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ORIGINAL ARTICLE

Systemic influence of immunosuppressive drugs on small and large bowel transport and barrier function

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Summary

Immunosuppressive drug (ISD)-associated gastrointestinal disorders are a relevant risk factor for graft loss or patient death. The pathomechanisms and the incidence of post-transplantation diarrhea remain to be fully understood. The aim of this study was to characterize the impact of cyclosporine A, tacrolimus (TAC), mycophenolate mofetil (MMF), enteric coated mycophenolic acid (EC-MPA), sirolimus, everolimus (EVE) and fingolimod (FTY 720) on small and large bowel transport and barrier function. Functions of the small bowel and distal colon of Wistar rats treated for 14 days with one of the drug were analyzed using Ussing chamber method. In detail, the glucose and sodium absorption, chloride secretion, and barrier function were compared. Bowel functions were investigated by inhibition or activation of the electrogenic epithelial transport, as well as by measuring transepithelial H^3-lactulose flux. TAC altered glucose absorption; EVE glucose absorption, small bowel barrier function and chloride secretion; MMF small bowel barrier function; and EC-MPA glucose absorption and the small bowel barrier function. Drug effects were partially dose-dependent. In conclusion, different ISD, such as TAC, EVE, MMF, or EC-MPA lead to different and specific patterns of pathophysiologic changes of small and large bowel barrier and transport function.

Introduction

Side effects caused by immunosuppressive drugs (ISD) evoke substantial problems after transplantation. They are a major factor influencing long-term quality of life and outcome. It is well known that most common side effects of nearly all ISD derive from local effects in the gastrointestinal tract [1], (Table 1). Bunnapardist *et al.* [2] analyzed retrospectively more than 40 000 kidney transplant recipients and found that the risk for graft loss and patient death is more than double in patients with noninfectious diarrhea. This is of particular importance because patient and graft survival can only be maintained with lifelong immunosuppressive therapy.

Gastrointestinal disorders in transplanted patients are caused by several specific factors such as ISD therapy, bacterial overgrowth and infections (*cytomegalovirus*, *Clostridium difficile*), graft-versus-host disease, post-transplant lymphoproliferative disease, or inflammatory bowel disease. The actual impact of ISD on gastrointestinal tract function has remained somehow unclear. The influence of ISD on small intestine function has already been examined in many studies. However, those reports have been very selective and do not provide consistent information. For example, sirolimus (SIR) is supposed to cause an increase in glucose absorption and in addition atrophy of the intestinal mucosa [3]. Moreover, inhibitory effects on a facilitated glucose transporter 2 (GLUT-2) trafficking have been described previously [4]. For cyclosporine A (CyA), divergent results have been published, i.e. depleting [5,6] and accelerating [3] influence on glucose absorption in the small bowel. The intestinal chloride secretion under ISD therapy was not analyzed until now, although this is important with regard to the pathophysiology of diarrhea (Table 2).

Table 1. Incidence of immunosuppressive drugs (ISD)-associated diarrhea in human: review of the literature.

ISD	Author	Group size	Application route	Study design (drug dose, transplanted organ or disease, study duration)	Diarrhea incidence (%)
CyA	*	266	Orally	250–400 ng/ml, liver, 1 year [40]	47
	Pirsch *et al.*	207	Orally	150–400 ng/ml for the first 3 months and 100–300 ng/ml afterwards, kidney, 1 year [41]	40.6
	Levy *et al.*	251	Orally	C_2 level within the target range of 0.8 to 1.2 μg/ml till month 3, and 0.7 to 0.9 μg/ml afterwards, liver, 6 months [42]	14
TAC	Pirsch *et al.*	205	Orally	10–25 ng/ml for the first 3 months and 5–15 ng/ml thereafter, kidney, 1 year [41]	43.9
	*	236	Orally	0.2–5 ng p/ml, liver, 1 year [40]	72
	Levy *et al.*	248	Orally	C_0 in the range of –15 ng/ml till month 3, 5–12 ng/ml afterwards, liver, 6 months [42]	29
MMF	Cantarovich *et al.*	19	Orally	1 g twice daily, liver, 12 months (1 year after transplantation) [43]	18
	Pfitzmann *et al.*	191	Orally	1–2 g twice daily, liver, 4 months [44]	24
	Rangel *et al.*	105	Orally	1 g twice daily, kidney, (?) [45]	79.2
	Darji *et al.*	118	Orally	500–3000 mg, kidney, (?) [34]	31.4
	Kamar *et al.*	93	Orally	500 mg twice daily, kidney, 1 year [32]	19.3
EC-MPA	Sumethkul *et al.*	12	Orally	720 mg once daily, kidney, 3–8 months [46]	15
	Darji *et al.*	118	Orally	360–2160 mg, kidney, 3–6 weeks after conversion from CellCept®, therapy [34]	20.1
	Kamar *et al.*	37	Orally	720 mg once daily, kidney, 1 year [32]	13.5
	Rangel *et al.*	60	Orally	720 mg twice daily, kidney, (?) [45]	62.3
SIR	Fairbanks *et al.*	21	Orally	9–12 ng/dl, liver, 64 weeks [47]	4.7
	Bissler *et al.*	18	Orally	1–5 ng/ml, patients suffering from tuberous sclerosis complex or sporadic lymphangioleiomyomatosis (not transplanted), 24 months [48]	38
	Moro *et al.*	14	Orally	(?), heart, 595 days median follow-up [49]	14.3
EVE	Moro *et al.*	42	Orally	(?), heart, 351 days median follow-up [49]	2.4
	Yao *et al.*	67	Orally	5 or 10 mg/day, patients suffering from low- to intermediate-grade neuroendocrine tumors (not transplanted) [50]	11
	Yee *et al.*	27	Orally	5 or 10 mg/day, patients with different type of leukemia (not transplanted) [51]	33
FTY 720	Kappos *et al.*	184	Orally	1.25 or 5 mg/day, patients suffering from multiple sclerosis (not transplanted), 12 months [52]	10–12

ISD, immunosuppressive drugs; CyA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mophetil; EC-MPA, enteric coated mycophenolic acid; SIR, sirolimus; EVE, everolimus; FTY 720, fingolimod.
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In summary, there is still no consensus on the pathophysiologic influence of ISD on the small bowel and colon function in the present literature. Our previously published results compared short-term exposure (1 h) of rat's small bowel with therapeutic and toxic ISD concentrations *ex vivo* [7]. The aim of the present study was to describe the influence of commonly used ISD on small and large bowel transport and barrier function after 14 days of treatment of the nontransplanted rat.

