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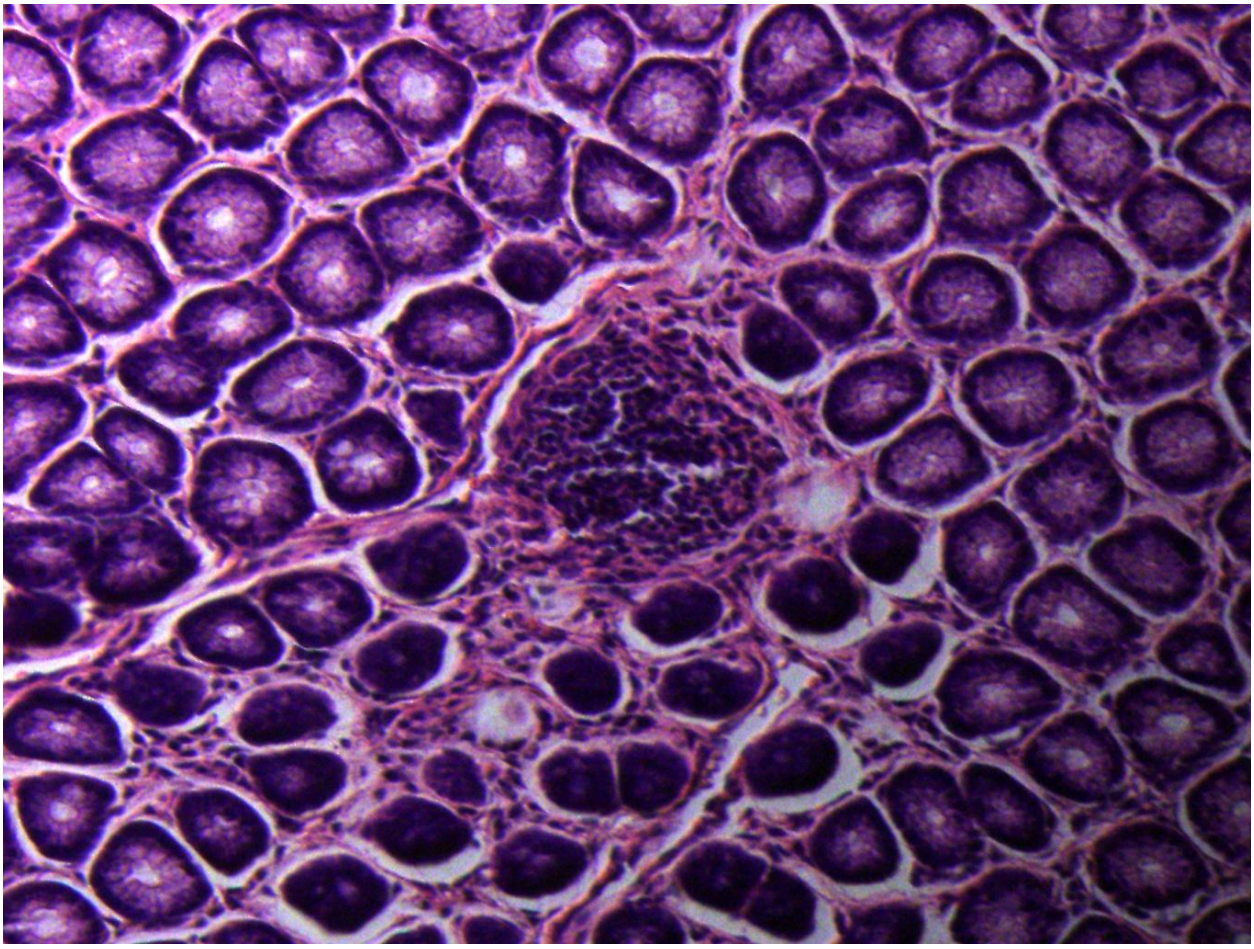
The impact of different diet compositions and caloric restriction on colon cancer probability in aged mice



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Abstract

Introduction Colorectal cancer incidence is epidemiologically related to a western life style. Since randomized controlled trials in human are lacking, the exact causes remain uncertain.

Materials and methods To examine the impact of diet quality and quantity on colon carcinogenesis, colons of 24 month old C57Bl/6J mice were obtained from a systems biology project. The mice received either a high fat *ad libitum* (HFAL), low fat *ad libitum* (LFAL) or low fat calorie restricted (LFCR) diet during their entire life time. The colons were fixed, embedded in paraffin, sliced and stained with hematoxylin & eosin (HE), CD45 and β-catenin. The inflammation foci were quantified. Body weights of these mice and body weights, fat mass, insulin and glucose measurements of mice housed under the same conditions were obtained from the project.

Results Body weight, relative total fat mass and relative visceral fat mass did not differ between LFAL and HFAL but did significantly differ between LFAL and LFCR. Glucose and insulin measurements were significantly different between LFAL and LFCR, but not between LFAL and HFAL. No carcinogenesis was found. Inflammation foci were detected and quantified in HE staining, using the CD45 staining as a confirmation. The number of foci was significantly different between LFAL and LFCR, but not between LFAL and HFAL. The mean foci size and summed total of all foci in an animal did not show differences. Alterations in β-catenin levels or localization were not visible. Remarkable large foci, expressing a lymphoid structure, were seen. More HFAL animals and less LFCR animals had these foci, both compared to LFAL.

Discussion and conclusion Based on literature, insulin resistance and inflammation are the major factors linking diet to colon carcinogenesis. Regarding to these factors, the results show LFCR versus LFAL differences but no HFAL versus LFAL differences. In adipose tissue, an important regulator of both insulin resistance and inflammation, differences were also only found between LFCR and LFAL. Although the *ad libitum* animals must be more prone to colon carcinogenesis than the calorie restricted animals, no carcinogenesis was found. In earlier studies, chemically induced colon carcinogenesis is enhanced, but not initiated, by dietary factors. It might be that no colon carcinogenesis initiating factor was present in these C57Bl/6 mice and that the mice were not prone to develop colon cancer spontaneously.

Key words: Colon cancer, colorectal cancer, adipose tissue, insulin, IFG-1, inflammation, caloric restriction, high fat diet

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List of abbreviations

AGE	Advanced Glycation End Products
AL	Ad Libitum
AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variances
BMI	Body mass index
CC	Colon cancer
CR	Calorie restriction
CRC	Colorectal Cancer
GALT	Gut-Associated Lymphoid Tissue
HF	High Fat
H&E	Hematoxylin and eosin
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
LF	Low Fat
mTOR	Mammalian Target of Rapamycin
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
SEM	Standard Error of the Mean
SNP	Single Nucleotide Polymorphism
STAT3	Signal Transducer and Activator of Transcription 3
Sq	Squares, representing 0,04167 x 0,04167 mm at 400x magnification
T2DM	Type 2 Diabetes Mellitus
TNF	Tumor Necrosis Factor
WHR	Waist-to-hip ratio

1. Introduction

Over the last decades, energy intake of humans has increased dramatically, as well as the intake of unhealthy nutrients like fat and sugar (Briefel & Johnson, 2004; Paeratakul et al., 2003). Moreover, technological advances allow a more sedentary lifestyle (Lakdawalla & Philipson, 2009). This higher energy input and lower energy output are imbalanced, which comes with consequences. The metabolic syndrome, obesity, diabetes and cardiovascular diseases are more common diseases in countries with this western lifestyle (Popkin, 2006). Cancer, and in particular colorectal cancer (CRC), appears to be related to the obesity epidemic (Center et al., 2009; Vano et al., 2009). The geographic variation of the incidence of colorectal cancer is remarkable, as shown in figure 1.

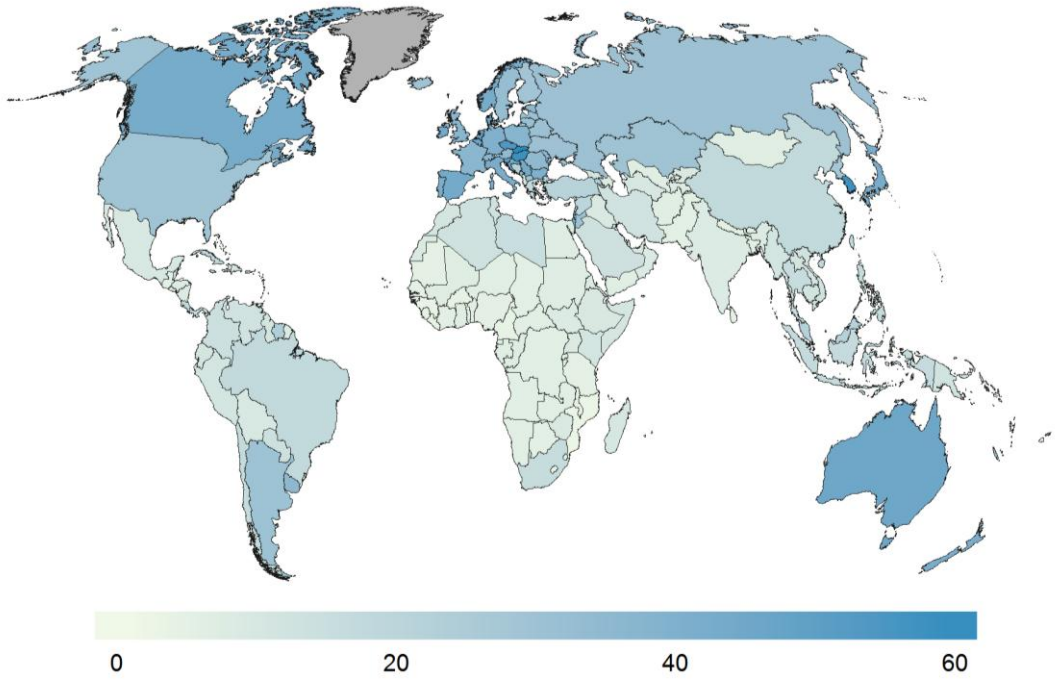

Figure 1 The incidence of colorectal cancers in men per 100.000 in 2012. The geographic pattern is very similar in woman. All data of the International Agency for Research on Cancer are corrected to take into account some degree of incompleteness or under coverage and are age-standardized to take into account variations in the age structure of the populations (International Agency for Research on Cancer, 2014).

Over time, it is observed that the largest rise in colorectal cancer incidence rates of individual countries occur during the economic transition, which is often accompanied by adaptation to the western lifestyle and behavior, and that the rates stabilize or decrease in long-standing economically developed countries

(Center et al., 2009). The incidence and mortality of CRCs in regions with a different developmental status are shown in figure 2.

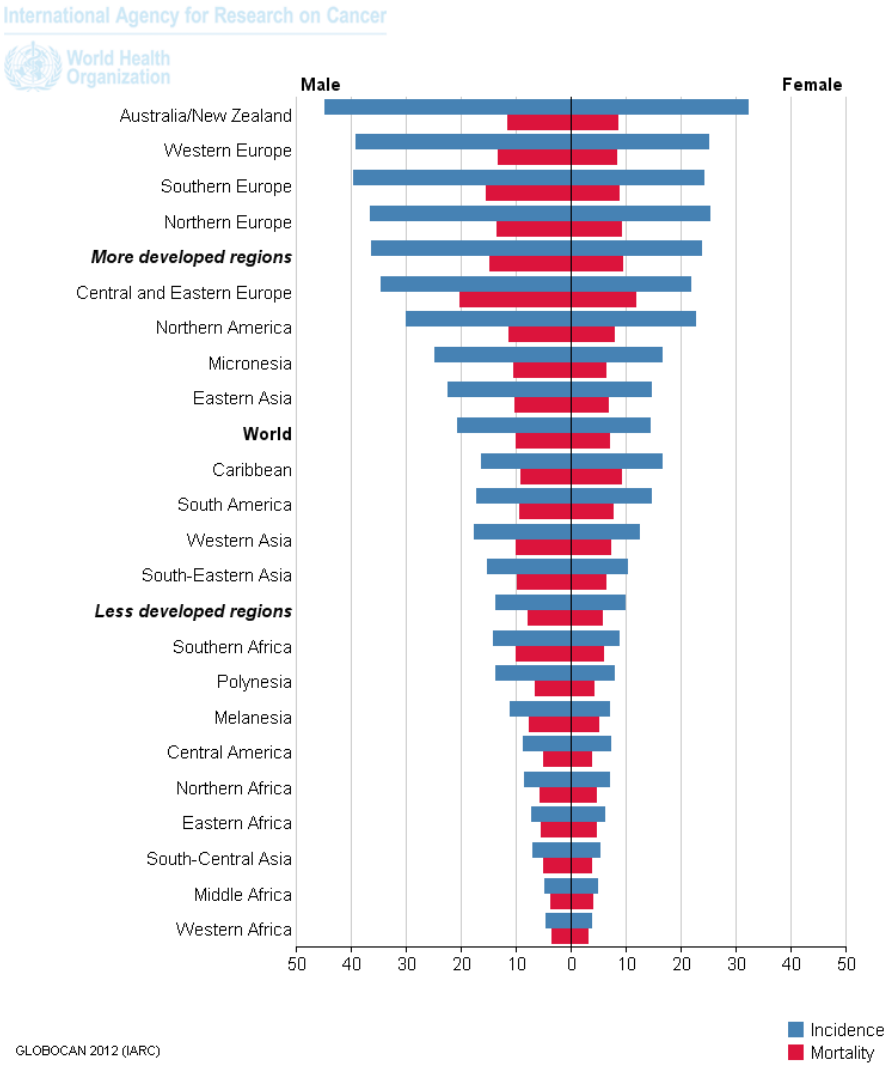

Figure 2 The incidence and mortality of colorectal cancers in men and women per 100.000 inhabitants. All data of the International Agency for Research on Cancer are corrected to take into account some degree of incompleteness or under coverage and are age-standardized to take into account variations in the age structure of the populations (International Agency for Research on Cancer, 2014).

