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ORIGINAL ARTICLE

Effect of mTOR inhibitor on body weight: from an experimental rat model to human transplant patients

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Summary

The aim was to study the influence of sirolimus (SRL) on body weight in a rat model and in kidney transplant patients. Wistar rats (15 weeks old) were either treated with vehicle (VEH; $n = 8$) or SRL ($n = 7$) 1.0 mg/kg three times per week for 12 weeks. Body mass and food intake were measured weekly. Adipocyte diameter was determined in hematoxylin–eosin stains. The body mass index (BMI) obtained from clinical kidney transplant trials comparing SRL-based with cyclosporine-based therapy was analyzed. Animals: SRL produced a decrease of the weight gain curve. At the end of the study, mean body weight in the SRL group was lower than in the VEH group (356 vs. 507 g, $P < 0.01$) in spite of comparable food intake normalized for body weight was not different. Mean adipocyte diameter was 36 μm in VEH and 25 μm in SRL rats ($P = 0.009$). Mean SRL blood trough concentration was 38 ng/ml. Kidney transplant patients: Two years after transplantation, BMI was significantly lower in the SRL-based treatment arm compared to cyclosporine (24.17 ± 2.99 vs. 25.97 ± 5.01 kg/m^2, $P = 0.031$). SRL treatment leads to less body mass. Adipocyte cell diameter was reduced in SRL-treated animals. A possible explanation may be the effects of SRL on metabolic regulation and cell growth.

Introduction

The use of immunosuppressive drugs, especially steroids, in transplant patients is often associated with an increase of body weight [1]. Furthermore, the prevalence of obesity increases from 19% to 36% at 1 year after transplantation [2,3]. Obesity increases the risk of new-onset diabetes mellitus after transplantation and the risk of cardiovascular morbidity, which itself is the most important cause of death after kidney transplantation.

The mammalian target of rapamycin (mTOR) inhibitors are structurally similar to tacrolimus and are used as immunosuppressive agents in transplant medicine. They block the cell cycle progression and inhibit lymphocyte proliferation. mTOR is a nutrient sensor and a

crucial key regulator for the signal pathways of factors of cellular growth and metabolism the p70 ribosomal S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) [4]. Both are implicated in protein synthesis. Furthermore, mTOR is a regulator of transcription by either inhibiting or activating cellular processes [5,6].

The hyperactivation of mTOR that is produced by chronic exposure to platelet-derived growth factor (PDGF), tumor necrosis factor-alpha (TNF-α), insulin, amino acids or fatty acids, which can lead to desensibilization to insulin and insulin resistance. Increased mTOR activity and subsequent over-activation of S6K1 can exert a negative feedback mechanism by phosphorylation of the insulin receptor substrate (IRS)-1, which can negatively influence the signaling of the PI3-K/Akt pathway [4,7]. The possible blockade of IRS-1 provokes a reduction of the translocation of the glucose transporter 4 (GLUT-4) to the cellular membrane in adipose and muscular tissue, which itself leads to hyperglycemia [8].

Mammalian target of rapamycin is an essential factor for adipocyte differentiation [9] that activates PPAR-γ [10], which is a transcription factor that plays a critical role in adipogenesis, accumulation of lipids. The mTOR pathway is overactivated in the liver and muscles of obese rats [11,12]. Treatment with rapamycin (sirolimus, SRL), an mTOR inhibitor, produces phosphorylation in the Thr308 and Ser473 of Akt, which is a downstream target for PI3K. Insulin induces the activation of Akt through the phosphorylation of both residues in HepG2 cells; while the insulin produces a temporary increase of Thr308 phosphorylation, the effect of the hormone maintains an increase for 30 min in the Ser473. Rapamycin preconditioning increases the phosphorylation of Akt in both places and a slow decrease of the dephosphorylation of Thr308. The inhibition of the mTOR pathway through rapamycin increases the signaling of Akt in HepG2 cells [12]. However, the absence of S6K1 in a knock-out mice model provokes hypoinsulinemia and hypersensitivity to insulin. Besides, the S6K1−/− genotype protects from obesity induced by nutrients [13].

In some patients and in experimental models, treatment with SRL has been observed to be associated with a lower body mass when compared with other immunosuppressive drugs such as calcineurin inhibitors (CNIs); however, so far no systematic study of this phenomenon has been published. The main objective of this study was to analyze systematically the potential effects of mTOR inhibitor treatment on body mass and adipocyte cell diameter in a rat model and to verify if similar effects were observed in kidney transplant patients receiving SRL-based therapy.

