ± 1.1 μIU/ml, $p<0.03$) but that the concentrations of triglyceride and glucose were not significantly different (1.11 ± 0.15 vs. 0.94 ± 0.06 mmol/l and 12.8 ± 0.5 vs. 11.8 ± 0.5 mmol/l, respectively).

4.4 Discussion

The results of this investigation are consistent with the IR hypothesis: there was evidence of IR before there was evidence of promotion of carcinogenesis. At 100 days, a time at which consistent evidence of promotion is seen, the HF diet promoted the growth of ACF initiated by AOM in the F344 rats. In all three experiments, the ACF size in animals fed the HF diet was about 1.4 times larger than in animals fed the LF diet ($p<0.001$ in each case). No difference in this parameter was observed among Experiments A, B, and C, showing that the OGTT as well as blood and urine collection procedures did not affect the measure of promotion. Before 100 days the HF diet affected IR. Glucose tolerance was significantly impaired in animals fed the HF diet compared with animals fed the LF diet. Fasting glucose and insulin were usually higher in the HF group, but these measures seemed less sensitive than the OGTT to detect the effects of IR. However, the temporal sequence (IR before promotion) does not prove causality or disprove the importance of other hypotheses linking diet and colon cancer.

The promoting effect of the HF diet observed is consistent with earlier studies

examining the effect of such diets on ACF growth and tumor promotion (Tang et al., 1996, Reddy and Maeura, 1984). The rapid effect of the HF diet on IR is also consistent with earlier studies. Colloidal lipid given intravenously affects IR in peripheral muscle within a few hours (Boden et al., 1991). HF diets increase IR as measured by the euglycemic clamp method relatively quickly, within a few days (Kraegen et al., 1986), and relatively rapid effects of HF diets, such as elevated glucose tolerance curves and triglycerides, have also been described (Boivin and Deshaies, 1995). These effects of diet on " IR" appear to occur much earlier than the fasting hyperinsulinemia, triglyceridemia, and glycemia observed in clinical "IR". Indeed, impaired glucose tolerance can precede clinical IR and non-insulin-dependent diabetes mellitus (NIDDM) by many years (DeFronzo and Ferrannini, 1991). The notion of "IR" is thus used in different senses in the physiological and clinical literature.

The hypotheses relating the association of IR and NIDDM, on the one hand, and CRC, on the other, are illustrated in Figure 4.6. Figure 4.6 begins with the close association of several factors, perhaps through some common mechanism, lead to a physiological resistance to insulin. Over a period of time, this may develop into clinical IR with hyperinsulinemia, a condition that puts the pancreatic β-cell at risk for developing NIDDM. In epidemiologic studies, there may be an association between NIDDM and CRC, but it is certainly weak (Williams et al., 1984, Adami et al., 1991, Weiderpass et al., 1997). Other possible hypotheses linking the development of IR and CRC are shown in Figure 4.6. Hypothesis 1 is the suggestion made by McKeown-Eyssen (McKeown-Eyssen, 1994) and Giovannucci (Giovannucci, 1995) that hyperinsulinemia (clinical IR) leads to increased CRC risk, perhaps through increased colonic cell proliferation; Hypothesis 2 is that high peak postprandial insulin or abnormal OGTT (physiological IR) leads to the risk; Hypothesis 3 is that the dietary

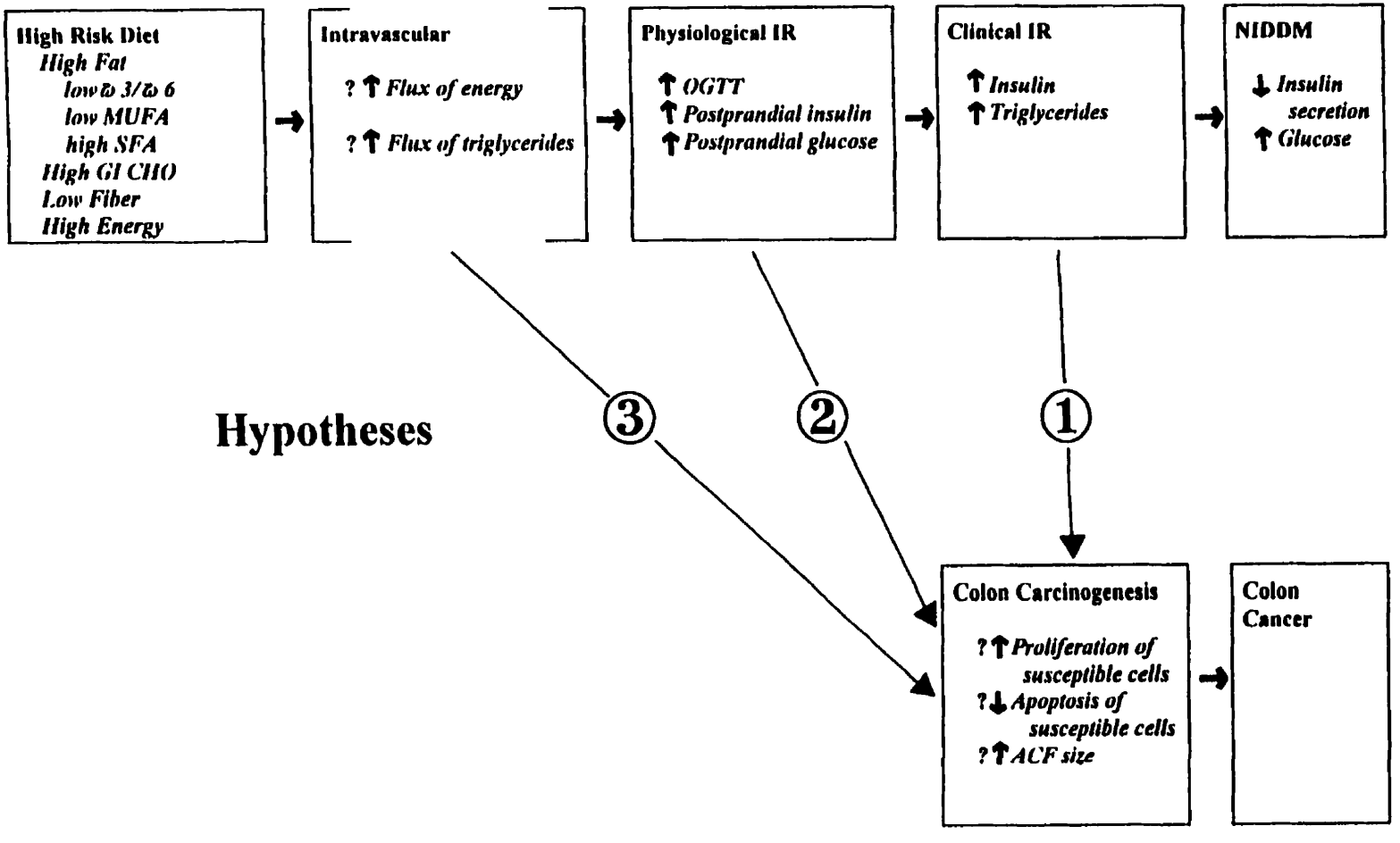


Fig 4.6. Possible hypotheses linking dietary risk factors, insulin resistance (IR), and colon carcinogenesis. MUFA, monounsaturated fatty acids; SFA, saturated fatty acid; GI, glycemic index; CHO, carbohydrate; ω-3 and ω-6, n-3 and n-6 fatty acids; NIDDM, non-insulin-dependent diabetes mellitus; ACF, aberrant crypt foci.

factors, or biochemical and physiological factors resulting from them, give rise to physiological IR and CRC risk.

The results of the studies are not compatible with Hypothesis 1, because consistent elevated levels of insulin were not found. However, the results could be compatible with Hypotheses 2 and 3. Hypothesis 2 is supported by the direct effect of exogenous insulin on the promotion of ACF (Corpet et al., 1997) and colonic tumors (Tran et al., 1996). It is, however, difficult to reconcile the marked effects of promotion with relatively small increases in insulin observed in the present studies with the limited promotion with massive increases of exogenous insulin. Perhaps the difference lies in the nature of the exposure: in the studies with exogenous lente insulin, there was a slowly developing, large increase in insulin associated with a decreased plasma glucose, whereas in this study with endogenous insulin there is a rapid but modest increase in postprandial insulin associated with a high glucose level. Perhaps the rapid increase in insulin concentration in the presence of high levels of glucose is effective in triggering a proliferative response or is toxic to colonic cells through increased glycosylation or oxidation. Hypothesis 3 assumes a common mechanism leading from diet to both IR and colon carcinogenesis. The mechanisms that lead from diet to IR are not well defined but may involve dietary energy and weight gain. One suggestion is that risk of IR is associated with high levels of FFAs (Williams et al., 1984), another is that high fluxes of triglycerides in chylomicrons or very-low-density lipoprotein particles are responsible (Boivin and Deshaies, 1995, Steiner, 1991). Possibly, transient increased glucose, fatty acids, and triglycerides, that is energy, reaching colonic cells affect cell-signalling pathways to increase the probability of cell proliferation and promotion. This might occur in the presence of an increased insulin level without acting through the increased insulin level itself.

The close association between the risk factors for IR and those for ACF promotion and CRC is nevertheless important. First, it shows that the link between diet and the physiological steps in the development of IR could provide a model for the study of the effect of diet on promotion. Diets high in energy, saturated fat, and glycemic carbohydrate and low in n-3 fatty acids could be deleteriously affecting cell signalling in colonic cells in ways that lead to IR and CRC. Second, it may mean that IR can be used as a readily applied marker of dietary risk in clinical studies. Dietary interventions that reduce physiological IR may also reduce CRC risk. Further animal and clinical studies are needed to assess these possibilities.

