

Genetic Polymorphisms of Human *Flavin Monooxygenase 3* in Sulindac-Mediated Primary Chemoprevention of Familial Adenomatous Polyposis

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ABSTRACT

Purpose: Sulindac is a nonsteroidal anti-inflammatory drug (NSAID) effective in regressing adenomas in patients with familial adenomatous polyposis (FAP). However, a recent randomized trial showed that sulindac, when compared with placebo, failed to prevent the development of adenomatous polyps in genotypically positive but phenotypically negative FAP patients. The present study determined whether polymorphisms in the gene encoding flavin monooxygenase 3 (FMO3), a hepatic microsomal enzyme that inactivates sulindac, played a role in determining the efficacy of sulindac in preventing polyposis in this cohort of FAP patients.

Experimental Design: Genotyping was performed on seven established *FMO3* polymorphisms previously shown to have functional relevance—*M66I*, *P153L*, *E158K*, *V257M*, *E305X*, *E308G*, and *R492W*—in 21 and 20 FAP patients, who received sulindac and placebo, respectively.

Results: None of the 41 patients exhibited heterozygous or homozygous *M66I* and *R492W* variant alleles, or homozygous *P153L*, *V257M*, and *E305X* variant alleles. Among sulindac-treated patients who did not develop adenomas

(“responders”), 4 (33%) were homozygous for *E158K* and 2 (17%) were homozygous for *E308G* variant alleles. In contrast, none of the patients on sulindac who developed adenomas (“nonresponders”) exhibited homozygosity for either of the two variant alleles. In addition, polymorphisms in the *E158K* or *E308G* allele were associated with a significant reduction in mucosal prostanoid levels in patients treated with sulindac.

Conclusions: Polymorphisms in *FMO3*, particularly at the *E158K* and *E308G* loci, may reduce activity in catabolizing sulindac and result in an increased efficacy to prevent polyposis in FAP.

INTRODUCTION

Enormous interest exists in using pharmacological or nutritional agents to prevent colorectal cancer, the second leading cause of cancer deaths in the United States (reviewed in ref. 1). This approach, known as chemoprevention, is directed at the prevention of adenomatous polyps and subsequent progression to colorectal cancer. In particular, epidemiologic evidence indicates that nonsteroidal anti-inflammatory drugs (NSAIDs) are effective chemopreventive agents in colorectal cancer (2). Results of randomized trials also support that aspirin, an NSAID, is effective in preventing colorectal adenomas (3, 4). The mechanism by which NSAIDs achieve this effect is thought to be mediated in part by inhibition of cyclooxygenase-2, which is often overexpressed in colorectal adenomas and carcinomas (5). The role of cyclooxygenase-2 in polyp formation is further confirmed by the ability of a cyclooxygenase-2-selective inhibitor, celecoxib, to reduce the number of polyps in patients with familial adenomatous polyposis (FAP; ref. 6).

Familial adenomatous polyposis (FAP) is an autosomal dominant disorder caused by germ-line mutations in the *adenomatous polyposis coli* (*APC*) gene. Affected individuals develop colorectal cancer in early adult life secondary to extensive adenomatous polyposis of the colon and rectum (7). NSAIDs, including sulindac and celecoxib, are effective chemopreventive agents in FAP (7). For example, sulindac reduces adenomatous polyp burden in randomized controlled trials of FAP patients who had polyps at study baseline (8–10). Moreover, the clinical efficacy of sulindac to induce polyp regression in these patients is correlated with a reduction in the mucosal prostanoid levels in the rectum (11, 12). These studies suggest that, in FAP, tissue prostanoids may serve as markers for polyp regression in response to sulindac (11).

In contrast to the adenoma regression models of chemoprevention described above, a recent study examined sulindac as a primary chemoprevention agent in FAP (13). Hence, a randomized controlled trial of sulindac was conducted in a group of FAP patients who were genotypically positive (*i.e.*, carried mutations in the *APC* gene) but phenotypically negative (*i.e.*,

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had not yet develop adenomatous polyposis). The study showed that sulindac failed to prevent the development of adenomas when compared with placebo (13). However, in patients treated with sulindac who did not develop polyps ("responders"), the levels of several prostanoids were substantially lower than in those who did develop polyps ("nonresponders"; ref. 14). These findings suggest that factors that influence tissue prostanoid formation in sulindac-treated patients, such as medication compliance, drug dosage, gut flora, and variations in metabolism of sulindac, may be important in determining the eventual outcome of such primary chemoprevention trials.

