Aberrant crypt foci in the colo-rectal mucosa as reliable markers of tumor development

with special reference to histomorphological and immunohistochemical characterization of aberrant crypt foci in rats

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Aberrant crypt foci in the colo-rectal mucosa as reliable markers of tumor development

With special reference to histomorphological and immuno-histochemical characterization of aberrant crypt foci in rats

Ph.D. Thesis

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DATA SHEET

Title: Aberrant crypt foci in the colo-rectal mucosa as reliable markers of tumor development.

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Abstract: The aim of the present thesis has been to evaluate a recently developed short term *in vivo* model, the aberrant crypt foci bioassay (ACF), for its ability to predict the development of colo-rectal tumors. Based on the knowledge obtained during the last decade, it can be stated that no simple connection exists between occurrence of ACF (neither qualitatively nor quantitatively) and later development of tumors. However, the literature has shown that part of the ACF show morphologic and genetic features characteristic for the tumorigenic process and a recent investigation indicate that all ACF belong to the same unity with basically the same chances for gradual progressing into tumors. It may be speculated that the progression depends on promotional conditions in the environment.

Key words: Aberrant crypt foci, ACF, colo-rectal cancer, histomorphology.

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Front cover: Longitudinal histological section of a HE stained aberrant crypt focus.

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1 PREFACE

The present work was initiated at the Institute of Toxicology to implement and evaluate a recently developed short term animal model, the aberrant crypt foci bioassay, which is currently being used to predict modulating effect of dietary components on development of colo-rectal cancer. One part of the work has dealt with the use of the short term model in dietary intervention studies. Another part has dealt with basic research concerning the relevance of the chosen end point parameter in relation to colon tumorigenesis.

The planning and conduction of the project were made in close collaboration with my good colleagues and friends Eva Kristiansen and Otto Meyer, and I wish to thank them for many enjoyable hours and for fruitful discussions within the fields of dietary related colo-rectal cancer. Further, a warmhearted thank to Otto Meyer for being my advisor during the project.

I also wish to thank Hanne Cathrine Bisgaard, Institute of Life Sciences and Chemistry, Roskilde University Center, Denmark for valuable comments to the manuscript.

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Copenhagen, November 1996

Inger Thorup

2 SUMMARY

The aim of the present thesis has been to evaluate a recently developed short term *in vivo* model, the aberrant crypt foci (ACF) bioassay, for its ability to predict the development of colo-rectal tumors.

Colo-rectal cancer is one of the most common malignant diseases in the Western industrialised countries and there is a general consensus that diets is an important aetiological component in development of this type of cancer. Thus, from a public health perspective it is of great interest to point out dietary components able to reduce or enhance the development of colo-rectal tumors. The ACF bioassay, which was introduced ten years ago, is able to, in a quick and easy way, to reveal microscopic alterated areas in the colo-rectal mucosa. These areas, known as the aberrant crypt foci (ACF), were suggested to represent a very early stage in the tumor development as they exhibited features characteristic for the tumorigenic process. The assay therefore was found very interesting in relation to the establishment of a screening test to evaluate the influence of dietary components on the occurrence of ACF, and thereby the risk for development of colo-rectal cancer.

However, based on the knowledge obtained during the last decade it can be stated that an unequivocal correlation between occurrence of ACF (neither qualitatively nor quantitatively) and later development of colo-rectal tumors has not been demonstrated. Thus, the literature has shown that testing of various dietary components in the ACF bioassay has led to contradictory results concerning the correlation between the number and size (crypt multiplicity) of the ACF and later development of tumors. Experiments aimed to investigate the presence of tumor markers in the ACF have mirrored ACF as a group of rather heterogeneous lesions of which some may be able to progress into tumors. Our own experiments concerning enumeration of ACF have supported the view that neither the total number nor the crypt multiplicity of the ACF is correlated with the tumor outcome. However, my most recently performed study which investigated the ACF at the cellular level may support the view that ACF possess preneoplastic properties.

It is concluded that no simple connection between occurrence of ACF and

development of tumors exists. However, the literature has shown that part of the ACF show morphologic and genetic features characteristic for the tumorigenic process. Based upon my most recent investigation it seems that all ACF belong to the same unity with basically the same chances for gradual progressing into tumors. Whether such a progression occurs, may depend on promotional conditions in the environment.

In the literature it has been mentioned that the fraction of 'larger' ACF is a reliable parameter for development of colon tumors. However, it seems most unlikely that ACF with a given number of crypts could be nominated as the valid marker for tumor development.

Even though a lot of knowledge about ACF has accumulated during the last decade, the outcome of the studies is still inconclusive concerning the neoplastic potential of the ACF.

3 SUMMARY IN DANISH (SAMMENDRAG)

Formålet med dette arbejde har været at evaluere en nyligt udviklet korttids-dyremodel, den såkaldte aberrante krypt foci (ACF) test, for dens evne til at forudsige udviklingen af tyk- og endetarmskræft.

Kræft i tyk- og endetarm er en af de mest hyppigt forekommende kræftlidelser i de vestligt industrialiserede lande, og kostens sammensætning har sandsynligvis stor indflydelse på udvikling af denne sygdom. Det vil derfor være af stor interesse at kunne påvise hvilke kostfaktorer, der kan virke hæmmede eller fremmende på tumorudviklingen.

ACF-modellen, som blev lanceret for ti år siden, kan hurtigt og let påvise mikroskopisk forandrede områder i tyktarmens slimhinde, de såkaldte aberrante krypt foci (ACF). Disse foci blev antaget at repræsentere meget tidlige stadier i udviklingen af tyk- og endetarmskræft, idet de udviste egenskaber, som er karakteristiske for tumor-udviklingsprocessen. Testen påkaldte sig derfor interesse med henblik på at få etableret en model, som indenfor en kort tidshorisont kunne benyttes til at vurdere forskellige kostkomponenters indflydelse på forekomsten af ACF og dermed risikoen for udviklingen af tyk- og endetarmstumorer.

Baseret på den akkumulerede viden gennem en ti-års periode må det dog konstateres, at det ikke har været muligt at påvise nogen entydig sammenhæng - hverken kvalitativt eller kvantitativt - mellem forekomst af ACF og senere udvikling af tumorer i tyk- og endetarmen. Således har litteraturen vist, at afprøvning af en række kostkomponenter i ACF-modellen har ført til modstridende resultater vedrørende sammenhængen mellem tilstedeværelse af ACF og udvikling af tumorer. I undersøgelserne er indgået registrering af såvel det totale antal ACF som størrelsen af disse (antallet af forandrede tarmkrypter per focus). Studier, hvis formål har været at undersøge tilstedeværelsen af tumormarkører i ACF, har gennemgående afspejlet ACF som en gruppe af heterogene forandringer, hvoraf nogle sandsynligvis har potentiale for tumorudvikling. Egne eksperimenter vedrørende kvantitering af ACF har kunnet understøtte opfattelsen af , at der ikke eksisterer en direkte korrelation mellem antallet og størrelsen af ACF og udvikling af tumorer. Min seneste

undersøgelse af ACF på cellulært niveau har dog vist, at ACF besidder egenskaber, som er forenelig med en hypotese om, at disse kan udvikles til tumorer.

Baseret på data fra litteraturen såvel som fra egne undersøgelser kan det konkluderes, at der ikke er nogen simpel sammenhæng mellem forekomst af ACF og udvikling af tumorer. Litteraturen afspejler, at en del af de undersøgte ACF udviser cellulære og genetiske forandringer karakteristiske for tumorudviklingsprocessen, og baseret på egen undersøgelse synes alle ACF at have de samme iboende muligheder for at udvikle sig til tumorer. Forhold i de nære omgivelser kan muligvis øve indflydelse på, hvorvidt en sådan tumorudvikling finder sted.

Det er i litteraturen blevet angivet, at tilstedeværelsen af store ACF er en god parameter for senere udvikling af tumorer. Det forekommer dog usandsynligt, at der skulle kunne fastsættes en præcis grænse for, hvor stor en ACF skal være for at være markør for tumorudvikling.

Selvom der gennem de seneste ti år er opnået meget viden om ACF, er det på indeværende tidspunkt ikke muligt at udtale sig definitivt om ACF´s rolle i udviklingen af tyk- og endetarms tumorer.

4 LIST OF PAPERS INCLUDED

The present Ph.D. Thesis is based upon the following five papers, which will be referred to through the report by their authors and Roman bold numerals.