CHAPTER 5

CORRELATION OF MARKERS OF INSULIN RESISTANCE AND COLON CANCER PROMOTION IN RATS ON DIETS DIFFERING IN ENERGY, FAT, n-3 FATTY ACIDS AND STARCH, AND FURTHER CORRELATION WITH ENERGY INTAKE

5.1 Introduction

As previously mentioned McKeown-Eyssen (McKeown-Eyssen, 1994) and Giovannucci (Giovannucci, 1995) noted the striking similarities of the dietary risk factors for insulin resistance (IR) and colorectal cancer (CRC) and hypothesized that these dietary factors first lead to IR, and that the hyperinsulinemia associated with IR then act as growth promoter to increase the growth of CRC.

As noted before, the dietary risk factors that increase risk of IR and CRC in animal studies are also similar. High saturated fat (Kern et al., 1990, Bull et al., 1979), and excess energy intake (Rizkalla et al., 1987, Clinton et al., 1992), increase the risk, while caloric restriction (Okauchi et al., 1995, Kumar et al., 1990), n-3 fatty acids (Storlien et al., 1991, Reddy et al., 1991) and the substitution of slowly digested for readily digested carbohydrate (Byrnes et al., 1995, Caderni et al., 1991), reduce the risk of both IR and CRC. Three direct tests of the IR hypothesis have been made with animal models. Tran et al (Tran et al., 1996) and Corpet et al (Corpet et al., 1997) found that exogenous insulin injections promoted the growth of colon tumors and aberrant crypt foci (ACF), putative precursors of CRC, in rats (Bruce et al., 1993) and suggested that the endogenous insulin associated with IR acts in the same manner. In chapter 4 the effect of a high fat diet was examined on both the development

of IR and promotion of ACF growth in the same F344 rats. It was found that the high dietary fat led to IR (impaired glucose tolerance) prior to emergence of evidence of ACF promotion.

Experiments described in this chapter mainly aim to answer question 2 in chapter 3: do different diets affect IR and CRC promotion to the same degree? Four major dietary factors that are thought to affect both IR and CRC: high and low dietary fat as before, dietary energy restriction and excess, n-3 fatty acid supplementation, and readily and less rapidly digested carbohydrate were used. First the effects of each of these factors on both IR and ACF promotion was determined in the same animals in a single experiment, to assure reproducibility of earlier results. Then the association between measures of IR and colon cancer promotion was examined for all the diets. It was reasoned that, if it was IR that led to promotion, the degree of promotion would necessarily be related to the degree of IR. It was asked first, do dietary fat, energy, n-3 fatty acids and carbohydrate digestibility affect both IR and CRC promotion when tested in the same experiment on the same groups of animals? Then it was asked, are the measures of IR correlated with the measures of CRC promotion for all these diets?

5.2 Materials and methods

5.2.1 Animals

112 male 8-week-old Fischer 344 rats (Harlan, Sprague-Dawley, Inc., Indianapolis, IN) weighing approximately 180 g were housed individually in wire-bottom cages in a temperature and humidity controlled environment (22°C and 50%, respectively). The cages

were arranged randomly in the room to minimize the possible confounding effects of light and noise. The room was maintained on 12-h dark/light cycle, with the dark cycle extended from 7 PM to 7 AM. Tap water from an automated system was provided *ad libitum*. Care of the animals conformed to the guidelines of the Canadian Council on Animal Care, and the protocol was approved by the University of Toronto Animal Care Committee.

5.2.2 Design

The protocol scheme is summarized in Figure 5.1. After 10-12 days of acclimatization when the animals consumed Rodent Chow (Ralston Purina International, Strathroy, Canada), the rats were individually identified and given an abbreviated oral glucose tolerance test (OGTT, 0 and 120 minutes only). Several days later the rats were initiated with the colon carcinogen, azoxymethane (AOM, Sigma, St. Louis, MO) at a dose of 20 mg/kg body weight between 9:00 and 11:00 AM and a week later randomized to the eight dietary groups of 14 animals each. These groups were designed to be compared in pairs. The average weights and the OGTT values obtained from the earlier determinations were found to be the same for each of the groups. Animal weights were then measured weekly and food consumption was measured over a week close to the end of the study. At sixty five days on the diets the OGTT was repeated. On the eighty second and eighty-seventh days blood was collected from the orbital sinus under light halothane anesthesia for determination of post-prandial glucose, insulin and triglyceride, and fasting insulin values, respectively. On the ninety-first day blood was again collected under anesthesia for determination of 1 h post-glucose gavage values and the animals were then killed and their colons removed for the assay of ACF.

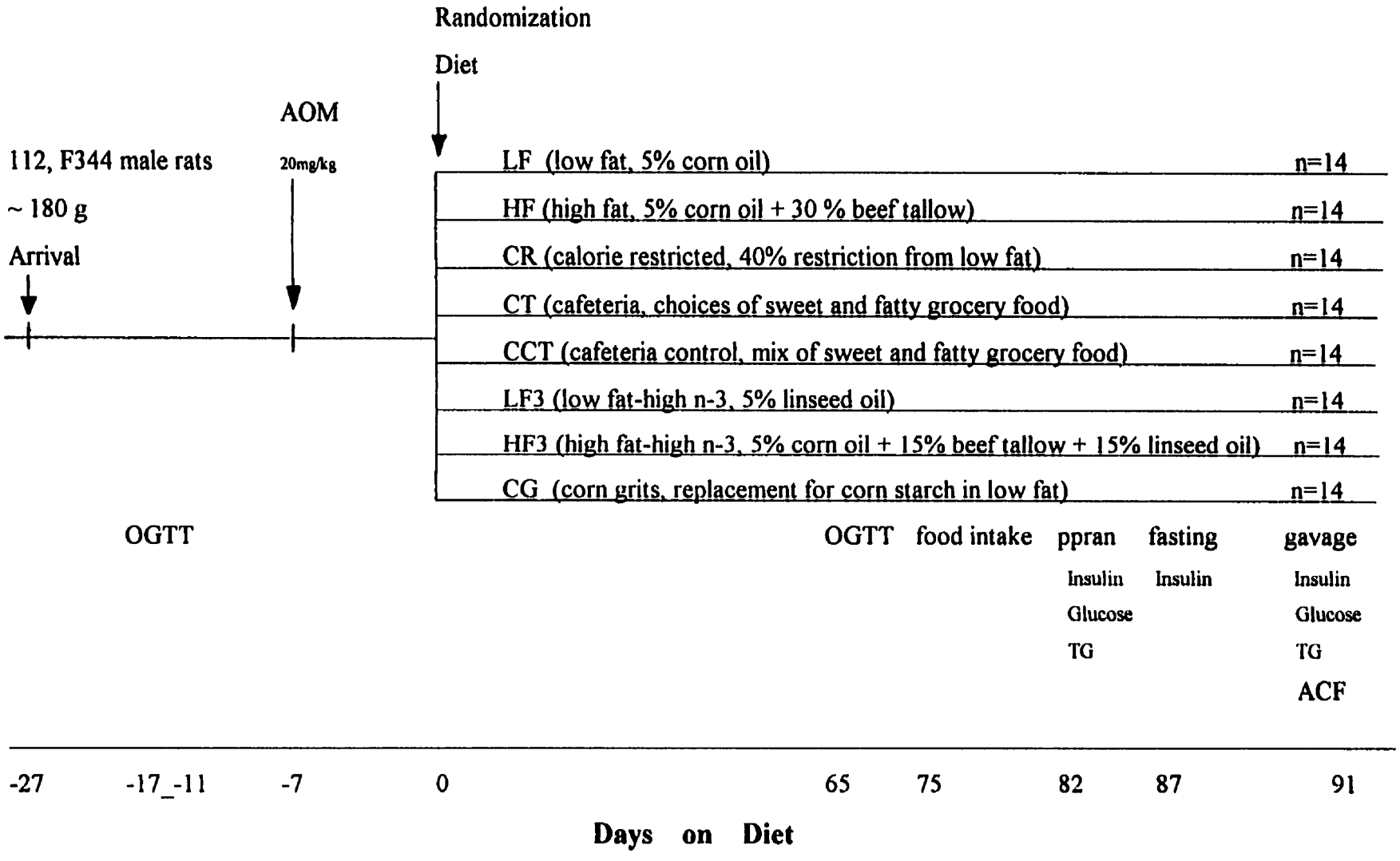


Fig 5.1. Diagrammatic representation of experimental protocol with time in days. AOM, azoxymethane; diet groups defined in table 1; OGTT, oral glucose tolerance test; ppran, postprandial measures of insulin, glucose and triglycerides; gavage, one hour post glucose gavage with a dose of 4mg glucose/g body weight; ACF, assay for aberrant crypt focus number and size.

5.2.3 Diets

Eight diets were developed to test the association of IR and ACF promotion with changes in dietary fat, dietary energy, n-3 fatty acids and reduced carbohydrate digestibility. They are detailed in Table 5.1.