Sulindac is a prodrug that contains a racemic sulfoxide moiety, which is reduced by the gut flora to the active sulfide form before being absorbed (15, 16). The active sulfide is in turn oxidized to the sulfoxide and, subsequently, the sulfone form; both are relatively inactive metabolites when compared with the sulfide form (15). These two oxidative steps are catalyzed by the flavin-containing monooxygenase subtype 3 (*FMO3*; ref. 17). *FMO3* belongs to the family of flavin-containing monooxygenases (*FMOs*) that catalyze the NADPH-dependent oxygenation of a wide variety of nucleophilic heteroatom-containing compounds including endogenous substances and xenobiotics (18, 19). There are six known *FMO* isoforms in human, all of which are localized on the long arm of chromosome 1 (20, 21). *FMO3* is abundantly present in the liver (22) and is responsible for metabolizing a variety of substrates including sulindac (17).

Studies of the *FMO3* gene have demonstrated substantial interindividual and interethnic differences that alter *FMO3* enzymatic activity (23). These variations are attributed to genetic polymorphisms, of which 24 have been identified to date (24). The identification of these polymorphisms has clarified the etiopathogenesis of trimethylaminuria, also known as the fish odor syndrome (24), and could facilitate the understanding of variations in the response of individuals to the chemicals and drugs metabolized by *FMO3*, including sulindac. The purposes of the present study are to characterize the *FMO3* genotypes in FAP patients who underwent the primary chemoprevention trial with sulindac (13, 14) and to determine whether *FMO3* polymorphisms play a role in the ability of sulindac to prevent polyp development.

MATERIALS AND METHODS

Study Population. The study population consisted of a group of 41 FAP patients who underwent a randomized, double-blind, placebo-controlled primary chemoprevention trial (13). These patients carried mutations in the disease-causing *APC* gene (*i.e.*, they were genotypically positive) but had no polyps at the time of entry into the trial (phenotypically negative). Patients were randomly assigned to receive sulindac or placebo for 4 years. The development of rectal polyps was assessed at baseline and at 4-month intervals for the duration of the trial by flexible sigmoidoscopy. Serum samples were collected from all of the subjects at the time of enrollment. The protocol was approved by the Johns Hopkins Medical Institution Joint Committee on Clinical Investigation (institutional review board).

***FMO3* Genotyping.** Genomic DNA was extracted from the sera of patients with the QIAamp DNA Blood minikit (Qiagen, Inc., Valencia, CA). We selected seven previously

established polymorphisms in *FMO3* that had been demonstrated to affect enzymatic functions for characterization: *M66I*, *P153L*, *E158K*, *V257M*, *E305X*, *E308G*, and *R492W* (25). Polymorphisms were determined by PCR amplification with published primer sequences and RFLP (25). Conditions for PCR were as follows: 94°C for 15 minutes; 45 cycles of 94°C for 30 seconds, 47°C for 30 seconds, and 72°C for 30 seconds; and 72°C for 4 minutes. The amplified product was digested with the following seven restriction endonucleases to assess the polymorphisms of interest: *FokI* at exon 3 (for the *M66I* variant), *BciVI* (*P153L*) and *HinfI* (*E158K*) at exon 4, *NlaIII* (*V257M*) at exon 6, *EcoRI* (*E305X*) and *BsaI* (*E308G*) at exon 7, and *BstXI* (*R492W*) at exon 9. The digested products were analyzed on either a 1% agarose gel or a 10-to-12% Tris-HCl polyacrylamide gel and were visualized with an α Innotech Chemiluminescence and Fluorescence Imaging system. Genotypes were classified as homozygous wild type (*WT/WT*), heterozygous polymorphic (*WT/P*), or homozygous polymorphic (*P/P*).

Composite Prostanoid Index. Levels of prostanoids in biopsied rectal specimens were determined as described previously (14) at baseline, at 4 months, and at 1, 2, 3, and 4 years after the beginning of the trial. The composite prostanoid index was calculated as the mean of percentage baseline prostanoid levels at each time point after the trial began, multiplied by 100. A composite prostanoid index of 1 indicates that there was no change in the prostanoid level during the trial as compared with the baseline value. An index of less or greater than 1 indicates a reduction or increase, respectively, in the prostanoid level when compared with the baseline value.

