Low intestinal tract adenomatous polyps regression in FAP patients by dietary-induced Erβ upregulation

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Summary

The two primary surgical options for familial adenomatous polyposis (FAP) are total colectomy with ileorectal anastomosis (IRA) or total proctocolectomy with ileal pouch/anal anastomosis (IPAA). A strict endoscopic surveillance of the rectal stump as well of the pouch and the removal of polyps are needed during all life for FAP operated patients. Attempts to control the proliferation and to reduce the risk of recurrent polyps and to prevent carcinomas in the rectum or pouch have been tried in the past years especially with NAIDs. A diet supplementation with a patented blend of phytoestrogens and indigestible and insoluble fibers (Adipol®) is able to control or reduce adenomatous polyps in FAP patients and to modify the ERs expression of the rectal or ileal pouch mucosa. At the basal time and every 6 months from the beginning of Adipol® administration, 17 patients were submitted to endoscopy and biopsy of the low intestinal tract for evaluation of the number, size and grade of adenoma dysplasia. After 6-12 months of treatment a significative decrease in the number of polyps was observed, while the dimension remains stable in 11 patients and decreases in 6. A passage from severe to mild dysplasia was present in 3 patients and a passage from adenomatous pattern to simple phlogosis in 4 patients. The proliferative and apoptotic activity was not significantly varied, while ER-β mRNA expression was progressively and significantly upregulated, during the treatment. Dietary supplementation with phytoestrogens can prevent the formation of new polyps with good tolerability and without side effects.

KEY WORDS: FAP, IRA, IPAA, colon cancer; estrogen receptors; phytoestrogens.

Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominant condition with a complete penetrance characterized by an early onset of multiple colorectal adenomas which with time develop into colorectal carcinomas. In absence of surgery, FAP patients have a high risk to die of metastatic colorectal cancer at young age. The two primary surgical options for colonic polyposis are total colectomy with ileorectal anastomosis (IRA) or total proctocolectomy with ileal pouch/anal anastomosis (IPAA). Advantage of IRA compared to IPAA are: the simplicity of the procedure, the low rate of postoperative complications and also the good functional results. However, major drawback of IRA is the occurrence of cancer in the rectal stump (1). In the last years IPAA was largely adopted thinking that this type of procedure could definitively abolish the occurrence of polyps and cancer in the distal intestinal tube. However, with time, in the majority of patients, one or more adenomatous polyps and sometimes large adenomas and also carcinomas can develop (2, 3). Attempts to control the proliferation of the FAP polyps, to reduce the risk of recurrent polyps and to prevent carcinoma in the rectum or pouch have been tried in the past years. Anti-COX drugs (sulindac, celecoxib) have been used in several randomized placebo-controlled trials showing a significative regression of the colorectal polyps in FAP patients (4, 5). However, concerns for their use were emerged since serious adverse effects and loss of action could occur after a prolonged administration (6, 7). High intake of certain dietary components such as ascorbic acid, eicosapentaenoic acid (the omega-3 fish oil derivative), association of curcumin and quercetin, have been also evaluated with a positive effect (8-10). The aim of the present study is to evaluate whether supplementation of the diet with a patented blend of phytoestrogens and indigestible and insoluble fibers (Adipol®) is able to control or reduce the adenomatous polyps in FAP patients and to modify the ERs expression of the rectal or ileal pouch mucosa.

Materials and methods

Subjects aged 18-65 years of both sexes were recruited between FAP patients undergoing surveillance. The patients had a diagnosis of FAP on the basis of at least two of the following features: 1) germ-line mutation of APC or MYH genes; 2) presence in the colon of more than 100 polyps; 3)
familiarity for the presence of multiple (>100 adenomatous colorectal polyps) colonic polyposis. Either patients with intact colon or patients submitted to subtotal colectomy and IPAA or total colectomy and IPAA were studied. The patients were enrolled in the study if the previous endoscopies documented the presence of at least 2 adenomatous polyps in the rectum or in the ileal pouch. Exclusion criteria were the use of NSAIDs during the 12 months prior to the study entry, pregnancy or lactation, hormonal contraception and/or therapy, use of phytoestrogens, administration of SERMs (specifically tamoxifen, raloxifene, toremifene), use of multivitamin supplement.