The *low fat diet* (LF) was based on AIN-76 D-65 (ICN catalogue, 1995) and provided 18% of energy as protein, casein, 70% of energy as carbohydrate, largely corn starch, and 12% energy as fat in the form of corn oil. The *high fat diet* (HF) provided 60% of its energy as fat with 40% of those calories provided by beef tallow. Vitamins, minerals and protein were at the same concentration as in the LF diet on an energy density basis. A low energy, *calorie restricted diet* (CR) provided 60% of energy of animals on the low fat diet. Feeding of the CR was based on consumption in the low fat diet group, and the results for the calorie restricted diet were compared with those on low fat diet. A high energy, *cafeteria diet* (CT), provided the animals with a daily variety of flavours and textures and encouraged the rats to increase their food and energy intake (Rogers and Blundell, 1984). This diet provided an average of 62% of calories as fat and the results obtained with this diet were compared to that of a separate cafeteria control diet. The *cafeteria control diet* (CCT) was developed by pretesting the cafeteria diet and preparing a mixture of the components that matched the feeding preferences of the rats, so that animals on the cafeteria control received the same dietary components as animals in the cafeteria group without the constantly changing flavours and textures. The *low fat n-3 diet* (LF3) was identical to the low fat diet except that all the corn oil in low fat diet was replaced with flax oil, an oil known to contain a large fraction of linolenic acid (Jenkins, 1995). The *high fat n-3 diet* (HF3) was identical to high fat diet except that one-half of the beef tallow in the high fat diet was replaced with flax oil. These diets

Table 5.1. Composition of the eight experimental diets

	LF	HF	CR	CT	CCT	LF3	HF3	CG
Protein (casien)	18	24.6	30	see	see	18	24.6	12.93
Protein (corn grits)	-	-	-	below	below	-	-	4.18
DL-Methionine	0.3	0.4	0.5			0.3	0.4	0.29
Fat (corn oil)	5	6.8	8.33			-	6.8	4.18
Fat (corn grits)	-	-	-			-	-	0.57
Fat (beef tallow)	-	30	-			-	15	-
Fat (flaxseed oil)	-	-	-			5	15	-
Starch (corn starch)	64	20.86	40.007			64	20.86	23.8
Starch (corn grits)	-	-	-			-	-	37.1
Sucrose	5	6.8	8.33			5	6.8	4.75
Fiber (cellulose)	3	4.1	5			3	4.1	2.1
Fiber (corn grits)	-	-	-			-	-	0.76
Mineral Mix	3.5	4.8	5.83			3.5	4.8	3.14
Vitamin Mix	1	1.37	1.67			1	1.37	0.95
Choline Bitartrate	0.2	0.27	0.333			0.2	0.27	0.19
TBHQ	0.001	0.007	0.00166			0.001	0.007	0.001
Moisture	-	-	-			-	-	4.75
Ash	-	-	-			-	-	0.19
Total weight	100	100	100	100	100	100	100	100
% calories								
Protein	18	18	30	11	11	18	18	18
Fat	12	60	19.5	62	62	12	60	12
Complex CHO	64	16	40	27	27	64	16	64
Simple CHO	6	6	10.5			6	6	6
Energy	401.5	552	403	426.2	426.2	401.5	552	381.9

LF, low fat diet; HF, high fat diet; CR, 40% calorie restricted diet based on composition of LF diet; CT, the cafeteria diet, consisted of 7 food items (peanut butter, weiners, cheese puff, Ritz cracker, kit kat, bologna, wafer) and a diet supplement. Food was provided in 3 containers: 3 food items provided daily, one new item at a time, and diet supplement every day. The diet supplement was designed from previous feeding studies so to provide minerals, vitamins and fiber in amounts similar to the other experimental diets; CCT, the cafeteria control diet, consisted of the food items and supplement used in cafeteria diet mixed together in order to reduce variety in texture and flavour. The proportion of items in the diet was the same as that of the cafeteria diet and was calculated from food intake in a similar previous experiment.; LF3, low fat with high n-3 diet; HF3, high fat with high n-3 diet; CG, the corn grits diet, the amount of protein, fat, carbohydrate, fiber, minerals and vitamins in this diet were adjusted according to the composition of corn grits.
All diets except CT, CCT, and corn grits were provided by Dytes, Bethlehem, PA.
The estimated ratio of n-6/n-3 fatty acids for LF, HF, CR, LF3, HF3, and CG diets were 56, 16.6, 56, 0.3, 0.8, and 56, respectively.
TBHQ; *tert*-butylhydroquinone.

were compared with the low fat and high fat diets respectively. A *corn grits diet* (CG) with a presumed reduced carbohydrate digestibility was developed in which one-half of the starch was replaced with corn grits - hulled, degermed, and coarsely ground white corn. This choice was based on the results of studies that had demonstrated that whole grain products, with a larger particle size, produced low plasma glucose responses in human subjects (Jenkins et al., 1988), although the corn grits were not tested for their glycemic responses in animal studies. The corn grits diet was compared with the low fat diet.

All of the semipurified diets were provided by Dyets (Bethlehem, PA). Flaxseed oil used in LF3 and HF3 was provided by Dyets from ACE Hardware (Bethlehem, PA). Cafeteria food was bought from local grocery stores (Appendix C). Corn grits were obtained from local suppliers and their composition was taken from USDA's table of food composition, 1996 (Appendix C). All foods were refrigerated (-20°C) prior to use and food in food containers were replaced regularly, from daily to twice weekly.

5.2.4 Measures of glucose, triglycerides and insulin

For the oral glucose tolerance test (OGTT), the animals were fasted for 7 hours (7 AM to 2 PM) and were then given an oral glucose gavage (4 mg/g anhydrous glucose). Blood glucose from the tail vein was determined by glucometer (Medisense Canada, Inc., Toronto, Canada), prior to and 30, 60 and 120 minutes after the gavage. The total area under the glucose tolerance curve (a.u.c.) was calculated from 0 to 120 minutes with the trapezoid rule (Wolever et al., 1991).

The post-prandial blood was collected at 8:00 AM, 1 h after food was removed, as a sample of convenience after the animals' usual early morning meal (Johnson et al., 1979). The

post glucose gavage blood was collected 1 hour after a glucose gavage which was administered as in the OGTT. Coded blood samples, obtained by orbital sinus and cardiac puncture, were centrifuged at 1800 rpm for 20 min at 4°C after the addition of EDTA. Glucose was measured by the glucose oxidase method (Kit 510 from Sigma, St. Louis, MO), and triglycerides by an enzymatic method (Kit 337 from Sigma). Insulin was measured by radioimmunassay using rat-specific antibody (RAT Insulin RIA kit, Linco Research Inc. St. Charles, MO) and a modification of the protocol described by Marban and co-worker (Marban et al., 1989) with the sample plasma volumes further reduced to 20 µl. As previously described, plasma samples and standards were diluted 1:5 with assay buffer and rat-specific antibody, ^{125}I-insulin tracer, and precipicating agent were all diluted 1:2 with assay buffer. Linearity was established to a dilution of 1:2 and the assay was assessed for inter-assay and intra-assay variation (coefficient of variation = 13%).

5.2.5 Measure of ACF promotion

ACF were assayed as previously described (Bruce et al., 1993, Bird, 1987). In brief, the colons were removed, rinsed in Krebs-Ringer bicarbonate buffer, cut longitudinally and fixed flat between filter papers in 10% buffered formalin. They were then stained briefly with 0.2% methylene blue and examined mucosal surface up under light microscope at x40 magnification for ACF. These were identified on the basis of darkly staining and enlarged crypts that appeared to bulge from the mucosal surface. The number of ACF per colon and the number of crypts comprising each foci were recorded to provide the mean number of ACF per colon and the average number of aberrant crypt per ACF (ACF size) as reported.

Table 5.2. Mean values of factors measured in the experiment ± SEM (n=11-14) for each of the eight dietary groups (upper panel), and pairwise comparisons of groups (lower panel)

Group	eng/d	final weight	a.u.c.	Glu-ppran	Ins-fast	Ins-gav	Ins-ppran	TG-gav	TG-ppran	ACF no./colon	log of ACF size
LF	64.5 ± 2.00	376 ± 7	15.3 ± 0.30	6.92 ± 0.18	244 ± 29	840 ± 107	355 ± 45	0.31 ± 0.05	0.84 ± 0.07	133 ± 11	0.40 ± 0.01
HF	72.5 ± 1.90	400 ± 7	16.7 ± 0.29	7.06 ± 0.15	284 ± 43	845 ± 107	413 ± 46	0.36 ± 0.04	0.94 ± 0.11	146 ± 13	0.55 ± 0.01
CR	40.3 ± 0.00	257 ± 3	14.1 ± 0.20	6.26 ± 0.17	153 ± 21	366 ± 74	172 ± 22	0.27 ± 0.03	0.63 ± 0.08	133 ± 9	0.37 ± 0.01
CT	98.6 ± 3.75	418 ± 6	17.4 ± 0.41	7.50 ± 0.16	283 ± 25	699 ± 113	473 ± 35	0.67 ± 0.34	0.91 ± 0.05	139 ± 13	0.55 ± 0.02
CCT	72.4 ± 1.10	384 ± 5	16.3 ± 0.27	7.25 ± 0.16	345 ± 114	551 ± 87	335 ± 27	0.26 ± 0.02	0.78 ± 0.08	124 ± 9	0.49 ± 0.02
LF3	57.4 ± 0.84	352 ± 4	14.8 ± 0.18	6.52 ± 0.13	277 ± 56	1015 ± 104	315 ± 25	0.24 ± 0.03	0.39 ± 0.04	112 ± 7	0.39 ± 0.01
HF3	62.9 ± 1.10	367 ± 3	15.6 ± 0.21	6.91 ± 0.10	177 ± 16	686 ± 189	280 ± 30	0.18 ± 0.02	0.57 ± 0.05	136 ± 9	0.44 ± 0.01
CG	60.6 ± 1.30	354 ± 5	15.3 ± 0.23	7.18 ± 0.14	292 ± 51	625 ± 89	346 ± 41	0.38 ± 0.05	0.77 ± 0.08	125 ± 12	0.39 ± 0.01
ANOVA	<0.001	<0.001	<0.001	<0.001	0.16	<0.006	<0.001	0.16	<0.001	<0.001	<0.001
HF vs LF	+ 0.008	+ 0.003	+ 0.003	+ NS	+ NS	+ NS	+ NS	+ NS	+ NS	+ NS	+ <0.001
HF3 vs LF3	+ NS	+ 0.04	+ 0.008	+ NS	- NS	- NS	- NS	- NS	+ 0.01	+ NS	+ 0.003
CR vs LF	- <0.001	- <0.001	- 0.005	- NS	- 0.007	- 0.001	- 0.002	- NS	- NS	0 NS	- 0.03
CT vs CCT	+ <0.001	+ <0.001	+ 0.03	+ NS	- NS	+ NS	+ 0.005	+ NS	+ NS	+ NS	+ 0.01
LF3 vs LF	- NS	- NS	- NS	- NS	+ NS	+ NS	- NS	- NS	- <0.001	- NS	- NS
HF3 vs HF	- <0.001	- <0.001	- 0.006	- NS	- 0.04	- NS	- 0.03	- 0.002	- 0.008	- NS	- <0.001
CG vs LF	- 0.005	- NS	+ NS	+ NS	+ NS	- NS	- NS	+ NS	- NS	- NS	- NS