I Thorup,I., Meyer,O. and Kristiansen,E. (1992) Effect of a Dietary Fiber (Beet Fiber) on Dimethylhydrazine-Induced Colon Cancer in Wistar Rats. *Nutr. Cancer*, **17**, 251-261.

II Thorup,I., Meyer,O. and Kristiansen,E. (1994) Influence of a Dietary Fiber on Development of Dimethylhydrazine-Induced Aberrant Crypt Foci and Colon Tumor Incidence in Wistar rats. *Nutr. Cancer*, **21**, 177-182.

III Kristiansen,E., Thorup,I. and Meyer,O. (1995) Influence of Different Diets on Development of DMH-Induced Aberrant Crypt Foci and Colon Tumor Incidence in Wistar rats. *Nutr. Cancer*, **23**, 151-159.

IV Thorup,I., Meyer,O. and Kristiansen,E. (1995) Effect of Potato Starch, Cornstarch and Sucrose on Aberrant Crypt Foci in Rats Exposed to Azoxymethane. *Anticancer Res.*, **15**, 2101-2106.

V Thorup,I. (1997) Histomorphological and immunohistochemical characterization of colonic aberrant crypt foci in rats. Relationship to growth factor expression. *Carcinogenesis*, **18** (3), 465-472.

Besides, in the text reference is given to the following publications concerning ACF, which is not directly included in the thesis.

1 Kristiansen,E., Meyer,O. and Thorup,I. (1996) Refined carbohydrate enhancement of aberrant crypt foci (ACF) in rat colon induced by the food-borne carcinogen 2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ). *Cancer Letters*, **105**, 147-151.

2 Kristiansen,E., Meyer,O. and Thorup,I. (1997) The ability of two cooked food mutagens to induce aberrant crypt foci in mice. *Eur. J. Cancer Prev.*, **6**, 1-5.

5 INTRODUCTION

5.1 Development of cancer

The development of malignant neoplasms, cancer, is named carcinogenesis. In the broadest possible sense it is the process of generation of benign as well as malignant neoplasms (tumors), but this process should more correctly be termed tumorigenesis. It is generally accepted that carcinogenesis is a multi step process which consists of different phases: *initiation, promotion,* and *progression*. The initiation takes place at the DNA level and alters the genetic information in the cell. As a result, the normal cell undergoes an irreversible change characterized by an intrinsic capacity for autonomous growth. This capacity may remain latent for weeks or years during which time the initiated cell may be morphologically indistinguishable from surrounding normal cells. In the promotion phase of the carcinogenic process the initiated, but normal looking cells are stimulated to divide and become morphologically abnormal. Thus, this phase enhances the development of clinically and pathologically detectable neoplasms. The progression is the phase in which the initiated, abnormal cells develop into malignant cells, that means the development of cancer. During this phase, the neoplasms show progressively increased invasiveness and develop the ability to metastasize.

During the last decade it has become possible to identify at least some of the molecular events that underlie the initiation, promotion and progression phases. The development of neoplasia may require changes in at least two classes of cellular genes, *the proto-oncogenes* and *the tumor suppressor genes.* During the process, the proto-oncogenes are activated to *oncogenes* which may lead to enhanced cellular growth as oncogenes have growth stimulatory properties. The tumor suppressor genes are inactivated which also result in enhanced cell proliferation since this type of genes inhibit cellular growth.

The molecular events during development of colo-rectal tumors have been the objects of many investigations and several gene alterations have been identified. As the colo-rectal cancer process develops through several histologically well defined stages, it has been possible to propose a sequential acquisition of the genetic alterations. The genetic changes that lead to colo-

rectal cancer tend to occur in a preferred order and it is suggested that the process begins with an inactivation of the *APC* (Adenomatous Polyposis Coli) tumor suppressor gene which may be responsible for transforming the normal colonic epithelium into a hyperproliferative stage. After inactivation of the *APC* gene, a mutational activation of the K-*ras* oncogene is seen and K-*ras* mutations show up in the early adenoma stage. The next genetic change is inactivation of the tumor suppressor gene *DCC* (Deleted in Colo-rectal Cancer) in the intermediate/late adenoma stage. The last event is inactivation of the tumor suppressor gene *p53* which is apparently involved in the change from the adenoma to the carcinoma stage. It should be emphasized that the order described is not absolute and the total accumulation of changes is maybe more important than their order in determining the carcinogenic process (1-4).

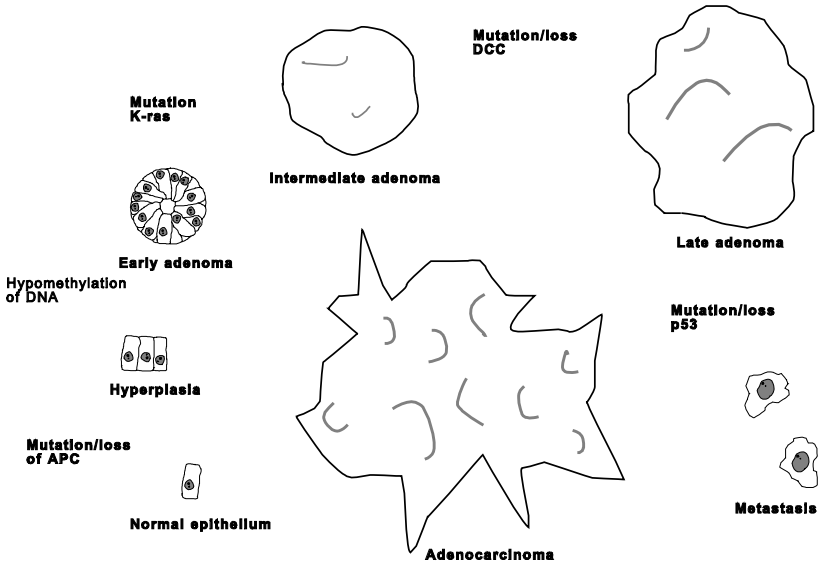

Figure 1: Proposed model for genetic alterations in colo-rectal carcinogenesis. Based on Fearon and Vogelstein (1).

5.2 **Colo-rectal cancer in humans**

Colo-rectal cancer is one of the most common malignant diseases in the Western industrialised countries. In Denmark where colo-rectal cancer ranks as the third leading cause of cancer death, approximately 3,300 new cases

occurred in 1993 (5). In the United States, approximately 152,000 new cases were thought to occur in 1993 (6). The prognosis of colo-rectal cancer remains poor with a 30-35% 5-year survival rate in population-based statistics (7-9). Thus, from a public health perspective this disease deserves serious attention. There is a general consensus that diets is an important aetiological component in development of cancer, especially cancer of the digestive tract and the hormone related organs (10,11). Thus, genetic and dietary factors have been mentioned as the major risk factors for development of colo-rectal cancer (10,12) and fat and red meat intakes have been correlated to elevated risk, whereas the intake of vegetable, fruit and, to lesser extent, dietary fiber has been inversely correlated with the development of colo-rectal cancer (8,13,14). That environmental factors, as the diet, influence the development of colo-rectal cancer has clearly been shown in experimental (11,15) and epidemiological (10,11,14) studies. Migrant studies and studies of religious subgroups with special dietary habits (13,16,17) have also supported the hypothesis that environmental factors, especially the diet, influences the development of colo-rectal cancer.

5.3 **Animal experiments in the colo-rectal cancer research**

Animal models are very useful tools for studying the influence of macro and micro nutrients on colon tumorigenesis, and for systematic studies of possible risk factors observed in epidemiological studies.

Development of cancer is a multi stage process which progresses over a very long time, and carcinogenicity studies in animals (including models of chemically induced colo-rectal cancer) should therefore cover most of their life span. This is the major reason for using mice and rats having a lifespan at maximum two to three years. These animal models are very valuable as the colo-rectal tumor itself is the end point and the histological features of the tumors developed are comparable to the benign and malignant tumors which develop spontaneously in humans (18-20).

However, systematic application of long term carcinogenicity tests to study a wide range of compounds in the human diet would be extremely time consum-

ing and expensive. Besides, such experiments needs a large number of animals as tumor development often is confined to a limited number of the animals tested (21). Further, in traditional carcinogenicity studies it is not normally possible to evaluate individual steps in the carcinogenic process, and characteristically they employ relative large doses of carcinogens to produce tumors which may mask any subtle effect of dietary components.