The age-standardized incidence of CRC per 100.000 in men differs between 44.8 in Australia and New-Zealand and 4.5 in Western Africa. The incidence in woman is slightly lower and differs between 32.2 and 3.8 per 100.000 in the same regions. CRCs are the third most common cancer in men, representing 10% of the total, and the second most common in woman, representing 9.2% of the total. The mortality rates of CRC are the highest in Central and Eastern Europe, 20.3 in men and 11.7 in woman per 100.000, and the lowest in Western Africa, 3.5 and 3.0, respectively (International Agency for Research on Cancer, 2014). Survival rates are highly depending on the stage of the carcinoma at diagnosis (Brenner et al., 2013), and are worse in the less developed regions of the world, probably due to poorer hygiene, poorer therapies and diagnosis in a later stage. Survival rates are improved by regular screening, since

this helps to detect the cancer at an earlier stage (Shaukat et al., 2013). Some countries have a screening program for people above a certain age and new methods for easier and lower cost screening are being examined (C. C. Chan et al., 2010; Chen et al., 2014). The broader use of screening methods could decrease the burden of disease. The costs of CRCs are high. Medicare, the USA social insurance program for people aged older than 65 years, paid an average of 41.134 USD for a CRC patient diagnosed in 2002 in the 2 months before and the first year after diagnosis (Warren et al., 2008). It is known that the costs of colon cancer are lower than the costs of rectal cancer (Luo et al., 2010).

Although several studies which associate lifestyle with colon cancer or colorectal cancer have been published, these epidemiological researches do not reveal the exact causes. It is known that being overweight is associated with the incidence of colorectal cancer, but whether this is caused by the westernized diet, causing overweight and colorectal cancer at the same time, or by the elevated body mass index itself, cannot be concluded (Brenner et al., 2013; Calle & Kaaks, 2004; Levi et al., 2011; Watson & Collins, 2011). Many factors, including obesity, diet composition, smoking, diabetes and physical inactivity, can contribute to colon cancer and these factors are often accompanied by each other in persons adapted to the western lifestyle. Since randomized controlled trials are lacking, the epidemiological studies cannot provide evidence about the exact causes of the association between a western lifestyle and colon cancer or colorectal cancer. To unravel how a western lifestyle leads to CC or CRC, possible mechanisms should be examined.

In order to become cancerous a cell needs to obtain several characteristics, called cancer hallmarks, including sustaining proliferative signaling, resisting cell death, evading growth suppressors and enabling replicative immortality. For a malign tumor, inducing angiogenesis and activating invasion and metastasis are needed. There is increasing evidence that deregulating cellular energetics and avoiding immune destruction are two addition cancer hallmarks. Genome instability and inflammation are known to enable the acquisition of the cancer hallmarks. An overview of the hallmarks is shown in figure 3 (Hanahan & Weinberg, 2011). There are different proposed mechanisms for the arousal of cellular cancer hallmarks due to a western diet.

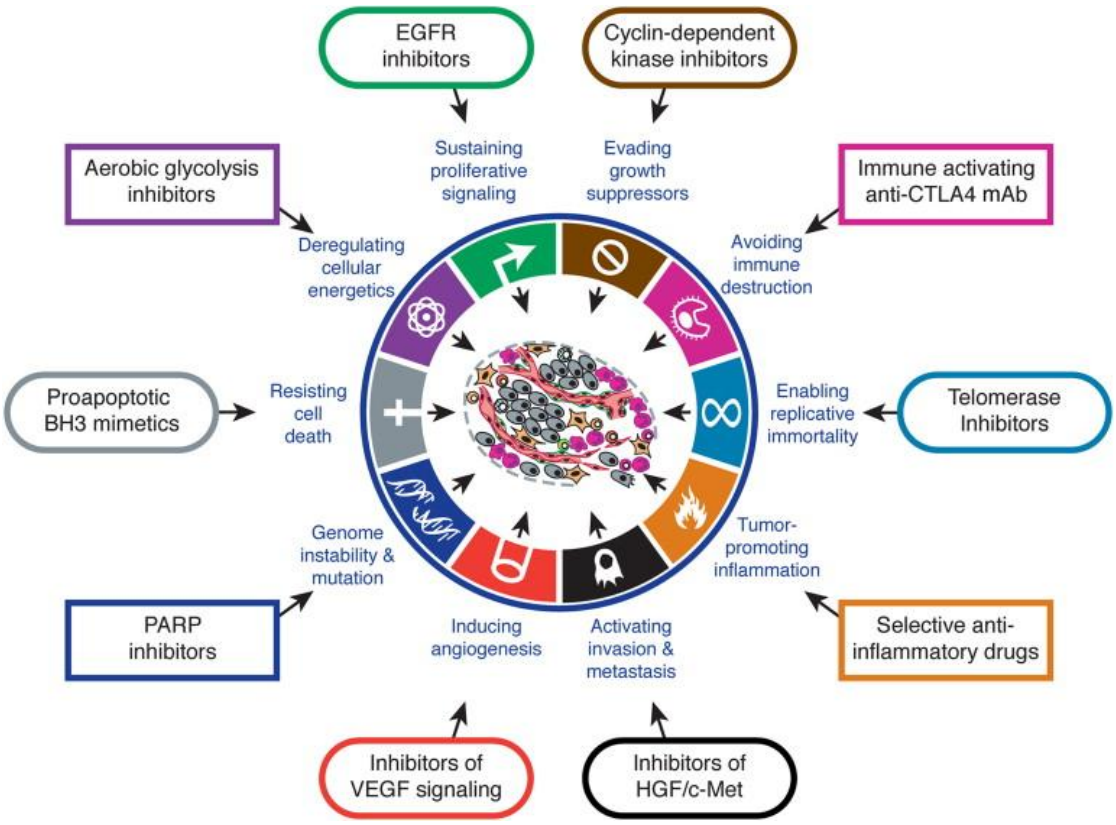

Figure 3 The hallmarks of cancer cells, including possible therapeutic targets (Hanahan & Weinberg, 2011).

One way for a cell to obtain these cancer hallmarks is by genetic mutations. Based on family history studies, population studies and an extensive twin study, inherited forms of CRC account for approximately 30% of all colorectal cancers. Approximately 5% of all CRC cases arise due to a highly penetrant inherited risk, including the Lynch syndrome or familial adenomatous polyposis (Grady, 2003; Jasperson et al., 2010; Lichtenstein et al., 2000). The remaining part of the familial cases is probably due to genes which are less penetrant but more common and are possibly triggered by gene-gene and gene-environment interactions. Genome-wide association studies have identified ten Single Nucleotide Polymorphisms (SNPs) that are associated colorectal cancer susceptibility (Tenesa & Dunlop, 2009). However, the known SNPs only explain a small part of the hereditability, and more common SNPs have yet to be identified (Jiao et al., 2014). Obesity and colorectal cancer may have shared genetic susceptibility since the same SNP variants are observed in both obesity and colon cancer (Roberts et al., 2010). Although the genetic component of CRC might not be ignored, the epidemiological evidence strongly indicates that lifestyle is an important factor in the onset of it and can provide cells cancer hallmarks. There were several risk and preventive factors proposed (Brenner et al., 2013).

Several nutrients are proposed to have an effect on the development and progression of colon cancer. Pericleous et al. have recently performed an extensive systematic literature review to reveal the role of different nutrients (Pericleous et al., 2013). Red meat might be associated with an increased risk of colon cancer, especially when it is cooked at high temperature. The haem component of meat can promote the formation of N-nitroso compounds, which are carcinogen (Lijinsky, 1999), and cooking can lead to more formation of these N-nitroso compounds, as well as the formation of mutagenic heterocyclic amines and polycyclic aromatic hydrocarbons (Cross & Sinha, 2004). The darker the meat is, the more haem it contains. The epidemiological evidence in combination with these proposed mechanisms endorse the carcinogenic potency of red meat. An association between alcohol intake and colon cancer is suggested by many studies, but the mechanism is still unknown. Proposed pathways include its ability to reduce folate levels, delay DNA reparation and promote mucosal cell proliferation and abnormal DNA methylation (Kune & Vitetta, 1992; Pericleous et al., 2013). Different epidemiological studies on the role of fat and unsaturated fat present conflicting results. However, a carcinogenic mechanism of dietary fat is proposed. Dietary fat induces formation of the bile acids deoxycholic acid and lithocholic acid, known carcinogens. Bile acids cause cell death, genomic instability and oxidative stress, which results in protein and DNA damage, and are thereby carcinogenic (Ajouz et al., 2014; Macrae, 1993; Payne et al., 2008). The evidence for the possible beneficial effects of omega-3 polyunsaturated fatty acids, dietary fiber, fruit and vegetables, folic acid, calcium and vitamin D, different polyphenols and other phytochemicals remains inconclusive, due to a lack of randomized controlled trials and many confounding factors such as smoking status, physical activity, obesity and diabetes, which can hardly be corrected for (Aune et al., 2011; A. T. Chan & Giovannucci, 2010; Pericleous et al., 2013). Overall, although the evidence is not striking yet, diet composition seems to have an important potency in modifying the colorectal cancer risk.

Besides the nutrient quality in the diet, diet quantity also seems to impact colon carcinogenesis. Diet quantity alteration can be reached by caloric restriction or overfeeding, both leading to an unbalanced energy balance and resulting in different fat masses. Overfeeding often results in obesity, which is associated with colorectal carcinogenesis in many epidemiological studies, as mentioned before. Obesity is also associated with colon carcinogenesis in mice (Tuominen et al., 2013). In APC mice, a mouse model of intestinal and colon carcinogenesis, it was noticed that the size of polyps was increased by a high fat diet (Day et al., 2013). Olivo-Marston et al. induced colon carcinogenesis in FVB mice using azoxymethane. Overfeeding, resulting in diet-induced obesity, enhanced colon carcinogenesis in these

mice, while 30% calorie restriction suppressed it (Olivo-Marston et al., 2014). Adipokines, insulin and inflammation factors are mainly proposed as mediators in the relationship between nutrient intake and colorectal carcinogenesis.

Many epidemiological human studies are available providing links between diet and colorectal carcinogenesis, but cannot conclude about the exact cause. In this research, the impact of diet quality and quantity on colon carcinogenesis in aged mice will be examined. Colons were obtained from a systems biology project. In this project, a large number of mice were divided in 2 diet groups. One group received a low fat diet, one group received a high fat diet. Both diet groups were divided in 3 subgroups, of which one received *ad libitum* food, one received ad libitum food and a running wheel and one received a restricted amount of food, 60% of what the *ad libitum* group ate. Food intake, water intake, running wheel activity and body weight were measured regularly. Furthermore, the mice were exposed to several challenges, including insulin measurements, indirect calorimetry and behavioral tests. Mice were sacrificed at different time points in their lifetime: 6, 12, 18 and 24 months, and one group was not subjected to terminal experiments to examine lifestyle effects on lifespan. For this research, the colons of the 24 month old low fat *ad libitum*, high fat *ad libitum* and low fat calorie restricted mice were available. It is hypothesized that a high fat diet will enhance colon cancer susceptibility, while a calorie restricted diet will decline colon cancer susceptibility, both compared to the control, low fat diet *ad libitum*, group.

2. Materials and Methods

2.1 Animals and Housing

C57BL/6J mice were obtained from a breeding colony at the Center for Behavior and Neurosciences (University of Groningen, Groningen). Only the male mice were used, since males, compared with females, appear to be more prone to develop metabolic and age-related diseases (Hwang et al., 2010). Furthermore, males do not have the fluctuating hormone levels due to the oestrus cycle. The nest size of the experimental mice was 5 to 8 and the nest has to have at least one female in it, to exclude developmental factors which can influence the metabolism during lifetime. The offspring of different couples was mixed into the different experimental groups. The mice were individually housed and their cages were provided with bedding material, nest material and a wooden stick and paper roll as cage enrichment. The mice were housed at 22°C and on a 12h dark, 12h light cycle (lights on at 7am UCT+1). Water was provided ad libitum.

Mice were kept under different dietary and experimental conditions throughout their lifetime from weaning onwards. There were 6 experimental groups.

1) *ad libitum* high fat food including access to a running wheel (HFRW)

2) *ad libitum* high fat food excluding access to a running wheel (HFAL)

3) *ad libitum* normal chow (low fat) food including access to a running wheel (LFRW)

4) *ad libitum* normal chow (low fat) food excluding access to a running wheel (LFAL)

5) calorie restricted high fat food excluding access to a running wheel (HFCR)

6) calorie restricted normal chow (low fat) food excluding access to a running wheel (LFCR)

Both high and low fat diets were obtained from ABDiets (Woerden, The Netherlands). The high fat diet contained 45% fat, the low fat diet contained 6% fat. The exact composition of these diets can be found in appendix I. The calorie restricted groups received 60% of the amount of food the *ad libitum* groups with the same diet ate. They received their meal every day between 4 and 5 pm (UCT+1). Every 3 weeks, body weight and water intake of all animals and the food intake of the *ad libitum* animals were

measured. Also, the running wheel activity of the LFRW and HFRW groups was measured for certain periods.

These six experimental groups made it possible to examine the effects of different diets, caloric restriction and physical activity on metabolism and biological ageing during lifetime. To make a full characterization possible, mice were sacrificed at the age of 6, 12, 18 and 24 months. One cohort of mice was not sacrificed, to examine the effects of the experimental conditions lifespan. This lifespan cohort started with a total of 16 mice per group, the other age cohort needed total of 16 mice per group at sacrificing age to reach statistical power. These 16 mice were divided in two groups of 8, undergoing different experiments during life time. Due to the predicted natural drop-out, based on earlier research, the total amount of animals at the beginning was 732. Only the mice sacrificed at 24 months from group 2 (n=6), 4 (n=6) and 6 (n=8) were used for this colon analysis. Fat mass data, fasting insulin data and the Area Under Curve data of glucose measurements were obtained from the project. These measurements were done in the other group of 8, but since both groups were housed under exactly the same conditions, values are representative for the colon analysis-group.

The protocol was approved by the Dutch Animal Experimentation Committee (DEC), under the license number DEC6120B.

2.2 Processing of the Colons

Right after the termination of the animals, the colons were collected, washed and the fecal pallets were removed. The colons were fixed in 4% formaldehyde and embedded in separate paraffin blocks using an automatic tissue processor (Leica TP1020). The paraffin-embedded colons were stored at 4°C.

The colons were remelted to make an entire colon examination possible. The colons were cut open in the longitudinal direction, fold open, flattened and embedded again, with the luminal side downwards. The remelted colons were cut into slices of 5 µm thin, using a microtome (Adama Instruments Microm HM340E). The slices were mounted onto adhesive glass microscope slides (StarFrost® Objektträger, Knittel Glass, Braunschweig, Germany), always two slices were mounted at one glass slide. The slides were dried overnight at 37°C. The slides were stored at room temperature. One colon from the HFAL group dropped out the paraffin block during slicing, which reduced the n of HFAL to 5.