Upper panel) Mean values of factors measured in the experiment ± sem (n=11-14) for each of the 8 diets shown in Table 1. The factors (first row) are: energy consumed in kcal/day/rat; final body weight in grams; a.u.c. as area under the OGTT curve (0-2 h) in (mmol/l)×h; glucose postprandial in mmol/l; insulin post-gavage, one hour after gavage, with a dose of 4mg glucose per each gram body weight, in μIU/ml; insulin postprandial in μIU/ml; triglyceride, one hour post gavage, 4mg/g bw in mmol/l; triglyceride postprandial in mmol/l; number of ACF, no. of aberrant crypt foci /colon; Log of ACF, logarithm of the number of aberrant crypts/crypt foci.

LF, low fat diet; HF, high fat diet; CR, calorie restricted diet; CT, cafeteria diet; CCT, control cafeteria diet; LF3, low fat-high n-3 diet; HF3, high fat-high n-3 diet; CG, corn grits diet.

Middle row) *p*-value for ANOVA.

Lower panel) Pairwise comparison of groups, direction of difference between means of two groups and the *p*-value.

5.2.6 Statistical measures

The descriptive data for each dietary group (Table 5.2) were presented as averages ± SEM with the exception of ACF size which was presented as average log of ACF size to obtain a more symmetric distribution with a variance not dependant on the mean. The pairwise comparisons of the results for the dietary groups (Table 5.2, lower panel) were made with the two-tailed Student's *t*-test after first comparing all groups with analysis of variance. In pairwise comparisons, a few groups were tested more than once. However, most of the differences cited had very low p-values ($p<0.005$ is significant in groups with multiple testing) and the significance is unlikely to be due to multiple testing and a lack of independence of comparisons. Correlations between the response variables were computed without adjusting for dietary group (raw or total correlation) and with adjusting for dietary group (partial correlation), (Table 5.3, above and below the diagonal, respectively). Multiple regression was used to establish which of the variables could be used to explain the variability in log of ACF size after accounting for the dietary group.

5.3 Results

Energy consumption, final weight and measures of the markers of IR and promotion observed for the 8 groups of animals on the different diets are detailed in Table 5.2. (upper panel). The curves of the OGTT for the 8 groups are shown in Fig. 5.2.

5.3.1 Pairwise comparisons of food consumption, weight and measures of IR and ACF promotion

The comparisons of pairs of diets designed to differ in fat, energy, n-3 fatty acids and

Table 5.3. Correlations of factors (total correlation, above diagonal and partial correlation, below diagonal)[a]

Factors	eng/day	final weight	a.u.c	Glu-ppran	Ins-fast	Ins-gav	Ins-ppran	TG-gav	TG-ppran	log of ACF size
eng/day	1	0.84	0.62	0.35	0.17	0.13	0.50	0.17	0.32	0.66
final weight	0.48	1	0.63	0.41	0.24	0.19	0.51	0.17	0.26	0.66
a.u.c.	-0.03	0.13	1	0.34	0.18	-0.05	0.39	0.21	0.27	0.65
Glu-ppran	-0.05	0.10	0.06	1	0.06	-0.03	0.38	0.13	0.41	0.34
Ins-fast	-0.01	0.05	0.06	-0.05	1	0.15	0.13	0.03	-0.01	0.15
Ins-gav	0.01	-0.04	-0.17	-0.04	0.12	1	0.20	0.13	0.25	0.03
Ins-ppran	0.10	0.11	0.06	-0.05	0.01	0.12	1	0.13	0.25	0.35
TG-gav	-0.14	0.08	0.10	0.05	-0.04	-0.06	-0.03	1	0.26	0.12
TG-ppran	0.05	-0.00	-0.04	0.33	-0.11	-0.03	0.05	0.12	1	0.34
log of ACF size	0.08	0.24	0.23	0.12	0.04	-0.04	0.01	-0.05	-0.02	1

a: correlations are based on data for all animals (n=108). The values above diagonal of the table show total correlations (r), values greater than 0.254 are statistically significant ($p<0.01$). The values below diagonal of the table show partial correlations (pr), values greater than 0.267 are statistically significant ($p<0.01$).

eng, energy; a.u.c., area under curve; Glu, glucose; ppran, post-prandial; Ins, insulin; fast, fasting; gav, one hour after glucose gavage; TG, triglycerides.

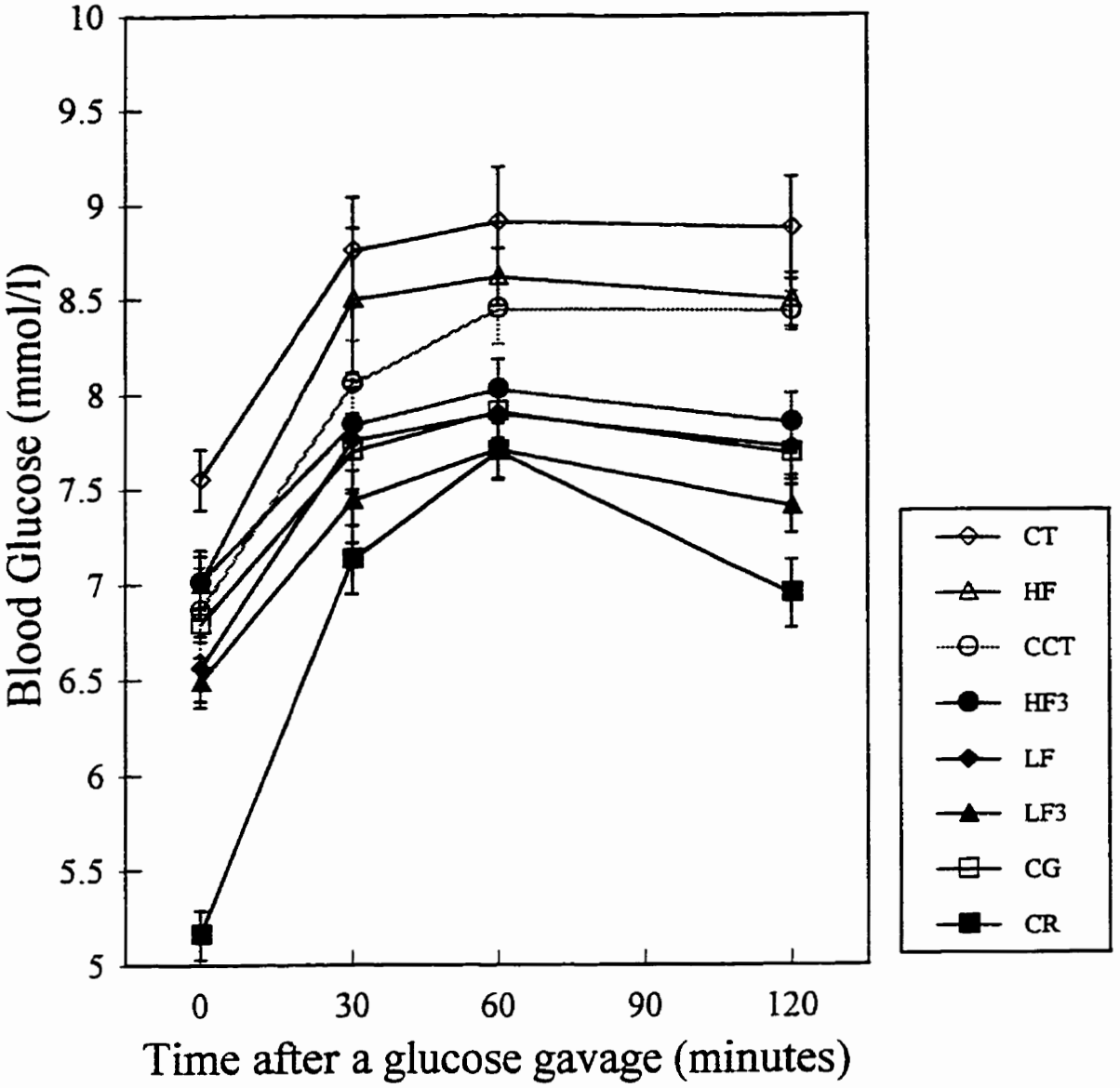


Fig 5.2. Results of the oral glucose tolerance test. values are mean ± SEM. Tail vein blood glucose was measured before and 30, 60 and 120 minutes after a glucose gavage (4mg/g bw). pairwise comparisons for 0 and 120 minutes:

	HF vs LF	HF3 vs LF3	CR vs LF	CT vs CCT	LF3 vs LF	HF3 vs HF	CG vs LF
Fasting	+ NS	+ $p<0.01$	- $p<0.001$	+ $p<0.02$	- NS	- NS	- NS
120 min	+ $p<0.001$	+ $p<0.04$	- $p<0.003$	+ NS	- NS	- $p<0.005$	+ NS

starch are shown in Table 5.2. (lower panel). The high fat diet (HF) resulted in a higher energy intake, a greater weight gain, an increased a.u.c. and an increased ACF size than the low fat diet (LF). The corresponding comparisons with the high and low fat diets containing increased amounts of n-3 fatty acids (HF3 and LF3) resulted in similar, though less marked differences. The cafeteria diet (CT) resulted in increased energy intake, final weight, a.u.c. and ACF size than its control (CCT), while the calorie restricted diet (CR) resulted opposite effect with decreased energy intake, final weight, a.u.c. and ACF growth. Post-prandial insulin was increased with the cafeteria diet, decreased with the calorie restricted diet. The substitution of n-3 fatty acids in the high fat diet resulted in reduced energy intake, final weight, a.u.c., and ACF growth and a reduced triglyceride after gavage and post-prandially. The corresponding comparisons with the n-3 low fat diet resulted in similar differences but most did not reach statistical significance. The corn grits diet (CG), which was expected to demonstrate a reduced carbohydrate digestibility of dietary starch compared with the corn starch in the low fat diet, resulted in a lower energy intake than the low fat diet, but none of the other measures approached statistical significance. Presumably the rats chewed the corn grits to starch quite efficiently so that the digestibility of the carbohydrate in the two diets was essentially the same.