5.3.1 Reasons for development and use of short term animal models

Introduction of short term studies using intermediate tumor markers as end points would make it possible to evaluate dietary components in a much quicker and less expensive way than by employing a traditional carcinogenicity study. In addition, if the end point parameter is present as multiple lesions per treated animal and moreover seen in most of the animals, the assay would require fewer animals compared to experiments that use tumors as an end point.

As mentioned above, the development of colo-rectal tumors progresses through a series of well defined pathological stages. The target cells of the colon tumorigenesis are assumed to be the crypt epithelial cells and much attention has been payed to the transition of normal colonic crypt cells to neoplastic cells. Initially epithelial hyperplasia is seen in the crypts followed by increasing degrees of dysplasia ending up with tumor development (22). There is considerable evidence that the adenoma-adenocarcinoma sequence is the predominant pathogenic pathway in colon tumorigenesis (14,23), and it has been suggested that benign tumors often will progress to malignancy. Thus, it seems reasonable to assume that dysplastic colonic crypts are precursors of colo-rectal tumors and focus on this type of alteration in the attempt to point out an applicable early preneoplastic marker.

5.3.2 The aberrant crypt foci (ACF) bioassay

The first step towards development of a short term *in vivo* bioassay for colon tumorigenesis was taken in 1987 by Bird (24), who introduced the visualization

of aberrant crypt foci (ACF) in blue-stained, unsectioned, formaldehyde fixed colons from carcinogen treated mice and rats. These aberrant crypt foci consisted of one to several colonic crypts that differed from the normal surrounding crypts by their increased size, thicker and deeply stained epithelial lining, and increased pericryptal zone (24,25). The number of aberrant crypt(s) per focus is described as the crypt multiplicity. At the discovery in 1987 the ACF were defined by their surface luminal features and not by their histological features, but it was speculated that these crypts might be dysplastic, since a histological investigation of a very limited number of HE stained transverse sections of ACF revealed the presence of dysplastic crypts (24).

The figures below illustrate diagrams of a topographic view of an ACF in an unsectioned blue-stained colonic mucosa (Figure 2) and a longitudinal histological section of an ACF surrounded by normal crypts (Figure 3).

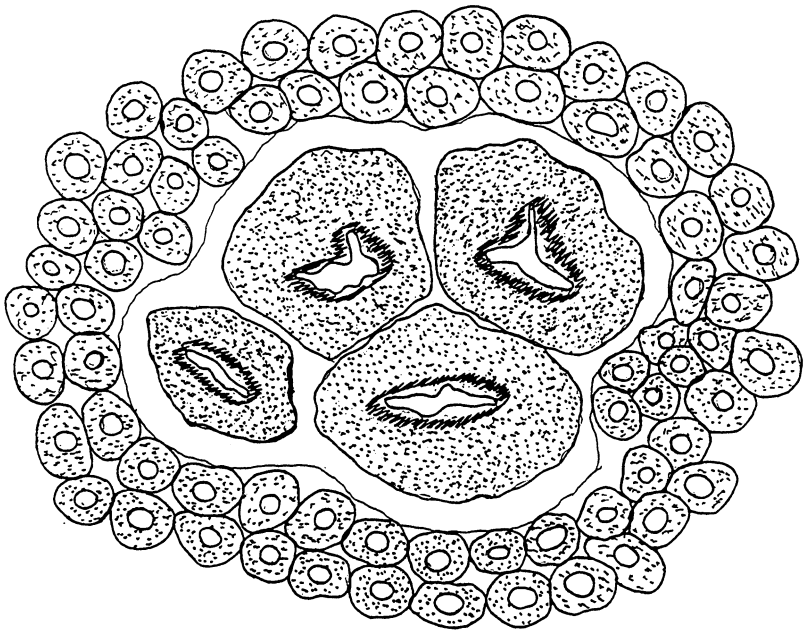

Figure 2: A schematic diagram depicting a topographic view of an ACF in an unsectioned blue-stained colonic mucosa. The ACF, which is surrounded by normal crypts, consists of four crypts. The aberrant crypts are often elevated above the normal mucosa.

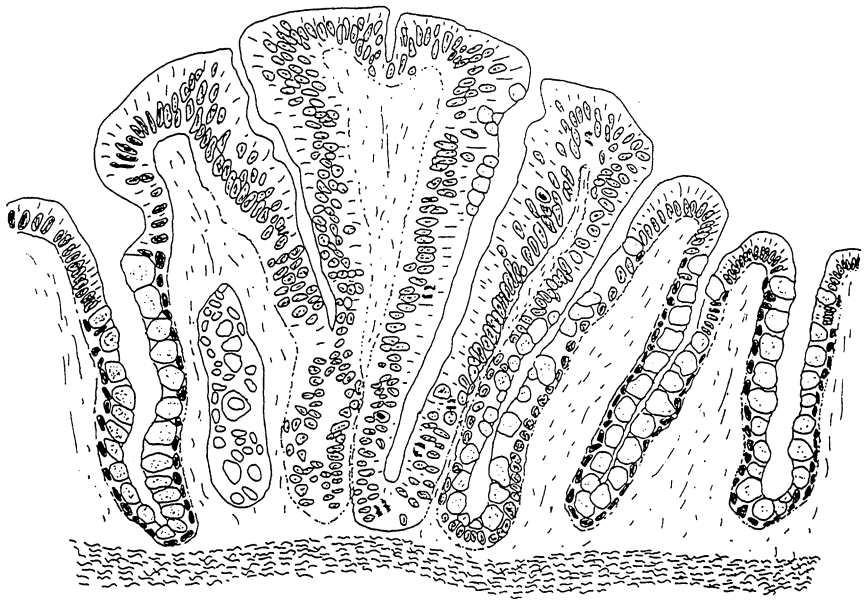

Figure 3: A schematic diagram depicting a longitudinal histological section of an ACF surrounded by normal crypts. Hypercellularity of the aberrant crypts is seen, and the colonic epithelial cells are hyperplastic or dysplastic (for further description, see paper **V**)

Recently it was shown that besides mice and rats also hamsters are able to develop ACF in the colon when treated with a colon carcinogen (26-28). The ACF are found predominantly in the distal colon (29,30), and they can be recognized in mice and rats as early as two to four weeks after dosing with a colon carcinogen (29,31-33).

Foci of atypical colonic crypts evidencing features of dysplasia have long been recognized in the colon from rats and mice following treatment with a colon carcinogen (22,34-38), but up to 1987 these lesions could only be identified by examining histological sections of the colon. This task to find these microscopic lesions in histological sections "is analogous to looking for a needle in a haystack" (25). As the blue-stain method (24,39) requires only that the colon is fixed, and the quantification of the lesions in the entire colon of rodents can be done in less than one hour, the ACF assay may provide a highly efficient *in vivo* bioassay for colon tumorigenesis. In addition, almost all the treated animals develop these foci and multiple lesions (up to several hundreds) are seen per animal.

During the last decade much evidence has accumulated pointing towards ACF as an early step in colon tumorigenesis. In the following these features are summarized:

a) *Induction and time of occurrence of ACF*. ACF can be seen in rodents two to four weeks after dosing with a colon carcinogen and generally multiple lesions are induced per animal. Mostly single crypt foci are observed at this early time point (29,31-33), but many of these foci become larger with multiple crypts with increasing time (29,31,40-44).

In some studies it has been noticed that branching of crypts or crypt bifurcation is more common in ACF than normal tissue (45-47), and it has been suggested that binary crypt fission plays an important role in the development of ACF (48,49). Yamashita et al. (45,46) have suggested that ACF are lesions of monoclonal expansion of mutated cells.

b) *Specificity of ACF formation*. Studies with rats and mice have shown that most of the colon carcinogens and some of the non-colon carcinogens tested are able to induce ACF, whereas the non-carcinogens are unable to do this (see Table 1).

Table 1. The ability of various test agents to induce ACF in rats or mice

Classification of test agents	Response (In mice and/or rats)
Colon carcinogens	
1,2-dimethylhydrazine (1,2-DMH)	+
Azoxymethane (AOM)	+
3,2´-dimethyl-4-aminobiphenyl (DMAB)	-
4-aminobiphenyl (4AB)	+
N-nitroso-N-methylurea (MNU)	+
3-methylcolanthrene (MCA)	+
2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ)	+
2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP)	+
Aflatoxin B1 (AFB1)	+
N-methyl-N-nitro-N-nitrosoguanidine (MNNG)	+
Non-colon carcinogens	
N-nitroso-dimethylamine (NDMA)	-
Benzo(a)pyrene (B(a)P)	+
1,1-dimethylhydrazine (1,1-DMH)	-
7,12-dimethylbenz(a)antrachene(DMBA)	+
Non-carcinogens	
Hydrazine sulphate (HS)	-
2-aminobiphenyl (2AB)	-
Benzo(e)pyrene (B(e)P)	-
Methylurea (MU)	-
Colcichine	-

+: a positive result; -: a negative result.