The study was conducted according to ICH Good Clinical Practice. Ethical approval was obtained from Careggi Hospital Committee.

**Endoscopic exams**

The endoscopies were performed with a flexible video-endoscope (Olympus GIF 165, Tokyo, Japan) at time 0 and every 6 months. Oral administration of PEG was used for the bowel preparation. All the rectum, the rectal stump from the ileo-rectal anastomosis to the anal verge or the ileal pouch, were examined counting the number of the polyps and their size. The size of the polyps was assessed by comparison with the diameter of the biopsy forcep (having respectively 4 mm when closed and 8 mm when open). Polyps which had a diameter around 10 mm or greater were removed by snare polypectomy 2-3 months before the baseline endoscopy. No polyps were removed by snare polypectomy 2-3 months before the baseline endoscopy. The genetic expression analysis has been carried out on biopsies of patients at the basal time and subsequently every 6 months from the beginning of Adipol® administration. It has been evaluated the expression of both estrogen receptors (ERα and ERβ) genes. Total RNA was extracted from biopsies with Qiazol reagent (Qiagen) according to the manufacturer’s instructions. One mg of total RNA was reverse transcribed using Quantitect Reverse Transcription-Kit (Qiagen) according to manual instructions. To verify the successful reverse transcription, qualitative PCR was performed using 1μl cDNA as template and 10 μM of each primer (forward and reverse) (described in Table 1) of the gene housekeeping β-Actin. The genetic expression analysis was performed by quantitative real time PCR (qRT-PCR) for ERα, ERβ and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as housekeeping gene, using Stratagene Mx3000-P Detection System (Stratagene, La Jolla, CA, USA). Reactions were performed using a TaqMan 5′- exonuclease assay and following the thermic profile according to manual instructions (Kapa probe fast qPCR Kit, Kapa Biosystems). Primers and internal labelled oligonucleotides TaqMan probes for each cDNA, described in Table 2, were designed by IDT integrated DNA Technologies. Target gene expression was normalized to GAPDH gene.

**Histopathology**

Histological samples were processed by standard methods and the biopsy sections of the visible polyps were examined in a blind manner. The pathologist classified the type of the polyps in negative (presence only of normal mucosa), presence of phlogosis, adenoma with low and high grade dysplasia, according to Vienna criteria.

**Determination of colorectal mucosal proliferation and apoptosis**

Three μm sections from paraffin-embedded colon and mounted on electrostatic-treated slides (Superfrost® Plus, Medite, Italy) were processed for determining Ki-67 and c-cas3 antigen immunoreactivity as described (11). The proliferative activity was expressed as labelling index (LI), number of labelled cells counted in all the crypts of the same subject/number of cells in all the crypt sections of the same subject x 100. The distribution of proliferating cells along the crypt was expressed as the percentage of labelled cells in each compartment over the total labelled cells in the crypt section. Apoptotic activity, expressed as apoptotic index (AI), was determined in sections processed with anti c-cas3 antibody, enumerating labelled cells in all the crypts of the same subject/number of cells in all the crypt sections of the same subject x 100.