5.3.2 Correlations of the measures

Average dietary energy, final weight, a.u.c. and ACF size for eight dietary groups were plotted as bar graphs, arranged in the order of ascending average of ACF size (Fig 5.3). It was evident from the data and comparisons in Table 5.2 and Fig. 5.3. that there were interesting associations between the average energy intake, final weight, a.u.c. and ACF size

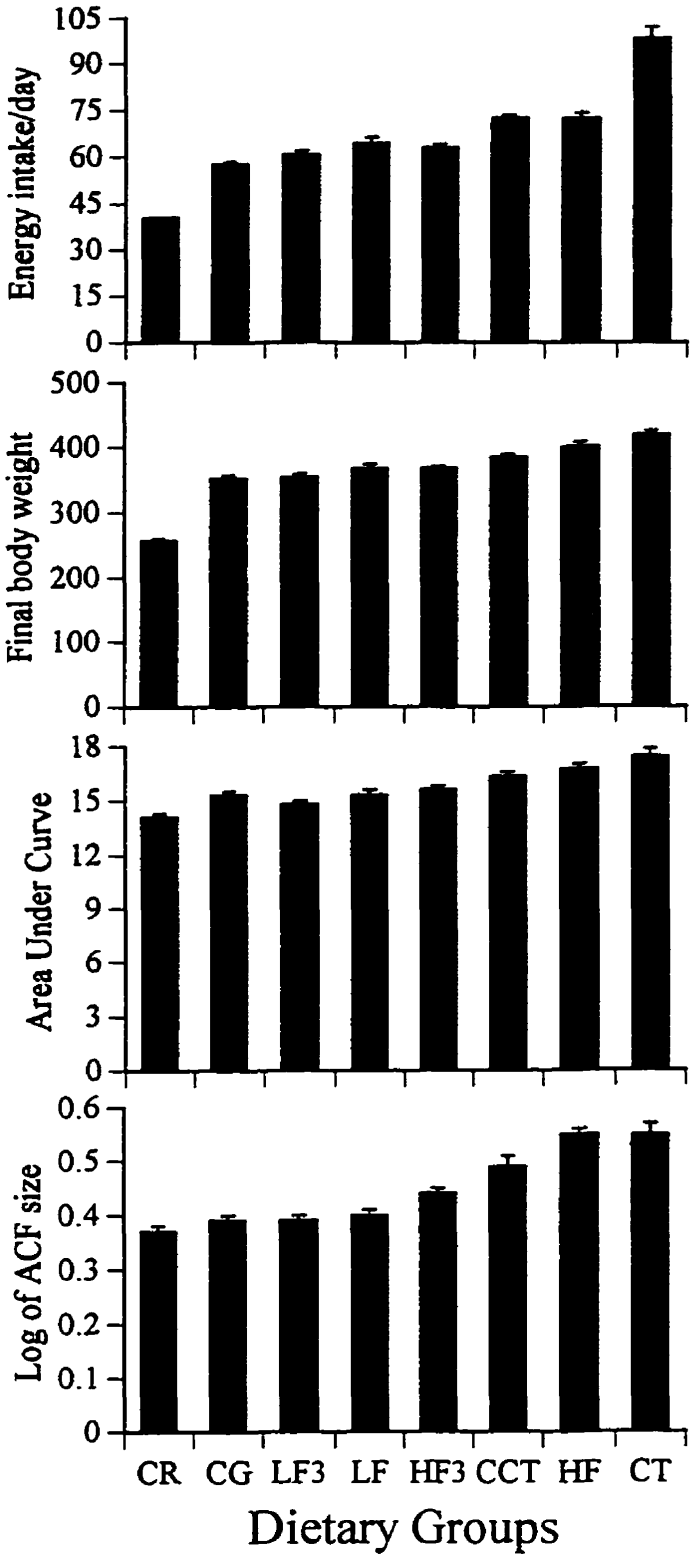


Fig 5.3. Energy intake/day (kcal/day), final body weight (g), area under curve ((mmol/l)×h), and log of ACF size (aberrant crypts/crypt foci) shown by dietary group from CR to CT as defined in Table 5.1.

of the eight dietary groups. ACF size was plotted as a function of these other measures in Fig 5.4. It was evident that ACF size was correlated with dietary energy, weight and a.u.c.

To examine the correlations further the raw or total correlation coefficients were determined (Table 5.3, above the diagonal). Raw correlations are based on deviations from the overall means. From the unadjusted or total correlations it was clear that energy consumption, final weight, a.u.c. and ACF size were all strongly associated with each other ($r = 0.62$ to 0.84, $p<0.001$). All four of these measures were also associated with the post-prandial measures of insulin, glucose and triglyceride concentrations ($r = 0.26$ to 0.51, $p<0.01$).

Partial correlation coefficients were also determined to adjust for dietary group variation (Table 5.3, below the diagonal). While the raw correlations are based on deviations from the overall means, the partial correlations, adjusting for dietary group, are based on deviations from the group means. These partial correlations provided an estimate of the correlation in a hypothetical population of animals that all received the same diet. The partial correlations indicated that even after taking into account the effect of dietary group, there were still substantial correlations between ACF size and a.u.c., ACF size and final body weight, and energy consumption and final weight ($r = 0.23$ to 0.48, $p<0.05$).

Stepwise linear regression (with forward selection) identified useful predictors for log of ACF size. This identified diet group, weight and a.u.c. (p-values: <0.001, 0.013 and 0.04, respectively). Jointly, these 3 variables explained 72.4% of the variation of log of ACF size. However, the additional explanation provided by weight and a.u.c. was limited since the R^2 for the regression with the categorical variable, diet group, as the only predictor was 69.4%.

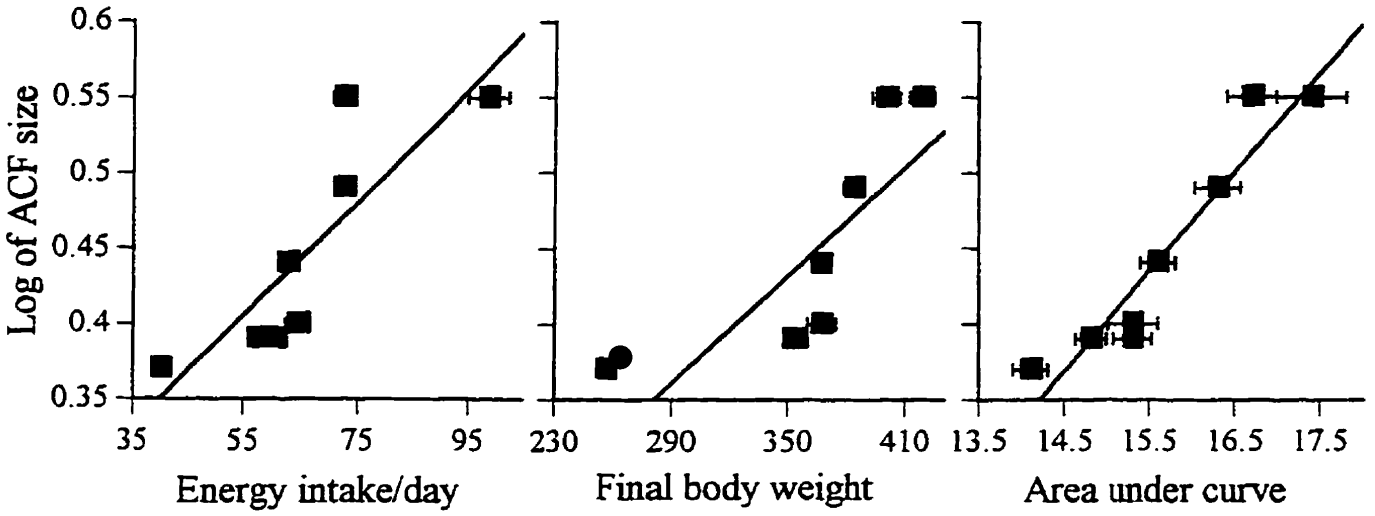


Fig 5.4. Log of ACF size as a function of energy intake, body weight and a.u.c. Log of ACF size (aberrant crypts/crypt foci) was shown as a function of energy intake (kcal) / day, final body weight (g), and area under curve ((mmol/l)×h) of the OGTT for all of the eight experimental dietary groups. Error bars larger than the symbols used, are shown in both directions. The lines are best mean square fits to the data. Correlations between log of ACF size and energy intake/day, final body weight, and area under curve are 0.66, 0.66, 0.65, respectively ($p<0.01$, for each), based on data for all animals ($n=108$).