2.3 Stainings

Hematoxyline-eosine staining

One out of ten slides were stained with hematoxylin and eosin (H&E) following the normal laboratory procedure, which can be found in appendix II. After the H&E staining, the slides were stored at room temperature.

CD45 and β-catenin immunohistochemistry

For immunohistochemistry, de paraffin slides were deparaffinized in xylene, followed by rehydration in ethanol. The antigen was retrieved by boiling in a citrate buffer (pH 6.0) and endogenous peroxidase blocking was performed with H_2O_2. After incubation of the primary and secondary antibody, the slides were treated with ABC reagent (Vector Laboratories, Burningame, CA, USA) and a DAB staining with SigmaFAST™ 3,3-diaminobenzidine tablets (Sigma-Aldrich, St. Louis, MO, USA) and hematoxylin counterstaining ware performed. The complete protocol can be found in Appendix III.

CD45 was used to stain inflammatory cells (Dahlke et al., 2004). Antibodies targeting CD45 were obtained from BD Biosciences (San Jose, CA, USA, material number: 550539) and were used in a 1:100 dilution. Different from the protocol, endogenous peroxidase blocking was performed after the incubation of the first antibody, to prevent CD45 epitope damaging by H_2O_2. The secondary antibodies, biotinylated polyclonal rabbit-anti-rat immunoglobulins (DAKO, Glostrup, Denmark), were used in a 1:300 dilution.

Primary antibodies targeting β-catenin were also obtained from BD Biosciences (material number: 610154), the secondary antibodies were biotinylated polyclonal rabbit-anti-mouse immunoglobulins (DAKO) and non-specific staining was blocked using 1% BSA. The primary antibodies were used in a 1:500 dilution and the secondary antibodies in a 1:300 dilution.

2.4 Microscope

Images of the stained colons were obtained using an Olympus BX50 light microscope and Cell^B software (Digital Image Systems, Athens, Greece).

2.5 Quantification

The inflammations in each colon were quantified in the H&E stained slices. One out of ten slides was H&E stained and the left coupe on each slide was examined, unless this coupe was incomplete; then the

right coupe was examined. So, every one out of twenty slides was examined, which equals every 5 µm out of 100 µm. Inflammations were defined using the CD45 staining for inflammatory cells. The researcher was blinded for the diet of the animals.

The slices were checked for inflammation using a normal light microscope at 100x magnification. When an inflammation spot was found, the size was measured using an ocular lens with a raster at 400x magnification. When the inflammation was too large to measure at 400x magnification, it was measured at 100x magnification. De raster was 10 by 10 squares. At 400x magnification, each square represented 0,04167 x 0,04167 mm. At 100x magnification, each square represented 0,1 x 0,1 mm. The size of the inflammations was measured in squares; when measured at 100x magnification, the size in squares at 400x magnification was calculated.

The study design only allowed an accurate 2 dimensional, cross-sectional measurement. Whenever an inflammation site was found, the size of this inflammation varied between 5 and 100 µm (i.e. between 1 and 20 slices). When the same inflammation spot was found in multiple slices, the largest measured area was used for analysis. Inflammation data used for analysis included the number of inflammation foci, mean size of the inflammations and a summed total of all inflammation sizes in each animal. Some aberrant structures were examined by a certified pathologist.

2.6 Statistical analysis

The inflammation data were analyzed using IBM SPSS Statistics version 20 for Windows (Armonk, New York: IBM Corp.) Testing for normality was performed using the Kolmogorov-Smirnov test and testing for homogeneity of variance was performed using Levene's test. Testing for outliers was performed using Grubb's test. The means were compared using one-way Analysis of Variances (ANOVA). When more than two groups were compared at the same time, Tukey's honest significant difference test was used as post-hoc analysis to find the means that are significantly different from each other. When data was not distributed normally, the Mann-Whitney U test was used to compare the means. For testing correlations, linear regression was used. $P<0,05$ was considered to be statistically significant. Graphs were produced using GraphPad Prism (GraphPad Software Inc.), with error bars representing the Standard Error of the Mean (SEM).

3. Results

3.1 Body weight and fat mass

The mean body weight of the HFAL group was 51,4 gr, of the LFAL group was 45,7 gr and of LFCR was 23,2 gr at sacrificing. HFAL did not differ significantly from LFAL, but LFCR did differ significantly from LFAL (p=0,000). All individual body weights are plotted in figure 5 and mentioned in table 1.

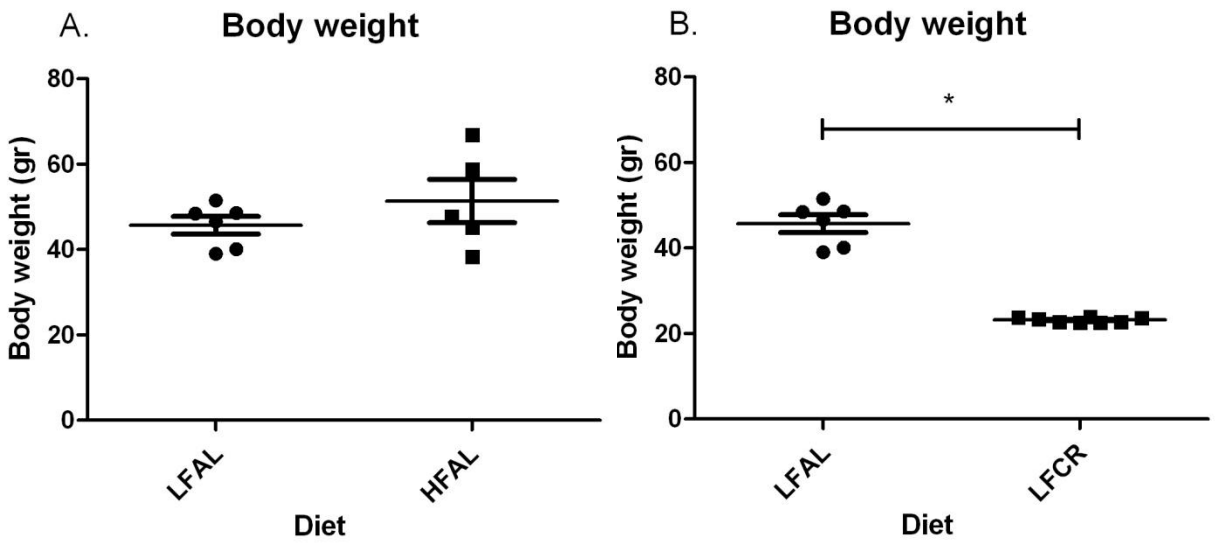

Figure 5 Body weights in grams at sacrificing age **A.** of LFAL and HFAL mice. **B.** of LFAL and LFCR mice (p=0,000). Data are shown ± SEM.

The mean body weights of all LFAL and HFAL age groups at sacrificing are shown in figure 6.

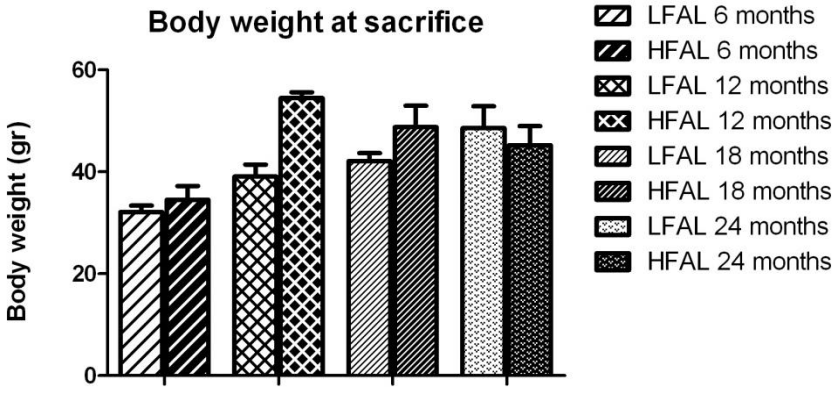

Figure 6 The mean body weights of HFAL and LFAL at 6, 12, 18 and 24 months at sacrificing. Data are shown ± SEM.

The fat mass of mice housed on the same conditions as the experimental mice was measured. The amounts of fat mass and visceral fat mass, corrected for body weight, did not differ between LFAL and HFAL, but was significantly different between LFAL and LFCR ($p<0,01$ and $p<0,01$), which is shown in figure 7 and 8.

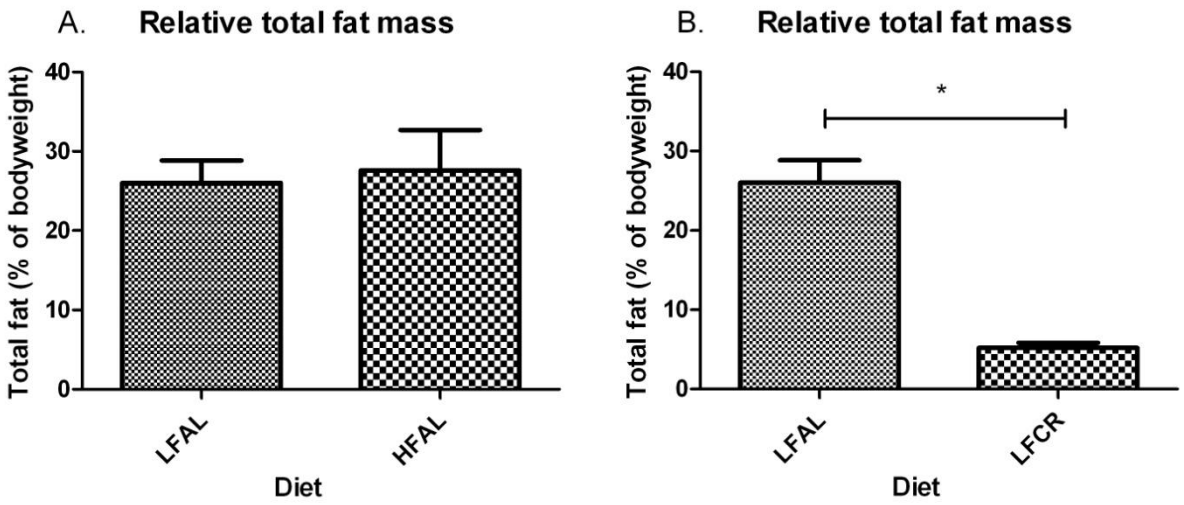

Figure 7 The total fat mass represented as percentage of total body weight **A.** of LFAL (n=3) and HFAL (n=4) **B.** of LFAL and LFCR (n=5) ($p<0,01$). Data are shown ± SEM.

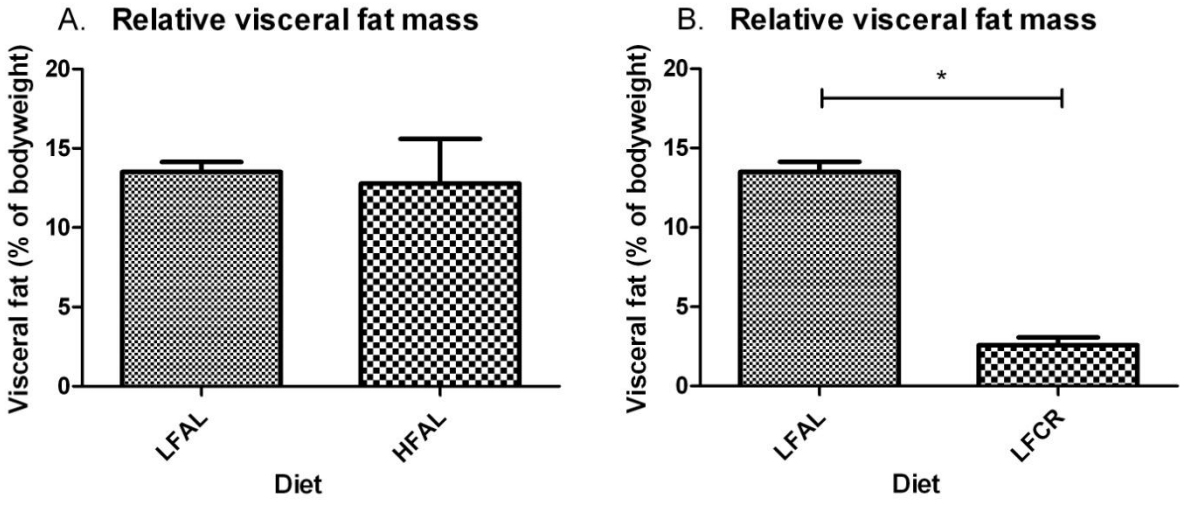

Figure 8 The total visceral fat mass represented as percentage of total body weight **A.** of LFAL (n=3) and HFAL (n=4) **B.** of LFAL and LFCR (n=5) ($p<0,01$). Data are shown ± SEM.

3.2 Fasting insulin and glucose

The fasting insulin and Area Under Curve glucose levels of mice housed under the same conditions as the experimental mice were measured and shown in figure 9 and 10. A significant difference in fasting insulin measurements was found between LFAL and LFCR ($p<0,05$), but not between LFAL and HFAL. Glucose levels were also significantly different between LFAL and LFCR ($p=0,001$), but not between LFAL and HFAL.