Materials and methods

Experimental animals and ISD

The 'Principles of Laboratory animal care' NIH publication Vol 25, No. 28 revised 1996; and the current version of the German Law on the Protection of Animals were followed. Male Wistar rats (280–350 g) were included in the experiments 10 days after delivery. They were kept in standard cages, between two and five animals per cage. Standard rat fodder and water were allowed *ad libitum*. Rats were daily treated orally using an adopted dull needle (intragastric gavage) with one of the examined ISD in the low (LD) or high (HD) dose ($n = 9$) for 14 days (Table 3). Low and high doses according to the therapeutic doses were used in rat-model transplantation [8–12]. The dosages used in rats are higher than those used in humans because of a different body area to volume ratio and a much faster hepatic metabolism. Drugs were diluted in 1 ml of tap water. As the control group ($n = 10$), rats received an injection of 1 ml of tap water.

Table 2. Pathomechanisms of immunosuppressive drug (ISD)-associated diarrhea: review of the literature.

ISD	Proposed pathomechanisms
CyA	None
TAC	-Activation of intestinal motilin receptor [30] -Increased intestinal permeability and impaired absorptive capacity as a result of inhibition of cellular energy production [27,28]
MMF	-Malabsorption resulting from small intestine villous atrophy [36,37] -Erosive colitis-like syndrome [53,54]
EC-MPA	None
SIR	Intestinal mucosa atrophy and malabsorption [3]
EVE	None
FTY 720	None

CyA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mophetil; EC-MPA, enteric coated mycophenolic acid; SIR, sirolimus; EVE, everolimus; FTY 720, fingolimod.

Table 3. Doses of the immunosuppressive drugs used in the study [mg/kg b.w./day, $n = 9$].

14 days treatment	TAC	CyA	EC-MPA	MMF	SIR	EVE	FTY 720
Low dose	0.3	1.5	10	15	0.25	0.5	0.3
High dose	1	5	30	40	0.5	3	1

CyA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mophetil; EC-MPA, enteric coated mycophenolic acid; SIR, sirolimus; EVE, everolimus; FTY 720, fingolimod.

The standard market forms of ISD were used. Small bowel glucose absorption, chloride secretion, and barrier function as well as colon barrier function, sodium absorption, and chloride secretion were measured consecutively after harvesting the intestine.

Small bowel functions

On the 15th day, the rats were fasted overnight, prepared for surgery and received an isofluran anesthesia. The abdomen and thorax were opened, and the heart apex was cut in order to sacrifice the animal and collect the blood sample. Approximately 2-cm pieces of the jejunum (10 cm distal from the hepato-duodenal ligament) were prepared and mounted into modified Ussing chambers with an exposed epithelial area of 0.28 cm^2 [13]. Short-circuit current (I_{sc} [μA]) and transmural resistance (R_t, [Ohm × cm^2]) were continuously recorded using a computer-controlled device (CVC8; Fiebig, Berlin, Germany) as described previously [14].

The absorption of glucose in the small bowel occurs mostly via sodium/glucose co-transporter (SGLT1) [15]. This transport is electrogenic and the measurement of the current flow allows quantifying the amount of absorbed glucose. Glucose absorption kinetics was measured using the nonmetabolized glucose analog, 3-*O*-methyl-D-glucopyranose (3OMG; Sigma-Aldrich, Steinheim, Germany) [16]. In brief, aliquots of standard medium [contained in mmol/l: Na^+ 140, Cl^- 123.8, K^+ 5.4, Ca^{2+} 1.2, Mg^{2+} 1.2 HPO_4^{2-} 2.4, $H_2PO_4^-$ 0.6, HCO_3^- 21 (gassed with 95% O_2 and 5% CO_2, pH 7.4, 37 °C)] supplemented with 3OMG at 10-min intervals resulting in the final concentrations of 4, 8, 16, 32, and 48 mmol/l, respectively, were added to both sides of the chamber. The maximal reaction rate (V_{max}) and Michaelis–Menten constant (K_M) were calculated from Lineweaver–Burk and Eady–Hofstee plots (data were corrected to reach similar V_{max} in both plots because of their different sensibility for either high or low substrate concentrations). To verify the integrity of the mounted epithelium, its secretory response was tested by adding 10^{-2} mol/l theophylline (Sigma-Aldrich) to both sides of the chamber. Samples which did not or weakly responded to theophylline, or samples the resistance of which was lower than 20 [Ohm × cm^2], were excluded from further analyses. All I_{sc} values were corrected for bath resistance as described by Tai *et al.* [17].

Two parameters commonly used for assessment of the small bowel barrier function were measured: ^{3}H-lactulose flux (J_{Lac}, [nmol/h cm^2]) and R_t [18]. ^{3}H-lactulose (D-[galactose-6–3H], 20 Ci/mmol) mucosal to serosal flux was determined as described by Schultz and Zalusky [19]. In brief, the tissue was clamped to 0 mV and an aliquot of ^{3}H-lactulose was added to the mucosal side of the chamber. Then the consecutive serosal and mucosal samples were taken and their radioactivity measured using a Tri-Carb 1900TR Liquid Scintillation Analyser (Perkin-Elmer, Waltham, MA, USA). Absence of significant drift of I_{sc} and R_t was proved by 30 min of calibration time before each experiment (chambers without tissue). Medium for J_{Lac} experiments was enriched by adding: (in mmol/l) D(+)-glucose 10, β-OH-butyrate 0.5, glutamine 2.5, D(+)-mannose 10, lactulose 20 and tobramycin (Brulamycin Ampullen 40) 50 mg/l. Mean R_t was calculated from a constant period of time (between 75 and 135 min during follow-up).