**Genetic expression analysis of Estrogen Receptors Alpha (ER-α) and Beta (ER-β)**

The genetic expression analysis has been carried out on biopsies of patients at the basal time and subsequently every 6 months from the beginning of Adipol® administration. It has been evaluated the expression of both estrogen receptors (ERα and ERβ) genes. Total RNA was extracted from biopsies with Qiazol reagent (Qiagen) according to the manufacturer’s instructions. One mg of total RNA was reverse transcribed using Quantitect Reverse Transcription-Kit (Qiagen) according to manual instructions. To verify the successful reverse transcription, qualitative PCR was performed using 1μl cDNA as template and 10 μM of each primer (forward and reverse) (described in Table 1) of the gene housekeeping β-Actin. The genetic expression analysis was performed by quantitative real time PCR (qRT-PCR) for ERα, ERβ and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as housekeeping gene, using Stratagene Mx3000-P Detection System (Stratagene, La Jolla, CA, USA). Reactions were performed using a TaqMan 5′-exonuclease assay and following the thermic profile according to manual instructions (Kapa probe fast qPCR Kit, Kapa Biosystems). Primers and internal labelled oligonucleotides TaqMan probes for each cDNA, described in Table 2, were designed by IDT integrated DNA Technologies. Target gene expression was normalized to GAPDH gene.

**Treatment**

The patients received 5 g of Adipol® twice a day, at break-

<table>
<thead>
<tr>
<th>Table 1 - Primers β-Actin.</th>
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<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>β-Actin for</td>
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<tr>
<td>β-Actin rev</td>
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Table 2 - Primers and Taqman Probes used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5'-3') and Taqman probes</th>
<th>Amplicon size (bp)</th>
<th>Tann (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACAGTCAGCCGCACTCTC AATCCGTTGACTCCGACCTTC F/CCACATGCC/ZEN/TCAGACACCATGGG/Q</td>
<td>87</td>
<td>60</td>
</tr>
<tr>
<td>ERα for</td>
<td>GACTATGCTTCAGGTACCATT</td>
<td>102</td>
<td>60</td>
</tr>
<tr>
<td>ERα rev</td>
<td>GGCTGGGAACATATAGTGTAT</td>
<td>F/TCTCTTGA/ZEN/GAAGGTGCTCAC/A/Q</td>
<td>60</td>
</tr>
<tr>
<td>ERβ for</td>
<td>TGGCAGTTATCATCATCTGTATGCGG</td>
<td>95</td>
<td>60</td>
</tr>
<tr>
<td>ERβ rev</td>
<td>GTGTCTCTGTTTACAGTAAAGTCGTTG F/TCCCTG/G/ZEN/TGAAAGGAAGATCGCTA/G/A/Q</td>
<td>60</td>
<td></td>
</tr>
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TaqMan probes with F as reporter fluorochrome (6-carboxyfluorescein [6-FAM]) and Q as quencher fluorochrome (Iowa Black® FQ); bp, base pairs of amplicon size; Tann, annealing temperature (°C).

fast and dinner. This patented blend was chosen for its composition of phytoestrogens (175 mg milk thistle extract, titered at 70% in silymarin and 30% silibin, 50 mg of flaxseed extract titered at 40%, secoisolariciresinol and insoluble and indigestible oat fiber containing cellulose, hemicellulose and lignin. Adipol® is a food supplement listed in the National Registry of Food Supplements of the Italian Ministry of Health, code: E1041842-Y. The compliance of the treatment was evaluated collecting the unused sachets every 3 months. Any side effects was recorded at the clinical controls.

Statistical analysis
A T test for paired data was utilized to compare the mean values of each parameter at the beginning and after the treatment with Adipol®. Differences were considered significant when P was ≤ 0.05. To test the reproducibility of the endoscopic findings interobserver agreement was calculated with kappa analysis. A score of >0.80 was judged as optimal.

Results

Endoscopic and histological exams
Seventeen patients [10 M/7 F; age mean 35.7 years (range 18-65)] met the inclusion criteria and were enrolled in the study. Fourteen of them were affected by FAP and 3 by AFAP. Ten of them were submitted to subtotal colectomy and IRA. In the last two years, all these patients except one were submitted to polypectomies of the rectum or pouch at the endoscopies performed for surveillance. Other 5 patients were submitted to IPAA. In some of these patients the ileal pouch was anastomized on a short (3-4 cm in length) rectal stump which harboured adenomatous polyps. Finally, 2 patients were not operated and presented an intact colon at the time of the study. The time elapsed from surgery to the beginning of the study was 12.4 and 7.2 year as a mean respectivley for IRA patients and IPAA patients. They were followed prospectively between 2013 and 2016. The length of the treatment lasted from 4 to 29 months (mean 17 months). The majority of the patients (10/17) undertook the treatment more than one year and seven of them more than 12 months.

