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# Mycophenolate Mofetil Alone and in Combination with Tacrolimus Inhibits the Proliferation of HT-29 Human Colonic Adenocarcinoma Cell Line and Might Interfere with Colonic Tumorigenesis

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**Abstract.** *Background/Aim: Familial adenomatous polyposis (FAP) was found to be completely reversed in a patient treated with mycophenolate mofetil (MMF) and tacrolimus following kidney transplantation. In this preliminary study, we assessed whether MMF and tacrolimus alone or in combination interfere with the cell cycle and proliferation in a human colonic adenocarcinoma cell line and in the colonic polyps of the patient with FAP. Materials and Methods: Human colonic adenocarcinoma HT-29 cells were treated with tacrolimus and MMF alone and in combination at different concentrations. Cell viability and proliferation were assessed using the MTT assay. Cell-cycle distribution was analyzed by flow cytometry. Expression of Ki-67, a marker of mitotic activity, was evaluated in the patient's colonic polyps before and under drug treatment. Results: MMF in combination with tacrolimus induced S-phase cell-cycle arrest and markedly inhibited HT-29 cell proliferation. Ki-67 expression in the patient's colonic polyps was significantly reduced following combined tacrolimus and MMF treatment. Conclusion: MMF and tacrolimus synergistically inhibited proliferation of a human colonic adenocarcinoma cell line and interfered with the expansion of colonic crypt proliferation in the polyp from a patient with FAP. The results confirm our clinical observation and indicate the possibility of novel approach to therapy of colorectal neoplasia.*

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Immunosuppressive therapy, which is used to prevent rejection of organ transplants and for treatment of autoimmune diseases, is known to increase the risk of malignancies and infections and, therefore, should be used cautiously or avoided in patients with cancer or pre-neoplastic lesions. As a matter of fact, neoplasia is a significant factor contributing to morbidity and mortality of renal allograft recipients, with up to 18% of post-transplantation death cases being attributed to malignancy. Frequent cases of cancer-related death in such patients were lymphoma, followed by lung and kidney cancer (1). Of note, risk of colon cancer is also elevated in patients post-transplantation (2).

It should be noted, however, that the choice of specific post transplantation therapy might differentially influence the risk of development of colorectal neoplasia. Recently, Safaeian *et al.* analyzed the risk of colorectal cancer in US patients post-solid organ transplantation and did not observe elevated incidence of colorectal cancer in those treated with tacrolimus and mycophenolate mofetil (MMF), as opposed to those treated with cyclosporine A and azathioprine post-transplantation (3). Consonant with these findings, we

recently described a case of complete regression of colonic polyps in a teenager with familial adenomatous polyposis (FAP) who was treated with tacrolimus and MMF following kidney transplantation (4).

These observations prompted us to examine whether tacrolimus and MMF treatment impedes proliferation of a human colonic adenocarcinoma cell line and FAP colonic polyps.

## Materials and Methods

*Cell line*. The human colonic adenocarcinoma cell line HT-29 (ATCC HTB-38; ATCC, Manassas, VA, USA), known to harbor non-sense adenomatous polyposis coli (APC) gene mutation (5), was grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 units/ml penicillin, 100 μg/ml streptomycin, and 12.5 units/ml nystatine at 37°C in a humidified chamber with 95% air/5% $CO_2$ (6). These cells are undifferentiated when grown in these conditions (6). Cell culture media and supplements were purchased from Biological Industries, Beit HaEmek, Israel.

For experimental purposes, cells were treated with tacrolimus (0.1 μM and 1 μM), MMF (1 or 10 μg/ml) or a combination of tacrolimus and MMF (0.1 μM and 1 μg/ml, 0.1 μM and 10 μg/ml, 1 μM and 1 μg/ml, and 1 μM and 10 μg/ml, respectively) as described elsewhere (7, 8). Control cells were treated with vehicle alone. It should be noted that MMF is a prodrug that is rapidly hydrolyzed into its active form, mycophenolic acid (MPA), by serum esterases *in vivo*, or by esterases in serum or plasma-containing cell culture media, or by tissue esterases (9-11). MMF and tacrolimus were purchased from Sigma-Aldrich Israel, Ltd, Rehovot, Israel.