5.4 Discussion

To determine whether diet affects the processes of IR and CRC promotion in the same way, the effect of diets high and low in fat, in energy, in n-3 fatty acids, and in readily digested carbohydrate were tested on markers of insulin resistance and colon cancer promotion in a single experiment with F344 rats. The results were consistent with the results of many earlier studies in which each of these factors was examined separately in individual studies with single dietary factors and with a measure of either insulin resistance or tumor promotion. Insulin resistance and colon cancer promotion are thus affected in a similar way by diet over a wide range of dietary variables. With the high and low fat diets, dietary fat comprised 60 or 12 percent of energy. With the calorie restricted and cafeteria diets, energy consumption represented from 40 to 100 calories per day. With the n-3 substituted diets, n-3 fatty acids make up an estimated 1% of beef tallow to nearly 15% of the flax oil/beef tallow mixture. With the corn grit diet, more than 50% of the readily digested corn starch was replaced with the presumably less easily digested corn grits. Clearly in these pairwise comparisons the dietary risk factors act in the same direction for both insulin resistance and colon tumor promotion. It is thus likely that a wide range of diets affect insulin resistance and tumor promotion in a similar way.

To examine the correlation between IR and CRC promotion, the average log of ACF size was plotted as a function of a.u.c. for each of the eight different diets (Fig. 5.4, last panel). The strong association was confirmed with the total and partial correlations, and linear regression studies. The major conclusion of this study is thus that the measures of insulin resistance (a.u.c. and post-prandial glucose, insulin and triglycerides) are correlated with the measures of colon cancer promotion (ACF size) for a wide range of diets.

In addition, the results showed correlations between energy intake and final weight, and a.u.c. and ACF size. The associations between a.u.c. and ACF size with both energy intake and final weight raised a further question: What mechanisms lead from the dietary factors to IR and tumor promotion? Two or more mechanisms may be involved: First, the different diets appear to affect energy consumption and final weight. With the calorie restricted diet, energy intake is enforced by the design, but with the other diets, the mechanism must involve appetite, satiety, and the regulation of food intake (Anderson, 1994). Variety in the cafeteria diet increases consumption; the beef tallow in the high fat diet increases consumption; the addition of n-3 fatty acids in the high fat diet seems to decrease consumption; the introduction of corn grits appeared to decrease energy consumption although it did not affect the other measures.

Second, the consumption of increased dietary energy, and possibly the increased body weight, may lead to IR and promotion. The surrogate measures of IR and promotion are each as closely related to dietary energy as to each other. Thus it is possible that the diets lead to a factor that independently leads to both IR and promotion. A possible factor and mechanism is suggested by the diabetes literature. Physiological studies undertaken over the past 30 years suggest that the post-prandial increase in chylomicra and very low density lipoproteins and the release of non-esterified fatty acids (NEFA) are likely important factors in the origin of IR. NEFA can affect insulin resistance in muscle and fat cells in vitro (Reaven and Chen, 1988, Paolisso et al., 1996) and triglycerides administered intravenously in a coloid form with heparin lead to increased NEFA and insulin resistance in peripheral tissues in both rats (Boivin and Deshaies, 1995, Kim et al., 1996) and human subjects (Boden et al., 1991) and also appear to increase oxidative stress (Paolisso and Giugliano, 1996). Dietary energy as

reflected in post-prandial triglyceride levels and release of NEFA could also affect epithelial cells. It is usual to attribute the promoting effect of dietary fat to the toxic effects of intraluminal fatty and bile acids on the luminal surface of the colonic epithelial cells. Exposure of these cells to NEFA from the basement membrane surface may be more important. Thus we suggest that increased dietary energy results in increased post-prandial lipemia and the peripheral release of NEFA which then leads to both insulin resistance and to a disruption of normal control mechanisms in epithelial tissues, perhaps through a mechanism involving oxidative stress, and to the increased tumor promotion of the epithelial tissues.

These proposed mechanisms suggest that three steps are involved in the effect of diet on IR and colon cancer promotion. First, diet composition affects satiety and the quantity of dietary energy consumed. Second, the diet composition and quantity of energy consumed affects the postprandial levels of energy in the bloodstream, in particular in the levels of lipoprotein triglycerides and also, perhaps, glucose. Third, the transient triglycerides and NEFA affect the response of fat and muscle cells to insulin, leading to IR, and affect the response of epithelial cells to other normal controls, leading to tumor promotion. An understanding of the mechanisms involved in the second and third steps pose interesting intellectual challenges, but studies of the first step could lead directly to approaches for diet control and, perhaps, to the prevention of these widespread diseases.

CHAPTER 6

GENERAL DISCUSSION

6.1 Introduction

As noted in chapter 3, if insulin resistance leads to colon cancer promotion, at least two conditions must be fulfilled. These conditions are necessary but not sufficient to prove causality. First, evidence of insulin resistance must appear before evidence of colon cancer promotion. Second, the degree of promotion or inhibition of colon cancer by different diets must be correlated with the degree of promotion or inhibition of insulin resistance by those diets. That is: **i)** Does evidence of insulin resistance appear before evidence of colon cancer promotion?

ii) Do different dietary risk factors affect insulin resistance and colon cancer promotion to the same degree?

The results of the experiments reported in chapters 4 and 5 answered these two basic questions in the affirmative. Throughout this research, colon cancer promotion was assessed on the basis of the average size of aberrant crypt foci (ACF), measured 100 days after carcinogen injection. Insulin resistance was assessed by oral and intravenous glucose tolerance tests (OGTT, IVGTT) and/or the area under the OGTT curve (a.u.c.). To answer question **i**, the effect of a high fat diet and a low fat diet on sequential development of insulin resistance and colon cancer promotion were compared. Impaired glucose tolerance was evident eight weeks before the assessment of ACF promotion. To answer question **ii**, the correlation between insulin resistance and colon cancer promotion for a range of diets was measured. The degree of impaired glucose tolerance and increased size of ACF were, in fact, correlated.

6.2 Overview of the results

Although the results of this research answered two questions asked in the affirmative, they do not prove that insulin resistance promotes colon cancer. McKeown-Eyssen and Giovannucci suggested that hyperinsulinemia was the cause of colon cancer promotion. However, here, fasting insulin concentrations were not elevated and not correlated with the size of ACF. Post-prandial (post morning meal) insulin concentrations were significantly correlated with ACF size, but this correlation was much weaker than the correlation between impaired glucose tolerance and ACF. Thus it is unlikely that post-prandial insulin is the primary cause of promotion. Furthermore, post-prandial triglycerides and glucose had the same correlation as post-prandial insulin with ACF (r=0.33-0.35, table 5.3). This further decreases the possible significance of insulin. The correlation between area under the curve (a.u.c.) and increased size of ACF was strong (r= 0.65, table 5.3), but this again does not prove that insulin resistance promotes colon cancer. Perhaps, the only way to prove that insulin resistance is or is not the cause of colon cancer promotion is to perform extensive experiments to discover the involved mechanisms.

A further possibility is that diet could be associated with other factors that lead to both insulin resistance and colon cancer promotion. An unexpected result from the experiment in chapter 5 was that the size of ACF had the highest correlation with energy intake/day and final body weight (r= 0.66, table 5.3). Energy intake and weight were also highly correlated with a.u.c. (r= 0.64, table 5.3). This raises a new question: Does energy intake induce IR and also promote CRC?

For the calorie restricted diet, energy intake is enforced by design, but for other diets different levels of energy consumption may involve appetite, satiety, and regulation of food

intake by diet composition (Anderson, 1994). The increased energy intake may lead to both insulin resistance and colon cancer promotion. Although the association between energy intake and final body weight was as expected closely related (r= 0.84, table 5.3), induction of IR and promotion of CRC can not be attributed to only obesity. IR (impaired glucose tolerance) appeared 4 weeks after starting the high fat diet at which time differences in body weights were not significant. High energy intake may lead to IR and CRC promotion, and at the same time to a higher body weight.

One possible mechanism linking energy intake to IR and CRC is that the quantity of energy consumed affects the post-prandial levels of energy in the bloodstream, particularly glucose and triglycerides. The flux of energy or flux of triglycerides may affect the response of adipose and muscle cells to insulin, leading to IR, and affect the response of epithelial cells to other normal controls, leading to tumor promotion. This process could involve a role for free radical-mediated oxidation (oxidative stress). Elevated levels of glucose are known to enhance oxidative stress (Hunt et al., 1988), and elevated levels of non-esterified fatty acids (NEFA) may also be involved in increasing oxidative stress (Paolisso and Giugliano, 1996). Increased post-prandial energy could thus lead to increased oxidative stress and result in both IR and CRC promotion.

6.3 Overview of diets and energy intake

Basically four types of dietary factors were used in the experiments in this thesis. They were dietary energy, dietary fat, type of dietary fat, and glycemic index of dietary starch. For dietary energy, the effect of a reduced and an excess energy intake were tested against a regular *ad libitum* energy intake. The calorie restricted diet was a straight forward restriction

from a standard low fat diet. Calorie restriction has been frequently used in both insulin resistance and colon cancer studies showing a reduction in both IR and CRC (Okauchi et al., 1995, Kumar et al., 1990) as well as body weight. These results are consistent with those of this thesis. In the cafeteria diet, variety of foods resulted in excess energy intake. The cafeteria diet is well known for its effects in insulin resistance studies, but has not been used in colon cancer studies. In the former, the cafeteria diet resulted in increased insulin resistance and body weight (Rizkalla et al., 1987), again a result which is consistent with those of this thesis. In cafeteria control diet, energy intake was reduced to a usual level by removing the variety factor. The cafeteria control diet was a new idea that was never used before. All three diets (calorie restricted, cafeteria and cafeteria control) worked successfully to provide different levels of dietary energy.