Chloride secretion was assessed after lactulose flux experiments. Phloridzin (Sigma-Aldrich), a selective inhibitor of SGLT1 [20], was added first to inhibit glucose transport (5 10^{-4} mol/l, mucosal side). After 20 min, theophylline (10^{-2} mol/l, mucosal and serosal side) together with prostaglandin E_2 (PgE_2, 10^{-6} mol/l, serosal side; Fluka, BioChemika, Seelze, Germany) was added. Theophyllin and PgE_2 both activate cyclic AMP-dependent chloride secretion [16]. The increase in I_{sc} was calculated afterwards (ΔcAMP). After another 20 min, bumetanide (10^{-5} mol/l, serosal; Sigma-Aldrich), an

inhibitor of a basolateral $Na^+2Cl^-K^+$ co-transporter (NKCC) [16], was added to maximally inhibit chloride secretion (ΔNKCC).

Colon functions

Besides jejunum, the late distal colon was harvested. Tissue preparation was performed under a microscope (7× magnification). Briefly, the tissue was placed on a silicone plate with the serosal side up, and the muscle layer (muscularis propria) was dissected (partial strip) [21]. Colon wall was then mounted into modified Ussing chambers, and I_{sc} and R_t were continuously measured. Colon barrier function was estimated by measurement of J_{Lac} and R_t (see the small bowel part).

To estimate the colonic sodium absorption, amiloride (Sigma, St. Louis, MO, USA) was used. Addition of amiloride to the mucosal and serosal sides of the chamber in the concentration of 10^{-4} mol/l inhibit the Na/K co-transport via a distal colon amilorid-sensitive epithelial sodium channel (ENaC) [22].

Chloride secretion was measured after J_{Lac} experiments: 20 min after the last J_{Lac} measurement, theophylline together with PgE_2 was added to the chamber as in the small bowel experiments to maximally stimulate the chloride secretion. After another 20 min, bumetanide was added to inhibit a NKCC co-transporter.

Statistical analysis

Mean values were calculated within the treated groups and compared with the control group. Some parameters were also compared between the groups, i.e. mycophenolate mofetil (MMF) versus enteric coated mycophenolic acid (EC-MPA). All parameters were compared using multivariable testing. According to the Bonferroni–Holm correction for the seven study groups, only $P < 0.007$ was defined as statistically significant.

Results

All animals survived the experimental period and did not show any obvious signs of ISD toxicity. Weight gain was not significantly different between the control and other groups (Fig. 1).

Small bowel

Glucose absorption kinetics curves as well as V_{max} were significantly lowered in the everolimus (EVE) HD and tacrolimus (TAC) HD groups. EVE LD and EC-MPA HD showed the same tendency, however, did not reach the significance level ($P < 0.05$ and $P < 0.03$, Fig. 2). V_{max} was significantly different between MMF and EC-MPA HD ($P < 0.005$).

J_{Lac} was elevated in the EVE HD group. In the MMF LD group, R_t was significantly decreased and in the high-

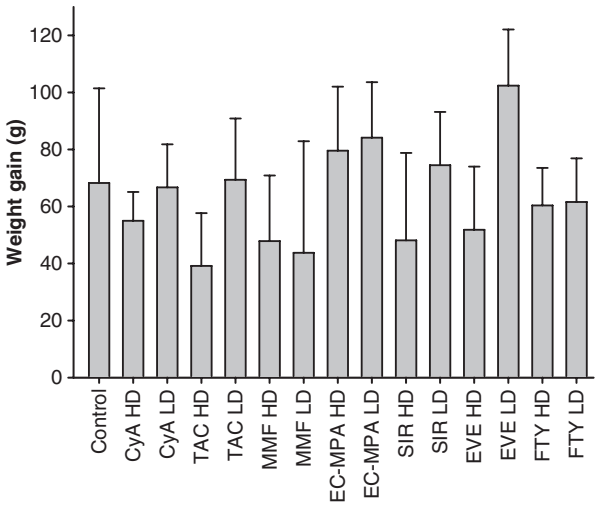

Figure 1 Weight gain of the study animals. None of the study groups showed a significantly different weight gain compared with the control group. LD, low dose; HD, high dose; CyA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mofetil; EC-MPA, mycophenolic acid; SIR, sirolimus; EVE, everolimus; FTY, fingolimod.

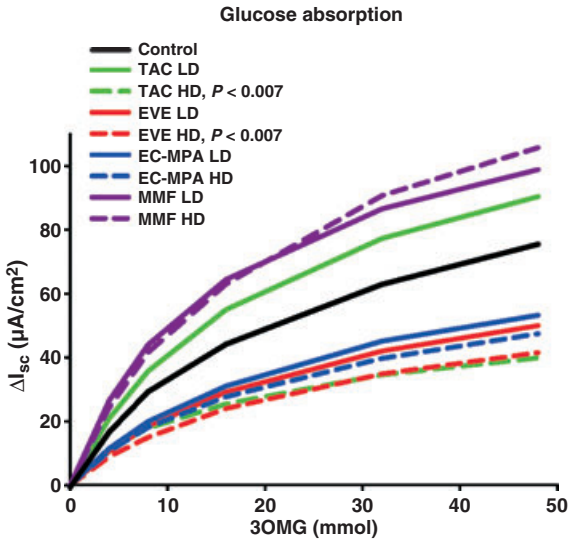

Figure 2 Glucose absorption kinetics. Nonmetabolized glucose analog (3OMG) was added in an increasing concentration to the mucosal chamber side. Increase in the current flow (ΔI_{sc}) as a result of the electrogenic Na^+/3OMG transport through SGLT1 co-transporter was recorded. Tacrolimus (TAC), everolimus (EVE) reduce significantly, and enteric coated mycophenolic acid (EC-MPA) shows a tendency to reduce glucose absorption rate. The influence of TAC was dose dependent. The *P*-value was calculated between the control and other groups and a Bonferroni–Holm correction for the seven study groups was applied (significance level $P < 0.007$).