Based on data from Bilbin et al., 1992 (50), Kristiansen et al., 1996 & 1997 (51,52), McLellan and Bird, 1988 (53) and Tudek et al., 1989 (33).

c) *Dose-response relationship*. Increasing doses of colon carcinogens administered to rodents led to an increase in total number of ACF per colon. A similar trend was seen with respect to the crypt multiplicity and size of ACF. However, some studies have shown that the different doses of carcinogen did not affect the relative distribution of ACF categorized with respect to crypt multiplicity (43,54). There seems to be a levelling out at high doses of 1,2-DMH and AOM (29,31,33,43,54).

d) *Persistence of ACF in the colon*. At least some of the ACF are stable in nature and do not seem to represent a transient response to exposure to a colon carcinogen, as they have been found to persist in the colon more than half a year after termination of carcinogen exposure (31,39,43). Part of the lesions seems to be of transient nature and is eliminated or remodelled to normal colonic crypts (42,53,55,56).

e) *Spontaneous occurrence and background level of ACF*. Our own investigations as well as studies performed by many other groups (29,31,33,57) have shown that ACF are never or only very seldom seen in the colon of untreated animals.

f) *Occurrence of ACF in humans*. Lesions that resemble rodent ACF have been observed in grossly normal, blue-stained, unsectioned human colonic mucosa (58-60). The ACF in humans have many features in common with those induced by carcinogens in rodents, including increased frequency in humans at increased risk for colo-rectal cancer, ranges of histopathological appearance (58-62), increased proliferation rate (63) and the presence of K-*ras* mutations (45,46,61,64-66).

g) *Morphological properties of ACF*. Histomorphological descriptions of rodent ACF (29,31,42,43,67-69) as well as human ACF (45,46,58-61,70-72) have revealed that at least part of the ACF show dysplastic properties.

h) *Similarities in mutations between ACF and colo-rectal tumors*. Investigations have revealed the presence of K-*ras* mutations or elevated expression of K-*ras* protein in both human (45,46,61,64-66) and rodent (67,68,73,74) ACF. Further, *APC* mutations have been demonstrated in human ACF (64,66). As it is assumed that alterations in the K-*ras* oncogene and the *APC* tumor suppressor gene are early events in the colon carcinogenesis (1,2), these

findings may suggest that ACF are early neoplastic lesions.

Based upon the above mentioned, it has been suggested that the aberrant crypts represent early preneoplastic lesions, and that the short term ACF assay may be a valuable tool in the evaluation of the possible influence of various dietary compounds on the development of colo-rectal tumors in humans. As a consequence, the assay is currently used to test a variety of possible colo-rectal cancer modulating dietary components.

5.4 Evaluation of the ACF bioassay as a predictor for development of colo-rectal tumors

Parallel to the use of the ACF assay as marker for potential tumorigenicity in dietary intervention studies, research has been devoted to pioneer work direct linking together the presence of ACF and later development of colo-rectal tumors. Even though there are many indications for ACF as an early marker for development of colo-rectal tumors, many of these studies have raised questions regarding the predictive ability of ACF in the pathogenesis of colo-rectal cancer. In the following, an attempt is made to evaluate the connection between induction of ACF and development of colo-rectal tumors, and the neoplastic potential of the ACF. The evaluation is primarily based upon **1**) studies which included data on enumeration of ACF and tumors within the same protocol, and **2**) basic research studies concerning ACF-biomarkers relevant in relation to colo-rectal tumorigenesis.

5.4.1 Studies including data on ACF and tumors within the same protocol

To investigate whether a correlation between the occurrence of ACF and colo-rectal tumors could be established, all studies which included both ACF and tumor data within the same protocol were evaluated. As the experimental induced carcinogenic process is highly susceptible to the experimental conditions, the ACF and tumor outcome depend on, e.g. 1) the age, sex, species and strain of the animal, 2) the type of carcinogen used to induce the carcino-

genic process, 3) the dose, frequency and route of carcinogen administration, 4) whether the carcinogen is given as an initiator, a promotor or both and 5) the duration of the study. Therefore, it is very important that comparison between the outcome ACF and tumors is confined to studies where these data are obtained under identical circumstances. Only nineteen studies were found to fulfil this criterion and the results are given in Table 2.

Table 2. Correlation between presence of ACF and development of colo-rectal tumors

Study no.	Correlation between ACF and tumor development based on:		Ref. no
	Total no. of ACF	ACF with crypt multiplicity ≥	
1	-	-	(41)
2	+	NR	(75)
3	+	4	(76)
4	-	4	(77)
5	+	NR	(78)
6	(+)	8	(79)
7	+	4	(80)
8	-	10 (14)	(81)
9	(-)	NR	(82)
10	(-)	-	(83)
11	-	-	(84)
12	-	-	(39)
13	+	4	(85)
14	+	NR	(86)
15	+	11	(87)
16	-	NR	(88)
17	-	14	(40)
18	-	-	(89)
19	-	-	(90)

+: Direct correlation demonstrated; -: No correlation demonstrated; NR: Not recorded
(+): Indication of direct correlation; (-): Conflicting results, most likely no correlation.
A common feature of the studies included is the presence of ACF in colon carcinogen treated animals and lack of ACF in untreated animals.

Although the ACF and tumor data were obtained under identical experimental conditions in the individual studies, great variations between the experiments exist and this should be kept in mind at the subsequent evaluation of the results. Besides variations as those already mentioned, differences were also seen with respect to the time point for evaluation of ACF. In study one to eleven, the protocol included sequential analyses of ACF until the tumors appeared and the interim sacrifices were performed within a range of four to 27 weeks after the first carcinogen treatment. The durations of the studies were 30 to 36 weeks. In study twelve to nineteen, only 'late' ACF were recorded, which means that no interim analyses of ACF were carried out. The ACF were only measured at the end of the study, 23 to 40 weeks after the start of carcinogen treatment. It could be argued that these studies cannot be characterized as precursor studies inasmuch as they assessed the ACF at the same time as tumors have appeared and therefore, in reality, investigated the association between ACF and tumor incidences.

In the first of our ACF-studies (39) only 'late' ACF were recorded as the enumeration was carried out on archived tissue from a previously performed tumor study (21), but in the second study (41) two intermediate evaluations of ACF were carried out. Neither of our studies could unequivocally support the hypothesis that the number of ACF and/or the crypt multiplicity correlated with colo-rectal tumor development. In the first study carried out on archived tissue, we observed significant differences between the investigated groups with respect to total number of ACF and number of small ACF, but the low amount of ACF observed in some groups were not mirrored by a lower colo-rectal tumor outcome (see paper **I** and **II**). However, the lack of correlation between the outcome of ACF and tumors could be explained by the fact that statistically significant differences were only seen in the total number of ACF and number of ACF with a crypt multiplicity at less than four, as it has been suggested that only ACF with a crypt multiplicity at four or more correlate with later development of tumors (56,77,81). In our second ACF study significant differences were revealed between the experimental groups, also with respect to ACF with a crypt multiplicity of four or more. But still, no statistically significant differences in tumor incidences were obtained (see paper **III**).

Thus, neither the enumeration of early nor late ACF was found to be a reliable parameter to predict colon tumor development. Consequently, the conclusion drawn from our studies is in accordance with that stated in about half of the ACF-tumor studies performed (see Table 2).

The other half of the studies cited in Table 2 found that the enumeration of ACF could be a reliable parameter for later tumor development, but mainly if the correlations were based on the 'larger' ACF. However, the definition of the term 'larger' varied from ACF with 4, 8, 10, 11 and up to 14 crypts per focus. In comparison, our study showed that even ACF with ten or more crypts were not conclusively predictable for tumor development (41).

