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ORIGINAL ARTICLE

Effects of chronic exercise on gut microbiota and intestinal barrier in human with type 2 diabetes

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ABSTRACT

BACKGROUND: Intestinal dysbiosis has been proposed as a possible contributor of the development of type 2 diabetes (T2D). Indeed, commensal fungi and opportunistic bacteria stimulate the local immune system, altering intestinal permeability with consequent leaky gut, which in turn activates systemic inflammation responsible for insulin resistance. It is also well known that chronic exercise improves glucose control and diabetes-induced damage. The aim of this study was to evaluate the role of chronic exercise on gut flora composition and leaky gut in T2D stable patients.

METHODS: Thirty clinically stable patients with T2D were studied before and after a six months program of endurance, resistance and flexibility training. Metabolic and anthropometric evaluations were carried out. Gut flora and intestinal permeability were measured in stools by selective agar culture medium and molecular biology measurements of zonulin, which is the protein that modulates enterocyte tight junctions.

RESULTS: Diabetes causes significant intestinal mycetes overgrowth, increased intestinal permeability and systemic low-grade inflammation. However, exercise improved glycemia, functional and anthropometric variables. Moreover, chronic exercise reduced intestinal mycetes overgrowth, leaky gut, and systemic inflammation. Interestingly, these variables are closely correlated.

CONCLUSIONS: Exercise controls diabetes by also modifying intestinal microbiota composition and gut barrier function. This data shows an additional mechanism of chronic exercise and suggests that improving gut flora could be an important step in tailored therapies of T2D.

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KEY WORDS: Exercise - Type 2 diabetes mellitus - Microbiota - Fungi - Dysbiosis - Zonulin.

Type 2 diabetes (T2D) is an increasingly epidemic metabolic disease which irreversibly damages many important body organs such as the kidneys, nerves and eyes. In addition, diabetes dramatically increases the risk of heart disease and bone disorders with a considerable social and economic impact.[1] Due to the socioeconomic impact of diabetes, research is actively looking at its intimate pathophysiological mechanisms responsible in order to propose innovative therapeutic strategies. Basic clinical evidence shows that both genetic and environmental factors can cause and worsen T2D.[2] Furthermore, recent research shows that low grade inflammation is

present in diabetes and is considered responsible for insulin resistance and can activate catabolic pathways, in turn causing T2D complications.[3]

Intestinal altered gut microbiota composition, or dysbiosis, has been proposed as a possible potential environmental contributor for the development of T2D.[4] Indeed, specific commensal fungi (mycetes), as well as opportunistic bacteria, can directly stimulate the gut immune system through the innate immure receptor Dectin-1. This causes local intestinal and clinically more importantly, systemic low grade of inflammation, as it allows translocation of gut microorganisms from the intestinal lumen to the blood circulation, thus stimulating an immune response.[5, 6] Recent data show local intestinal inflammation influences fundamental intestinal functions such as epithelium permeability, causing the so-called leaky gut (LG), which is characterized by modulation of intestinal intracellular tight junction.[7, 8] In addition, and particularly important in T2D, the link between endogenous glucagon-like peptide-2 and intestinal tight junction has been recently documented.[9] Evidence suggests that increased circulating and/or fecal concentrations of zonulin (Zon, haptoglobin-2 precursor), a protein that modulates the permeability of tight junctions between cell walls of the digestive tract, is also associated and is a marker of LG.[7, 8]

Nowadays, robust evidence shows that exercise plays a significant role in preventing the disease and controlling glycemia, as well as in diabetes-related organ complications. Indeed, aerobic exercise is often prescribed to prevent and manage diabetes. Data show that aerobic training increases the responsiveness of skeletal muscles to insulin by increasing the expression and/or the activity of the enzyme involved in cell glucose use. Moreover, physical activity positively influences the blood lipid profile, blood pressure and body weight by reducing cardiovascular risk and mortality in both healthy and affected people.[10] Interestingly, recent work shows that regular physical activities also influences microbiota composition and immune response.[11]

Therefore, the aim of this study is to evaluate the role of chronic exercise on gut flora composition and intestinal permeability in patients with stable T2D. The effects on host biochemical markers (including inflammatory status), functional and anthropometric measurements were also evaluated.