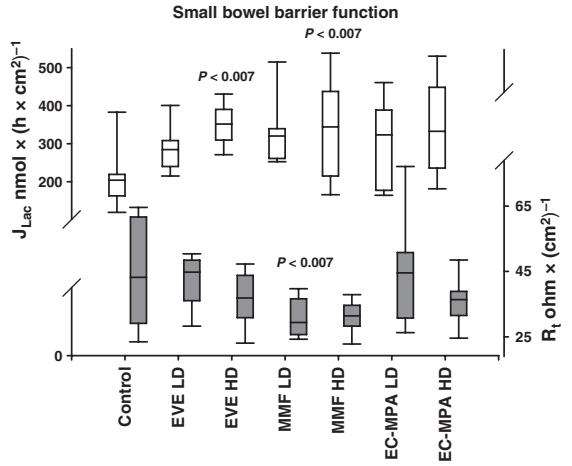

Figure 3 Small bowel barrier function. ^{3}H-lactulose flux (J_{Lac}) and transepithelial resistance (R_t) are markers for big and small molecules epithelial barrier function. In the everolimus (EVE) and myophenolate mofetil (MMF) groups, small bowel barrier function was significantly altered (J_{Lac} increased or R_t reduced). In rats treated with enteric coated mycophenolic acid (EC-MPA), tendency to influence both parameters can clearly be seen, however, did not reach statistical significance. The *P*-value was calculated between the control and other groups and a Bonferroni–Holm correction for the seven study groups was applied (significance level $P < 0.007$).

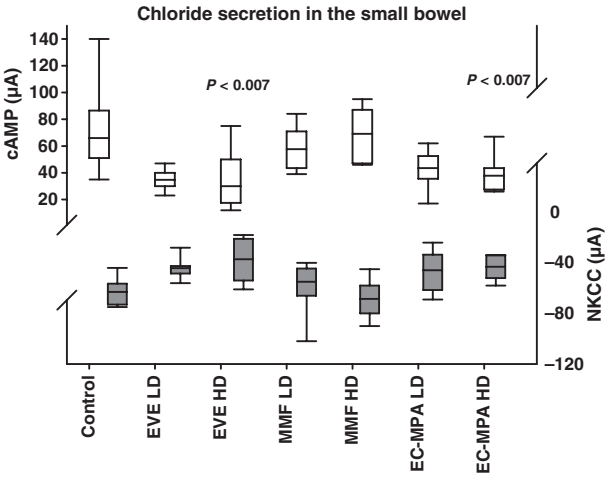

Figure 4 Chloride secretion was measured using cAMP-dependent chloride secretion activation by theophylline and PgE_2 (cAMP) as well as the basolateral $Na^+2Cl^-K^+$ co-transporter (NKCC) inhibition with bumetanide. cAMP was significantly increased in everolimus (EVE) HD versus control group (26.3 ± 11.1 vs. 6.3 ± 4.9 µA, $P < 0.0004$). EVE LD and enteric coated mycophenolic acid (EC-MPA) HD group increased reaction to amiloride versus control but did not reach the significance level (23.3 ± 15.0; 18.0 ± 10 vs. 6.3 ± 4.9 µA, $P < 0.05$). There were no significant differences in the NKCC and cAMP values between the control and other groups. The *P*-value was calculated between the control and other groups and a Bonferroni–Holm correction for the seven study groups was applied (significance level $P < 0.007$).

dose group, R_t decreased ($P = 0.024$) and J_{Lac} significantly increased. In the EC-MPA high-dose group, only J_{Lac} was increased but did not reach the significance level ($P < 0.03$). The altered small bowel barrier function parameters did not differ significantly between MMF and EC-MPA groups (Fig. 3).

In the EVE HD group, small bowel chloride transport after stimulation with theophylline and prostaglandin E_2 was significantly reduced in comparison with the control group. In the LD group, the same tendency was observed, but only a significance level of $P < 0.05$ was reached. Similar results were found in the EC-MPA groups: HD $P < 0.007$, LD $P < 0.05$. There was no significant difference in case of inhibition of NKCC co-transporter with bumetanide between the control and other groups (Fig. 4).

Large bowel

In none of the groups, colon barrier function was changed significantly compared with the control group.

Epithelial sodium channel function was significantly increased in EVE HD group versus control (26.3 ± 11.1 vs. 6.3 ± 4.9 µA, $P < 0.0004$). EVE LD and EC-MPA HD group increased reaction to amiloride versus control, however, did not reach the significance level (23.3 ± 15.0; 18.0 ± 10 vs. 6.3 ± 4.9 µA, $P < 0.05$, Fig. 5).

Discussion

Many studies have reported a high incidence of gastrointestinal symptoms in transplanted patients treated with ISD. However, the reported incidence of diarrhea varies significantly among the different studies (Table 1). This variability occurs as a result of the complex clinical situation after transplantation, as well as the multifactorial pathophysiology of the gastrointestinal disorders. Nevertheless, appearance of diarrhea is an important prognostic factor for donor and graft survival [2].

The aim of this study was to elucidate the influence of several, commonly applied ISD on a small and large bowel transport and barrier function, and its pathomechanisms. The different intestinal functions were investigated using an advanced Ussing chamber method, which can specially characterize nearly the entire bowel function, and is well-known for over 50 years [23]. This method was used to discover the pathologic mechanism of zonula occludens toxin in infection caused by *Vibrio cholerae* strains [24], or to measure the cystic fibrosis transmembrane conductance regulator chloride transporter [25,26]. Diarrhea might occur as a result of dysfunction of the different bowel functions (Table 4). The