In most of the above mentioned studies the comparisons were made between the different treatment groups included in the experiment. Only a few of the studies have compared the presence of ACF between tumor-bearing and tumor-free animals (40,77,81,90) and evaluated the presence of ACF and tumors at the individual animal level (39,41,83). Neither did any of these two ways of evaluation lead to consistency between the presence of ACF and tumors. One study found no differences in the occurrence of ACF between groups of tumor-bearing and tumor-free animals (90), one observed a higher number of ACF with four or more crypts in animals with tumors (77), whereas others observed that only the number of ACF with more than thirteen crypts were significantly increased in the tumor-bearing animals (40,81). With respect to the presence of ACF and colon tumors at the individual animal level, our own studies did not reveal any correlation between a high number of total ACF or 'large' ACF and presence of tumors (39,41). In one study a significant correlation between tumor size and ACF with three or more crypts has been demonstrated (83). It could be argued, that a correlation between a high number of ACF and occurrence of tumors was not achieved because the presence of a tumor could prevent, e.g. by influence of cytokines, the development of new ACF or even lead to regression of already existing ACF. However, our studies have shown that some of the colons which harboured tumors had high amounts of ACF, including ACF of high multiplicity, whereas others had low amounts of ACF. If the assessment of ACF is indicative for later tumor development, the time point for the intermediate evaluation of ACF may be very crucial. Thus, one of

the studies revealed that the total number of ACF parallelled tumor frequency when measured six weeks after last carcinogen treatment, but not when measured at the termination of the study 14 weeks later (83). It cannot be excluded that the variations in the results obtained could be related to the different time point for the ACF assessment in the nineteen studies.

In addition to the above-mentioned studies, two more experiments deserve attention, although they do not fulfil the requirements for being included in this evaluation. A recent study dealing with different strains of transgenic mice treated with dimethylhydrazine showed no agreement between the susceptibility to develop ACF and adenomas, which indicated that an ACF does not necessarily progress to an adenoma (91). It was stated that when evaluating the predictive role of ACF for colon tumorigenesis, genetic factors must be taken into account. In another study, a very unique *in vivo* technique was applied which delivered a direct evidence that ACF are able to progress to tumors (92). One hundred days after carcinogen treatment, rats were anesthesized whereupon colotomy was performed and individual ACF selected and labelled. Another hundred days later it was shown that although many of the ACF regressed with time, some persisted and developed into tumors. The number of tumors observed in the labelled areas was 17 times higher than expected by random chance. This study thus supported the hypothesis that some, but not all ACF are precursor lesions for tumors.

In summary, about half of studies did not find enumeration of neither early nor late ACF a reliable parameter to predict colon tumor development and/or modulation of colon tumorigenesis. The other half found the parameter reliable, but mainly if the calculations were based on the 'larger' ACF. However, the definition of the term 'larger' varied from ACF with 4, 8, 10, 11 and up to 14 crypts per focus. One reason for the inconsistency in the outcome of the studies could be differences in the experimental design. Thus, based on the above mentioned studies it is not possible to correlate the enumeration of ACF with the possibility for later development of colo-rectal tumors.

5.4.2 Studies including basic research on ACF-biomarkers indicative for

To further elucidate whether ACF are precursors for tumor development, all studies which described features of ACF indicative for the carcinogenic process were evaluated. Such features include, e.g. morphological, genetic and enzymatic alterations in the ACF.

Dysplastic features of ACF

As described earlier, hyperplastic and dysplastic lesions may progress into tumors, and therefore the demonstration of hyperplasia and/or dysplasia in the ACF may support the hypothesis that ACF represent preneoplastic lesions. When Bird in 1987 (24) introduced her screening method for the rapid detection and enumeration of ACF, no thorough histological examination was included. In the following year and up to now, some detailed histological examination of the ACF has been performed. However, taken into account the widespread use of the ACF bioassay (more than two hundred published papers), only little attention has been payed to the histological evaluation of the lesions and only a rather limited number of studies including histomorphological investigations have been published.

From observations in rodents (29,31,42,57,67-69) as well as humans (45,46,-48,58,59,61,70-72) it is evident that some of the ACF are dysplastic in nature, but it is also found that they vary morphologically from near normal to overtly dysplastic. Therefore, it has been suggested that ACF are biologically heterogeneous lesions. Differences in degree of dysplasia have been observed among ACF from the same colonic tissue, among ACF with the same crypt multiplicity, and even among crypts within the same focus (29,42,57,59,61,62). The heterogeneous appearance of the ACF at the histological level is supported by observations in blue-stained, unsectioned colons as the luminal morphology of the aberrant crypts is variable (33,56,70). It has been proposed that topographic features of the ACF can be used to identify the degree of atypia in human colonic tissue (59,60), although this observation could not be confirmed in a later study (70). It has also been speculated that a certain group of ACF consisting of dilated crypts represent unstable lesions prone to regress or

remodel (93).

As only rather limited information is accessible concerning the histomorphological characterization of ACF, a study covering this field was recently carried out at our institute (see paper **V**). This study includes, among other things, evaluation of the degree of dysplasia in a large number of ACF developed under different experimental conditions, which lead to disparities in the number as well as the crypt multiplicity obtained. In the study, the histological characterization of ACF is based on longitudinally sectioned foci. This is in contrast to most of the existing studies which are primarily based on cross-sectioned foci (29,42,59,60,67-69). In my experience, the best results are obtained when the ACF are evaluated on longitudinally sectioned foci as the crypts behave differently from bottom to top. For instance, the degree of dysplasia was most obvious in the lower region of the crypt whereas the alterations in the expression of certain growth factors were seen only in the upper part. When crypts are cut horizontally in cross sections, a smaller proportion of the cells are available for morphological evaluation and unless serial sections are performed through the whole ACF, the score values will depend on the distance from bottom. This view is in agreement with Pretlow et al. (70).

In the study described in paper **V** I have shown that different experimental conditions had no influence on the severity of dysplasia in the ACF investigated, but a strong correlation between degree of dysplasia and crypt multiplicity was seen. Although this marked association was found, varying degrees of dysplasia were seen within ACF belonging to the same category of crypt multiplicity and even within crypts belonging to the same ACF. As our previous study has shown that ACF increased in crypt multiplicity over time (41), the observation in my study (paper **V**) may support the view that experimentally induced dysplastic crypts in rats may have the potential for gradually progressing into tumors. This is also in accordance with observations in human colonic tissue as Otori et al. (61) have suggested that hyperplastic ACF may develop into adenomatous ACF. However, other investigations have demonstrated that the degree of dysplasia was not (42,45) or only weakly (70) related to the crypt multiplicity. The lack of correlation seen in these studies

could be due to the lower number of ACF examined compared with the present study.

During the evaluation of the degree of dysplasia an increased mitotic activity, which has been proposed as a biomarker of the early stages of colon cancer (94), was noted in the ACF (paper **V**). The study design did not make it possible to quantify the proliferative activity, but in the ACF a higher mitotic activity was seen compared with the surrounding normal tissue. The major part of the foci showed a mitotic pattern similar to that of normal appearing adjacent crypts in that the mitotic cells were restricted to the lower two-thirds of the crypt rather than distributed throughout the crypts. This observation is in agreement with most of the published data concerning ACF and mitotic activity (43,63,95). In some of the foci, primarily those exhibiting the highest degree of dysplasia, the mitotic activity was seen distributed throughout the crypts as reported for adenomas (22,96). This finding may further support the suggestion that ACF are early neoplastic lesions. In one published study it has been reported that all the ACF showed an alterated mitotic pattern as the mitotic activity was present in the middle portion of the aberrant crypts, but in the bottom of the normal crypts (44). One publication (31) reported that the heterogeneity of ACF was reflected in the proliferative activity as some crypts exhibited marked increase in mitotic activity whereas others did not.

In summary, data from the literature indicate that ACF are heterogeneous lesions of which some are dysplastic in nature and maybe able to develop into tumors. My most recent study confirmed the rather heterogeneous appearance of the ACF, but in addition, it demonstrated a clear and strong correlation between the degree of dysplasia and crypt multiplicity, which has not previously been reported. This finding supports the suggestion that ACF, at least in the rat, may have the potential for gradually progressing into tumors.

Genetic alterations in ACF

During the last few years studies concerning genetic alterations relevant in relation to colo-rectal tumorigenesis have been added to the morphological studies to further elucidate the importance of the ACF in the tumorigenic process. The major part of the studies has focused on mutations in the K-*ras*

oncogene which are suggested to be an early event in the colon carcinogenesis. Besides, sparse literature exists concerning alterations in the *APC* and p53 tumor suppressor genes, which represent an early and late event, respectively, in the process (see section 5.1).