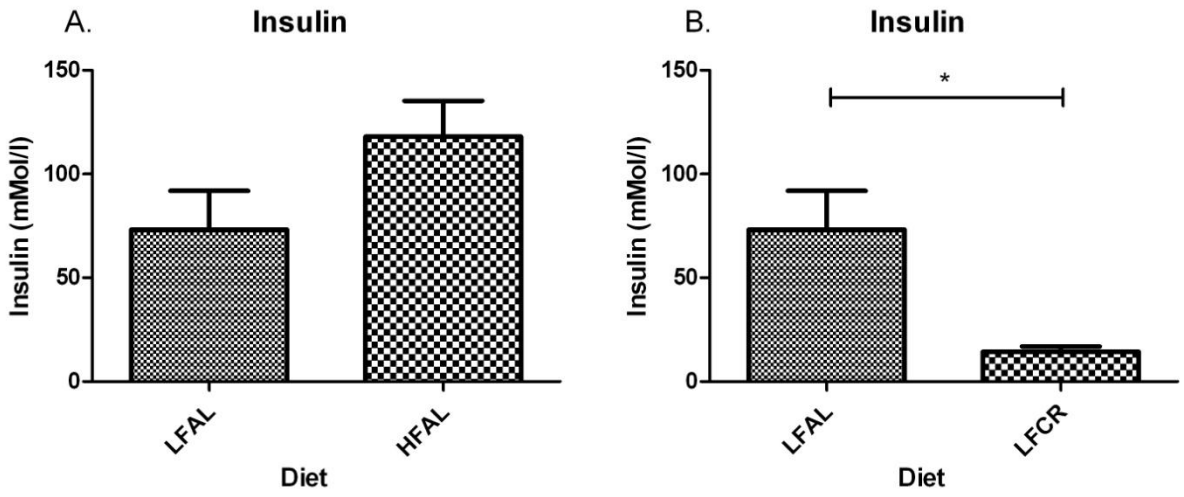

Figure 9 Fasting insulin values of **A.** LFAL (n=7) and HFAL (n=4). **B.** LFAL and LFCR (n=8) ($p<0,05$). Data are shown ± SEM.

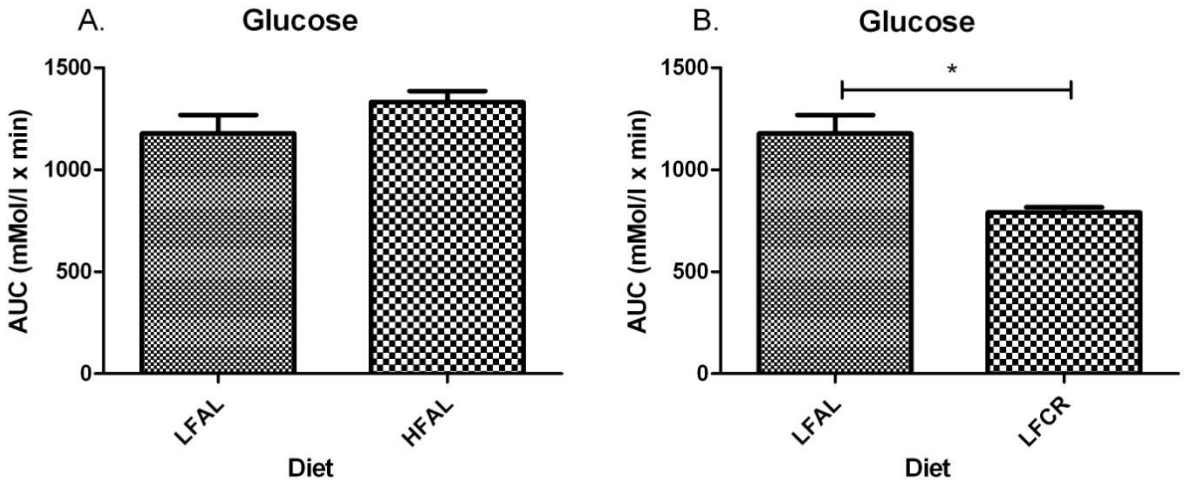

Figure 10 Areas Under Curve measured in the oral glucose tolerance test **A.** of LFAL (n=8) and HFAL mice(n=8). **B.** of LFAL and LFCR mice (n=8) ($P=0,001$). Data are shown ± SEM.

3.4 Hematoxyline-Eosine staining

Due to the protocol, both cross sectioned and longitudinal sectioned crypts were found. Examples of normal tissue at different magnifications are shown in figure 11.

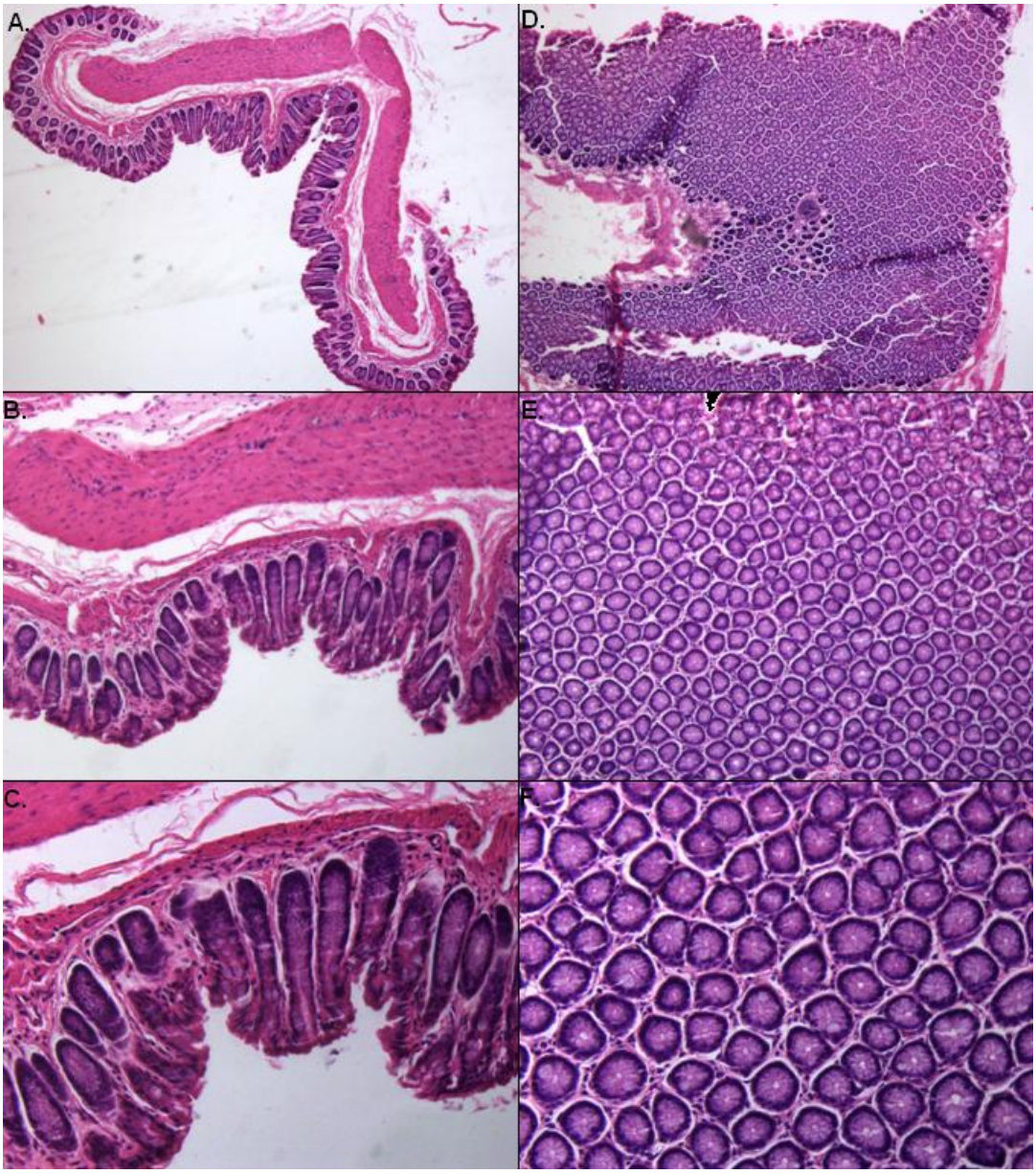

Figure 11 Hematoxylin-Eosin stainings of normal colonic tissue of a **A.** longitudinal section at 40x magnification. **B.** longitudinal section at 100x magnification. **C.** longitudinal section at 200x magnification. **D.** cross section at 40x magnification. **E.** cross section at 100x magnification. **F.** cross section at 200x magnification.

3.4 CD45 staining

The result of a CD45 staining was shown at different magnifications in figure 12. The negative control did not shown any antibody-specific staining, which is shown in figure 12D. This staining was used to determine inflammatory cells.

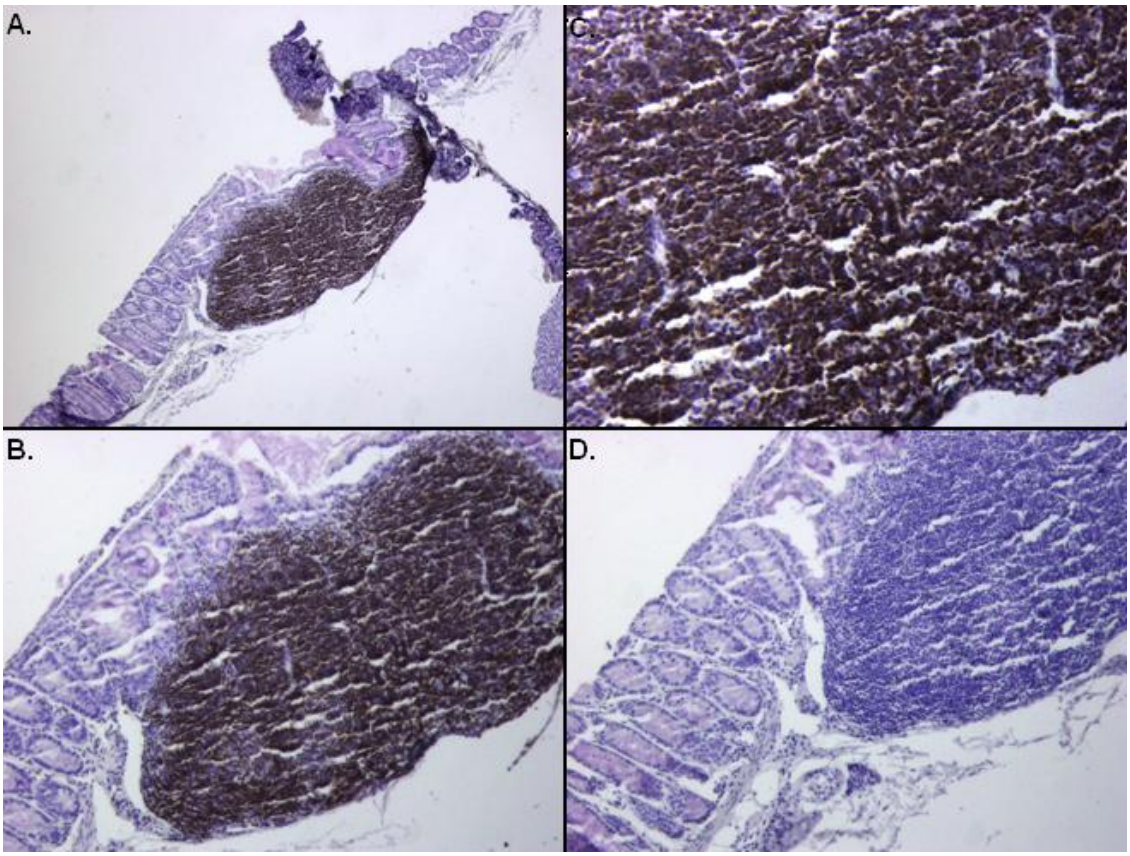

Figure 12 CD45 staining at **A.** 40x magnification. **B.** 100x magnification. **C.** 200x magnification. **D.** Negative control of a slice next to B at 100x magnification

3.6 β-catenin staining

The result of a β-catenin staining was shown at different magnifications in figure 13. The negative control did not shown any antibody-specific staining, which is shown in figure 13D. The baseline levels of β-catenin in the colonic crypts are high and because of that no alterations were visible.

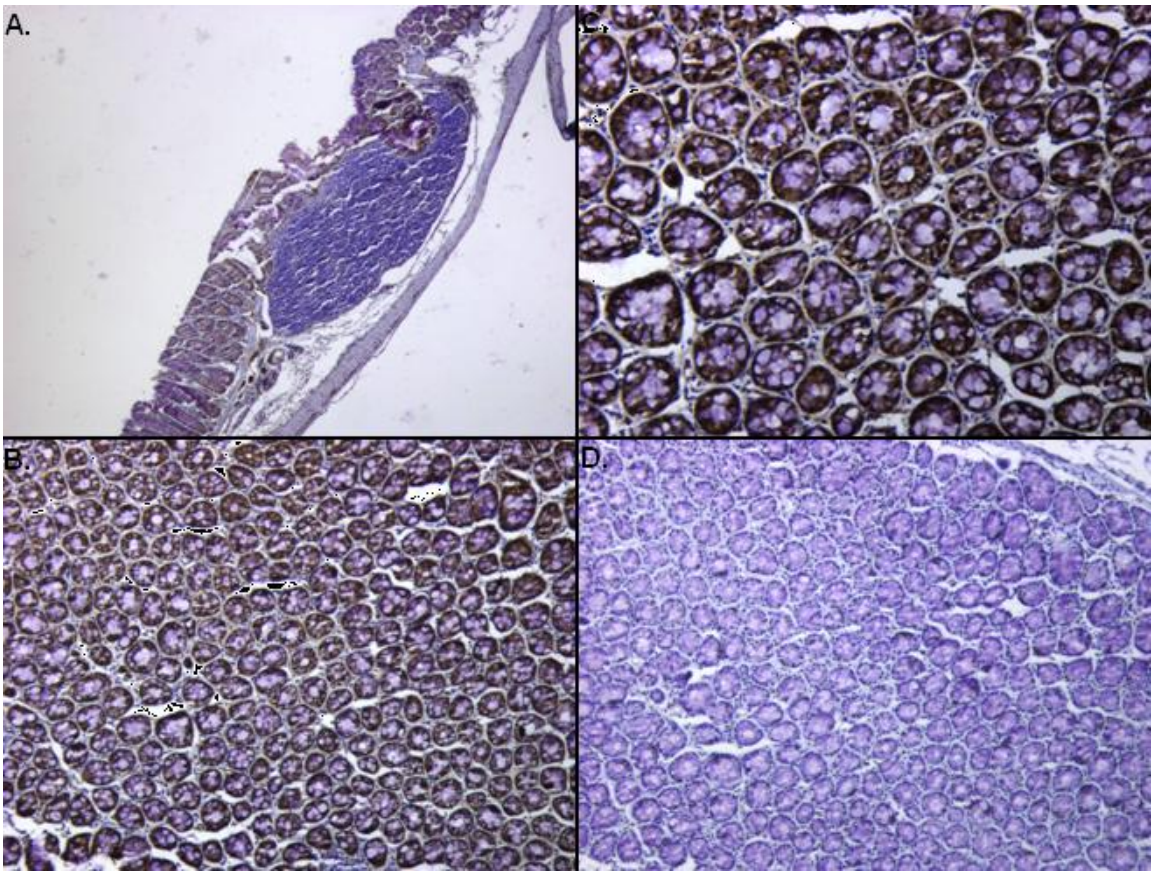

Figure 13 β-catenin staining at **A.** 40x magnification. **B.** 100x magnification. **C.** 200x magnification. **D.** Negative control of a slice next to B at 100x magnification.