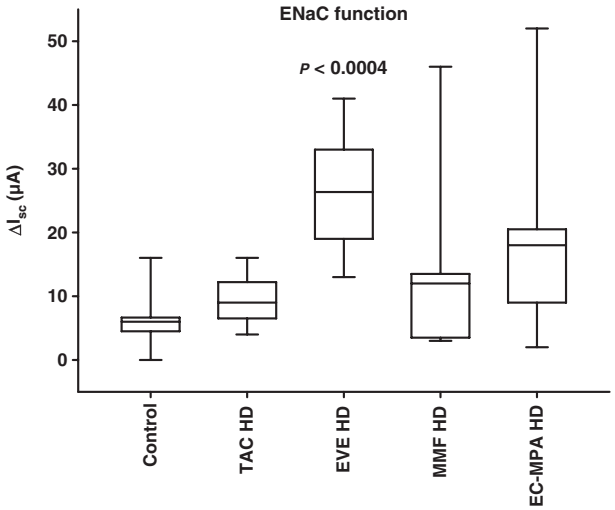

Figure 5 Sodium re-absorption via a distal colon amiloride-sensitive epithelial sodium channel (ENaC). Inhibition of the ENaC channel by amiloride resulted in a decrease in sodium absorption and in the current flow (ΔI_{sc}). Sodium absorption was significantly increased in the everolimus (EVE) and enteric coated mycopenolic acid (EC-MPA) high-dose groups versus control group. In the high-dose tacrolimus (TAC) group, the significance level was not reached ($P < 0.05$). The same drugs altered the glucose/sodium absorption in the jejunum. The *P*-value was calculated between the control and other groups and a Bonferroni-Holm correction for the seven study groups was applied (significance level $P < 0.007$).

Table 4. Diarrhea types and its mechanisms.

Diarrhea type	Mechanism
Motility disorder-dependent diarrhea*	Hypermotility: i.e. hyperthyroidism Hypomotility: i.e. hypothyroidism
Malabsorptive diarrhea†	Malabsorption of nourishment: i.e. glucose
Osmotic diarrhea‡	Lack in absorption mechanisms for i.e. lactulose or mannitol
Secretory diarrhea§	Increased Cl^- secretion by i.e.: *E. coli* enterotoxin
Leak-flux diarrhea¶	Increased intestinal barrier permeability by i.e.: *Vibrio cholerae*

*Motility disorder diarrhea can take place in a hyper- or hypomotility situation. Hypermotility can be caused for example by hyperthyroidism and leads to a reduced contact time between nourishment and the bowel absorptive area. Hypomotility (i.e. postoperative hypomotility, hypothyreoidism) on the other hand leads to the prolonged presence of the nutriment in the bowel lumen, causing bacterial overgrowth followed by diarrhea.

†Malabsorptive diarrhea is caused by solutes, which have not been absorbed in the bowel. Malabsorption of glucose, galactose or tropical sprue are examples of this disorder.

‡Some of the authors rule out osmotic DIA, which takes place due to *a priori* absent transport mechanisms for definite substances as lactulose or mannitol [55].

§Secretory diarrhea is caused by increased net Cl^- secretion in the bowel lumen. This is due to cAMP, cGMP, PKC or Ca^{2+}-dependent activation of Cl^- channels and/or inhibition of the Na^+ and Cl^- resorption in the apical membrane of enterocytes. This mechanism is activated for example by different enterotoxins produced by *E. coli.*

¶Leak-flux diarrhea occurs because of a defect of the intestinal barrier function and increased permeability of the intestinal mucosa to small or big molecular solutes [56]. *Shigella flexneri*, *Clostridium* spp. or *Vibrio cholerae* induce through their toxins alterations in the tight junction complex and lead to a massive loss of water and solute [57].

leak flux, secretory and malabsorptive pathomechanisms can be fully analyzed using the Ussing chamber method. However, this method has some limitations, as it is only possible to analyze the bowel functions *in vitro*. The complex intestinal functions like motility or influence of hormones such as gastrin or motilin cannot be assed by this method.

It has been previously shown by our group that a direct exposure to the therapeutic ISD concentrations does not cause significant alterations in glucose absorption, chloride secretion or barrier function in the small bowel. Nevertheless, high concentrations of EVE, mycophenolate mophetil (MMF), and EC-MPA in the small bowel lumen had a relevant influence on mucosal function [7]. These high concentrations could occur after oral application and might be a relevant factor during the genesis of gastrointestinal disorders.

Calcineurin inhibitors (e.g. CyA and TAC) are widely used in the field of transplantation. A significant spectrum of side effects has been described for both drugs [1]. Diarrhea seems to occur more often in TAC – than in CyA-treated patients (Table 1). Nevertheless, the influence of CyA on the bowel function has not been well-described in the present literature. Dias *et al.* [3] showed that glucose absorption was increased in the jejunum and (probably) secondary reduced in the ileum of rats treated for 20 days with CyA. They also found increased absorptive surface of the small bowel villi. Furthermore, only slightly decreased glucose absorption after direct exposure of the small bowel to the toxic CyA concentrations was noticed [7]. In the present study, however, neither a correlation for that finding nor any other pathophysiologic influence on the intestinal barrier and transport function was observed in those rats treated with CyA. The mechanism by which bowel function might be altered by CyA remains unclear. Results of this study confirm those from Dias *et al.* (in case of CyA, glucose malabsorption does not take place). Moreover, bowel barrier function, as well as chloride secretion, is not altered after 2 weeks of therapy with CyA. Longer exposure to CyA should be analyzed to explain the relative high incidence of diarrhea in patients treated with CyA.

Tacrolimus has been suspected to decrease the intracellular adenosine triphosphate (ATP) level [27–29]. Yanchar *et al.*, describe increased small bowel permeability *in vivo* (99 T_c-DTPA) in rats treated for 6 weeks with different TAC doses. In the present study, glucose malabsorption in the jejunum was observed. As 1-h exposure even to the high TAC concentrations [7] does not cause any alteration in the small bowel function, a direct inhibitory effect of TAC on the glucose–sodium co-transport seems to be unlikely. Thus low energetic status of enterocytes caused by TAC seems possible, as proposed by Gabe *et al.* [27]. The dose dependency of this effect has to be taken into account during the clinical application.

Sirolimus and EVE are both macrocyclic lactones, while SIR occurs naturally and EVE is its chemical modification. Both drugs cause comparable side effects [1], although EVE influences physiologic function of the small bowel more potently than SIR [30]. In our experiments, EVE showed the most severe influence of all analyzed drugs on bowel transport and barrier function. SIR did not influence the small or large bowel transport or barrier function.