Materials and methods

This research project was a controlled open-label trial. It was conducted involving the Sports and Exercise Medicine Centre and the Diabetology Service, Spedali Civili di Brescia Hospital Trust, and Hospital of Montichiari, Brescia, Italy. The research protocol was approved by the Ethics Committee of the Spedali Civili and participants gave written informed consent. All experiments were performed in accordance with the Declaration of Helsinki.

Participants

To avoid confusing factors, we selected 30 male patients with a mean age of 70±2.3 years, non-smokers without nutritional supplements. The patients' diet was energy restricted to 7949 kJ (1900 kcal). More specifically, diets derived energy from 40-60% carbohydrates, 30% fat and 10-20% protein. The diets were adapted to Mediterranean style consumption with >20 g/1000 kcal fiber. Alcohol and sucrose consumption was not permitted. Daily diet administration was divided into three principal meals (breakfast, lunch and dinner) and one or two snacks in the morning and/or afternoon.

Eligibility criteria included diagnosis of T2D for at least 2 years, no need for insulin therapy, arterial hypertension and dyslipidemia controlled by statins and either ACE-inhibitors or angiotensin receptor blockers, absence of diabetes-specific complications and ischemic heart disease and the ability to perform physical activities. Exclusion criteria included endocrine disorders and inflammatory or malabsorption intestinal diseases. Moreover, subjects were not administered antibiotic, steroid, laxative, antidiarrheal and/or probiotic treatment over the previous 3 months or during the study.

Measurements

Medical examination, biochemical analyses, cardiopulmonary exercise test, anthropometric mea-

surements (Body Mass Index [BMI], waist circumference, and body composition by bioelectrical impedance analysis), gut flora and intestinal permeability were examined at baseline (T0) and after six months of supervised and tailored exercise training (T1). Biochemical analyses and cardiopulmonary exercise test (CPX) were performed as described elsewhere.[12]

The gut flora was determined as previously described.[6] Briefly, stool samples were collected with a sticker and inserted into hermetic vials with a specific medium. The microbiota were then plated using a selective agar culture medium and measured after 48 hours of incubation. The results were expressed in colony-forming units (c.f.u.) per millilitre of stool. Further proof of isolation was performed by using bacterial metabolic test on isolated organisms through the BBL Crystal™ Identification System (Becton Dickinson, Franklin Lakes, NJ, USA).

Intestinal permeability was measured as a fecal Zon concentration (ng/mL) using commercial ELISA kits (Immunodiagnostic AG, Bensheim, Germany). The normal amount of Zon in feces of healthy subject is considered to be <60 ng/mL.

Interventions

All T2D patients received standard medical care aimed at achieving optimal glycemic, lipid, blood pressure and body weight targets, as established by existing guidelines. Medication includes glucose-, lipid- and blood pressure-lowering agents and a dietary regimen prescribed by the diabetologist. No further nutritional intervention was given throughout the study. Medication was adjusted throughout the study to account for potentially reduced needs.

The training program consisted of six months of endurance, resistance and flexibility training, following the most recent guidelines of the Italian Society of Diabetology and Medical Diabetology Association (www.standarditaliani.it) and previously described in detail.[12] Training sessions were performed in a hospital-based setting and supervised by specialized personal. Physical activity was performed 3-times a week for about 90 minutes each session.

Endurance training involved cycling on mechanically braked cycle ergometers while wearing heart rate monitors, at an intensity individually prescribed according to CPX results. In the first 3 months, endurance training was performed approximately 5 bpm below the heart rate gas exchanged threshold (HR_{GET}). From the third month, training heart rates were allowed to temporary increase above HR_{GET}, gradually reaching but not exceeding the heart rate ventilator compensation point (HR_{VCP}) in an interval-training fashion. Time per session was increased progressively in the first 3 months, starting from 15 minutes and reaching the target of 35 minutes.