*Ki-67 staining*. Specimens of tubular adenoma with low-grade dysplasia obtained from FAP patient prior to initiation of tacrolimus and MMF treatment and tubular adenoma specimens obtained after 6 months of tacrolimus and MMF treatment were analyzed. Formaline-fixed paraffin-embedded 5 μm-thick tissue sections were deparaffinized, rehydrated and stained for Ki-67 using a monoclonal antibody to human Ki-67 (MIB1) (Dako, Agilent, Santa Clara, CA, USA). Immunostaining was performed using an automated platform (BenchMark XT, Ventana Medical Systems Inc., Tucson, AZ, USA) according to the manufacturer's instructions. The staining was evaluated by counting positively stained colonocytes in 20 randomized fields per slide. Scoring was expressed as the average count of Ki-67-positive cells per 100 colonocytes counted.

*Viability and proliferation assay*. Aliquots of $5\times10^3$ cells per well were seeded into the 96-well plates and incubated with tacrolimus, MMF or combination of tacrolimus and MMF at concentrations indicated above. Cells were grown for 24, 48, 72 and 96 hours. At the end of each time period the cell viability was assessed using the MTT assay (CellTiter 96® Assay; Promega Corp, Madison, WI, USA) according to the manufacturer's instructions. Briefly, 15 μl of the Dye Solution was added to each well and the plate was incubated at 37˚C for 4 hours in a humidified, atmosphere with 5% $CO_2$. Afterwards, 100 μl of the Solubilization Solution/Stop Mix were added to each well. Following additional incubation of 1 hour, the contents of each well were gently pipetted in order to obtain a uniformly colored solution and absorbance was read at 570 nm wavelength using a 96-well plate reader. The results are expressed as a percentage of the respective control absorbance at each time point.

*Cell-cycle analysis*. Cell-cycle analysis was performed by flow cytometry using Propidium Iodide Flow Cytometry Kit (Abcam, Cambridge, UK). Briefly, aliquots of $2\times10^5$ cells per well were seeded into 6-well plates and incubated with tacrolimus, MMF, or combination of tacrolimus and MMF. Cells were grown for 48 and 96 hours. At the end of each respective time period, cells were harvested, washed twice with phosphate buffered saline, pH 7.4, fixed with 70% ice-cold ethanol, treated with RNase A, stained with 50 μg/ml of propidium iodide for 20 minutes, according to the manufacturer's instructions. Data acquisition was performed by FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). Ten thousand cells per sample were collected, and propidium iodide staining intensity was plotted on a linear scale using BD FACSDiva Software version 6.1.3 (BD Biosciences). Cell-cycle profiles were analyzed using Weasel v3.4.2 software (WEHI, Parkville, Victoria, Australia).

*Statistical analysis*. Cell proliferation experiments were performed in triplicates and repeated three times. Cell-cycle experiments were performed in quadruplicates and repeated twice. Statistical significance was evaluated using two-tailed Student's *t*-test. Differences were considered statistically significant with $p<0.05$. Results are presented as the mean±standard deviation.

## Results

*Expression of Ki-67 antigen*. Expression of Ki-67 antigen, a known proliferative marker (12), was assessed in low-grade tubular adenoma specimens obtained pre-kidney transplantation from a patient with FAP, i.e. prior to initiation of tacrolimus and MMF treatment, and in low-grade tubular adenoma specimens that were obtained 6 months after kidney transplantation (4), and expressed as the percentage of Ki-67-positive colonocytes. The Ki-67 score prior to initiation of tacrolimus and MMF treatment (Figure 1A) was 35.5±5.0% (range=31-44%), whereas 6 months post-kidney transplantation under tacrolimus and MMF treatment, the score significantly decreased to 16.6±6.6% (range=7-30%), $p<0.001$ (Figure 1B). The results indicate decreased expression of Ki-67 antigen in FAP polyps following drug treatment, suggesting decreased cell proliferative activity and impaired expansion of the crypt proliferative compartment in FAP colonic adenomas, a hallmark of hereditary and sporadic colonic cancer.