Consistent with other studies, the high fat diet resulted in insulin resistance and colon cancer promotion (Kern et al., 1990, Bull et al., 1979). The low fat and high fat diet in this thesis were designed to only manipulate the amount of dietary fat. However, the high fat diet also resulted in a higher energy intake and a higher final body weight. This overlap has also been observed in some other studies (Narisawa et al., 1991, Bird and Lafave, 1995). The reason for it may be:

i) The high fat diet had 1.37 times more caloric density compared to the low fat diet. In order to ingest the same amount of calories, rats on high fat diet must consume 28% less grams of food compared to the low fat group. This reduction in the quantity of food may not be possible for rats. In this research, rats on high fat diet reduced the food ingestion by 20%.

ii) It has been suggested that fat provides less satiety compared to other macronutrients (Geliebter, 1979). This may be the reason for higher energy intake in high fat diet.

The effect of n-3 fatty acids was tested in a low fat diet and a high fat diet. Adding n-3 fatty acids inhibited both insulin resistance and colon cancer promotion as was suggested by other studies (Storlien et al., 1991, Reddy et al., 1991). Although n-3 fatty acids resulted in lower energy intake in both cases, only high fat n-3 diet reduced energy intake and final body weight significantly. The observed energy intake reducing effect of n-3 fatty acids was unexpected and the reason for it is not clear. A previous experiment has reported a reduction of body weight with n-3 fatty acids in animals, but the effect has not been attributed to difference in energy consumption (Cunnane et al., 1986).

The effect of glycemic index was tested by replacing most of the starch in the low fat diet with corn grits. Corn grits have larger particle size compared to powdered corn starch used in the low fat diet, and it was presumed that it reduces rate of digestibility and therefore glycemic index. However, the diet did not reduce a.u.c. It reduced energy intake to a small degree, but this reduction did not affect body weight significantly.

6.4 Conclusion

McKeown-Eyssen and Giovannucci suggested that hyperinsulinemia, possibly through the growth promoting effect of insulin, promotes colon cancer. The results of studies in this thesis do not support a strong role for hyperinsulinemia. The association between impaired glucose tolerance and colon cancer promotion is much stronger than that for hyperinsulinemia and colon cancer promotion. However, this association does not prove that insulin resistance (impaired glucose tolerance) promotes colon cancer. Moreover, the association between energy intake and insulin resistance as well as colon cancer promotion is the strongest of all. This suggests that circulating energy supply may have an important role in inducing IR and

promotion of CRC. Further experimental studies are needed to investigate the relationship between energy intake, insulin resistance and colon cancer.

6.5 Possible future work

Two kinds of studies might be considered in the future: First are studies investigating mechanism(s) involved between diet composition, energy intake, insulin resistance and colon cancer. Second are studies testing a range of other dietary or life-style risk factors on energy intake, IR and colon cancer promotion in order to find those factors with the strongest correlations and to rank the degree of risk.

6.5.1 Investigating mechanisms involved

This could involve considerable experimental work. Mechanisms could be divided into two parts: **i)** those that link diet composition and energy intake, and **ii)** those that link energy intake to IR and CRC. The first relationship, between diet composition and food/energy consumption, has been a subject under investigation for years (Anderson, 1994, Geliebter, 1979). For the second relationship, although there are many mechanisms suggested for linking diet and IR or diet and CRC, a mechanism (s) linking dietary energy with both IR and CRC, is a new concept. Both IR and CRC studies offer a number of mechanisms to explain the relationship between dietary factors and these two clinical conditions. It will be a great challenge to find a broader based mechanism that explains the diverse effect of diet composition and energy intake on IR and CRC. One suggestion as it was mentioned in section 6.2, is to test for the effect of diet on oxidative stress and the effect of oxidative stress on IR and CRC.

6.5.2 Testing different dietary risk factors

The advantages of testing further risk factors for IR and CRC , and also for energy intake and weight are:

i) This type of research provides further strong and reproducible correlations, which could lead to new hypothesis.

ii) They would allow a ranking of dietary risk factors based on the degree of ability of these factors to inhibit or promote IR and CRC, which could be important in clinical studies.

One of the most controversial dietary factors in terms of both IR and CRC is dietary fat. The literature has consistently shown that increased dietary fat promotes both IR and CRC (Kraegen et al., 1991, Tang et al., 1996, Reddy et al., 1977). However, the effect of type of dietary fat is not as clear cut. In most studies oils containing n-3 fatty acids, such as fish oil, flaxseed oil and perilla oil, have inhibitory effects on both IR and CRC promotion (Zampelas et al., 1995, Reddy and Sugie, 1988, Storlien et al., 1991, Narisawa et al. 1991). Olive oil with mono-unsaturated fatty acids has an inhibitory effect only on CRC (Reddy and Maeura, 1984), and not on IR (van Amelsvoort et al., 1988). A role for the ratio of n-3/n-6 fatty acids has been suggested (Storlien et al., 1993, 1991). Testing the effect of different types of fat and different ratios of n-3/n-6 on measures of IR and CRC promotion, as well as energy intake and weight could provide a valuable insight.

One other important dietary component is carbohydrate. It has been shown that slowly digested carbohydrate inhibits IR and CRC promotion (Byrnes et al., 1995, Caderni et al., 1991). In this thesis the study of starch digestibility was not satisfactory. A dose response study between carbohydrate digestibility and measures of IR, CRC, and energy intake could be useful to clarify the relationship.

The relationship between dietary protein and IR or CRC seems much weaker compared to fat and carbohydrate. However, It has been shown that protein may have an effect on food consumption and energy intake for a certain period of time (Anderson et al., 1994, 1988, 1983). The long term effect of dietary protein on energy intake and body weight (Clinton et al., 1992, Ratnayake et al., 1997) is controversial. Therefore, it might be interesting to test the effect of dietary protein on energy intake, IR induction and CRC promotion, and correlations between them.

The importance of information from testing dietary risk factors is to provide recommendations for obese and insulin resistance patients. In western countries, food consumption and energy intake is usually higher than requirements, and this energy is usually provided by fat and simple carbohydrates (Rizek et al., 1983). This high energy intake promotes obesity and insulin resistance that subsequently can progress to NIDDM. Since colorectal cancer usually occurs later in life, insulin resistance could be used as a biomarker for CRC risk. Proper experimental based recommendations are vital for prevention of these widespread diseases.

CHAPTER 7

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Appendix A:

Experiment: Effect of two different type of caging on insulin resistance measures

Background: Two type of caging system are used for housing rats. The first type is metal cages with wire mesh at the bottom. In wire bottom cages, rats do not have access to bedding or their feces. The second type is plastic cages used with wood chips or corn cob bedding. In Study 1, chapter 4, rats were placed on plastic cages with wood chips bedding. Rats were suspected to consume wood chips and their feces. Therefore, in the Study 2, chapter 4, rats were placed on wire bottom cages and at the same time a control group was placed in plastic cages with wood chips bedding. The experiment that is described below was an extension of experiment D, in Study 2, in chapter 4.

Objective: To determine the effect of type of caging on body weight and insulin resistance measures.

Materials and Methods: Male F344 rats were acclimatized for 19 days and were initiated with the colon carcinogen azoxymethane (AOM) at 20mg/kg body weight between 9 and 11AM. One week later they were randomized into two groups (n=10), one on wire bottom cages and another on plastic cages with wood chips bedding and at the same time a low fat diet (the low fat diet is described in chapter 4) was started. Rats were weighed weekly. At the end of the experiment (4-5 weeks after randomization), an oral glucose tolerance test (as described in chapter 4) was performed, and fasting blood samples (7 AM-2 PM) were collected .

Results: Body weight and fasting insulin, triglycerides and glucose were not different between two groups with different type of caging (with or without bedding). Oral glucose tolerance was also the same in two groups.

Appendix B:

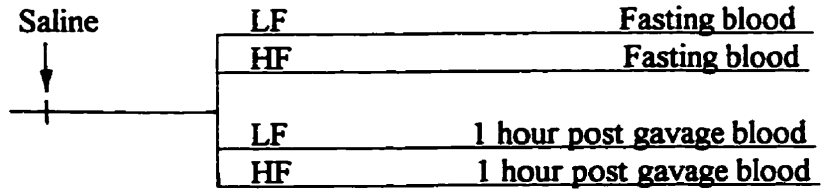
Experiment: The effect of carcinogen azoxymethane (AOM) on insulin resistance

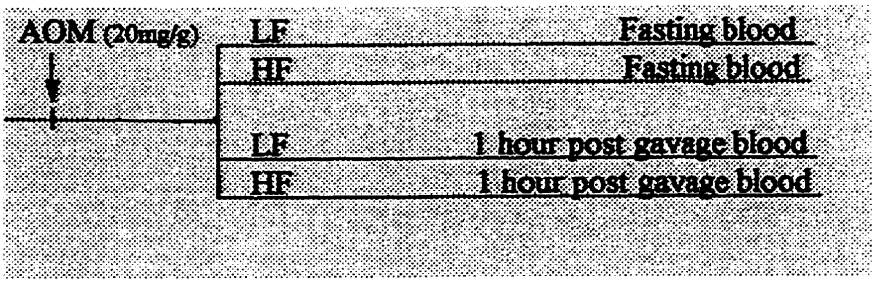
Background: Study 1 in chapter 4 showed that azoxymethane initiated rats on the high fat diet developed impaired glucose tolerance and had increased ACF growth. The effect of carcinogen (AOM) injection on impairment of glucose tolerance and increasing insulin resistance was under question. The following experiment was an extension of experiments D and E, in Study 2, chapter 4. In the following experiment rats were not initiated with AOM.