Resistance training consisted of 40 to 50 minutes of various exercises involving the major muscle groups (upper limb, lower limb, chest, back and core). Exercises consisted both in calisthenics and repetitions with ankle weights, dumbbells and elastic bands. Subjects began with 3 sets of 8 repetitions, then progressively improved to 3 sets of 12-15 repetitions. For exercises requiring dumbbells, weights started from 1-3 kg and were increased up to 2-6 kg depending on the subject and type of exercise.

Flexibility training consisted of static stretching exercises that involved upper and lower body, before and after the resistance training sessions.

Statistical analysis

Baseline to end-of-study changes (expressed as mean and standard deviation) were analyzed using Student's *t*-test for paired samples. To assess the statistical significance of differences between groups at each time point we used Student's t-test for independent samples. The relation between variables were assessed by using a simple regression analysis. A value of $P<0.05$ was considered statistically significant.

Results

The anthropometric, body composition and biochemical function of patients are shown in Table I and II. Untrained T2D patients had a high BMI (29.8 ± 3.7 kg/m^2), altered glycemic control and low levels of systemic inflammation with normal renal function and visceral proteins concentration. They also had dysbiosis with reduced fecal

TABLE I.—*Anthropometric values and body composition before (T0) and after (T1) chronic exercise training.*

Parameters	T0 (N.=30)	T1 (N.=30)	*t*	P value
Weight, kg	90.82±14.3	83.1±12.9*	2.196	0.032
Height, cm	174.3±2.6	175.12±2.9*	2.559	0.013
BMI	29.89±3.7	27.07±2.8**	3.329	0.002
Waist circumference, cm	105.22±10	99.47±8.06*	2.459	0.017
Fat mass, %	27.67±5.15	24.17±4.04**	2.929	0.005
Lean mass, %	72.47±5.19	75.83±4.04**	2.809	0.007

Data are expressed as mean±SD.
BMI: Body Max Index.
*P<0.05 and **P<0.01 by Student's *t*-test.

TABLE II.—*Biochemical and functional measurements before (T0) and after (T1) chronic exercise training.*

Parameter	T0 (N.=30)	T1 (N.=30)	*t*	P value
Glucose, mg/dL	144.9±11.74	128.53±12.07*	5.325	0.000
HbA_{1c}, %	7.10±0.65	6.9±0.87	1.009	NS
HOMA index	4.32±0.9	3.83±0.78*	2.253	0.028
CRP, mg/dL	6.89±1.02	5.23±1.08**	6.121	0.000
Triglycerides, mg/dL	149±85.7	129±55.24	1.074	NS
Total cholesterol, mg/dL	168.13±9.8	159.38±19.18*	2.225	0.03
LDL cholesterol, mg/dL	88.63±16.1	83±13.3	1.477	NS
HDL cholesterol, mg/dL	49.63±19.3	52.13±17.35	0.528	NS
WBC, $\times 10^3$/uL	7.8±1.89	7.57±1.72	0.493	NS
Hemoglobin, g/dL	14.16±0.94	14.06±0.73	0.460	NS
Creatinine, mg/dL	0.91±0.23	0.93±0.25	0.322	NS
AST, U/L	27.75±11.82	24.88±9.23	1.048	NS
ALT, U/L	38.63±20.58	32.25±20.5	1.203	NS
Total protein, g/dL	7.38±0.45	7.28±0.34	0.971	NS
VO_{2max}, mL/min/kg	16.87±2.74	19.43±2.69*	3.652	0.043
Watts	152.67±17.54	170.23±17.53**	3.812	0.000

Data are expressed as mean±SD.
HbA_{1c}: glycate hemoglobin; CRP: C-reactive protein; WBC: white blood cells; AST: aspartate transaminase; ALT: alanine aminotransferase; NS: not statistically significant.
*P<0.05 and **P<0.01 by Student's *t*-test.