3.7 Inflammation data

During the dissection of the animals, two animals, 24-65 and 24-33, were mixed up which was noticed afterwards. 24-33 was a sick animal with an enlarged spleen and a liver tumor. Remarkably, this animal had the least amount of inflammation in its colon of his diet group and had a low amount of inflammation compared to all animals. We assume that the researcher dissecting the colons did not notice the mention of the mixing up. Therefore, the data of animals 24-65 and 24-33 were interchanged. The mean size of the inflammations was calculated in squares of 0,04167 x 0,04167 mm (sq), as well as

the summed total of all inflammations found in one animal. Typical examples of different sizes of inflammation foci are shown in figure 14. The inflammation data are shown in table 1.

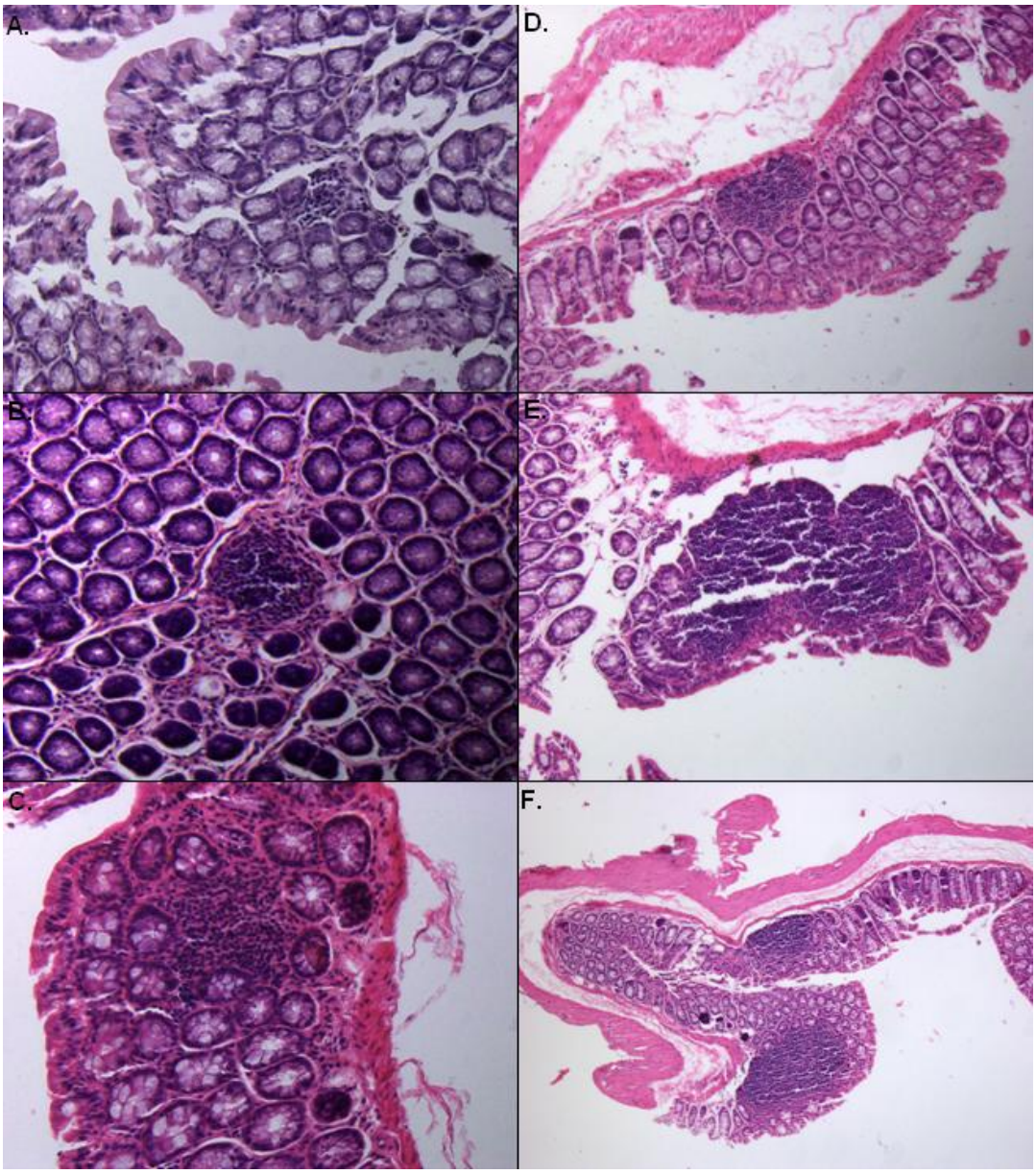

Figure 14 Inflammations of different sizes at different magnifications **A.** 1 square, 200x magnification. **B.** 4 squares, 200x magnification. **C.** 5 squares, 200x magnification. **D.** 27 squares, 100x magnification. **E.** 60 squares, 100x magnification. **F.** 22 and 81 squares, 40x magnification.

Animal no.	Group	Diet	Quantity	Total foci	Mean size (sq)	Summed (sq)	Body weight (gr)
24-30	1	HF	AL	56	16,0	897,9	47,8
24-21	1	HF	AL	35	6,8	246,5	66,8
24-22	1	HF	AL	60	14,1	848,8	58,8
24-27	1	HF	AL	42	13,3	560,0	45,2
24-33	1	HF	AL	71	5,9	418,0	38,3
24-63	2	LF	AL	46	7,4	339,5	40,1
24-62	2	LF	AL	50	6,5	324,0	48,4
24-66	2	LF	AL	58	13,9	804,6	51,5
24-65	2	LF	AL	26	2,8	71,5	46,6
24-64	2	LF	AL	45	3,7	164,5	48,6
24-61	2	LF	AL	48	11,0	529,5	39,0
24-104	3	LF	CR	20	3,3	65,0	22,8
24-101	3	LF	CR	9	19,9	179,0	23,7
24-102	3	LF	CR	27	13,2	355,5	23,9
24-110	3	LF	CR	36	4,2	152,5	23,8
24-105	3	LF	CR	8	18,3	146,5	22,8
24-107	3	LF	CR	15	8,3	124,0	22,6
24-109	3	LF	CR	21	8,8	185,0	23,4
24-108	3	LF	CR	28	5,7	158,5	22,6

Table 1 Inflammation data and body weights of all experimental subjects.

The total number of inflammations and the mean size of the inflammations did not differ from a normal distribution ($p>0,05$) and the variances were tested for homogeneity ($p>0,05$). No outliers were detected.

As shown in figure 15, no significant differences were found in the mean size of the inflammations neither between HFAL and LFAL nor between LFAL and LFCR.

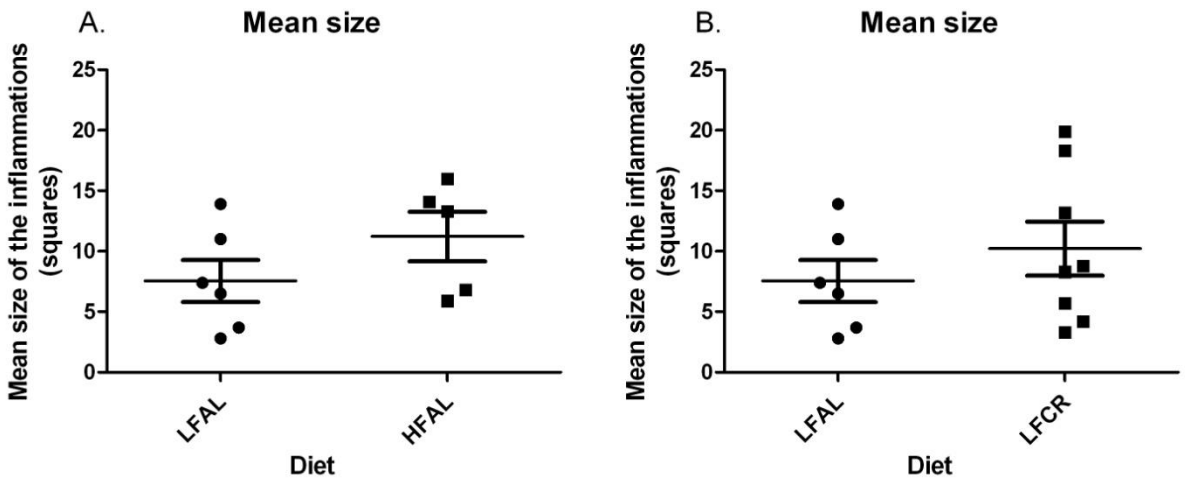

Figure 15 Mean size of the inflammations **A.** in LFAL and HFAL diet. **B.** in LFAL and LFCR diet. Data are shown ± SEM.

One-way ANOVA with post-hoc Tukey's test revealed a significant difference in total number of foci between LFAL and LFCR, but not between LFAL and HFAL as seen in figure 16.

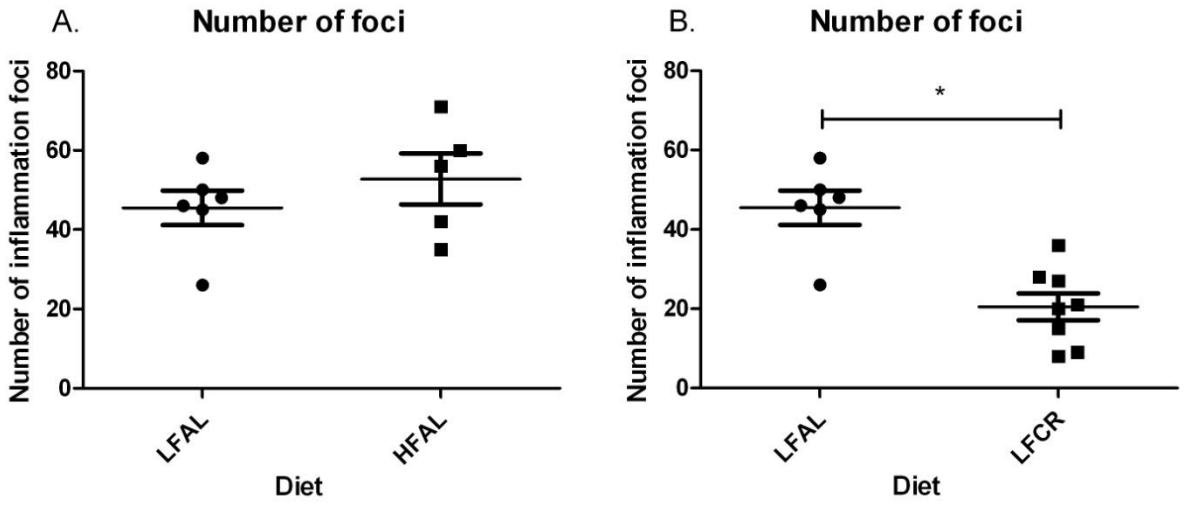

Figure 16 The number of inflammation foci found **A.** in LFAL and HFAL diet. **B.** in LFAL and LFCR diet (p=0,002). Data are shown ± SEM.

The summed sizes of all inflammations in each animal are shown in figure 17. They were not normally distributed. Mann-Whitney U test revealed no significant difference in summed total between LFAL and LFCR.

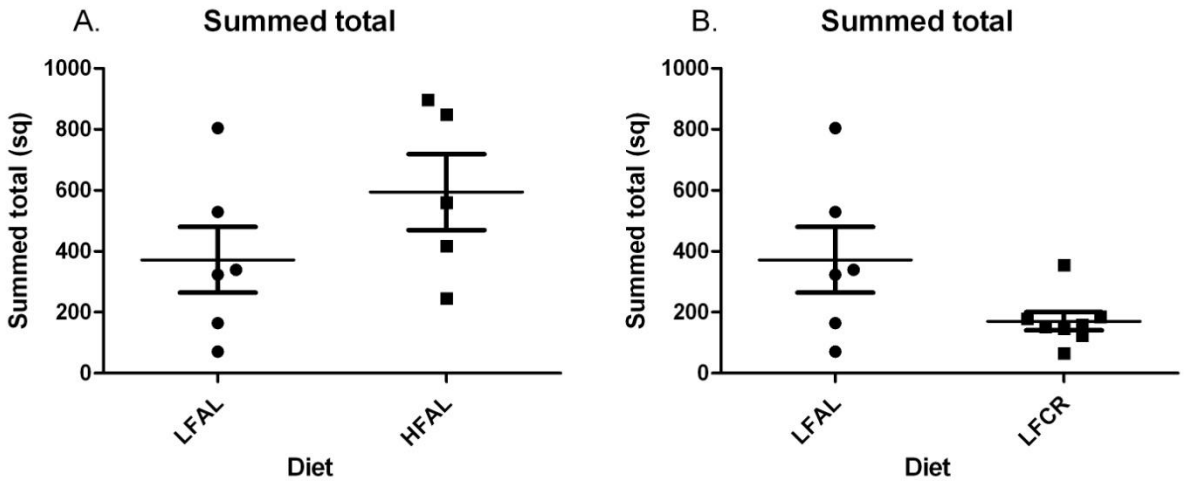

Figure 17 The summed total sizes of all inflammations found. **A. i**n LFAL and HFAL. **B.** in LFAL and LFCR. Data are shown ± SEM.

There was a significant linear regression line found between body weight and number of inflammations (β=0,838, R^2=0,434, p=0,0022). However, when only the results from the *ad libitum* group were tested, no significant result was found (β =-0,157, R^2=0,0246, p=0,645). This is shown in figure 18.