Statistical Analysis. Frequencies of the *WT/WT*, *WT/P*, and *P/P* genotypes for each of the seven polymorphic sites were calculated. Comparisons were made between the sulindac and placebo groups. Comparisons within the sulindac group were further made between those patients who did not develop polyps (responders) and those who did (nonresponders) at the conclusion of the trial. Statistical analysis was performed with the χ^2 and Fisher's Exact tests. Comparison between genotypes and composite prostanoid index was also conducted and analyzed with Student's *t* test.

RESULTS

Table 1 summarizes the clinical outcome of the primary chemoprevention trial in FAP as published previously (13). Among the group of 21 patients treated with sulindac, 12 remained polyp-free at the end of trial. These patients were designated as responders because they did not develop any

Table 1 Clinical outcome of the primary chemoprevention trial in FAP

| | Total <i>n</i> = 41 | Sulindac group <i>n</i> = 21 | Placebo group <i>n</i> = 20 |
|----------------------|------------------------|---------------------------------|--------------------------------|
| Polyp development* | | | |
| Yes (non-responders) | 20 (49) | 9 (43) | 11 (55) |
| No (responders) | 21 (51) | 12 (57) | 9 (45) |

NOTE. Values are expressed as number (%).

* The outcome of the trial was assessed by whether patients developed polyps as determined by examination of the rectum with a flexible sigmoidoscopy at the end of the 4-year trial.

polyps while on treatment. Nine of the 21 patients who received sulindac did develop polyps during the trial and were designated as nonresponders. The proportion of patients who did not develop polyps was not statistically different between the sulindac and placebo groups (13).

Among the seven polymorphic sites examined, there were no identifiable polymorphisms at the *M66I* and *R492W* loci (results not shown). Polymorphisms were identified among the remaining five loci and were compared between the patients receiving sulindac and those receiving placebo (Table 2). The overall distribution of the five variant alleles in the 41 FAP patients studied was similar to that previously observed in a population with a similar demographic (26). None of the 41 patients had any homozygous *P153L*, *V257M*, or *E305X* variant alleles. Of the two remaining polymorphic sites, the homozygous *E158K* variant allele was present in eight patients (20%), and the homozygous *E308G* variant allele in six patients (14%). There was no statistical difference in the presence of either the homozygous *E158K* ($P = 1.00$) or *E308G* ($P = 0.41$) variant allele between the sulindac and placebo groups.

Table 3 compares the five polymorphic *FMO3* alleles between the nonresponders (patients who developed polyps) and responders (patients who did not develop polyps) in the group of patients that received sulindac in the trial. As shown, all 4 of the homozygous *E158K* variant allele and 2 of the homozygous *E308G* variant allele were present in the responder group. None of the nonresponders exhibited homozygosity in either variant allele. The P values of the proportion of homozygous variant *E158K* and *E308G* alleles between the responder and nonresponder groups were 0.10 and 0.49, respectively.

Table 4 shows the three most common *FMO3* genotype combinations for the patients in the study. Genotype combination 1 was the most common and includes heterozygous or homozygous variant alleles in both *E158K* and *E308G*. Table 5

Table 2 *FMO3* genotypes in FAP patients who underwent primary chemoprevention trial

| Polymorphisms | Total <i>n</i> = 41 | Sulindac group <i>n</i> = 21 | Placebo group <i>n</i> = 20 |
|---------------|------------------------|---------------------------------|--------------------------------|
| <i>P153L</i> | | | |
| WT/WT | 39 (95) | 19 (90) | 20 (100) |
| WT/P | 2 (5) | 2 (10) | 0 |
| P/P | 0 | 0 | 0 |
| <i>E158K</i> | | | |
| WT/WT | 16 (39) | 7 (33) | 9 (45) |
| WT/P | 17 (41) | 10 (48) | 7 (35) |
| P/P | 8 (20) | 4 (19) | 4 (20) |
| <i>V257M</i> | | | |
| WT/WT | 36 (88) | 19 (90) | 17 (85) |
| WT/P | 5 (12) | 2 (10) | 3 (15) |
| P/P | 0 | 0 | 0 |
| <i>E305X</i> | | | |
| WT/WT | 28 (68) | 14 (67) | 14 (70) |
| WT/P | 13 (32) | 7 (33) | 6 (30) |
| P/P | 0 | 0 | 0 |
| <i>E308G</i> | | | |
| WT/WT | 13 (32) | 2 (10) | 11 (55) |
| WT/P | 22 (54) | 17 (80) | 5 (25) |
| P/P | 6 (14) | 2 (10) | 4 (20) |

NOTE. Values are expressed as number (%).