Lactobacillus spp. (103.7±65.5 $\times 10^5$ CFU/mL) compared to normal values (>150 $\times 10^5$ CFU/mL), the presence of pathogenic gut flora with low or occasionally presence of *Campylobacter* spp., *Salmonella* spp., *Shigella* spp., and *Yersinia enterocolitica*, and more importantly, massive concentrations of mycetes and *Candida albicans* (Figure 1), which in healthy subjects should not be present in the stool. The number of microbiota CFU in feces of T2D patient are shown in Table III. In addition, these patients had higher fecal Zon concentrations compared to the normal value of healthy subjects, suggesting the presence of leaky gut (Table III, Figure 1).

Chronic physical activity significantly reduced body weight, BMI, fat mass and waist circumference and increased lean mass of T2D patients (Table I). As expected, chronic exercise decreased glycemia, HOMA index and total cholesterol, whereas increased oxygen consumption and watts produced. Moreover, exercise reduced systemic inflammation measured as CRP. The biochemical and functional measurements after training are summarized in Table II.

Interestingly, chronic training program had a significant effect on the composition of gut flora. Specifically, chronic exercise significantly reduced the amount of mycetes and *Candida albicans* without modifying the presence of *Lactobacillus* spp., *Bifidobacterium* spp., and/or *Streptococcus* spp. Moreover, exercise also significantly reduced Zon concentrations (Table III, Figure 1).

It is interestingly to note that the amount of mycetes, Zon, and systemic inflammation evaluated as CRP, strongly correlated both before and after training. This would suggest a link between gut mycetes, intestinal barrier function and consequent systemic inflammation (Figure 2).

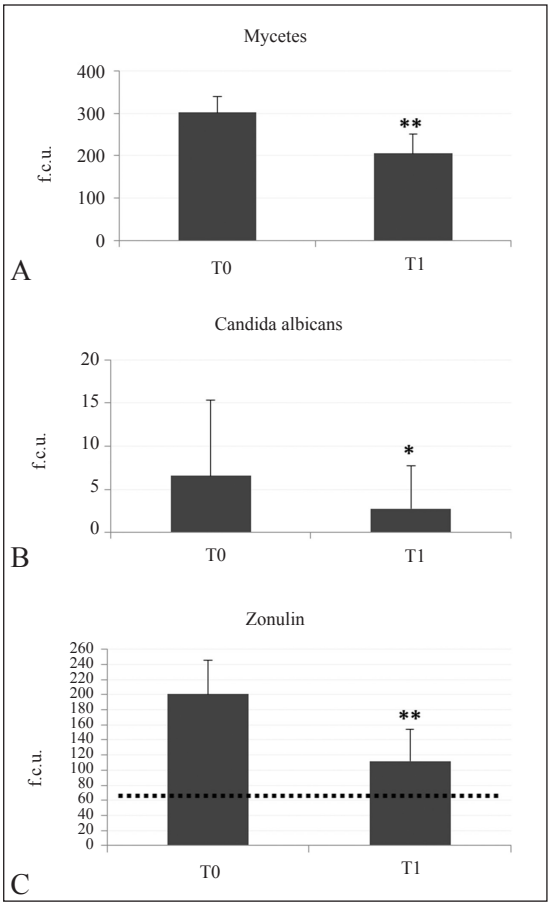

Figure 1.—Concentration of (A) Mycetes, (B) *Candida albicans* and (C) Zonulin in stool before (T0) and after (T1) chronic exercise training. Normal value: below the dotted line.
*P<0.05, **P<0.01 by Student's *t*-test.