*Proliferation assay*. Significant inhibition of proliferation of HT-29 cells was observed following 72 and 96 hours of treatment with 10 μg/ml MMF alone ($p<0.002$ compared to combination with 1 μM tacrolimus (56.9±20.9%, 76.4±24.4%, respectively, $p<0.001$ compared to respective control; Figure 2A). Treatment of HT-29 cells with a combination of 10 μg/ml MMF and 0.1 μM tacrolimus resulted in marked inhibition of proliferation of HT-29 cells

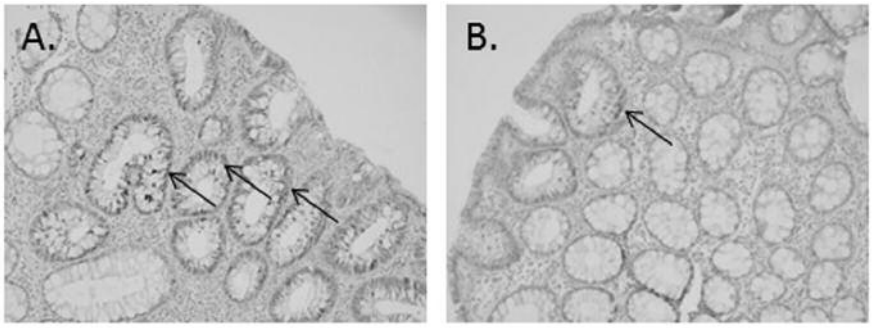


Figure 1. *Immunostaining for Ki-67 in colonic tubular adenoma samples obtained before initiation of tacrolimus and mycophenolate mofetil (MMF) treatment (A) and 6 months after commencement of treatment (B). Selected positively stained nuclei are indicated with black arrows. Marked decrease in Ki-67 expression following combined treatment can be seen. Representative slides are presented (original magnification ×100).*

at 24, 48, 72 and 96 hours (82.5±11.7%, 58.5±11.3%, 49.8±29.4%, 54.8±8.7%, respectively, $p<0.005$ compared to respective control). It should be noted that the combination of 10 μg/ml MMF and 0.1 μM tacrolimus inhibited the proliferation of HT-29 cells significantly more effectively than treatment with 10 μg/ml MMF alone ($p<0.01$ at all respective time points; Figure 2A).

Treatment of cells with combination of 1 μg/ml MMF and 0.1 μM tacrolimus also resulted in significant inhibition of growth of HT-29 cells at 72 and 96 h (48.4±8.1%, 86.1±25.45%, $p<0.02$, compared to respective control or to treatment with 1 μg/ml MMF alone). Likewise, combination of 1 μg/ml MMF and 1 μM tacrolimus impeded proliferation of HT-29 cells at 72 and 96 h (73.39±10.38%, 89.2±21.05, $p<0.05$ compared to respective control; $p<0.02$ compared to treatment with 1 μg/ml MMF alone at 96 h). Transient inhibition of proliferation at 72 h was observed following treatment with 1 μg/ml MMF (71.6±35.7%, $p<0.002$ compared to respective control; Figure 2B).

Tacrolimus alone at both concentrations used did not influence the growth of HT-29 cells (Figure 2C), the distinct trend for acceleration of cell proliferation under influence of 1 μM tacrolimus was not statistically significant.

*Cell-cycle analysis*. To understand whether the inhibitory effect of MMF and tacrolimus on the growth of HT-29 cells results from cell mitotic arrest, we analyzed the effect of these drugs on cell-cycle transition using propidium iodide staining and flow cytometric analysis.

Flow cytometric analysis of cell-cycle distribution of control cells demonstrated 49.7±7% of cells in $G_1$ phase, 32.6±3.6% cells in S phase, and 18.1±4.9% cells in $G_2$/M phase. The accumulation of HT-29 cells in the S-phase of the cell cycle was detected following 48-h exposure to 10 μg/ml MMF (50.8±22.3%, $p<0.04$ compared to control), 1 μM tacrolimus with 10 μg/ml MMF (60.5±17.5%, $p<0.001$, compared to control), 1 μM tacrolimus with 1 μg/ml MMF (38.4±5.2%, $p<0.03$, compared to control), 0.1 μM tacrolimus with 10 μg/ml MMF (52.6±10.6%, $p<0.001$, compared to control), and 0.1μM tacrolimus with 1 μg/ml MMF (59.0±20.6%, $p<0.001$, compared to control; Figure 3). Tacrolimus alone and 1 μg/ml MMF failed to induce cell-cycle mitotic arrest.

Cell-cycle alterations were not observed following 96 h of treatment (data not shown).