Materials and methods

Animals

Male Wistar rats (Charles River Laboratories España, Barcelona, Spain) weighing approximately 225 g were kept at constant temperature and humidity with a 12-h light/dark cycle. The animals had free access to standard rat chow (Harlan Interfauna Ibèrica, S.L., Barcelona, Spain) and water.

This study was approved by and conducted according to the guidelines of the local animal ethics committee (Local animal studies ethics committee, Decret 214/97).

Experimental design

Male Wistar rats at 15 weeks of age were distributed between two groups according to intraperitoneal administration of vehicle (VEH; $n = 8$) or SRL ($n = 7$) 1 mg/kg three times per week, during 13 weeks. The animals were housed in metabolic cages; body weight, food intake and water intake were determined weekly.

After 10 weeks of treatment with SRL, rats were kept without food for 12 h overnight before measuring serum insulin, glucose, cholesterol and triglycerides. Insulinemia was measured using the RIA kit INSULIN-CT from CIS bio international (Gif-sur-Yvette, France). Glucose, cholesterol, and triglycerides levels were measured using standard methods in the central laboratory of the Hospital Clínic Barcelona.

Sirolimus concentrations

Sirolimus whole blood trough concentrations were measured using a high-performance liquid chromatography assay coupled with a triple quadrupole mass spectrometer (Quatro Micro, Micromass, Waters, Milford, MA, USA) with a limit of detection of 1 ng/ml and a linearity from 1 to 75 ng/ml. Blood samples for these measurements were obtained at the time of killing, 12 h after the last dose of SRL.

Adipocyte histology

At the end of the study, animals were killed and periepididymal adipose tissue samples were harvested, fixed in formalin, and embedded in paraffin by routine methods. Hematoxylin–eosin (HE) staining was performed. For each animal 50 randomly selected adipocytes were evaluated.

Histological analysis was performed using a laboratory upright microscope (Zeiss Axiolab, Oberkochen, Germany) and camera RT-KE Color Mosaic (Diagnostic instruments, Inc., Sterling Heights, MI, USA). Diameters were measured using specific software SPOT 4.6 (Diagnostic Instruments,

Inc.) and Image-Pro® PLUS (Media Cybernetics Inc., Bethesda, MD, USA).

Tibial length

After killing the animals, tibial length was determined using a sliding caliper.

Patients

Patient data on body weight was obtained from a pooled data analysis of two controlled, randomized studies comparing de novo kidney transplant patients who received either a SRL-based or a cyclosporine (CsA)-based treatment, both in combination with antimetabolites (either azathioprine or mycophenolate mofetil) and steroids. The principal results of these studies were published by Groth *et al.* [14] and Kreis *et al.* [15].

Statistical analysis

Statistical analysis was performed using the SPSS 14.0 statistics package (Microsoft, Redmond, WA, USA). Values are given as mean ± standard deviation. The Mann–Whitney *U*-test, ANOVA, or ANCOVA were used as applicable.

Results

Results of the rat model

No animal died as a result of drug treatment during this study, and the physical aspect of the animals remained good during the entire study period, in both the SRL treatment and the vehicle group. The tibial length was not significantly different between the VEH and SRL groups (49 ± 4 and 47 ± 1 mm, respectively). The whole blood trough SRL concentrations were 38.8 ± 7.9 ng/ml in the SRL group, at the end of study.

During treatment with SRL, animals in the SRL group did not gain weight and consequently at the end of the study this group presented with 30% less weight ($P < 0.01$) when compared with the VEH group, which continued to have normal weight gain (Fig. 1). Throughout the study, the animals of the SRL group consumed the same or higher amount of food and water (Fig. 2).

Glycemia measured after 10 weeks of treatment was significantly lower in the SRL group (Table 1), whereas insulin concentrations were unaltered. Animals treated with SRL presented higher cholesterolemia but no significant differences in triglyceridemia at the end of the study (Table 1). Table 2 shows the hepatic function parameters, AST and ALT, which did not differ between the SRL and VEH groups. The adipocyte cell diameter was 36.6 ± 6.3 μm in VEH and 25.0 ± 1.8 μm in SRL rats ($P = 0.009$). HE stains of adipose tissue are shown in Fig. 3.

Results from kidney transplant trials

The mean body mass index (BMI) of the SRL-treated patients was significantly lower than that of the CsA-treated patients at all time points after transplantation up to 2 years (Table 3). Interestingly, the SRL-treated patients had better renal function at all time points after transplantation in both the studies.