Dias *et al.*, proposed (analyzing rabbits treated with SIR for 20 days) a malabsorption diarrhea mechanism attributable to the atrophy of intestinal mucosa. Rabbits significantly lost weight during therapy, while this was not the case in the present study [3]. Morphologic assessment of the mucosa was not performed in the present study. However, such changes are unlikely, as no alterations of the intestinal transport and barrier function in the SIR groups were observed. While effects of ISD are often dose dependent, we suppose that the lack of similar effects could be explained because of faster metabolism/lower exposure in the rat than in the rabbit as a result of the different body surface to volume ratio. There are no clinical reports on mucosal atrophy in patients treated with therapeutical SIR doses, however, as rapamycin possesses a strong antiproliferative potential, such effect cannot be excluded.

No data concerning the possible pathophysiology of diarrhea in patients treated with EVE are yet available. We have reported about impaired small bowel barrier function after direct exposure to the high EVE concentration [7]. A corresponding effect occurs after treatment over 14 days with therapeutic dosages of EVE. Moreover, malabsorption of glucose accompanied with the increased re-absorption of sodium in the colon, as well as reduced chloride secretion capacity in the small bowel was observed. The increased Na^+ absorption in the colon can be interpreted as a self-regulation mechanism as was exclusively determined in groups in which glucose/Na^+ absorption was impaired (Fig. 5). Such a regulation has been already described by Bhala *et al.* [31]. Taken together, the observed pathologic changes in small and large bowel transport and barrier function might be a sign of a general enterocyte dysfunction because of a low energetic status.

Mycophenolic acid (MPA) is the active substance of as well the MMF as the EC-MPA. The enteric coating of EC-MPA dissolves and release MPA in the late small intestine, while MMF is de-estrificated and MPA absorbed in the earlier part of gastrointestinal tract [1]. It was shown that the gastrointestinal side effects of MMF and EC-MPA appear with a similar frequency [32,33]. On the contrary, recently Darji *et al.* [34] in a multicenter, open-label, prospective study, observed an improvement in the incidence of gastrointestinal side effects after conversion from MMF to EC-MPA therapy. Not much is known about the direct MPA influence on enterocyte function. An MPA acyl glucuronide as a product of its metabolism is supposed to be toxic and responsible for the MPA side effects [35]. Till today, its connection to diarrhea remains unclear. Patients suffering from MPA-associated gastrointestinal side effects present either duodenal small villous atrophy [36,37], or erosive colitis changes. In a previous study of our group, a decrease in maximal chloride secretion capability in the small bowel as a reaction to a direct influence of high mycophenolate concentrations was observed [7]. After 14 days of treatment, impaired small bowel barrier function in both the MMF and EC-MPA groups, and impaired glucose absorption followed by slightly decreased sodium re-absorption in the colon of rats treated with EC-MPA was observed. We suppose that the higher impact of EC-MPA in comparison with MMF on the small bowel functions might be caused by a longer exposure of the small bowel mucosa because of the slower release of the drug (enteric coating). Thus the role of MPA acyl glucuronide in the pathophysiology of diarrhea seems unlikely because MMF and EC-MPA had significantly different influence on the small bowel function (glucose absorption).

Taken together, MPA impairs global enterocyte function. Reduced glucose transport capacity, as well as either higher apoptotic rate or impaired function of the tight junctions (altered small bowel barrier function), could suggest the influence on the early stage of the cellular protein production. The reduction in enterocyte energetic status (like EVE) might also take place in case of MPA.

FTY720 (FTY, fingolimod) is a new immunosuppressive agent currently used in clinical studies [38]. Presently, only some clinical findings about its side effects are available. Preliminary results show that FTY is free of typical side effects of ISD [38], but the appearance of diarrhea could be comparable even with MMF [39]. Recently, an incidence of diarrhea of 10–12% was observed in a study, where FTY 720 was used as a monotherapy in the treatment of patients with multiple sclerosis. In the

Table 5. Pathomechanisms of ISD-associated diarrhea in this study.

ISD	Possible pathomechanisms of ISD-associated diarrhea: results of this study
CyA	**No significant influence** on small bowel and colon transport and barrier function
TAC	Reduced glucose absorption and secondary increased Na^+ colon re-absorption (n.s.) – **malabsorptive diarrhea**
MMF	Altered small bowel barrier function – **leak-flux diarrhea**
EC-MPA	Reduced glucose absorption and secondary increased Na^+ colon absorption (n.s.) – **malabsorptive diarrhea** Altered small bowel barrier function – **leak-flux diarrhea**
SIR	**No significant influence** on small bowel and colon transport and barrier function
EVE	Reduced glucose absorption and secondary increased Na^+ colon re-absorption Impaired small bowel barrier function Reduced chloride secretion overall diminished mucosal small bowel function – **malabsorptive and leak-flux diarrhea**
FTY 720	**No significant influence** on small bowel and colon transport and barrier function

ISD, immunosuppressive drugs; CyA, cyclosporine A; TAC, tacrolimus; MMF, mycophenolate mophetil; EC-MPA, enteric coated mycophenolic acid; SIR, sirolimus; EVE, everolimus; FTY 720, fingolimod.

present study, no influence of FTY on small and large bowel transport or barrier function was found.

In conclusion, several pathomechanisms potentially leading to diarrhea in patients treated with ISD are shown in this study (Table 5). All experimental animals survived the 14-day treatment period; however, those treated with TAC, EVE, MMF, and EC-MPA had pathologic alterations of the small or large bowel barrier and transport function. Those changes were dose dependent and might lead to gastrointestinal disorders by treated patients even without bacterial overgrowth or other complications of the immunosuppressive therapy.

Authorship

MM: participated in performing the research, data analysis, and writing of the paper. PM: participated in statistical analysis. JFL: participated in writing of the paper. PN: participated in writing of the paper. MS: participated in performing the research, data analysis, and writing of the paper.