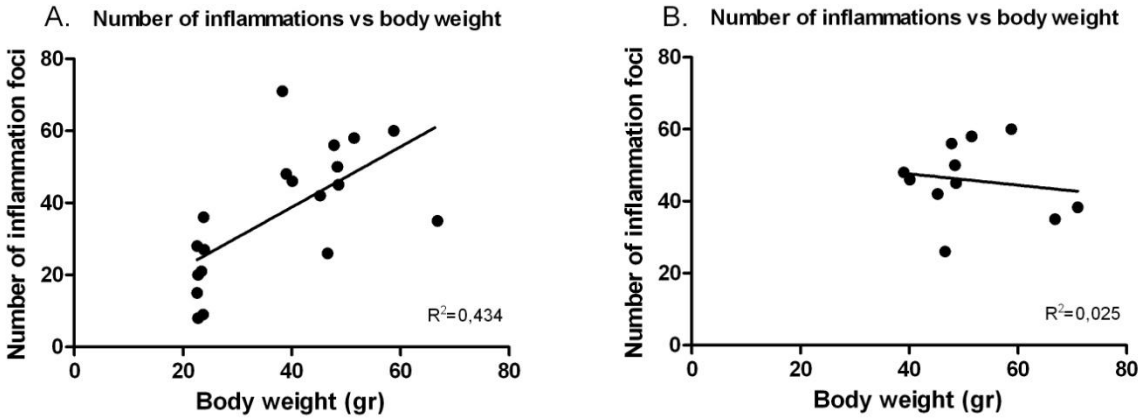

Figure 18 Linear regression line of the number of inflammation foci depending on body weight. **A.** all groups included. **B.** LFCR excluded.

Although the mean sizes of the inflammation foci showed no significant results, notable large inflammations were observed in some animals. These large structures showed a structure comparable to lymphoid tissue, although this could not be confirmed. Examples of these large foci are shown in Figure 19. For a more detailed view, magnifications of 19A and 19B are shown in figure 20. Roughly, foci larger than 100 squares of size showed lymphoid structures, while foci under 100 squares of size did not

show lymphoid structures. Therefore, the amount of large foci (defined as foci >100 squares) was examined as well. These results are shown in figure 21.

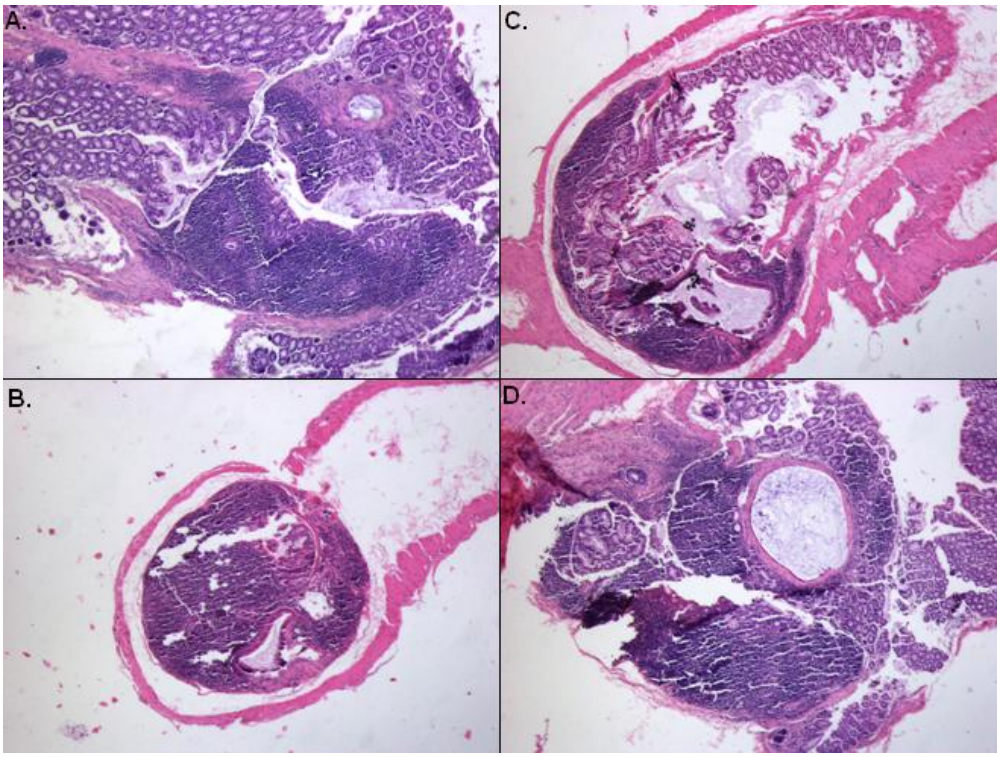

Figure 19 Examples of lymphoid structures found in inflammations in different animals at a 40x magnification.

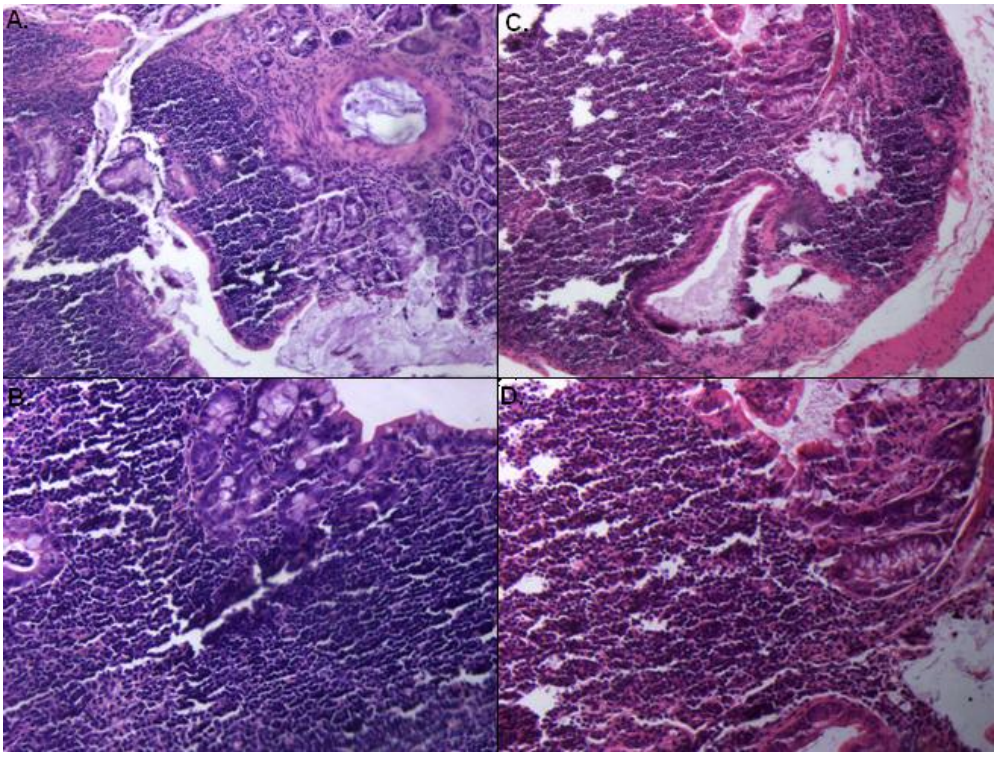

Figure 20 Two foci of figure 19 at a higher magnification. **A.** 5A 100x magnificated. **B.** 5A 200x magnificated. **C.** 5B 100x magnificated. **D.** 5B 200x magnificated.

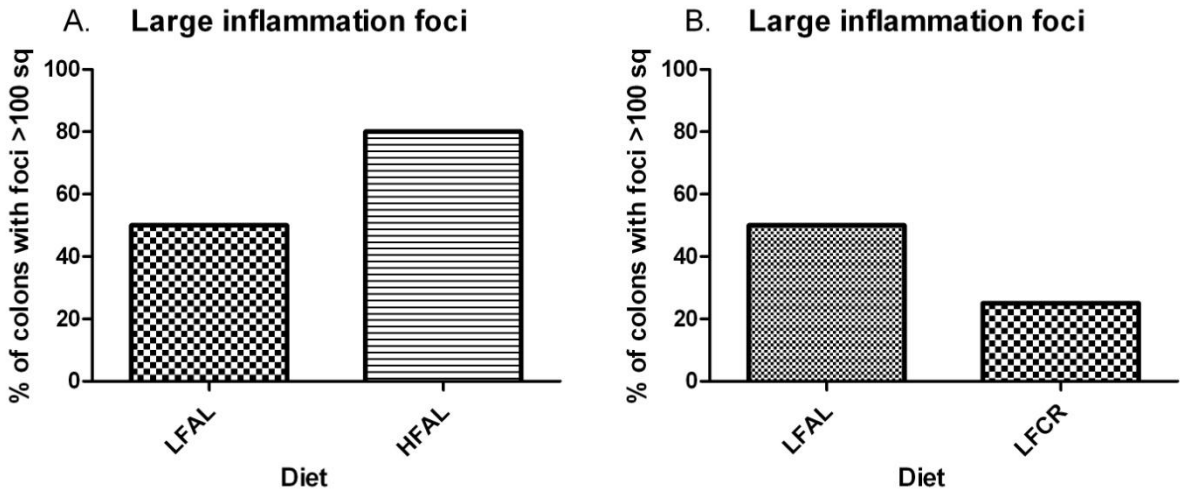

Figure 21 The percentage of animals with inflammation foci larger than 100 squares of **A.** LFAL and HFAL. **B.** LFAL and LFCR.

4. Discussion

Adipose tissue is an important regulator of many homeostatic and metabolic processes, including the insulin metabolism and inflammation. Both the insulin metabolism and inflammation are associated with colon carcinogenesis. Other adipokines are also known to impact carcinogenesis, but insulin resistance and inflammation seems to have the greatest role (van Kruijsdijk et al., 2009). In this study, relative adipose tissue mass and body mass did not differ between both *ad libitum* groups, although that would be likely based on their different diet compositions. However, when body mass at earlier age was examined, a difference between HFAL and LFAL was observed, which is shown in figure 6. Significant differences were found between both the body mass and relative fat mass of LFCR compared to LFAL.

Elevated levels of insulin and hyperactivation of the Insulin-like Growth Factor (IGF) axis are associated with colon cancer in human (Forte et al., 2012). A case-controlled study by Jiang et al showed that the levels of serum leptin, insulin, IGF-1 and the IGF-1/IGF Binding Protein-3 (IGFBP-3) ratio were significantly higher in colorectal cancer patients compared to healthy controls. The body mass index (BMI) and waist-to-hip ratio (WHR) of colon cancer patients were positively correlated with leptin and insulin levels and with the IGF-1/IGFBP-3 ratio. BMI and WHR were negatively correlated with the IGFBP-3 levels (Jiang et al., 2014). A cross sectional study by Rampal et al. showed that increasing levels of glucose, homeostatic model of risk assessment (HOMA) values, levels of hemoglobin A1c and C-peptide, and metabolic syndrome are significantly associated with prevalence of adenomas in the colon (Rampal et al., 2014). There is epidemiological evidence that Type 2 Diabetes Mellitus (T2DM) and pre-diabetes are risk factors for developing colorectal adenoma, independent from the other shared risk factors like obesity and physical inactivity (Cha et al., 2013; Deng et al., 2012; Khalili & Chan, 2012; Sun & Yu, 2012). T2DM is characterized by insulin resistance, leading to hyperinsulinemia which results in overactivation of the IGF-axis and to hyperglycemia. In the many human studies demonstrating an association between altered glucose metabolism and colon cancer or colorectal cancer, a causal relationship has not been proven yet (Gao et al., 2012; Jiang et al., 2014; Rampal et al., 2014; Wei et al., 2005). However, when examining the mechanisms involved, a causal relationship will be likely.

Circulating levels of insulin and IGFs have cellular properties of tissue growth factors and properties at the organismal level as hormones that regulate growth and energy metabolism (Pollak, 2008). IGF-1 is an important mitogen, it promotes cell proliferation and inhibits apoptosis. IGF Binding Protein 3 (IGFBP-3) induces apoptosis by competing with IGF-1 for receptor binding and is also able to induce apoptosis

by itself. IGFBP-3 influences the activity of IGF-1 by regulating its local concentration (Pollak, 2008). The tumor suppressor p53 inhibits proliferation by stimulating IGFBP-3 synthesis and inhibiting IGF-1 Receptor synthesis (Neuberg et al., 1997). It was demonstrated that IGF-1 might be required for the oncogenic action of oncogenes. Furthermore, IGF-1 enhanced the Vascular Epithelial Growth Factor and vessel abundance in tumors. Inducing angiogenesis is another hallmark of cancer cells, since the tumor cells need to be provided with nutrients in order to survive and proliferate (Pollak, 2008). The key pathways at cellular and organismal levels are shown in figure 4. The several ways by which overnutrition can provide cells cancer hallmarks via elevated levels of insulin and IGF are summarized in figure 5A.

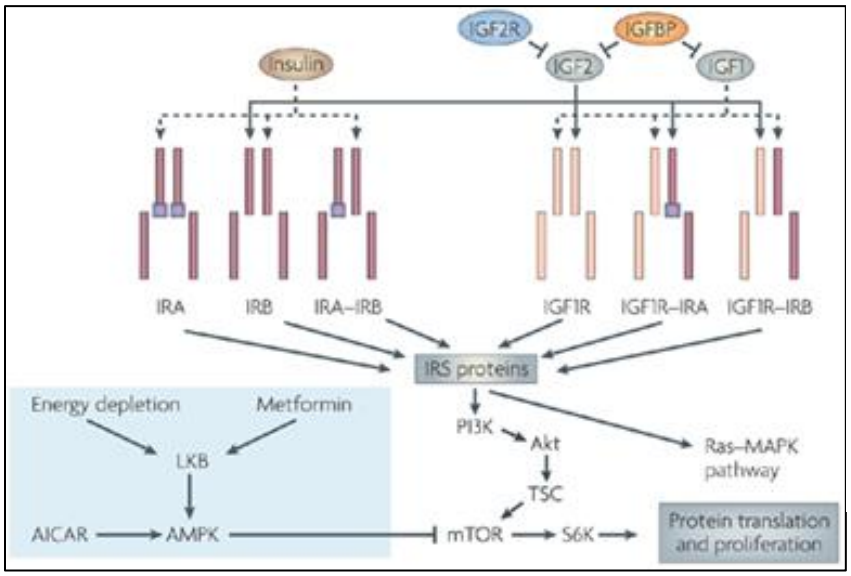

Figure 4 Insulin binds to an insulin receptor (IRA, IRB or IRA-IRB), while IGF binds to a member of the IGF-1 receptor family. The bioavailability of IGF-1 and IGF-2 is influenced by IGF Binding Proteins (IGFBPs). IGF-2 bioavailability is also influenced by the IGF2-receptor, which binds IGF-2 but does not transduce a signal. Via Insulin Receptor Substrates, the receptors activate the Ras - Mitogen Activated Protein Kinase and phosphoinositide 3-kinase/Akt/mammalian Target of Rapamycin (mTOR) signalling pathways, resulting in cellular survival, proliferation, growth and reduction of apoptosis (Pollak et al., 2008).