Table 3 Association between *FMO3* genotypes and clinical outcome in patients treated with sulindac

| Polymorphisms | Polyps (nonresponders) <i>n</i> = 9 | No polyps (responders) <i>n</i> = 12 |
|---------------|---|--|
| <i>P153L</i> | | |
| WT/WT | 8 (89) | 11 (92) |
| WT/P | 1 (11) | 1 (8) |
| P/P | 0 | 0 |
| <i>E158K</i> | | |
| WT/WT | 4 (44) | 3 (25) |
| WT/P | 5 (56) | 5 (42) |
| P/P | 0 | 4 (33) |
| <i>V257M</i> | | |
| WT/WT | 9 (100) | 10 (83) |
| WT/P | 0 | 2 (17) |
| P/P | 0 | 0 |
| <i>E305X</i> | | |
| WT/WT | 6 (67) | 8 (67) |
| WT/P | 3 (33) | 4 (33) |
| P/P | 0 | 0 |
| <i>E308G</i> | | |
| WT/WT | 2 (22) | 0 |
| WT/P | 7 (78) | 10 (83) |
| P/P | 0 | 2 (17) |

NOTE. Values are expressed as number (%).

shows the association between the clinical outcome of the primary chemoprevention trial and the frequency of these three genotype combinations. As shown, genotype combination 1 was five times more prevalent in the sulindac group of patients who did not develop polyps (responders) than those who developed polyps (nonresponders). However, because of the relatively small sample size, the difference in the frequency of genotype combination 1 between responders and nonresponders did not reach statistical significance ($P = 0.18$).

Genotype combination 1 contains a variant *E158K* or *E308G* allele, both of which have been associated with a reduced capacity to oxidize substrates (24, 31–33). To determine whether genotype combination 1 may be associated with altered ability to metabolize sulindac, we compared the composite prostanoid index for each of the five prostanoids, prostaglandin D2 (PGD₂), prostaglandin E2 (PGE₂), prostaglandin F2 α (PGF_{2 α}), thromboxane B₂ (TXB₂), and 6-keto-PGF_{1 α} , between patients with genotype combination 1 and those with all other genotype combinations in the sulindac group. As shown in Fig. 1, genotype combination 1 is associated with a statistically significantly lower composite prostanoid index when compared with that of all other genotype combinations for four of the five prostanoids. These results suggest that patients with genotype combination 1 are more sensitive to the action of sulindac, thus resulting in lower prostanoid formation.

DISCUSSION

FAP is a disease that predisposes affected individuals to develop colorectal cancer if prophylactic colectomy is not performed after the onset of adenomatous polyposis (7). To date, several randomized placebo-controlled trials have demonstrated that certain NSAIDs, including sulindac (10, 27, 28) and celecoxib (6), reduce polyp burden (size and number) when com-

Table 4 The three most common *FMO3* genotype combinations in the trial patients

| Genotype combination | <i>M66I</i> | <i>P153L</i> | <i>E158K</i> | <i>V257M</i> | <i>E305X</i> | <i>E308G</i> | <i>R492W</i> | <i>n</i> | Overall frequency, % |
|----------------------|-------------|--------------|--------------|--------------|--------------|--------------|--------------|----------|----------------------|
| 1 | WT/WT | WT/WT | WT/P or P/P | WT/WT | WT/WT | WT/P or P/P | WT/WT | 9 | 22 |
| 2 | WT/WT | WT/WT | WT/WT | WT/WT | WT/WT | WT/P or P/P | WT/WT | 6 | 15 |
| 3 | WT/WT | WT/WT | WT/WT | WT/WT | WT/WT | WT/WT | WT/WT | 5 | 12 |

Table 5 Association between *FMO3* genotype combinations and clinical outcome of the primary chemoprevention trial

| Genotype combination | Sulindac | | Placebo | |
|----------------------|------------------------|----------------------------|------------------------|----------------------------|
| | Polyps <i>n</i> = 9 | No polyps <i>n</i> = 12 | Polyps <i>n</i> = 9 | No polyps <i>n</i> = 11 |
| 1 | 1 (11) | 5 (42) | 2 (22) | 1 (9) |
| 2 | 3 (33) | 2 (17) | 1 (11) | 0 |
| 3 | 1 (11) | 0 | 3 (33) | 1 (9) |
| All others | 4 (44) | 5 (42) | 4 (44) | 9 (82) |