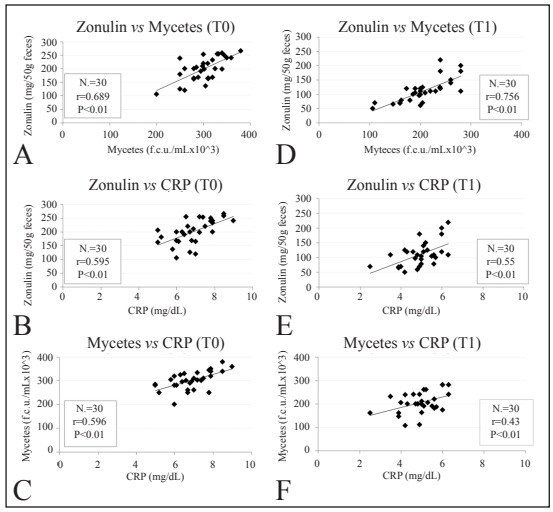

Figure 2.—Correlation between fecal concentration of zonulin, mycetes, and systemic inflammation (CRP), before (T0) (A-C) and after (T1) (D-F) chronic exercise training.

Discussion

This study found that patients with T2D had intestinal overgrowth of mycetes and altered intestinal barrier functions. These data are concomitant with scarce glycemic control and increased systemic inflammation. Chronic exercise significantly decreased mycetes in the gut flora and improved leaky gut. Moreover, exercise increased lean mass, reduced fat mass and inflammation with better glycemic control and physical performance.

TABLE III.—*Gut flora composition before (T0) and after (T1) chronic exercise training.*

Genus/species (×10⁵ CFU/mL)	Normal value	T0 (N.=30)	T1 (N.=30)	*t*	P value
Lactobacillus spp.	>150	103.7±65.5	122±97.9	0.851	NS
Bifidobacterium spp.	>200	211.8±58	198.7±88.6	0.678	NS
Enterococcus spp.	<150	0.6±2.2	0	1.494	NS
Streptococcus spp.	<150	0	0	–	–
Bacteroides spp.	>150	153.1±87.3	174.3±56	1.120	NS
Escherichia coli	<150	175.5±95.6	146.2±85.5	1.251	NS
Candida albicans	<5	6.57±8.8	2.77±4.92*	2.064	0.043
Mycetes spp.	<200	301.6±38.76	205±45.24**	8.876	0.000
Campylobacter spp.	0	3.2±7.2	3.7±8.6	0.244	NS
Clostridium difficile	<50	1.0±3	2.5±6.6	1.133	NS
Salmonella spp.	0	8.1±28	6.5±6.6	0.305	NS
Shigella spp.	0	16±31.6	18.3±22.3	0.326	NS
Yersinia enterocolitica	0	22.33±39.8	28.1±40.4	0.560	NS

Data are expressed as mean±SD.
CFU: colony-forming units; NS: not statistically significant.
*P<0.05 and **P<0.01 by Student's *t*-test.

Pathogenic gut flora overgrowth and intestinal permeability

Recent studies in T2D patients showed that gut microbiota is modified and different ratio of *Firmicutes* to *Clostridium* spp., *Lactobacillus* spp., and *Clostridum* spp. correlated with T2D.[13] However, these data are partial, and the differences observed may be due to different ethnicity, age and/or gender. In addition, the mechanisms responsible for dysbiosis, as well as the mechanistic link between altered gut flora and diabetes, is not yet clear.

Interestingly, we found intestinal dysbiosis with reduced concentrations of *Lactobacillus* spp. and the massive presence of mycetes in T2D patients. These data agree with previous recent results showing the massive presence of mycetes (predominantly *Candida albicans*) in patients with T2D.[14] We suggest that fungi intestinal colonization is probably secondary to diabetes and not its cause. Indeed, elevated glycemia might create specific local conditions which favors intestinal mycete colonization, as confirmed by studies which show a correlation between blood glucose concentration and mycete colonization.[15] However, we have to consider that mycetes might also stimulate global inflammation, present in diabetes and which itself causes metabolic disarrangement including scarce control of glycemia.

Indeed, it has been documented that mycetes interact with the innate immune receptor Dectin-1. Dectin-1 is a C-Type lectin receptor which recognizes B-1,3 glucans present in the majority of fungal cell walls. These receptors stimulate intracellular caspase signaling, which causes pro-inflammatory cytokines production and activation of lymphocyte T helpers.[16]

Interestingly in our study, the presence of mycetes was associated and correlated with more low-grade inflammation as documented by the elevated CRP. It is well known that low grade inflammation is responsible for insulin resistance. Indeed, inflammatory cytokines counteract muscle insulin actions. For instance, TNF-α induce insulin resistance inactivating insulin receptor substrate-1 by receptor serine phosphorylation.[17] In addition, activation of the innate immune system could cause inflammation of intestinal enterocytes with altered function of the intestinal barrier and fungi translocation from the intestinal lumen to the patient's blood, thus amplifying the immune response.