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References

1. Taylor AL, Watson CJ, Bradley JA. Immunosuppressive agents in solid organ transplantation: mechanisms of action and therapeutic efficacy. *Crit Rev Oncol Hematol* 2005; **56**: 23.
2. Bunnapradist S, Neri L, Wong W, *et al.* Incidence and risk factors for diarrhea following kidney transplantation and association with graft loss and mortality. *Am J Kidney Dis* 2008; **51**: 478.
3. Dias VC, Madsen KL, Mulder KE, Keelan M, Yatscoff RW, Thomson AB. Oral administration of rapamycin and cyclosporine differentially alter intestinal function in rabbits. *Dig Dis Sci* 1998; **43**: 2227.
4. Helliwell PA, Rumsby MG, Kellett GL. Intestinal sugar absorption is regulated by phosphorylation and turnover of protein kinase C betaII mediated by phosphatidylinositol 3-kinase- and mammalian target of rapamycin-dependent pathways. *J Biol Chem* 2003; **278**: 28644.
5. Sigalet DL, Kneteman NM, Thomson AB. The effects of cyclosporine on normal bowel. *Transplantation* 1991; **51**: 1296.
6. Dias VC, Madsen KL, Yatscoff RW, Doring K, Thomson AB. Orally administered immunosuppressants modify intestinal uptake of nutrients in rabbits. *Transplantation* 1994; **58**: 1241.
7. Malinowski M, Martus P, Neuhaus P, Stockmann M. The influence of commonly used immunosuppressive drugs on the small bowel functions – a comparative experimental study. *Ann Transplant* 2009; **14**: 38.
8. Nielsen FT, Ottosen P, Starklint H, Dieperink H. Kidney function and morphology after short-term combination therapy with cyclosporine A, tacrolimus and sirolimus in the rat. *Nephrol Dial Transplant* 2003; **18**: 491.
9. Schuler W, Sedrani R, Cottens S, *et al.* SDZ RAD, a new rapamycin derivative: pharmacological properties *in vitro* and *in vivo*. *Transplantation* 1997; **64**: 36.
10. Heller T, Geide A, Bonitz U, *et al.* Effect of the antioxidant idebenone on adverse events under mycophenolate mofetil therapy in a rat model. *Transplantation* 2008; **85**: 739.
11. Takeichi T, Uemoto S, Minamiguchi S, *et al.* Effect of ONO-4057 and tacrolimus on ischemia-reperfusion injury of the liver. *World J Gastroenterol* 2009; **15**: 5712.
12. Fuller TF, Hoff U, Kong L, *et al.* Cytoprotective actions of FTY720 modulate severe preservation reperfusion injury in rat renal transplants. *Transplantation* 2010; **89**: 402.
13. Schulzke JD, Fromm M, Hegel U. Epithelial and subepithelial resistance of rat large intestine: segmental differences, effect of stripping, time course, and action of aldosterone. *Pflugers Arch* 1986; **407**: 632.