Not only elevated levels of the insulin and IGF ligands can be mitogenic, the number of receptors is important as well. There is extensive evidence that IGF-1 receptor is commonly expressed by neoplastic cell lines and human cancers, causing these cells to mitogenically respond to normal physiological levels of IGF-1 (Zhang et al., 2013). Elevated levels of insulin can lead to insulin resistance and thereby to hyperglycemia, which is also mitogenic. High glucose levels provide energy for cell proliferation, which is sensed by AMP-activated Protein kinase α (AMPK). Furthermore, the level of advanced glycation end products (AGEs) is elevated by hyperglycemia and binding of AGEs to their receptors causes inflammation and oxidative stress and is thereby promoting carcinogenesis (Rojas et al., 2010).

In mice IGF-1 plays an important role in carcinogenesis as well, since liver-specific IGF-1 deficient mice, which only have 25% of serum IGF-1 levels compared to the controls, have significantly decreased tumor development compared to the controls after transplantation of adenocarcinoma tissue fragments in the cecum (Wu et al., 2002).

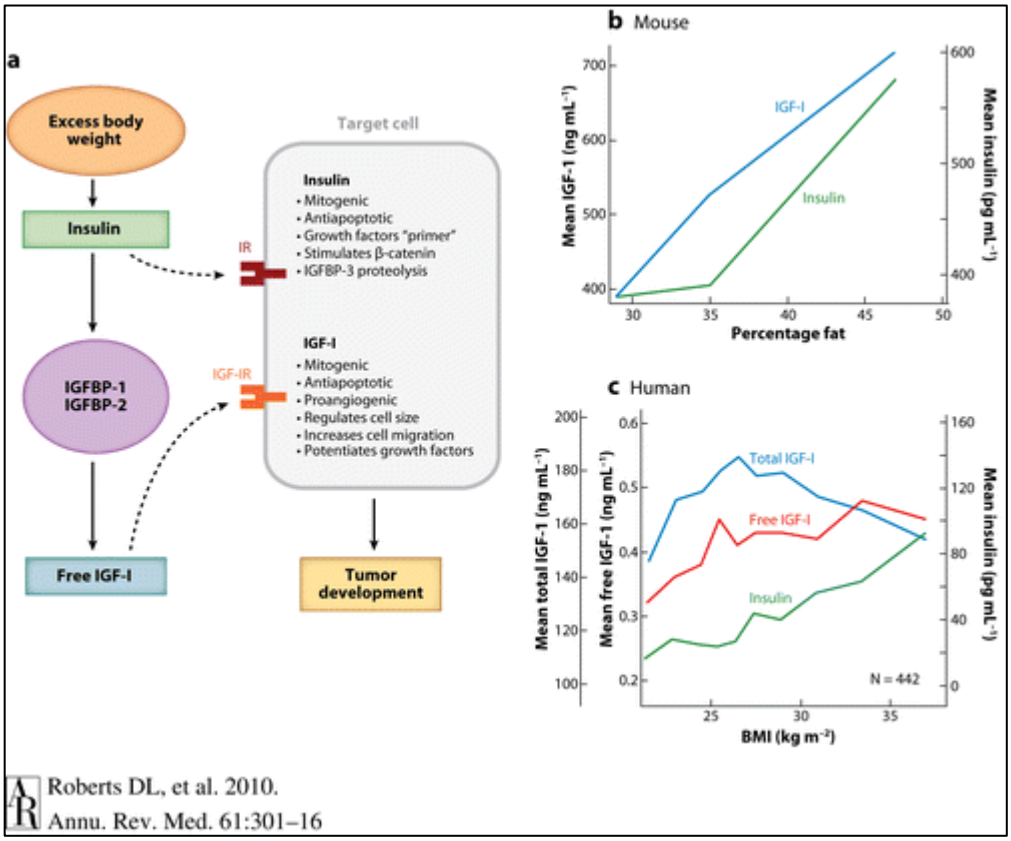

Figure 5 A. Obesity results in hyperinsulinemia and thereby in decreased IGFBP-1 and IGFBP-2 production and increased IGF-1 levels. The effects of elevated insulin and IGF-1 levels may be tumor promoting in different ways. **B.** The relationship between fat percentage in mice or BMI in human and insulin and IGF-1 levels is shown (Roberts et al., 2010).

Besides high nutrient intake, high adiposity also contributes to elevated levels of insulin, glucose and free IGF-1 and decreased levels of IGFBP-1 and IGFBP-2. This is shown in figure 5B (Roberts et al., 2010). Serum levels of IGF-1 were found decreased and of IGFBPs were found increased after 10 weeks of caloric restriction compared to a high fat diet (Fenton et al., 2009). As seen in figure 5, body weights differ significantly between LFAL and LFCR diet, but not between HFAL and LFAL diet. Also, the percentage of total fat mass did not differ between HFAL and LFAL but did differ between LFAL and LFCR. Intake of a high fat diet leads to elevated levels of insulin and thereby to increased insulin resistance compared to a low fat diet in mice, but this effect was not found in this study (Seo et al., 2014). In this study, fasting insulin and glucose levels after a glucose tolerance test did not differ

significantly in HFAL compared to LFAL, although a slight increase was visible. The fat masses of LFAL and HFAL were of the same relative size, which might have overruled the effect of nutrient quality intake. Fasting insulin and glucose levels after a glucose tolerance test were found lower in LFCR compared to LFAL, which is corresponding with the theory that elevated fat mass results deregulation of the insulin signalling. In this way, *ad libitum* fed animals will be more prone to develop neoplasia than calorie restricted animals because of their increased fat mass, while nutrient quality seems to play a minor role. Furthermore, insulin signalling is one of the regulatory mechanisms which communicate energy status to cells to control their behavior. In the presence of enough nutrients, insulin receptors are activated to stimulate cell proliferation. Other regulatory mechanisms involved in this process are mTOR and AMPK signalling (Pollak, 2008). This also indicates that nutrient quantity and thereby energy balance can impact carcinogenesis, apart from nutrient quality.

Another hallmark of cancer cells is inflammation (Hanahan & Weinberg, 2011). On the one hand, oncogenic cells or oncogenes create an inflammatory microenvironment, which is tumor-promoting (Mantovani, 2010). On the other hand, inflammatory cytokines from an external source predispose cells to cancer. The latter pathway might be involved in diet-induced carcinogenesis. Inflammation is tumor promoting, since it leads to proliferation and survival of malignant cells, angiogenesis, metastasis, inhibition of anti-tumor adaptive immunity and a reduced response to hormones and chemotherapeutic agents. It is suggested that cancer-related inflammation also induces genetic instability (Colotta et al., 2009). Inflammation occurs both systemically and locally.

Adipocytes and macrophages in adipose tissue are potent external sources of pro-inflammatory factors and induce a systemic, low-grade inflammation in obesity. Adipocytes secrete CCL2 which induces macrophage infiltration in the adipose tissue and thereby more secretion of pro-inflammatory factors. Macrophages and adipocytes can secrete many pro-inflammatory factors. Systemically, levels of pro-inflammatory factors leptin, TNF, IL-6, IL-1β and CCL2 are elevated in obese persons compared to lean persons, which can contribute to insulin resistance and other inflammatory diseases (Tilg & Moschen, 2006). After 10 weeks of a high fat diet, serum levels of IL-6 and leptin were found increased compared to a calorie restricted diet in mice (Fenton et al., 2009). Deregulation of insulin signalling also results in a systemic chronic inflammation with elevated levels of inter alia TNF-α and IL-6 (Sciacca et al., 2013).

Cytokines can activate the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3), which are both mitogenic.

STAT3 is required for transformation, enhances transformation and blocks apoptosis. This makes it carcinogenic in nude mice and human (Bromberg et al., 1999; Niu et al., 2002). NF-κB induces the transcription of inter alia anti-apoptotic and angiogenic genes (Pikarsky et al., 2004; Setia et al., 2014). Furthermore, inflammation causes Reactive Oxygen Species (ROS) formation and thereby lipid, protein and DNA damage (Sciacca et al., 2013). Thus, adipose tissue causes systemic inflammation which is tumor-promoting. In that way *ad libitum* fed mice will be more prone to colon carcinogenesis than calorie restricted mice. No separation seems to be made between LFAL and HFAL fed mice, since their relative fat mass and body weight did not differ. However, there is no evidence that systemic inflammation alone is sufficient for tumor initiation (Sciacca et al., 2013).

Besides enhancing tumor growth and invasion, leukocyte recruitment and angiogenesis, TNF-α also promotes Wnt signalling, of which β-catenin is a key regulator (Liu et al., 2012). Activated β-catenin moves from the cytosol to the nucleus of the cells, acts there as a growth promoter and is thereby tumor promoting (Oguma et al., 2008; Setia et al., 2014). Nuclear and cytosolic β-catenin accumulation might be an essential step in colon carcinogenesis in mice and human, since it was found in all colonic cancers examined by Iwamoto et al. (Iwamoto et al., 2000). To examine alterations in β-catenin expression and localization between the different diet groups, an immunohistochemical β-catenin staining was performed. However, this did not result in any visible alterations.

Locally, the immunosuppressive IL-10 and TGF-β are found in high levels in the tumor micro-environment. They prevent the tumor for break down by the immune system, for instance by blocking of the maturation of the dendritic cells. The growth-promoting and anti-apoptotic IL-6 is also found in high levels in colon cancer patients (Germano et al., 2008). Tumor-associated macrophages accumulate in the hypoxic regions of the tumor and are known to secrete molecules which promote angiogenesis, a hallmark of cancer cells. The levels of different CCL and CXCL cytokines are found altered in tumor tissue as well (Germano et al., 2008).

In this study, inflammation foci were found in the colons. Although the composition of these foci is not known, it was assumed that the foci contribute to a pro-inflammatory environment in the colon and are therefore potentially mitogenic. As seen in figure 8, LFCR fed mice had a significantly lower number of inflammation foci in their colons compared to their LFAL controls. This is as expected, since LFCR mice had a significantly lower body weight and fat mass, than LFAL mice. The number of foci did not differ

significantly between HFAL and LFAL, which corresponds with their equal relative fat masses and body weights. The relative visceral fat mass, shown in figure 8, was also significantly different between LFAL and LFCR, but not between HFAL and LFAL. The visceral fat mass is closest to the colon and therefore the most potent source of foci-inducing inflammatory factors. The difference in number of inflammation foci between *ad libitum* fed animals and caloric restricted animals could be enhanced by the fact that calorie restricted animals had less feed in their colons than *ad libitum* fed animals. The friction between feed and colonic mucosa can result in irritation and thereby in inflammation. Many studies have shown an association between colitis and colon cancer. Inflammatory diseases such as ulcerative colitis are known to come with an elevated colon cancer risk in human (Jess et al., 2012). In mouse studies, it was proven that colitis can enhance tumor growth induced by a carcinogen (Clapper et al., 2007).

Notably, the number of large foci (>100 squares) was higher in HFAL compared to LFAL and was lower in LFCR compared to LFAL, which was shown in figure 21. Large foci often displayed a lymphoid structure. However, the exact number of foci with a lymphoid structure was not examined due to practical reasons. It seems that a high fat diet, compared to a low fat diet, did enhance the growth of some inflammation foci towards a lymphoid structure while it did not increase the total number of foci in one colon. Gut-associated lymphoid tissue (GALT) protects the body from harmful food and bacteria (Chandran et al., 2003). This is apparently more necessary in HFAL than in LFAL and less necessary in LFCR compared to LFAL. B and T lymphocytes and macrophages are present in the GALT, making it thereby a potential source of lymphomas and inflammation (Kuper, 2006). This could make the HFAL animals more prone to colon cancer than the LFAL animals. A comparable observation was made by Day et al. Day et al. described a high-fat diet enhancement of chemical induced colon cancer. This did not result in a larger number of polyps, but did result in an increase in the number of large polyps (Day et al., 2013). The increased amount of large inflammation foci in HFAL compared to LFAL could also be caused by the fact that HFAL fed animals do have a higher body weight during their life time than LFAL animals.

Although there are many mechanisms known by which diet quantity and amount can promote colon carcinogenesis, no pre-malignant or malignant structures were found in this study. There is proof that colon carcinogenesis can be enhanced by adiposity, but it is not proven that this can initiate carcinogenesis. In all animal studies known, carcinogenesis is initiated by a carcinogen or transplantation of neoplastic tissue and enhanced by dietary factors, but not initiated by the dietary factors itself (Day et al., 2013; Olivo-Marston et al., 2014; Tuominen et al., 2013). This indicates that other factors, for instance accumulation of genetic mutations, are needed for initiation of carcinogenesis. It might be that no colon carcinogenesis initiating factor is present in the C57Bl/6J mice. In human, it could be that pre-neoplastic stages are worsened by a western lifestyle and therefore detected, while they would remain unnoticed without the enhancement by a western lifestyle.

Another explanation for the absence of aberrant structures is that they might have remained unnoticed and this might have been the case of potential changes in the amount of lymphoid structures in the tissue either. The conventional way of counting aberrant crypt foci, which are premalignant structures, is a whole colon staining with methylene blue. Although the method used in this study gives insight in the colon histology, the methylene blue staining might be preferred for an accurate quantification of aberrant crypt foci. McGinley et al described a method for processing a rat's colon with different staining including a whole colon methylene blue staining, and an immunohistochemistry β-catenin staining in microtome slices all on the same tissue (McGinley et al., 2010).

To summarize, pathways by which adipose tissue could lead to the acquisition of cancer hallmarks in cells are shown in figure 6, and these have been mentioned individually above already. For the pathways examined, adipose tissue effects overrule diet quality effects in these aged mice. This is consistent with what is found in literature about the important role of adipose tissue (van Kruijsdijk et al., 2009). The main regulator of inflammation is adipose tissue and the effects of adipose tissue on insulin metabolism also overrule the effect of high fat versus low fat diet.