At baseline (T0), the mean number (±SD) of detected polyps was 22 (±15) in the rectal stump of IRA patients, 5 (±4) in the rectum of patients with intact colon and 1.5 (±0.5) in the ileal pouch. In 2 of the patients with a mechanical IPAA polyps were detected in the residual rectal stump. The dimension of the polyps varied from 1 to 3 mm. Histology confirmed the presence of tubular adenomatous polyps with a low grade dysplasia in all the patients except 3 in whom high grade dysplasia was found. Histology was negative for the presence of adenoma in one of the patients.

After 6 months of treatment with Adipol® a significative decrease in the number of polyps was observed (Figure 1) moving from 14±17 to 9±10. After at least 12 months of treat-
ment the number of polyps remain stable in 5 patients and decreases in 5 patients. In the 7 patients in whom the treatment lasts more than 12 months, the number of polyps decrease with a mean of 8.5±7.5 (Figure 2). The dimension of the polyps remains stable in 11 patients and decreases in 6 (Figure 3). Only 1 patient showed recurrence of new polyps during the first 12 months of treatment. In this patient no modification of expression of ERβ was observed. However, after 24 months of treatment the polyps decreased concomitantly to the increased expression of ERβ. This is also the only patient in whom polypectomies of the rectal stump were needed during the first year of treatment. The histological evaluation shows stable adenomatous pattern with mild dysplasia in 10 patients, a passage from severe to mild dysplasia in 3 patients and a passage from adenomatous pattern to simple phlogosis in 4 patients. A good degree of concordance was encountered for the number and dimension of the polypoid lesions by the two endoscopists (k value 93%).

The study was completed by all the patients. No patients complain of disturbances and any adverse effect was referred. No one stopped the treatment voluntary.

Proliferative activity of the normal mucosa was determined in seven FAP patients before and after treatment with Adipol® for at least 12 months. The results showed that proliferative activity was not significantly varied by Adipol® (LI was 28.3±1.9 and 27.8±2.5, before and after treatment, respectively; means±SE) (Figure 4). Since a deregulated pattern of proliferation along the crypt is a defect associated with carcinogenesis (12), we also determined the distribution of proliferating cells along the crypt before and after Adipol® treatment. As expected, the majority of proliferating cells was
found in the lower and mid parts of the crypt, while only a small percentage of proliferating cells was present in the upper crypt compartment. This pattern of proliferation was not varied by Adipol® treatment (Figure 4, panel B). Similarly, apoptotic activity in the mucosa was not significantly varied by Adipol® (AI: 0.36±0.15 and 0.18±0.06, before and after treatment, respectively; means±SE).

Genetic expression analysis of Estrogen Receptors Alpha (ER-α) and Beta (ER-β)

The expression of both estrogen receptors α and β genes during the administration of the dietary supplement with Adipol®, has been evaluated in 17 patients on a treatment time of 6, 12, 18 and in some cases 24 months. The quantitative analysis has demonstrated that in intestinal mucosa either of the rectum or of the ileal pouch, basal ERβ mRNA significantly increased (p<0.01) at 12, 18, and 24 months from the administration of Adipol® (Figure 5). The biological effect increased with time in the majority of patients (Figure 6). In some patients the upregulation of ERβ happened only after several months and this data was correlated with a progressive decrease of the number and size of the polyps. On the contrary, ERα mRNA expression has not changed over the months during the treatment (Figure 7).