NOTE. Values are expressed as number (%).

pared with placebo in FAP patients with polyps before therapy. Whereas the mechanisms by which NSAIDs accomplish this chemopreventive effect are still unclear, it may depend in part on the inhibition of cyclooxygenase-2, which is elevated in colorectal neoplasia (29). Levels of prostanoids, the main cyclooxygenase products, are elevated in the adenomas of FAP patients in a size-dependent manner (30). Moreover, reduction in the levels of prostanoids in the rectum of FAP patients treated with sulindac correlates with increased effectiveness in the ability of the drug to regress adenomas in several studies (11, 12). The reduction in mucosal prostanoid levels may have prognostic implication because it is also associated with a favorable clinical response in a subgroup of patients who received sulindac in the recently concluded primary chemoprevention trial of FAP (13, 14).

Several reasons may explain the heterogeneity by which

sulindac reduced mucosal prostanoid levels in patients who received it. They include medication compliance, drug dosage, difference in gut flora, and variation in the inherent ability to metabolize sulindac once absorbed. Sulindac, when ingested orally, is a sulfoxide that contains an equal mixture of *R*- and *S*-enantiomer (16, 17). In the gut, sulindac sulfoxide is converted by bacterial flora to the pharmacologically active metabolite sulindac sulfide that is primarily responsible for the inhibition of cyclooxygenases (15). Once absorbed, sulindac sulfide is reoxidized to the *R*- or *S*-sulfoxide enantiomer, which is further irreversibly oxidized to the pharmacologically inactive sulindac sulfone before excretion (17). Both oxidizing steps are primarily carried out by the flavin monooxygenases, of which *FMO3* is the predominant isoform in the hepatic microsomes (17). Because the *FMO3* gene is highly polymorphic with some polymorphisms encoding enzymes with altered activity (24, 31–33), we examined *FMO3* polymorphisms as a possible mechanism responsible for the clinical outcome of patients who underwent sulindac-mediated primary chemoprevention of FAP (13).

Seven common *FMO3* polymorphisms were characterized in this study, some of which result in decreased enzymatic activity and cause trimethylaminuria or fish odor syndrome (24, 31). The overall distribution of the wild-type and seven variant alleles in this group of predominantly Caucasian patients is similar to that previously described in a Caucasian population (25, 26). Among these seven loci, only two, *E158K* and *E308G*, demonstrated the presence of homozygous variants in the pa-