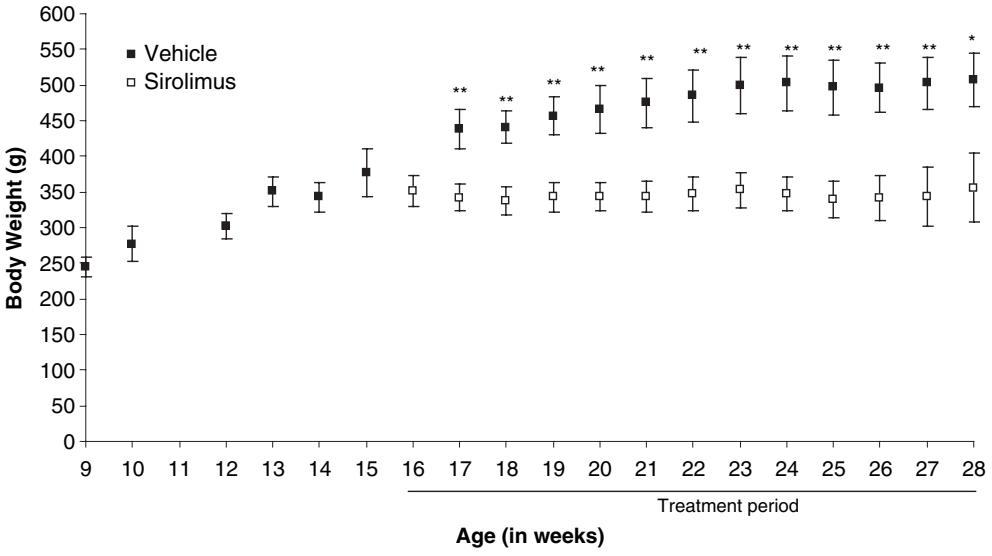

Figure 1 Effect of sirolimus treatment on body weight. Significantly different versus vehicle animals, $*P < 0.05$; $**P < 0.01$.

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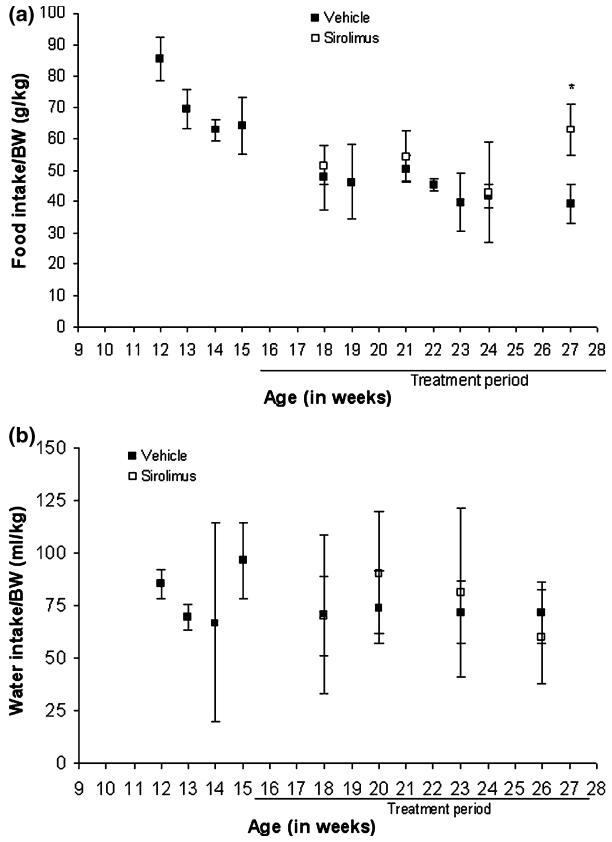

Figure 2 Effect of sirolimus treatment on food intake normalized for body weight (a) and water intake normalized for body weight (b). Significantly different versus vehicle animals, $*P < 0.05$.

Table 1. Effects of sirolimus on biochemical parameters after fasting for 12 h overnight.

	Group	Mean ± SD	*N*	*P*-value
Insulin (ng/ml)	Vehicle	0.73 ± 0.20	8	NS
	Sirolimus	0.77 ± 0.21	7	
Glucose (mg/dl)	Vehicle	137.9 ± 25.2	8	0.018
	Sirolimus	104.0 ± 11.3	7	
Cholesterol (mg/dl)	Vehicle	86.3 ± 15.5	8	0.04
	Sirolimus	114.6 ± 9.6	7	
Triglycerides (mg/dl)	Vehicle	114.6 ± 40.5	8	NS
	Sirolimus	96.4 ± 20.9	7	

Insulin, glucose, cholesterol and TG serum levels are at 15 weeks of treatment.