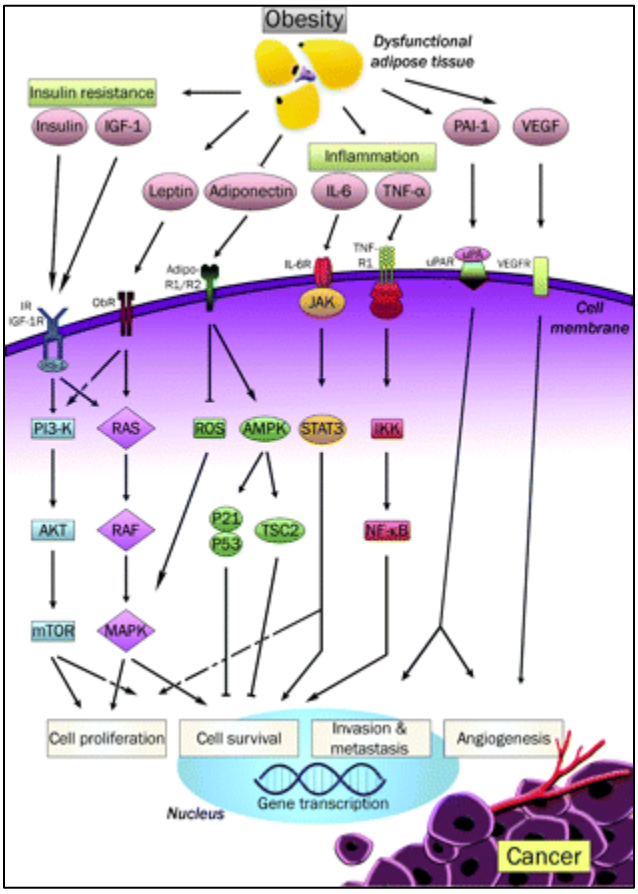

Figure 6 Potential pathways linking adiposity with cancer. Obesity can lead to elevated insulin resistance, inflammation and levels of adiponectin, leptin, plasminogen activator inhibitor-1 and vascular endothelial growth factor. Via their downstream signalling pathways, all these factors can promote the occurrence of different cancer hallmarks (Van Kruijsdijk et al., 2009).

Further research on these slices can be done to examine the composition of the inflammation foci. This could give useful insights in impact of the presence of these structures. Quantification of the amount of lymphoid tissue could reveal a high fat diet impact. Furthermore, systemic cytokine profiles of the mice could be compared to local inflammation foci in the colon to examine whether or not they are related. In future research towards this subject, the earlier mentioned method of tissue processing designed by McGinley et al. should be preferred. This method allows an accurate counting of aberrant crypt foci, which are pre-malignant structures and are therefore a good quantification of colon carcinogenesis (Orlando et al., 2008). With quantified aberrant crypt foci, it can be examined whether or not colon carcinogenesis in mice is correlated with the different pathways by which diet could induce it. Furthermore, to make a translation to the human situation possible, an animal model with the same susceptibility to colon cancer should be used.

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Appendices

Appendix 1: Diet compostitions

Diet composition low fat diet

2141 AM II Diet (AB diets, Woerden, The Netherlands)

Samenstelling		Gewicht	Aandeel
4011	HAVER Gepeld.	240.00	8.00
7009	LUZERNE K.G. 16%re	60.00	2.00
7013	HAVER	60.00	2.00
7020	LIJNZAAD.	90.00	3.00
7123	LUZERNE Z.G. 8%re.	90.00	3.00
7078	WEIPOEDER M.S.A.	112.50	3.75
7122	MELASSE 46% Suiker.	60.00	2.00
7132	KANENMEEL.	450.00	15.00
9001	gelitaflex jonker	75.00	2.50
7162	BIERGIST	112.50	3.75
7165	SOJA-OLIE GERAFF.	60.00	2.00
7209	TARWE HF.	1431.90	47.73
7375	HF PREMIX AM.	158.10	5.27
		3000.00	100.00

	Analysis		Amount		Analysis		Amount
1	Cr protein	g.	259,38	50	C14:0	g.	0,47
2	Cr fat	g.	63,13	51	C16:0	g.	9,14
3	Cr fibre	g.	34,69	52	C16:1	g.	0,78
4	Ash	g.	51,95	53	C18:0	g.	3,55
5	Moisture	g.	99,01	54	C18:1	g.	15,82
6	Sug + St	g.	432,52	55	C18:2	g.	19,76
7	Nfree ex	g/kg	27,86	56	C18:3	g.	7,64
8	Dry matter	g.	901,87	61	Vit A	mg.	16105,12
9	Lysine	g.	12,04	62	Carotene	IE	2,80
10	Methion.	g.	3,88	63	Vit D	IE	1561,50
11	Meth+Cys	g.	7,08	64	Vit D3	mg.	1561,50
12	Cystin	g.	3,20	65	Vit E	mg.	88,64
13	Threonin	g.	8,37	66	Vit K	mg.	0,00
14	Thryptoph	g.	2,36	67	Vit K3	mg.	2,45
15	Isoleuc.	g.	8,49	68	B1 thiam	mg.	15,61
16	Arginine	g.	15,73	69	B2 ribofl	mg.	9,61
17	Phenylal	g.	9,55	70	B6 pyrid	mg.	13,79
18	Histidin	g.	4,92	71	B3 niacin	mg.	40,77
19	Leucine	g.	15,75	72	B5 panth a	mg.	21,84
20	Tyrosine	g.	6,16	73	Vit B12	ug	52,55
21	Valine	g.	11,42	74	Folic ac	mg.	4,01
22	Alanine	g.	16,96	75	Choline	mg.	315,29
23	Asp acid	g.	18,74	76	Biotin	ug	565,26
24	Glut acid	g.	40,74	77	Inositol	mg.	0,00
25	Glycine	g.	27,18	78	Vit C		0,00
26	Proline	g.	21,72	82	Starch	g.	363,51
28	Serine	g.	10,66	83	Sugar	g.	43,28
29	Taurine	g/kg	0,00	84	Glucose	g/kg	5,78
30	Calcium	g.	8,08	85	Starch B	g.	43,78
31	Phos total	g.	5,53	86	Lactose	g.	18,00
32	Phos abs	g.	3,05	87	Onv-rc	g.	17,57
33	Phos dig	g.	2,51	88	NDF	g.	96,87
34	Potassium	g.	8,64	89	ADF	g.	37,62
35	Magnesium	g.	1,25	90	Hemicell	g.	66,84
36	Sodium	g.	2,70	91	OOS	g.	10,88
37	Chlorine	g.	4,17	92	ADL	g.	2,20
38	Sulfur	g.	0,35	93	OOS-ADF	g.	4,54
39	IP	g.	1,27	96	Iron	mg.	119,71
44	Fos	g.	471,98	97	Mangan	mg.	56,38
45	Me	MJ	11,27	98	Zinc	mg.	80,05
46	Ew		110,20	99	Copper	mg.	18,54
47	Nev	MJ	9,70	100	Cobalt	mg.	1,78
48	C10	g.	0,02	101	Iodine	mg.	0,17
49	C8-C12:0	g.	0,03				

Diet composition high fat diet

4031.09 High fat diet with lard (AB diets, Woerden, The Netherlands)

Samenstelling		Gewicht	Aandeel
7060	CERELOSE/dextrose	3,057.50	20.38
7082	METHIONINE SYNTH. DL	45.00	0.30
7083	ZOUT (GEZAKT).	55.00	0.37
7514	CHOLINE CL 50%	60.00	0.40
7530	SOYA-OLIE.	450.00	3.00
7546	KCl.	130.00	0.87
7547	KH2PO4	130.00	0.87
7552	MgO SCHWER REINST	37.50	0.25
7559	CaHPO4.2H2O (LAAG F)	240.00	1.60
7560	CaCO3 REINST/Me.2069	180.00	1.20
7579	MAISZETMEEL GEL INST	2,800.00	18.67
7584	DICACEL2+4/cellulose	900.00	6.00
7586	REUZEL.	3,150.00	21.00
7599	ZURE CASEINE.	3,600.00	24.00
7611	MgSO4.7H2O	75.00	0.50
8500	STAND.VIT.PREMIX	45.00	0.30
8503	ST.SPOR.PREMIX	45.00	0.30
		15,000.00	**100.00**

Analyse		Eenheid	Aandeel	Analyse		Eenheid	Aandeel
1	Cr.Prot	g/kg	214.41	53	C18:0	g/kg	34.53
2	Cr. Fat	g/kg	234.90	54	C18:1	g/kg	96.06
3	Cr.Fiber	g/kg	61.57	55	C18:2	g/kg	34.35
4	Minerals	g/kg	22.45	56	C18:3	g/kg	4.37
5	Moisture	g/kg	62.75	57	C20-C22	g/kg	12.69
6	Sug.+St.	g/kg	350.46	61	Vit. A	IU/g	21.60
7	Nfree ex	g/kg	368.47	64	Vit. D3	IU/g	2.40
8	Dry Mat.	g/kg	933.77	65	Vit. E	mg.	75.20
9	Lysine	g/kg	14.90	67	Vit. K3	mg.	12.00
10	Methion.	g/kg	8.73	68	Vit. B1	mg.	24.00
12	Cystine	g/kg	0.62	69	Vit. B2	mg.	13.87
13	Threonin	g/kg	8.54	70	Vit. B6	mg.	18.40
14	Tryptoph	g/kg	2.78	71	Niacin	mg.	47.04
15	Isoleuc.	g/kg	12.10	72	Pant.ac.	mg.	19.08
16	Arginine	g/kg	7.34	73	Vit.B12	mcg.	60.00
17	Phenylal	g/kg	8.74	74	Folic.ac	mg.	9.41
18	Histidin	g/kg	5.57	75	Choline	mg.	1,491.56
19	Leucine	g/kg	22.85	76	Biotin	mcg.	367.98
20	Tyrosine	g/kg	11.33	77	Inositol	mg.	599.97
21	Valine	g/kg	14.11	82	Starch	g/kg	146.53
22	Alanine	g/kg	4.75	83	Sugars	g/kg	185.97
23	Asp.acid	g/kg	11.11	86	Lactose	g/kg	0.48
24	Glut.ac.	g/kg	42.12	91	Cellulos	g/kg	54.00
25	Glycine	g/kg	6.14	93	Glucose	g/kg	189.93
26	Proline	g/kg	19.27	96	Iron	mg.	155.03
28	Serine	g/kg	6.74	97	Mangan.	mg.	76.20
30	Calcium	g/kg	8.63	98	Zinc	mg.	21.16
31	Phos.tot	g/kg	5.17	99	Copper	mg.	0.17
34	Potass.	g/kg	7.04	100	Cobalt	mg.	0.57
35	Magnes.	g/kg	1.96	101	Iodine	mg.	0.22
36	Sodium	g/kg	1.41	102	Selenium	mg.	0.59
37	Chlorine	g/kg	6.73	104	Chromium	mg.	0.08
38	Sulfur	g/kg	0.68	105	Nickel	mg.	2.57
49	C8-C12:0	g/kg	-	107	Fluorine	mg.	0.08
50	C14:0	g/kg	3.39	109	Arsenic	mg.	0.96
51	C16:0	g/kg	54.99	111	Lead	mg.	3.97
52	C16:1	g/kg	5.55	113	Alumin.	mg.	
53	C18:0	g/kg	34.53				
54	C18:1	g/kg	96.06				
55	C18:2	g/kg	34.35				
56	C18:3	g/kg	4.37				
57	C20-C22	g/kg	12.69				
61	Vit. A	IU/g	21.60				
64	Vit. D3	IU/g	2.40				

Appendix II: Hematoxyline-eosine staining

The hematoxyline-eosin (H&E) staining took place in a fume hood.

- Deparaffination of the coupes:
- 2 times 5 minutes xylene
- 5 minutes 100% alcohol
- 5 minutes 96% alcohol
- 5 minutes 70% alcohol
- 3 times 5 minutes demineralized water

- Staining of the coupes:
- 3 minutes Hematoxyline
- 20 minutes rinse with running water
- Wash with demineralized water
- 2 minutes eosine
- Rinse with 100% ethanol

- Dehydrating of the coupes:
- 5 minutes 100% alcohol
- 2 times 5 minutes xylene

- Mounting of the coupes:
- Add some Eukitt Mountain Medium
- Cover with coverslip

Appendix III: Immunohistochemistry protocol for paraffin sections

- Deparaffinize: 2x 5”” xylene, 1x 5”” 100% ethanol, 1x 5”” 96% ethanol, 1x 5”” 70% ethanol, 3x 5”” demi-H_2O
- For antigen retrieval:
  - Citrate buffer: 2,9410 g NaCitrate (N19a) + 2,10 g Citric Acid (C32) + 1000 ml demi-H_2O. Set pH at 6,0 using NaOH.
  - Pre-heat buffer 8”” in microwave at 100% power
  - cook slides for 8”” at 80% power, 15”” cool down
- After antigen retrieval wash with Phosphate Buffered Saline (PBS) 3x 5””
- Blocking endogenous peroxidases with 0,3% H_2O_2 in PBS (49,5 ml PBS+0,5 ml H_2O_2) for 30”” in the dark
- Was 3x 5”” with PBS
- First antibody on (in PBS), 200 µl on slide, and keep overnight in cold room with wet tissues in a closed box to prevent the slides from drying out.
- Wash 3x with PBS
- Second antibody (ABC procedure)
  - Anti-rat or mouse biotin (1:300), 1,5 hrs at room temperature
- Wash 3x with PBS
- Make ABC complex and keep 30”” on ice before use
  - Make A: 1.8% A in PBS (18 µl A + 1 ml PBS)
  - Mix well
  - Add 1,8% B (18 µl)
  - Mix well
  - Keep on ice
- Wash 3x with PBS
- Put ABC complex on tissue for at least 45””
- Wash 3x with PBS
- Dissolve 2 dablets of DAB (gold&silver) in 1 ml demi-H_2O
- Vortex very well (gas formation, release lid of the Eppendorf cup during vortexing)
- DAB staining: put on the tissue until the tissue turns brown (seconds to minutes)
- Wash 3x with PBS
- Hematoxylin-staining: 45”
- Dehydration: 1x 5”” 70% alcohol, 1x 5”” 96% alcohol, 1x 5”” 100% alcohol, 2x 5”” xylene
- Mounting: Eukitt on It, cover with a cover slip