Investigations have been carried out on animal as well as human ACF. Davies and Rumsby (97) investigated ACF from rats treated with AOM and did not detect K-*ras* mutations in any of the ACF examined. In contrast, Shivapurka et al. (98) detected K-*ras* mutations in ACF from 25-37% of AOM treated rats (foci from the same rat were pooled) and in colonic tumors from 39% of the tumor-bearing rats. In a similar study, Vivona et al. (74) demonstrated K-*ras* mutations in 7% of the ACF, 4% of the colonic adenomas, and 37% of the carcinomas investigated. No mutations were seen in normal mucosa. From these studies it was concluded that the demonstration of the genetic mutations in the ACF provided further evidence for the significance of these lesions as precursor of malignant potential during colo-rectal carcinogenesis, but Shivapurka et al. (98) stated that only part of the ACF and tumors seemed to arise from K-*ras* mutations. In the above mentioned studies the ACF are induced by AOM. However, in a rat study Tachino et al. (73) demonstrated K-*ras* mutations in 11% of ACF induced by the cooked food mutagen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). They mentioned that the observation was of interest as no *K-ras* mutations were found in 11 tumors induced by IQ. In a recent study with transgenic mice overexpressing the *MGMT*-CD2 gene which codes for O^6-alkylguanidine-DNA alkyltransferase (an enzyme active in DNA repair), Zaidi et al. (99) observed the presence of ACF without mutations in K-*ras* and presence of K-*ras* mutations in apparent normal-looking mucosa. This lead to the suggestion that the presence of ACF and K-*ras* mutations are not co-dependent in this model system and to the proposal that carcinogen treatment induces at least two overlapping but not identical premalignant lesions, i.e. ACF and K-*ras* mutations. They speculated that K-*ras* mutant ACF is more likely to progress to malignant lesions than ACF without K-*ras* mutations. In a number of ACF-studies with AOM-induced rats Stopera et al. demonstrated K-*ras* mutations in 32% of the aberrant crypts examined (67), elevated expression of K-*ras* *m*RNA and K-*ras* protein (68), and c-*fos* *m*RNA and c-*fos*

protein (100), and occurrence of mutated p53 protein (69). In a paper by Yamashita et al. (45) reference was given to a study, reported in Japanese, which found K-*ras* mutations in 5% of the ACF from mice.

In a study on human colonic tissue Jen et al. (64) have analyzed ACF for the presence of K-*ras* and *APC* mutations. The ACF were histologically examined, and categorized as hyperplastic or dysplastic. K-*ras* mutations were seen in 100% of the hyperplastic ACF, but were not detected in the one dysplastic ACF found. In contrast, no *APC* mutations were identified in any of the hyperplastic lesions while such mutations were observed in the dysplastic ACF. A similar picture was seen in hyperplastic and dysplastic polyps investigated in the same study. The authors suggested that *APC* are closely associated with the advent of dysplasia, a hallmark of malignant potential. In contrast, mutations of K-*ras* seemed to be independent of dysplasia. They proposed a model where, if a K-*ras* gene mutation occurs as the first genetic event a hyperplastic (non-dysplastic) ACF which has little potential to progress will form. However, if an *APC* mutation occurs first, a dysplastic ACF will result. Smith et al. (66) examined the same type of genetic alterations and identified K-*ras* mutations in 13% and *APC* mutations in 4.6% of the ACF. In addition, K-*ras* mutations were found in 45% of the tumors evaluated. They concluded that both K-*ras* and *APC* mutations occurred in the ACF, but the K-*ras* oncogene activation was more common than *APC* tumor suppressor gene inactivation at the ACF stage, and that activation of K-*ras* may be one of the key phenomena in the generation of the ACF phenotype. Their observations supported the role of the ACF as a colo-rectal cancer precursor. In this study the genetic alterations were not correlated with the morphology of the ACF. In another study, Pretlow et al. (65) observed that mutations in K-*ras* were present in 73% of ACF, but not in morphologically normal mucosa from the same patients. They concluded that ACF are the earliest identified precursors of human colon cancer. Yamashita et al. (45,46) have looked for K-*ras* mutations and accumulation of p53 protein in human ACF and tumors. They demonstrated K-*ras* mutations in 58% of the ACF and in 44% of the adenocarcinomas. No mutations were seen in normal mucosa. The presence of K-*ras* mutations could neither be correlated with the crypt multiplicity nor histopathological features, and the base pair alterations

were not necessarily correlated with those of coexistent adenocarcinomas. No accumulation of p53 protein in nuclei was found in any ACF, but was seen in 52% of the colo-rectal carcinomas. They proposed that K-*ras* mutations are essential and sufficient for ACF formation, but that K-*ras* mutation does not always lead to formation of neoplasms in the colo-rectal mucosa. Otori et al. (61) have in their study analyzed large ACF (50 or more crypts per focus) for the presence of K-*ras* mutations and nuclear accumulation of p53 protein. K-*ras* mutations were detected in 85% of the foci. However, the larger and more dysplastic the ACF were, the lower was the amount of K-*ras* mutations detected. The authors hypothesized that ACF consist of two different lesions: a "*de novo* adenoma" and a K-*ras* dependent lesion. p53 protein accumulation was not found in any of the ACF. In another type of study Augentlich et al. (101) have investigated DNA from ACF for genomic instability. They analyzed for alterated allele lengths characteristic for a defect in the DNA repair, and in 10-15% of the ACF they detected alterated allele length between ACF and adjacent normal colonic tissue. They concluded that instability could be detected in some ACF, but the contribution of this finding to the morphological and histological phenotype of ACF, and the possibility of progression is not clear.

In summary, K-*ras* mutations are assumed to be one of the earliest events in the colon tumorigenesis and demonstration of these types of mutations in the ACF could contribute to the suggestion, that ACF are early precursors of colon tumors. Although K-*ras* mutations were a rather consistent finding in most of the studies, the results did not form a clear picture of the genetic alterations in the ACF. Thus, the reported incidences of K-*ras* mutations were in the range of 0% to 32% in ACF from rodents and 13% to nearly 100% in human ACF, and in two studies it was found that hyperplastic rather than dysplastic ACF contained K-*ras* mutations. In some of the papers it was suggested that different subsets of ACF exist. The findings in the ACF should be considered together with observations on K-*ras* mutations in colo-rectal tumors. K-*ras* mutations have only been observed in about 50% of the colo-rectal carcinomas and adenomas greater than one cm in size, and only in about 10% of adenomas

less than one cm (102). The very high frequency of K-*ras* mutations seen in the ACF in some of the studies may indicate that only a subset of tumors could arise from ACF or that the activated *K-ras* oncogene is deleted during the progressing of ACF to tumors. The few studies on *APC* mutations (64,66), accumulation of p53 protein (45,46,61,69) and c-*fos* protein (100) and genomic instabilities (101) did not allow any conclusions.

Enzyme alterations in ACF.

Hepatic enzyme alterated foci have been detected in rodents after treatment with carcinogens and it has been suggested that they represent preneoplastic lesions. It could be of interest to establish whether similar changes in the enzyme expression could be demonstrated in the ACF. A few papers concerning this item have been published (30,57,58,62,103) in which various histochemical procedures were applied. The data showed that no single marker could identify all the foci (57,62) and the most consistent result observed was a marked decrease in hexosaminidase activity in ACF of rats (30,57,103). In contrast, an increased activity was shown when human ACF were investigated (58). Further, studies in rats have shown that two populations of hexosaminidase decreased foci exist, one type which contained morphologically altered crypts and one in which the crypts appeared normal (103). Alteration in hexosaminidase has been linked with colon neoplasms in rodents as well as humans, and therefore, the enzyme alterated foci might be related to the tumorigenic process (57). In a chemopreventive study the hexosaminidase staining procedure was applied on ACF (104) and all the foci were negative, but in addition, some normal looking crypts lacked hexosaminidase activity.

Thus, it can be concluded that no consistency exists with respect to altered expression of enzyme activities in ACF, and the foci seem to be a group of rather heterogeneous lesions. Based upon the existing data it is not possible to make any correlation with a neoplastic potential of ACF.