Table 2. Effects of sirolimus on liver enzymes, AST – ALT (IU/l).

	Group	Mean ± SD	*N*	*P*-value
AST	Vehicle	169.4 ± 26.0	8	NS
	Sirolimus	184.6 ± 79.8	5	
ALT	Vehicle	46.6 ± 6.2	8	NS
	Sirolimus	48.1 ± 17.8	7	

Discussion

This is the first study evaluating the influence of mTOR inhibition on the evolution of body weight in an animal model independent of association with any other pathophysiologic condition (e.g. renal insufficiency). The animals in our model were healthy and received no further intervention except for treatment with SRL. In our model, we showed that prolonged SRL treatment attenuated the otherwise physiologically normal gain of body weight [16]. Surprisingly, this alteration could not be attributed to a change in the amount of food or water intake, because no difference of food or water intake normalized for body weight could be detected. In human studies gastrointestinal side-effects of SRL namely diarrhea were observed. These side-effects could be treated with SRL dose reduction [17]. In our study in rats, diarrhea was not observed. Bravo *et al.* [18] did not observe differences of body weight after mycophenolate mofetil treatment during 14 weeks in an experimental rat model. Although we did not observe an influence of SRL on bone length, Alvarez-Garcia *et al.* [19] identified an influence a bone growth, an explanation for this could be that in their study, SRL was introduced at an earlier stage of bone growth. In a qualitative analysis of tibialis anterior muscle using HE stains, no morphologic differences could be observed (data not shown). In order to exclude hepatic damage induced by the drug as a possible reason for altered metabolism, we analyzed the hepatic enzymes, but did not find any significant differences between the treatment and control groups. Nevertheless it is well known that SRL treatment can be associated with slightly elevated liver enzymes in humans [14].

Mammalian target of rapamycin inhibitors have been used in other rat models, and in some of the studies, alterations of body weight have been reported [20–22]. However, in all of these studies weight was analyzed in diseased animals, and none of these studies evaluated the amount of food intake.

Therefore, the alteration of body weight could be attributed to a metabolic alteration such as a change in energy intake. Indeed, an increased metabolic rate was observed by Um *et al.* [13] in a S6K1 knock-out mouse model. These animals did not develop obesity in spite of being fed a high-fat diet. In our rat model, the mTOR inhibition might have shown a similar phenotype as the S6K1 knock-out. In addition, Le Bacquer *et al.* [23] demonstrated that increased activation of the eIF4E in mice, which lacked the 4E-BP1 and 4E-BP2 proteins that bind and inhibit eIF4E, caused the mice to be more sensitive to diet-induced obesity. The inhibition of mTOR would also result in reduced activation of eIF4E, which is situated downstream of mTOR, and thus might have