From our data, we cannot say whether mycete colonization is the cause or consequence of T2D. We believe that the truth lies somewhere in the middle. Probably, mycetes dysbiosis alone does not cause T2D. However, it may contribute to perpetuate and amplify inflammation which does influence T2D. Other interesting data obtained from our analysis, was the higher concentrations of Zon in feces of our T2D patients. Zon is the protein that modulates integrity of tight junctions (TJ) between enterocytes, and consequently influences the anatomical and functional status of intestinal cells including digestive, absorptive, neuroendocrine and immunological activity. Particularly important is the role of TJ in regulating intestinal permeability, which is fundamental to allow molecular trafficking between the gut lumen and blood through the para-cellular space.

High concentrations of fecal Zon suggest severe alteration of intestinal permeability, also called LG. This condition not only causes the impairment of physiological intestinal functions but also allows translocation of gut microorganisms from the intestinal lumen to the circulating blood, stimulating the immune response found in our patients.[7, 8, 16]

Taking our data together, we showed that mycetes overgrowth in T2D patient microbiota was concomitant with both massive increases of Zon, indicating the presence of LG, and a low grade of systemic inflammation influencing glucose

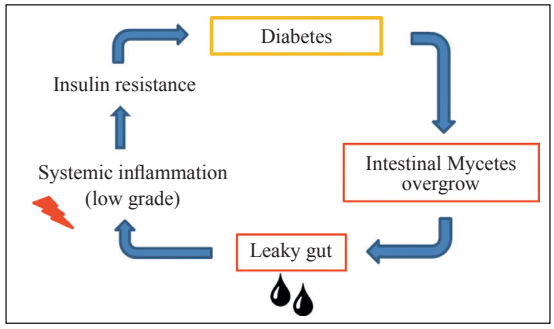

Figure 3.—The vicious circle that binds diabetes to the alteration of the gut microbiota, to the increase of intestinal permeability, to chronic inflammation and therefore to insulin resistance.

metabolism that generates intestinal mycetes overgrowth, thus creating a vicious circle. This pathophysiological hypothesis is suggested by the significant correlation between mycetes, Zon, and low systemic inflammation that we found as illustrated in Figure 3.

Effects of chronic exercise

Nowadays, it is known that exercise controls glycemia and inflammation, increases physical performance and modifies the body composition of T2D patients.[10] Here, we showed that exercise modified dysbiosis by also reducing intestinal mycete colonization and LG, without modifying the concentrations of other microorganisms. We do not know whether these improvements are due to enhanced diabetes control or whether they occurred independently. Interestingly, many studies performed in animals and a few in humans, show that exercise influences both immunological gut function and microbiota in both T2D and healthy subjects.[11, 18]

The mechanisms by which exercise influences gut flora is not yet fully understood. However, some data suggest that several factors may explain why exercise could influence microbiota. Animal data show that exercise may modify bile acid profiles.[19] These compounds are knows to have some anti-microbial properties influencing the composition of intestinal flora. These observations are also supported by significant changes in microbiota profile induced by supplementation with colic acid.[20]

Other experimental research shows that physical activity may modify fecal short-chain fatty acids (SCFAs), which increases the presence of fecal butyrate and in turn butyrate-producer intestinal bacteria.[21] Interestingly, SCFA activate muscular AMPK, an enzyme which regulates muscle metabolism of glucose and lipids. These metabolic effects may be important in diabetes and confirm cross-communication between microbiota, exercise and global metabolism.[22]

Moreover, exercise may influence gut immunological function. Animal studies show that long-term moderate physical activity increases the intestinal presence of immunoglobulin A (IgA), and decreases lymphocytes-B and $CD4^+$ T cells influence over gene expression of cytokines, such as IL-6, Il-4, IL-10, and TGF-B, involved in IgA production.[23] These modifications increase mucosal immunity that can counteract intestinal pathogen colonization.