Alterations in growth factor expression in ACF

Today it is assumed that changes in the expression and distribution of different

growth factors, their receptors and the signal transduction system are involved in the tumorigenic process. However, as no detailed information has been published on this item in relation to ACF it was decided that further investigation into this subject might help to clarify the role of ACF in the tumorigenesis. ACF from an earlier performed study (41) were investigated for the expression of two well described growth factors, the transforming growth factor α (TGF-α) and ß (TGF-ß), and their respective receptors, the epidermal growth factor receptor (EGFR) and transforming growth factor ß receptors I and II (TGFßR I and II) as well as phosphorylated cellular tyrosine (P-tyr) (see paper **V**). Based upon data from the study it could be stated that the ACF deviated from the surrounding normal tissue with respect to expression of the TGFs, especially TGF-α. With respect to the growth factor receptors, no altered expression was observed concerning EGFR, whereas neither of the TGFßRs showed positive reaction in the colonic tissue at all. Regarding P-tyr no altered expression was observed between ACF and adjacent normal tissue. All the ACF reacted in the same way despite of differences in degree of dysplasia, crypt multiplicity or developmental conditions. Thus, the present study showed an altered expression of TGFs in all the ACF investigated which may be consistent with the assumption that ACF are preneoplastic lesions, but no single ACF showed particular characteristics indicating specific proneness to tumor development.

Other alterations in ACF

A close link between cell proliferation and apoptosis exists (105) and in rats it has been demonstrated that after acute cytotoxic effect on colonocytes induced by AOM, the cells of ACF are more resistant to apoptotic cell death than normal crypt cells (106). It was found that the largest ACF were associated with the lowest number of apoptotic bodies per crypt, which led to the suggestion that ACF of higher crypt multiplicity were more likely to progress to colonic tumors than ACF of low crypt multiplicity. No attempt was made to correlate degree of dysplasia with number of apoptotic bodies.

Elevated expression of carcinoembryonic antigen (CEA) has been demonstrated in 93% of human ACF analyzed (70). This antigen is normally

expressed at high levels in the fetal colon and at low levels in the adult colon and has found an important place in relation to human colon cancer. The expression of CEA was correlated with the size, but not to the degree of dysplasia of the ACF. It was concluded that the demonstration of increased expression of CEA supported the hypothesis that ACF are preneoplastic lesions.

Over all conclusion: Based upon the many types of studies described above ACF appear as a group of heterogeneous lesions and the relationship between ACF and tumorigenesis seems to be rather complicated: **1**) no simple correlation exists between enumeration of ACF (whether based on the total number or number of 'large') and the risk for colon tumor development. Attempts have been made to correlate 'larger' ACF to the outcome of tumors, but no consistent pattern has been demonstrated, **2**) at the histological level ACF were found to vary morphologically from near normal to overtly dysplastic, although correlation between degree of dysplasia and crypt multiplicity has been described and, **3**) analysis for gene mutations in the ACF did not reflect an unequivocal picture of the genetic alterations and suggestions have been made that more subsets of ACF exist. On the other hand, many of the features characteristic of the carcinogenic process, with respect to morphological and genetic alterations as well as alterations in growth factor expression, have been demonstrated in the ACF.

Irrespective of the inconclusive outcome of the studies, the fact remains that the total number as well as the crypt multiplicity of ACF are currently being used as early markers for the risk of later colo-rectal tumor development.

6 **SPECIFIC STUDIES**

The fact that colo-rectal cancer is one of the most common malignant diseases in the Western industrialised countries combined with the generally accepted view that the disease is influenced by dietary habits, acted as an incentive to our investigations concerning the influence of diet on the development of colo-rectal cancer. Further, when the ACF assay was introduced it was of great interest to establish, whether this short term model could be used as a reliable screening test in the evaluation of the influence of dietary components on the development of colo-rectal cancer.

The aim of our first experiment was to investigate the influence of a dietary fiber on colon tumorigenesis (paper **I**) and it was carried out as a carcinogenicity study with tumor development as the end point. The study was designed to evaluate the possible modulating effect of the dietary fiber when given during preinitiation, initiation, promotion and progression. It was concluded that no effect of the dietary fiber was found at any stage of the colorectal carcinogenic process. Even though differences (but not statistically significant) in tumor incidences between the groups were seen, these did not reflect any effect of the dietary fiber intake. Statistically significant differences in tumor incidence between the groups were neither detected with respect to total, malignant, nor benign tumors. Most of the tumors were seen in the proximal part of the colon and often located near lymphoid nodules. The tumor incidence obtained in the study was low (13%-31%) compared with data reported in the literature, and this may have resulted in an insufficient sensitivity of the design used, but it should be pointed out, that a rather large number of animals (30 per group) were included.

When knowledge of the ACF assay was obtained from the literature, we applied this model on archived tissue from the above-mentioned study to investigate whether any correlation between occurrence of ACF and tumors could be established (see paper **II**). The method stated by Bird (24) to examine the ACF was slightly modified as we preferred the Giemsa to the methylene-blue in the staining procedure. Both of the staining procedures result in blue coloured tissue. Only part of the middle colon was used for the investigation

as all the colonic tissue was not longer available, but investigations at our institute (unpublished data) had shown that the majority of ACF were located in the area chosen for the investigation. Contrary to the results regarding tumors obtained from the earlier study (paper **I**), an effect of the dietary fiber on the occurrence of ACF was seen. A significant inverse relation was observed between duration of the intake of a fiber-rich diet and number of animals with ACF, as well as the total number of ACF and number of small ACF per affected animal. Based on group level no consistency existed between the presence of ACF and colon tumors. Neither could any correlation between ACF and tumors be established when assessed on the individual animal level. Although the lack of correlation between the outcome of ACF and tumors might be explained by the observation that statistically significant differences between groups were only seen in the total number of ACF and number of small ACF, we concluded that the hypothesis that ACF represent preneoplastic lesions needed to be supported by further experimental data.

Therefore, we decided to perform a study (paper **III**) which should cover the following requirements: **1**) the duration of the study should be long enough to obtain tumors, **2**) the study should include interim sacrifices for sequential measurements of ACF, and **3**) the tumor frequency in the study should be high enough to obtain sufficient sensitivity. The two first mentioned requirements were met by interim sacrifices 10 and 20 weeks after the start of dosing and final sacrifice after 31 weeks. The third request was met partly by prolonged dosing period compared to earlier studies, partly by offering the animals 'high-risk' diets, i.e. diets reflecting the diet habits in the Western countries where the incidence of colo-rectal cancer is high. One diet had a high content of refined carbohydrates and another had a high content of lard. Both of these diet components are assumed to be risk factors of the colon tumor development. In a third diet, the dietary fiber tested in paper **I** and **II** was mixed into the lard diet to see whether it could give protection against tumor development when added to a high risk diet. Overall, the requests were met (although the tumor incidence was still rather low), but the results did not conclusively show that the total number or the crypt multiplicity of ACF was predictor for tumor outcome. Enumeration of ACF revealed significant differences between some

of the groups at all time points. The diet rich in refined carbohydrates enhanced the occurrence of ACF in all the crypt multiplicity categories, whereas the fiber-rich diet caused a reduction. A similar picture was not clearly reflected in the tumor outcome. Even though the highest tumor incidence and the largest tumors appeared in the group with the very high number of ACF, no statistically significant increase in tumor incidence was seen compared with the other groups. In addition, the group with the lowest number of ACF showed no reduction in tumor incidence compared with the other groups. On the other hand, it cannot be ruled out that a higher number of animals in the group given refined carbohydrates would have led to a statistically significant increase in tumor development. When assessed on individual animal basis, lack of correlation between the total number and crypt multiplicity of ACF and occurrence of tumors was demonstrated. Thus, neither in this study did the tumor picture unequivocally support the ACF data. The investigation did not include parameters to differentiate between ACF at the cellular level, but the results were in accordance with the suggestion that ACF is a heterogeneous group of lesions.

Lack of consistency between ACF and tumor outcome could also be observed in paper **IV**. Even though the intention of this study was to evaluate the influence of different kinds of starches on the development of ACF (encouraged by paper **III**) and not the correlation between ACF and tumor development, it indicated a rather complicated correlation between late ACF and tumor development. Four groups of animals were offered diets with mixed carbohydrates, sucrose, potato starch, and cornstarch, respectively, as the carbohydrate pool. Concerning the animals given the potato starch diet, a good correlation between the outcome of ACF and tumors was seen. This type of diet lead to a decrease in the total number of ACF as well as number of medium and larger ACF and no tumors were seen at sacrifice. On the other hand, neither were tumors seen in the animals offered the cornstarch diet, which caused high values of ACF in all categories. Tumors were found in the groups offered diets with mixed carbohydrates or sucrose, but the ACF values in these two groups did not differ at all from the values obtained in the corn starch diet group. When assessed on individual animal basis, no correlation between the total

number and crypt multiplicity of ACF and the occurrence of tumors could be found. Thus, like the previous study it revealed no simple connection between the occurrence of ACF and tumor development.