Discussion

FAP represents a human model of adenoma-carcinoma sequence in which all the glandular mucosa of the intestine is genetically predisposed to the development of adenomas and carcinomas with time. The high content of phytoestrogens in the eastern diet is considered among factors responsible for the lower colorectal cancer incidence and mortality in western population in contrast to USA population (13). It has been also shown that dietary intake of phytoestrogens is inversely related to the colorectal cancer risk (14). Phytoestrogens are an heterogeneous group of polyphenolic plant-derived compounds which on the basis of their chemical structure are classified into four classes: isoflavones, flavonoids, lignans and coumestans. The dietary compounds are present in the food as inactive precursors and are processed by the intestinal microflora into active hormonal molecules. Eviendep® is a patented blend of phytoestrogens (silymarin and lignans) and non-starch insoluble fibers which has been tested both in APC mutated mice and in FAP patients. The number and the degree of dysplasia of the intestinal polyps significantly decreases in the animals after 3 months of phytoestrogens supplementation (15). Also the number and the size of the duodenal adenomatous polyps of FAP patients are reduced after 3 months of oral supplementation with Eviendep® (16). From 2014 Eviendep® has been changed name in Adipol®. The effect of Adipol® on the rectal or ileal pouch mucosa has not been tested until now. The protective effect of the phytoestrogens is due to their structure which resembles that of the estrogens and can bind to estrogen receptors (ERs). ERs are widely distributed in human colorectal cells. Two subtypes of ERs are identified: ERg and ERβ. ERβ is the predominant subtype. The expression of ERβ is progressively reduced in the colorectal tumors and its decrease is inversely proportional to the degree of
the dysplasia and the stage of the cancer (17). Conversely, ERα remains substantially unchanged between normal mucosa and neoplastic mucosa. Therefore, the protective effect of the estrogens against colorectal carcinogenesis seems related to a low ERα/ERβ ratio and the consequent prevalent estrogen binding to ERβ. It has also been shown on cultures of the colorectal cancer cells that the over-expression of ERβ in human colon cancer cells inhibits their proliferation by modulation of some key molecular regulators of the cell cycle (decrease in cyclin E and increase in the CDK inhibitor p21(CIP1)) (18). Dietary supplement with Eviendep® administered for 2 months is able to increase the expression of ERβ in the colonic mucosa of patients scheduled to undergo surveillance colonoscopy for previous sporadic colonic adenomas (19). Similarly an upregulation of ERβ has been shown in the duodenal polyps of patients with FAP after administration of Adipol®. Interestingly, the changes of molecular factors involved in the carcinogenesis after Adipol® supplementation have been studied on biopsies of the duodenal adenomas showing a decrease of mRNA expression of some oncogenes such as COX-2, PCNA and MUC1 and an increase of mRNA expression of inhibitors genes such as MUC. Also microRNAs could be modified after supplementation with Adipol®: the expression of miR-101 which suppresses growth and invasiveness of colorectal cancer have been found to increase after Adipol® supplementation (20).

The present experience shows that the treatment with Adipol® allows to abolish the growth of new adenomatous polyps in all patients and can reduce the number and size of the adenomas observed at the beginning of the treatment. It is also important to note that none of the patients (except 1) required to perform removal of recurrent polyps for over two years, differently from what was observed before starting treatment with Adipol®. Furthermore, the positive effect is maintained during all the period of supplementation of Adipol®, showing that there is not an escape of its effect. Of note, Adipol® does not induce or potentiate ERα expression in the rectal mucosa, demonstrating its selective effect on the ERβ. In our study the decrease of the polyps was accompanied by a decrease of dysplasia and an increase of ERβ expression. As in other reports, this effect seems not due to the modification of the proliferative activity or apoptotic activity in the rectal mucosa (21).

Our data confirm the absence of collateral effects and the tolerability of this patented blend. Currently, there are no approved therapies for the primary chemoprevention of FAP (22). Despite the evidence that NSAIDs may regress adenomas after colectomy with IRA, their effect is lost with time (6, 22).

In conclusion, taking in account that surgery does not eliminate the recurrence of adenomatous polyps in the lower intestinal tract of operated FAP patients, dietary supplementation with phytoestrogens can prevent the formation of new polyps with good tolerability and in absence of side effects. In the future, it will be necessary a controlled clinical trial to confirm our findings.

References