## Discussion

The salient finding of this report is the significant decrease in the number of proliferating epithelial cells and in the expansion of the proliferative crypt compartment in the colonic crypts of colonic adenomas of a patient with FAP who underwent treatment with MMF and tacrolimus compared to control untreated FAP colonic adenomas. We believe that the present *ex vivo* finding, albeit preliminary, in conjunction with histological observations of colonic polyps biopsies from the same FAP patient showing the absence of dysplasia and typical adenomatous changes after 6 months of the drug treatment (4), strengthens the view that combined MMF and tacrolimus treatment impedes the development of colonic cancer. On the basis of these observations, the question arises: What are the molecular mechanisms underpinning the anticancer effect of these immunosuppressive drugs?

*In vivo* the morpholino ethyl prodrug MMF is completely converted to the active moiety, MPA, by the action of esterases present in serum and in tissues, including the gastrointestinal tissues (9-11). MPA is a potent, selective, uncompetitive, and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme in the synthesis of purine nucleotides and, therefore, essential for building nucleotide pools. MPA is very effective in blunting T- and B-lymphocyte proliferation and, thereby, is

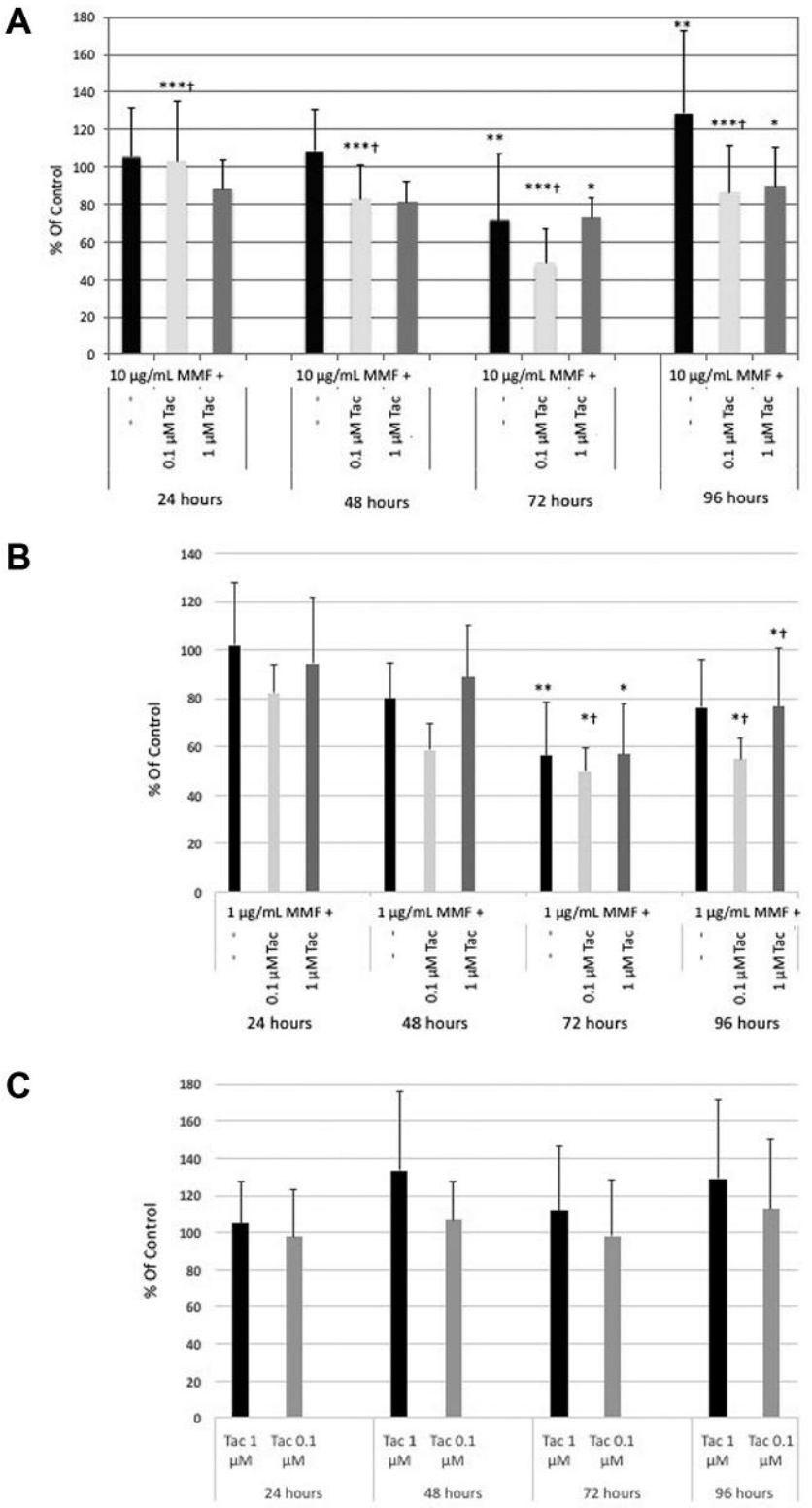