The attainment of our somewhat conflicting results concerning the correlation between ACF and tumor development and the circumstance that ACF seem to be heterogeneous lesions prompted the investigation of paper **V**. In this study it was decided to investigate, by the use of conventional histomorphological and immunohistochemical procedures, whether any variations with respect to variables indicative for tumor development could be detected among ACF developed under different circumstances leading to disparities in enumeration of ACF. For the experiment, archived tissues from the study described in paper **III** were used. The variables measured were the degree of dysplasia, the type of mucus production, and the expression of TGF-α and TGF-β, and their respective receptors, EGFR and TGFβR I and II, and P-tyr. In addition, it was analysed whether there was a correlation between crypt multiplicity and the different parameters investigated. Hitherto, no regular investigations concerning expression of growth factors and their receptors in ACF have been published. The study showed that for all parameters investigated, but sialomucin the different experimental conditions had no effect on the individual ACF, irrespective the number and distribution of the different categories of ACF varied among the diets. However, the study revealed a strong correlation between the degree of dysplasia and crypt multiplicity as well as a correlation between changes in mucus production and degree of dysplasia and crypt multiplicity. Further, an altered expression of TGFs in all the ACF investigated was seen. These findings may be consistent with the assumption that ACF are preneoplastic lesions. Over all, the study therefore supports the suggestion that ACF, at least in the rat, may have the potential for gradually progressing into tumors, but no single ACF showed particular characteristics indicating specific proneness to tumor development. The suggestion that ACF gradually progress into tumors is supported by two recently published abstracts concerning evaluation of degree of dysplasia of human ACF (71,72). In these studies it was found that morphological characteristics allowed the definition of groups of ACF with respect to degree of dysplasia. These groups may represent

sequential steps in the development of human colo-rectal tumors.

Thus, the parameters investigated in this study do not point towards the existence of more subsets of ACF. Rather it could be suggested that all the ACF developed have a neoplastic potential, but it could be speculated that the ultimate progression to colon tumors depends on promotional conditions in the environment.

In all the above-mentioned studies the ACF were induced by the traditional chemical carcinogens 1,2-DMH and AOM. Recently we have commenced a study to investigate the development of ACF as well as their predictive value in relation to tumor development when the diet-related colon cancer initiator IQ is used as inducer. If a natural occurring component in the human diet could be used as initiator, the ACF bioassay would be more useful as a model in screening for dietary modulation of colon carcinogenesis. At present, only interim results are available from the study and they have shown that not only were low dose of IQ able to induce ACF, but the diets offered to the animals were also able to modify the number of ACF obtained (51). Whether correlation between the occurrence of ACF and tumors exists will be established within the next couple of months.

6.1 **Paper I**

Thorup,I., Meyer,O. and Kristiansen,E. (1992) Effect of a Dietary Fiber (Beet Fiber) on Dimethylhydrazine-Induced Colon Cancer in Wistar Rats. *Nutr. Cancer*, **17**, 251-261.

6.2 **Paper II**

Thorup,I., Meyer,O. and Kristiansen,E. (1994) Influence of a Dietary Fiber on Development of Dimethylhydrazine-Induced Aberrant Crypt Foci and Colon Tumor Incidence in Wistar rats. *Nutr. Cancer*, **21**, 177-182.

6.3 **Paper III**

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6.4 **Paper IV**

Thorup,I., Meyer,O. and Kristiansen,E. (1995) Effect of Potato Starch, Cornstarch and Sucrose on Aberrant Crypt Foci in Rats Exposed to Azoxymethane. *Anticancer Res.*, **15**, 2101-2106.

6.5 **Paper V**

Inger Thorup (1997) Histomorphological and immunohistochemical characterization of colonic aberrant crypt foci in rats. Relationship to growth factor expression. *Carcinogenesis*, **18** (3), 465-472.

7 **MAIN CONCLUSIONS**

Based upon own experiments and a thorough literature search it can be concluded, that there is no simple connection between occurrence of ACF and development of tumors. However, it is obvious that ACF show morphologic and genetic features characteristic for the carcinogenic process and hence, may possess a potential for neoplastic progression. Over the years it has been discussed whether the ACF represent true preneoplastic lesions which may progress via a multi step process to tumors or whether ACF and colon tumors represent end points of two parallel but independent pathways resulting as a consequence of a common colon cancer initiation (39,84,89,107). If a direct relationship between presence of ACF and development of colo-rectal cancer exists, it still remains to be elucidated.

Many of the investigations carried out have described the heterogeneity of the ACF and it has been suggested that more subsets of ACF exist. However, based upon the parameters investigated in paper **V** it seems that all ACF belong to the same unity with basically the same chances for gradually progressing into tumors. Whether such a progression occurs, may depend on promotional conditions in the environment. Thus, in our studies (21,41) the colo-rectal tumors were often located near lymphoid nodules. As it is well known that lymphoid tissue is able to produce a lot of different cytokines, it could be suggested that various promotional factors are present in the micro environment surrounding the ACF located over lymphoid nodules. It could be of interest to investigate the expression of various cytokines in the ACF as well as the surrounding normal mucosa. Cytokines which may be of relevance are the tumor necrosis factor (TNF), some of the interleukines and the eicosanoids (e.g. the prostaglandins), which may all in one way or another be implicated in the immune/inflammatory response in the lymphoid nodules (108,109). Not only could variations in the pattern of expression of lympho- and monokines and prostaglandins along the length of the large intestine be of importance. Other kinds of cytokines in the environment may also be speculated to exert an influence on the development of ACF, e.g. in my own study, the expression of TGF-α was found to vary along the colo-rectal mucosa. It was abundantly

expressed in the proximal colon, but towards the rectum a gradual reduction in expression was observed. Further studies are planned to take place at our institute to investigate the influence of various environmental factors on the possible progression of ACF to tumors.

If indeed all ACF have the ability to progress to tumors provided that they get the right promotional conditions, it could be speculated that somehow the total amount of ACF would be of importance. Thus, the possibility of obtaining an ACF which fulfils all the requirements for tumor development will increase with increasing number of ACF. On the other hand, it does not seem that the total number of ACF is a valid parameter for the risk of colon tumor development. Maybe the total number of ACF in specific areas of the large intestine, e.g. those situated over lymphoid tissue (84,89,110), are of particular interest.

It has been suggested that the fraction of 'larger' ACF is a reliable parameter for development of colon tumors. However, this term cover crypt multiplicities from four to fourteen and in some of the studies the correlation between occurrence of ACF and tumor outcome was calculated at more crypt levels, and only the very large foci correlated with tumor development. This observation is in accordance with my last study (paper **V**) which showed that the degree of dysplasia gradually increased with increasing crypt multiplicity. It seems most unlikely that ACF with a given number of crypts could be nominated as the valid marker for tumor development as ACF probably gradually progress into tumors.

Even though a lot of knowledge about ACF has accumulated during the last decade, including the studies in the present thesis, data are still inconclusive concerning the neoplastic potential of the ACF. Nevertheless, the fact remains that the total number as well as the crypt multiplicity of ACF are currently being used as early markers for the risk of later development of colon tumors. However, interpretation of the results of intervention studies that use ACF as end points should take into account the questionable value of enumeration of ACF as valid predictors of colon cancer risk. On the other hand, as the ACF exhibit many of the features characteristic of the carcinogenic process with respect to morphological as well as genetic alterations, this bioassay may

contribute to further understanding of the pathogenesis of colon cancer.

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9 LIST OF ABBREVIATIONS

ACF	Aberrant crypt foci
AOM	Azoxymethane
APC	Adenoma polyposis coli
CEA	Carcinoembryonic antigen
1,2-DMH	1,2-Dimethylhydrazine
EGFR	Epidermal growth factor receptor
HE	Hematoxylin and eosin
IQ	2-amino-3-methyl-imidazo[4,5-*f*]quinoline (IQ)
P-tyr	Phosphotyrosine
TGF	Transforming growth factor
TGF-α	Transforming growth factor α
TGF-ß	Transforming growth factor ß
TGFßR I and II	Transforming growth factor ß receptors I and II