Objective: To determine the effect of carcinogen (AOM) on body weight and measures of insulin resistance in rats on low fat or high fat diets.

Materials and Methods: 40 male F344 rats were acclimatized for 19 days and were injected with saline at the same time that animals in Study 2, chapter 4 were initiated with AOM. One week later they were randomized in 4 groups (n=10), and two groups were placed on a low fat diet, and two others on a high fat diet (low fat and high fat diets are described in chapter 4). At the end of experiment (4-5 weeks after randomization), one low fat group out of two and one high fat group out of two were fasted (7 AM-2PM) and blood samples were collected. The second low fat and high fat groups were fasted and then they were given a gavage of glucose, and one hour after gavage blood samples were collected. Fig. B.1 clarifies the design of the experiment.

Results: The high fat groups had impaired glucose tolerance, but fasting insulin, triglycerides, and glucose were not elevated compared to low fat groups. Post gavage insulin was significantly higher in high fat groups. AOM injected and saline injected groups were not different in weight and insulin resistance measures for either low fat or high fat groups.





Study 2
(Chapter 4)

Fig. B.1. Diagrammatic representation of experiment testing the effect of saline injection vs. AOM injection on insulin resistance in rats. LF, low fat; HF, high fat

Appendix C:

C.1 Diet preparation and composition of diet supplement for cafeteria diet (chapter 5)

Three food items were presented for rats every day. The schedule and food items were:

Mon	*Tues*	*Wed*	*Thur*	*Fri*	*Sat*	*Sun*
wiener						
wafer	wafer					
cheese puff	cheese puff	cheese puff				
	bologna	bologna	bologna			
		pnut butr	pnut butr	pnut butr		
			Ritz	Ritz	Ritz	
				Kit Kat	Kit Kat	Kit Kat
					wiener	wiener
						wafer

Brands of the food items were: bologna and wiener, Oscar Myer; cheese puff, Hostess Fritolay; peanut butter, Kraft; KitKat, Nestle; Ritz cracker, Christie; wafer, Hollandia.

The composition of the supplement diet is shown in table C.1.1. This diet is specially supplementing DL-Methionine, choline bitartrate, fiber, vitamins and minerals. About 2.5 grams of supplement diet was sprinkled on top of other foods each day.

Average food and energy consumption of 8 food item, and average fat, protein and carbohydrate intake of rats on cafeteria diet is summarized in table C.1.2. Cafeteria control had the same food composition except that every thing was mixed together, therefore food and energy consumption was reduced compared to cafeteria diet.

Table C.1.1. Composition of diet supplement

	g/100g
Casien	18
DL-Methionine	1.8
Corn starch	24
Sucrose	5
Corn oil	5
Cellulose	18
Mineral Mix	21
Vitamin Mix	6
Choline bitartrate	1.2
TBHQ	0.006
Total	100

Table C.1.2. Average food and macronutrient intake of rats on cafeteria diet

	pnut butr	wiener	cheese puff	Ritz	Kit	bologna	wafer	supl.	Average
food (g)/wk	11.3	40.1	9.1	10.6	20.4	44.6	15.8	15	166.8
eng (kcal)/wk	79	128	52	55	108	142.5	83.5	42	690
								fat/wk (g)	48
								protein/wk (g)	19.1
								CHO/wk (g)	46
								%eng as fat	62
								%eng as protein	12
								%eng as CHO	27

eng, energy; pnut butr, peanut butter; supl., supplement diet; CHO, carbohydrate; wk, week.

C.2 Composition of corn grits diet

Almost half of corn starch in the low fat diet was replaced with corn grits. Corn grits was bought from distributors of Grain Process Enterprises Ltd. (Scarborough, Canada). Composition of corn grits was taken from table of food composition provided at USDA's web site (Table C.2.1). The low fat diet that was mixed with corn grits was adjusted according to corn grits composition. The composition of this diet is shown in table C.2.2.

Table C.2.1. Composition of corn grits (USDA)

	g/100g
Protein	8.800
Lipids	1.200
Carbohydrates	78.000
Fiber	1.600
Moisture	10.000
Ash	0.400
Minerals:	*mg/100g*
Calcium	2.000
Iron	1.000
Magnesium	27.000
Phosphorus	73.000
Potassium	137.000
Sodium	1.000
Zinc	0.410
Copper	0.075
Manganese	0.106
Vitamins:	*mg/100g*[a]
Vitamin C	0.000
Thiamin	0.130
Riboflavin	0.040
Niacin	1.200
Pantothenic acid	0.485
Vitamin B-6	0.147
Folate, *mcg*	5.000
Vitamin B-12, *mcg*	0.000
Vitamin A, *IU*	0.000
Vitamin A, *RE*	0.000
Vitamin E, *ATE*	0.260

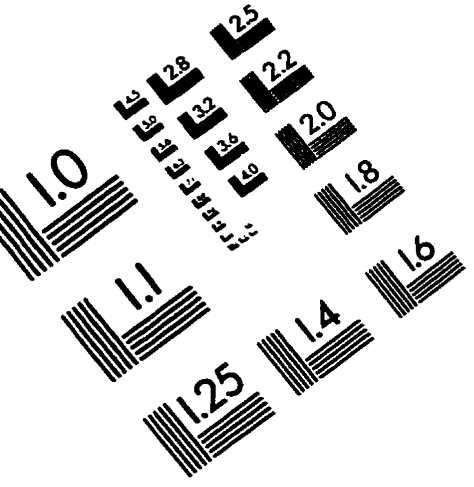
a: unless otherwise noted

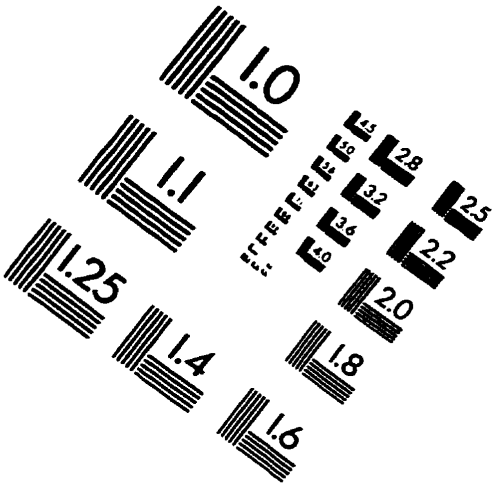
Table C.2.2. Composition of low fat diet mixed with corn grits

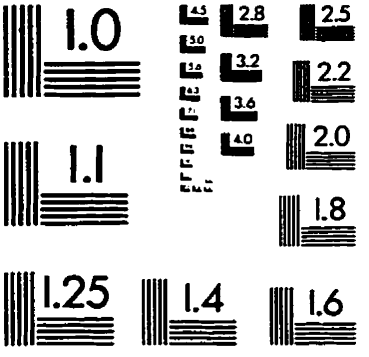
	g/100g
Casien	24.65
DL-Methionine	0.54
Corn starch	45.3
Sucrose	9.1
Corn oil	8
Cellulose	3.9
Mineral Mix*	6.34
Vitamin Mix*	1.81
Choline bitartrate	0.36
TBHQ	0.0018
Total	100

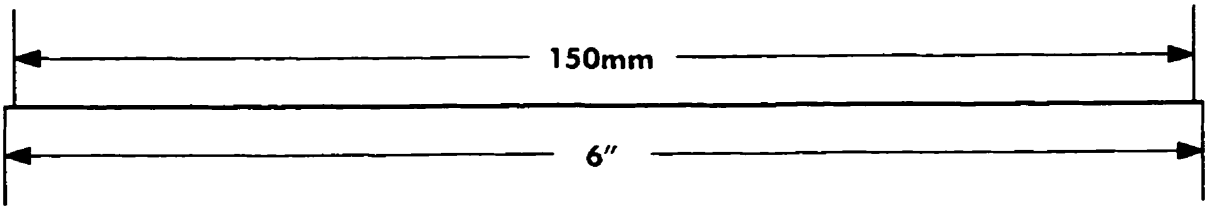
*, adjusted according to corn grits minerals and vitamins

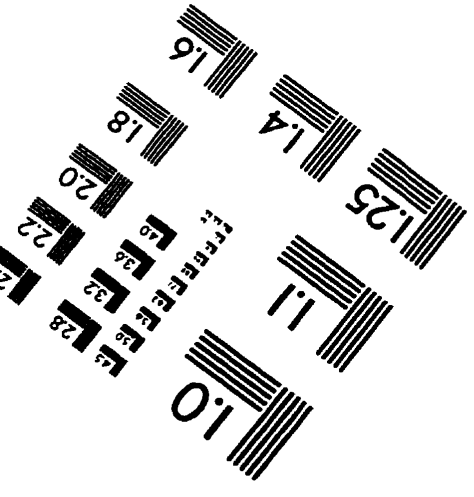
IMAGE EVALUATION
TEST TARGET (QA-3)

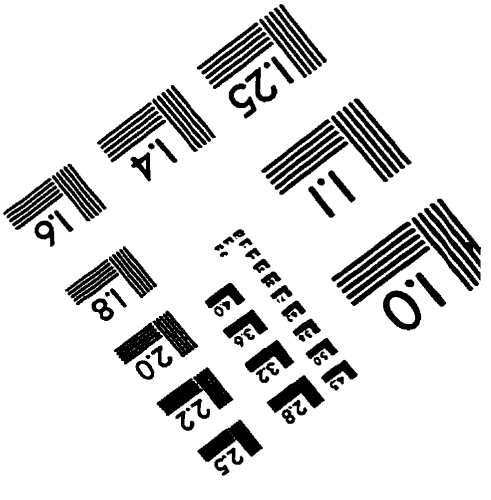












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