It is also interesting to consider the possible indirect influence of exercise on gut flora and intestinal permeability. Previous data show that exercise stimulates muscle myokine release, which increases glucose muscle metabolism by AMPK activity and reduces body inflammation.[24] Body inflammation influences enterocytes' metabolic homeostasis, which in turn influences both intestinal permeability and microbiota composition. Exercise also causes weight loss. Human data show that microbiota composition varies between obese and non-obese subjects, although it is not known whether weight loss influences microbiota or if gut flora induces weight loss.[25] Our data are in line with previous observations and indicate that even in patients with T2D, exercise is an effective strategy to support conventional therapies improving patient quality of life.

Lastly, but not less important, exercise shortens intestinal transit time (ITT).[26] Interestingly, human data show that ITT changes microbiota composition, which is probably related to modified intestinal water and/or nutrient availability. Exercise might also influence the hypothalamic-pituitary-adrenal axis. It is believed that exercise-induced hormone-release may influence, gastric secretion, mucus production, mesenteric blood flow and intestinal cells metabolism influencing microbiota changes.[27, 28] Taken together, these data suggest a link between microbiota, muscle, the brain and human metabolism, which can be influenced by exercise.

Clinical implications

We believe that our results have practical clinical implications. For the first time, we demonstrated that patients with T2D have dramatic intestinal mycete colonization, LG, and increased systemic low-grade inflammation, which are all interrelated (Figure 3). In addition, we showed that exercise reduces both mycete gut colonization and the presence of LG, probably allowing better intestinal function, which influences nutrient metabolism, hormonal production and the absorption of oral administered drugs.

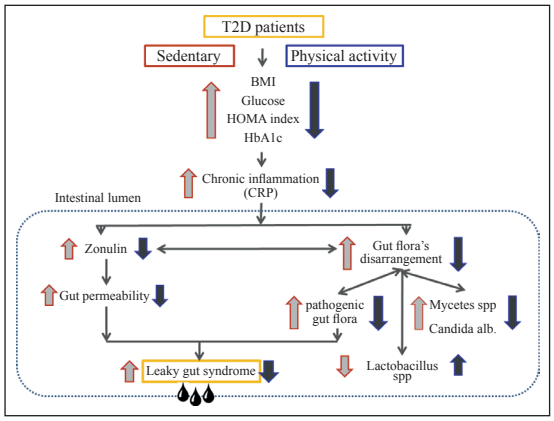

Figure 4.—The influence of physical activity on gut flora and zonulin concentration in sedentary (grey arrows) and trained (black arrows) T2D patients.
Downward arrows: decrease; upward arrows: increase.

The cure of intestinal microbiota with physical exercise and/or specific therapies, could be an important step for tailored therapy allowing traditional therapy and global patient metabolism to function properly. A schematic representation of the influence of physical activity on gut flora and Zon concentrations in T2D patients is shown in Figure 4.

Limitations of the study

This study has some limitations. Firstly, we used a selective culture medium to identify bacteria and mycetes rather than molecular biology techniques. We did not want to provide a "fecal fingerprint" of T2D patients. Our aim was to identify saprophytes and minor intestinal pathological and mycete species able to activate immunological responses without any evidence of gastrointestinal symptoms using a simple, universally available and cheap technique.

Secondly, we did not measure circulating inflammatory molecules such as TNF-α and/or IL-1α or IL-6, but we measured CRP. Indeed, CRP is the balance between pro-inflammatory and anti-inflammatory molecules and it also provides accurate information on inflammatory body status.[6]

Conclusions

Exercise may help control diabetes by modifying intestinal microbiota composition and gut barrier function. Our data show an additional mechanism of chronic exercise and suggests that improving gut flora is an important step in tailored therapies of T2D.

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