Figure 2. *Influence of 10 µM (A) and 1 µM (B) mycophenolate mofetil (MMF) alone and in combination with tacrolimus (Tac) at different concentrations on the growth of HT-29 cells; tacrolimus alone did not influence proliferation of HT-29 cells (C). Results are expressed as percentage of the control values at the respective time point. Significantly different in A: at *p<0.001, **p<0.002, ***p<0.005 compared to control, and †p<0.01 compared to MMF alone; in B: at *p<0.02 and **p<0.002 compared to control; †p<0.02 compared to MMF alone.*

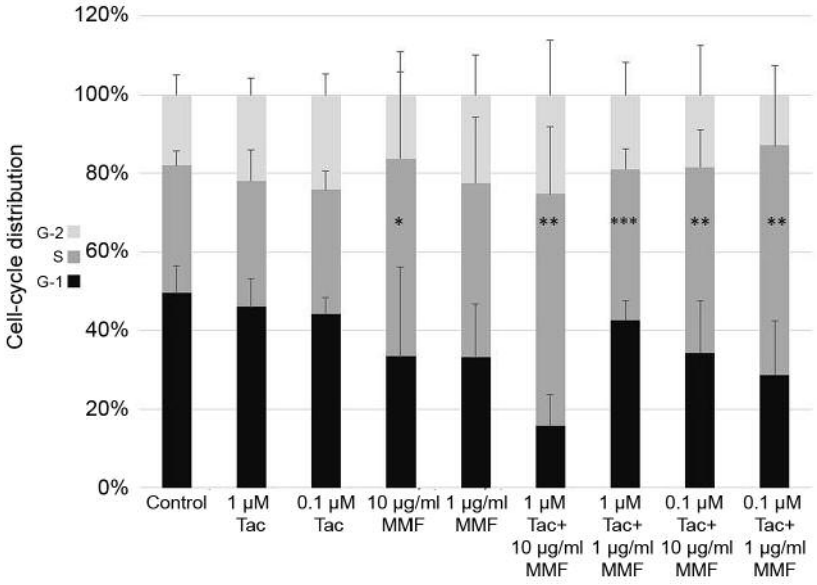


Figure 3. *Mycophenolate mofetil (MMF) alone and in combination with tacrolimus (Tac) induced S-phase mitotic arrest of HL-29 cells after 48 h of treatment. Data are presented as mean of two independent experiments performed in quadruplicates. Significantly different of *p<0.04, **p<0.001 and ***p<0.03 compared to control.*

a potent immunosuppressive drug (9). Of note, MPA is completely metabolized in the liver, kidney and gastrointestinal tract by uridine disphosphate glucuronidase transferases (UDPGT) (13). It is noteworthy that in many tumors, the expression of IMPDH, particularly the isoform IMPDH2, is markedly up-regulated [reviewed in (14)].

To explore putative mechanisms involved in the anticancer action of MMF and tacrolimus on FAP adenomas, we selected the human colonic adenocarcinoma cell line HT29 for the following reasons. Firstly, these cancer cells, like FAP cells, harbor a nonsense *APC* gene mutation (5). Moreover, the HT29 cell line possesses the glucuronidation system capable of inactivating MPA (15), and, thereby, represents a versatile *in vitro* microsystem for assessing the biological effect of MMF or tacrolimus alone and in combination. Notably, an early work used the HT-29 cell line *in vitro* to study the metabolism of analogs of MPA (16).