14. Stockmann M, Schmitz H, Fromm M, *et al.* Mechanisms of epithelial barrier impairment in HIV infection. *Ann N Y Acad Sci* 2000; **915**: 293.
15. Wright EM, Loo DD. Coupling between Na+, sugar, and water transport across the intestine. *Ann N Y Acad Sci* 2000; **915**: 54.
16. Kroesen AJ, Stockmann M, Ransco C, Schulzke JD, Fromm M, Buhr HJ. Impairment of epithelial transport but not of barrier function in idiopathic pouchitis after ulcerative colitis. *Gut* 2002; **50**: 821.
17. Tai YH, Tai CY. The conventional short-circuiting technique under-short-circuits most epithelia. *J Membr Biol* 1981; **59**: 173.
18. Van Itallie CM, Anderson JM. Molecular structure and regulation of tight junctions. *Current Topics in Membranes*. New Haven: Academic Press, 2001.
19. Schultz SG, Zalusky R. Ion transport in isolated rabbit ileum I. Short-circuit current and Na fluxes. *J Gen Physiol* 1964; **47**: 567.
20. Helliwell PA, Kellett GL. The active and passive components of glucose absorption in rat jejunum under low and high perfusion stress. *J Physiol* 2002; **544**: 579.
21. Fromm M, Schulzke JD, Hegel U. Control of electrogenic Na+ absorption in rat late distal colon by nanomolar aldostreone added *in vitro*. *Am J Physiol* 1993; **264**: E68.
22. Amasheh S, Barmeyer C, Koch CS, *et al.* Cytokine-dependent transcriptional down-regulation of epithelial sodium channel in ulcerative colitis. *Gastroenterology* 2004; **126**: 1711.
23. Ussing HH, Zerahn K. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Phys Scand* 1951; **23**: 110.
24. Fasano A, Baudry B, Pumplin DW. *Vibrio cholerae* produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci USA* 1991; **88**: 5242.
25. Blouquit S, Regnier A, Dannhoffer L, *et al.* Ion and fluid transport properties of small airways in cystic fibrosis. *Am J Respir Crit Care Med* 2006; **174**: 299.
26. Hamilton KL, Kiessling M. DCEBIO stimulates Cl- secretion in the mouse jejunum. *Am J Physiol Cell Physiol* 2006; **290**: C152.
27. Gabe SM, Bjarnason I, Tolou-Ghamari Z, *et al.* The effect of tacrolimus (FK506) on intestinal barrier function and cellular energy production in humans. *Gastroenterology* 1998; **115**: 67.
28. Madsen KL, Yanchar NL, Sigalet DL, Reigel T, Fedorak RN. FK506 increases permeability in rat intestine by inhibiting mitochondrial function. *Gastroenterology* 1995; **109**: 107.
29. Yanchar NL, Riegel TM, Martin G, Fedorak RN, Kneteman NM, Sigalet DL. Tacrolimus (FK506) – its effects on intestinal glucose transport. *Transplantation* 1996; **61**: 630.
30. Ginsburg PM, Thuluvath PJ. Diarrhea in liver transplant recipients: etiology and management. *Liver Transpl* 2005; **11**: 881.
31. Bhalla V, Hallows KR. Mechanisms of ENaC regulation and clinical implications. *J Am Soc Nephrol* 2008; **19**: 1845.
32. Kamar N, Oufroukhi L, Faure P, *et al.* Questionnaire-based evaluation of gastrointestinal disorders in de novo renal-transplant patients receiving either mycophenolate mofetil or enteric-coated mycophenolate sodium. *Nephrol Dial Transplant* 2005; **20**: 2231.
33. Chanda SM, Sellin JH, Torres CM, Yee JP. Comparative gastrointestinal effects of mycophenolate mofetil capsules and enteric-coated tablets of sodium-mycophenolic acid in beagle dogs. *Transplant Proc* 2002; **34**: 3387.
34. Darji P, Vijayaraghavan R, Thiagarajan CM, *et al.* Conversion from mycophenolate mofetil to enteric-coated mycophenolate sodium in renal transplant recipients with gastrointestinal tract disorders. *Transplant Proc* 2008; **40**: 2262.
35. Shipkova M, Beck H, Voland A, *et al.* Identification of protein targets for mycophenolic acid acyl glucuronide in rat liver and colon tissue. *Proteomics* 2004; **4**: 2728.
36. Kamar N, Faure P, Dupuis E, *et al.* Villous atrophy induced by mycophenolate mofetil in renal-transplant patients. *Transpl Int* 2004; **17**: 463.
37. Ducloux D, Ottignon Y, Semhoun-Ducloux S, *et al.* Mycophenolate mofetil-induced villous atrophy. *Transplantation* 1998; **66**: 1115.
38. Segoloni GP, Quaglia M. New immunosuppressive drugs for prevention and treatment of rejection in renal transplant. *J Nephrol* 2006; **19**: 578.
39. Tedesco-Silva H, Mourad G, Kahan BD, *et al.* FTY720, a novel immunomodulator: efficacy and safety results from the first phase 2A study in de novo renal transplantation. *Transplantation* 2004; **77**: 1826.
40. The U.S. Multicenter FK506 Liver Study Group. A comparison of tacrolimus (FK 506) and cyclosporine for immunosuppression in liver transplantation. The U.S. Multicenter FK506 Liver Study Group. *N Engl J Med* 1994; **331**: 1110.
41. Pirsch JD, Miller J, Deierhoi MH, Vincenti F, Filo RS. A comparison of tacrolimus (FK506) and cyclosporine for immunosuppression after cadaveric renal transplantation. FK506 Kidney Transplant Study Group. *Transplantation* 1997; **63**: 977.
42. Levy G, Villamil F, Samuel D, *et al.* Results of lis2t, a multicenter, randomized study comparing cyclosporine microemulsion with C2 monitoring and tacrolimus with C0 monitoring in de novo liver transplantation. *Transplantation* 2004; **77**: 1632.
43. Cantarovich M, Tzimas GN, Barkun J, Deschenes M, Alpert E, Tchervenkov J. Efficacy of mycophenolate mofetil combined with very low-dose cyclosporine microemulsion in long-term liver-transplant patients with renal dysfunction. *Transplantation* 2003; **76**: 98.
44. Pfitzmann R, Klupp J, Langrehr JM, *et al.* Mycophenolatemofetil for immunosuppression after liver transplantation: a follow-up study of 191 patients. *Transplantation* 2003; **76**: 130.

45. EB R, CS M, JR S, *et al.* Mycophenolate mofetil versus enteric-coated mycophenolate sodium after simultaneous pancreas-kidney transplantation. *Transplant Proc* 2009; **41**: 4265.
46. Sumethkul V, Na-Bangchang K, Kantachuvesiri S, Jirasiritham S. Standard dose enteric-coated mycophenolate sodium (myfortic) delivers rapid therapeutic mycophenolic acid exposure in kidney transplant recipients. *Transplant Proc* 2005; **37**: 861.
47. Fairbanks KD, Eustace JA, Fine D, Thuluvath PJ. Renal function improves in liver transplant recipients when switched from a calcineurin inhibitor to sirolimus. *Liver Transpl* 2003; **9**: 1079.
48. Bissler JJ, McCormack FX, Young LR, *et al.* Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. *N Engl J Med* 2008; **358**: 140.
49. Moro JA, Almenar L, Martinez-Dolz L, Sanchez-Lazaro I, Aguero J, Salvador A. Tolerance profile of the proliferation signal inhibitors everolimus and sirolimus in heart transplantation. *Transplant Proc* 2008; **40**: 3034.
50. Yao JC, Phan AT, Chang DZ, *et al.* Efficacy of RAD001 (everolimus) and octreotide LAR in advanced low- to intermediate-grade neuroendocrine tumors: results of a phase II study. *J Clin Oncol* 2008; **26**: 4311.
51. Yee KW, Zeng Z, Konopleva M, *et al.* Phase I/II study of the mammalian target of rapamycin inhibitor everolimus (RAD001) in patients with relapsed or refractory hematologic malignancies. *Clin Cancer Res* 2006; **12**: 5165.
52. Kappos L, Antel J, Comi G, *et al.* Oral fingolimod (FTY720) for relapsing multiple sclerosis. *N Engl J Med* 2006; **355**: 1124.
53. Maes BD, Dalle I, Geboes K, *et al.* Erosive enterocolitis in mycophenolate mofetil-treated renal-transplant recipients with persistent afebrile diarrhea. *Transplantation* 2003; **75**: 665.
54. Papadimitriou JC, Cangro CB, Lustberg A, *et al.* Histologic features of mycophenolate mofetil-related colitis: a graft-versus-host disease-like pattern. *Int J Surg Pathol* 2003; **11**: 295.
55. Lingappa VR. Gastrointestinal disease. In: Nogueira I, ed. *Pathophysiology of Disease, An Introduction to Clinical Medicine*. Columbus: Lange Medical Books/McGraw-Hill, 2003: 340–380.
56. Bjarnason I, Macpherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 1995; **108**: 1566.
57. Guttman JA, Finlay BB. Subcellular alterations that lead to diarrhea during bacterial pathogenesis. *Trends Microbiol* 2008; **16**: 535.