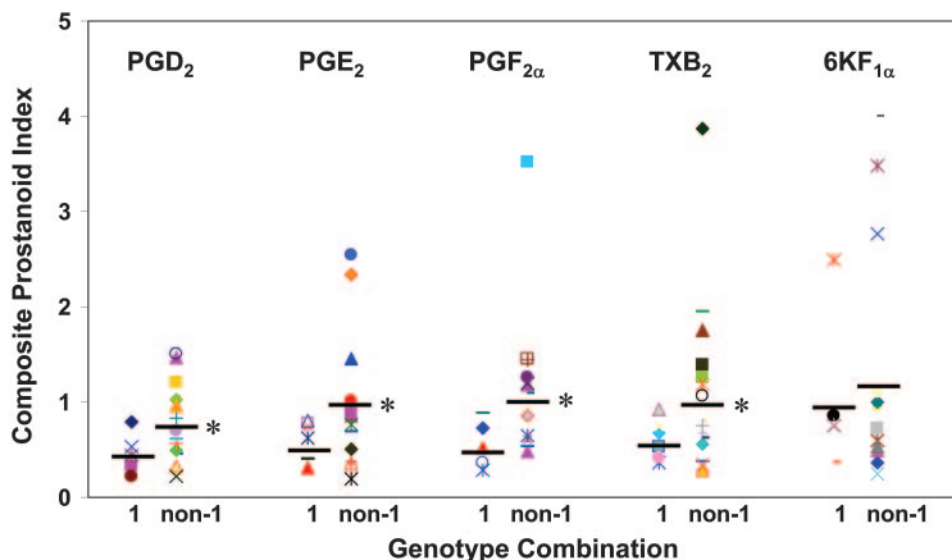


Fig. 1 Comparison of composite prostanoid index and genotype combinations in the sulindac group of patients. The composite prostanoid index for each of the five prostanoids is compared between the sulindac group of patients with genotype combination 1 (*n* = 6) and the group of patients with all other genotype combinations (*non-1*; *n* = 15). The thick horizontal bars are means. *, *P* < 0.05 by Student's *t* test. *PGD*₂, prostaglandin D₂; *PGE*₂, prostaglandin E₂; *PGF*_{2α}, prostaglandin F_{2α}; *TXB*₂, thromboxane B₂; *6KF*_{1α}, 6-keto-PGF_{1α}.

Table 6 Effects of known *FMO3* polymorphisms on substrate affinity

| Polymorphisms | Substrates | References |
|--|------------------------------|------------|
| <i>E158K</i> | Trimethylamine | 24, 32, 35 |
| <i>E158K</i> and <i>E308G</i> | Trimethylamine | 34 |
| <i>E158K</i> and <i>E308G</i> | Ranitidine and thiobenzamide | 36 |
| <i>E308G</i> | Methimazole | 37 |
| <i>E158K</i> , <i>E308G</i> , and <i>V257M</i> | Clonazepam and caffeine | 25 |
| <i>E158K</i> | Benzylamine | 38 |

tients tested (Table 2). Importantly, in the group of patients receiving sulindac, the two homozygous variant alleles were present only in those who did not develop polyps (responders; Table 3). Moreover, a particular genotype combination (type 1) that contains either heterozygous or homozygous *E158K* and *E308G* alleles was preferentially present in the responders (Table 5). These results suggest that this particular genotype combination may be protective against polyp formation in patients treated with sulindac. It is, therefore, of great interest to note that genotype combination 1 is associated with substantially lower levels of prostanoids for four of the five prostanoids measured when compared with all other genotype combinations in patients who received sulindac (Fig. 1). This finding is consistent with our hypothesis that specific polymorphisms in *FMO3* are associated with an increased ability of sulindac to reduce mucosal prostanoid levels, resulting in a favorable clinical response.

The effects of certain polymorphisms on *FMO3* to metabolize substrates have been described. For example, the Michaelis constant (K_m) for trimethylamine was significantly elevated in the homozygous *E158K* variant compared with the wild-type enzyme (32). Also, individuals with compound heterozygous or homozygous *E158K/E308G* alleles had decreased trimethylamine *N*-oxygenation after oral trimethylamine challenge (34). In addition, both variant alleles have been found in patients affected with trimethylaminuria or fish odor syndrome, in which there is an inability to metabolize trimethylamine (23). Table 6 is a summary of the effects on some of the known polymorphisms of *FMO3* on the affinity for some of the established substrates. Although sulindac is a known substrate of *FMO3* (17), the effects of these polymorphisms on the metabolism of sulindac have not been characterized. Conceivably the polymorphisms could reduce enzyme activity and lead to a reduced ability to oxidize sulindac sulfide. This, in turn, may lead to a longer half-life of sulindac sulfide (the active form of sulindac) with increased inhibition of cyclooxygenase activity and lower levels of tissue prostanoids. Importantly, the effect of the *E158K* and *E308G* variants on lowering tissue prostanoid levels was demonstrated by the present study.

Several limitations of our study exist. First, the sample size was relatively small, which provided limited statistical power. Second, we genotyped only a subset of polymorphisms identified in *FMO3*. Others may have additional impact on the eventual enzymatic activity. However, this study highlights the principle of using pharmacogenetics to determine the mechanism by which sulindac may prevent the development of adenomatous polyps in a rare population of individuals who are at high risk for colorectal cancer. Additional investigation in other cohorts of FAP patients who received sulindac for chemoprevention may validate our observations.

In summary, our study identified a higher prevalence of both *E158K* and *E308G* *FMO3* variant alleles in FAP patients who were treated with sulindac and did not develop polyps. Patients with these two variant alleles had reduced mucosal prostanoid levels when compared with patients with other genotypes. These results, therefore, indicate a protective effect of the *E158K* and *E308G* variant alleles on the development of polyps in FAP patients who receive sulindac as a means of primary chemoprevention. Our studies suggest that consideration of pharmacogenetics is important in the subsequent design of sulindac-mediated and other chemopreventive trials of colorectal neoplasia.

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