In the present report, we demonstrated a dose-dependent antiproliferative effect of MMF alone and a synergistic antiproliferative effect of the combination of tacrolimus and MMF on human colonic adenocarcinoma cell line HT-29. The analysis of the cell cycle of HT-29 cells following 48 h of treatment with 1 or 10 μg/ml MMF alone or in combination with tacrolimus demonstrated cell-cycle arrest in the S-phase, a cell-cycle stage associated with stalling of DNA replication.

A tenable possibility is that the restraining action of MMF and of its combination with tacrolimus on the human colonic adenocarcinoma cell line HT-29 and on FAP colonic crypt proliferation derives from the poor supply of purine nucleotides as a consequence of MMF-induced inhibition of IMPDH activity (14). One may suggest that that salvage of guanine *via* the salvage pathway is probably inadequate to satisfy the demanding needs of the rapidly dividing cancer cells. The depletion of guanine nucleotides is believed to underpin the action of IMPDH inhibitors; of note, guanine nucleotides serve as precursors for RNA and DNA and are involved in a vast array of biological events.

Current evidence attributes the anti-neoplastic effects of MMF to its ability to inhibit angiogenesis (17) and cell migration (18), and induce caspase-dependent apoptosis through both the intrinsic mitochondrial and extrinsic cytosolic pathways of apoptosis as a consequence of its interference with DNA synthesis. Of note, induction of apoptosis by IMPDH inhibitors has frequently been reported (19, 20). Although the analysis of kinetics of apoptosis in HT-29 cells following MMF and tacrolimus treatment was beyond the scope of this study, we noted expansion of a hypoploid sub-$G_1$ cell population (data not shown), concomitant with the observed S-phase arrest in the HT-29 cells following MMF and tacrolimus treatment, suggesting an enhanced rate of apoptosis.

A consistent finding in our study was the failure of tacrolimus on its own to interfere with the growth of the cancer cells and its ability to potentiate the anticancer action of MMF. It is pertinent at this point to mention that although the inhibitory effect of MMF on proliferation of colorectal

cell cancer cell lines was noted three decades ago (21), the enthusiasm for using this drug for treatment of colorectal cancer has somewhat abated following the discovery of a mechanism of intrinsic resistance of intestinal cancer cell lines due to inactivation of MPA by glucuronidation (15). In this context, and of relevance to our study, Zucker *et al*. (22) observed increased bioavailability of MMF, as judged by the elevation of plasma concentration of MPA, in renal transplant patients treated with MMF in combination with tacrolimus and attributed it to the ability of tacrolimus to inhibit glucuronidation of MPA with a corresponding decrease of MPAG, the inactive glucuronide metabolite of MPA. Interestingly, this tacrolimus-mediated inhibitory effect on MPA degradation was shown *in vitro* to be exerted *via* the inhibition of UDPGT (23).

On the basis of these observations, it may be suggested that the contribution of tacrolimus to restraining the growth of colonic cancer cells, when used in combination with MMF, is related to the inhibition of intracellular MPA degradation, thus providing a sustained anti-growth effect of MPA. There is not, however, firm consensus as to whether tacrolimus is indeed involved in limiting MPA degradation, and until this is firmly proven, our mechanistic explanation, although attractive in its simplicity, remains to be validated.

Of interest is the observed discrepancy between the consistent cytopathic effect of MMF in combination with tacrolimus at 48, 72 and 96 h, and the apparent normal cell-cycle distribution in all tested cell groups at 96 h. It is worth noting that Chen *et al*. recently demonstrated the presence of fast- and slow-cycling cell subpopulations in tumors, including colonic tumors, with differential sensitivities to IMPDH inhibition by MPA (24). Whether the differential response of HT29 cells to prolonged exposure to the drugs observed in our study represents drug-induced selection of HT-29 subpopulations with a different phenotypic background determining the responsiveness to MPA remains to be investigated.

Herein we presented the result of a small pilot study aimed at understanding the intriguing clinical case of complete reversion of colonic pre-neoplastic syndrome following treatment with combination of two immunosuppressive drugs. To the best of our knowledge, this study and our previous publication (4) represent the first direct evidence of an *in vivo* anti-growth effect of tacrolimus and MMF treatment of hereditary colon cancer. Further studies, aimed at shedding light on the molecular events underlying the observed phenomena, that include analysis of transcriptosomic changes induced by MMF and tacrolimus in our experimental setting, and the use of mouse models of colon cancer, are underway.

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## Conflicts of Interest

None.

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