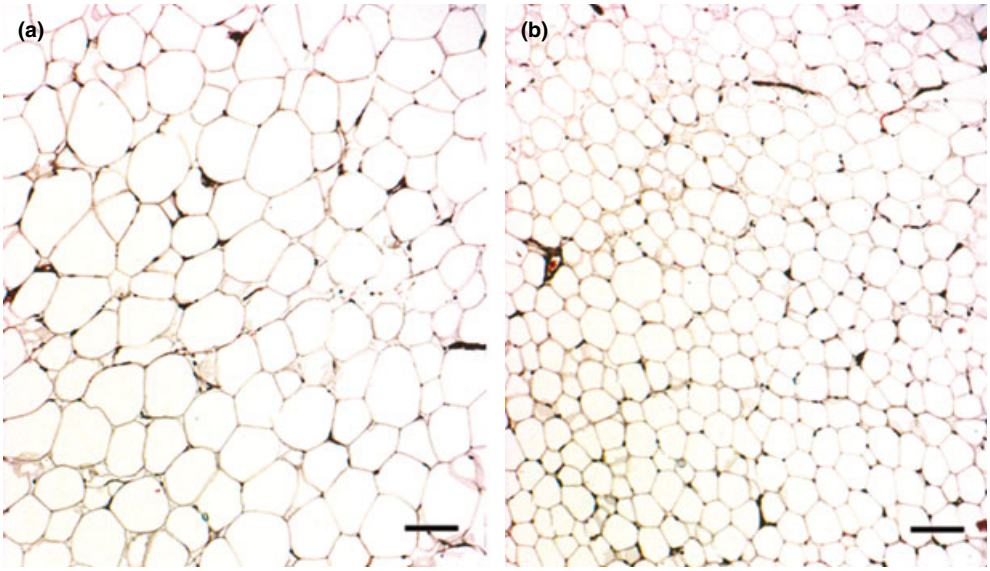

Figure 3 HE stains of adipocytes in VEH (a) and SRL (b) animals. Magnification are 100×, Metering bar corresponding 50 μm. SRL animals present a significantly lower adipocyte diameter compared with vehicle animals ($P = 0.009$).

Table 3. Body mass index (kg/m^2) in all randomized kidney transplant recipients On-Therapy data.

	Baseline		Value		Change from baseline		ANCOVA
	Ctl	SRL	Ctl	SRL	Ctl	SRL	*P*-value
Baseline							
N			79	81			
Mean ± SD			23.97 ± 4.06	23.66 ± 3.48			
Median (Min–Max)			24.00 (15.4–32.7)	23.40 (15.7–33.0)			
Month 1							
N	65	68	65	68	65	68	0.004
Mean ± SD	23.95 ± 4.22	23.22 ± 3.31	23.85 ± 4.39	22.54 ± 3.44	−0.11 ± 1.10	−0.68 ± 1.14	
Median (Min–Max)	23.70 (15.4–32.7)	23.15 (15.7–31.1)	24.10 (14.1–36.1)	22.10 (15.2–30.7)	0.00 (−2.8–3.4)	−0.65 (−3.1–2.5)	
Month 6							
N	60	51	60	51	60	51	0.006
Mean ± SD	23.53 ± 4.05	23.11 ± 3.06	24.64 ± 4.11	23.35 ± 3.07	1.11 ± 1.65	0.24 ± 1.85	
Median (Min–Max)	23.35 (15.4–32.4)	23.00 (15.7–29.3)	24.90 (15.6–33.0)	23.40 (16.8–31.1)	1.30 (−3.1–4.2)	0.30 (−4.2–4.1)	
Month 12							
N	53	43	53	43	53	43	0.015
Mean ± SD	23.47 ± 4.08	23.22 ± 3.10	25.24 ± 4.38	24.03 ± 3.20	1.77 ± 1.83	0.80 ± 2.08	
Median (Min–Max)	23.10 (15.4–32.4)	23.00 (15.7–29.3)	25.20 (16.8–35.9)	23.90 (16.6–32.1)	1.60 (−1.9–6.2)	0.60 (−4.5–5.1)	
Month 24							
N	43	30	43	30	43	30	0.031
Mean ± SD	23.79 ± 4.21	23.10 ± 3.19	25.97 ± 5.01	24.17 ± 2.99	2.17 ± 2.31	1.07 ± 1.80	
Median (Min–Max)	23.80 (15.4–32.4)	22.30 (15.7–29.3)	25.70 (17.5–38.7)	24.40 (17.6–29.3)	2.00 (−2.3–8.9)	0.80 (−3.3–4.2)	

an effect contrary to the effect described by Le Bacquer *et al.* [23].

One of the possible causes of the differences of body mass could be a reduction of adipocyte tissue. When growth conditions permit, rapamycin-sensitive mTOR signaling promotes anabolic processes and antagonizes catabolic processes [24]. Therefore, we measured the adipocyte cell diameter in the two groups of animals. Inter-

estingly, the adipocyte diameter was reduced in the SRL group, which may represent an increase catabolic processes or inhibition of cell growth. Rapamycin inhibits the clonal expansion and differentiation of the pre adipocytes 3T3-L1 and induces the de-differentiation of the mature adipocytes (the adipogenesis), these actions take place through the blockade of the expression and the transactivation of S6K1, 4E-BP1 and the transcription factors C/EBP-α and PPAR-γ [10].

The blockade of mTOR/S6K1 through rapamycin quickly leads to a substantial increase of glucose transport as a result of the stimulation for insulin in human and mice adipose cells. mTOR is not the only factor implicated in the establishment of insulin resistance in adipocytes 3T3-L1, but it regulates the feedback of the action of insulin, placing mTOR as a modulator of glucose transport in the signal transduction pathway of insulin [25].

Furthermore, we could show that SRL-treated rats had lower glycemia in spite of showing the same insulin concentration, thus suggesting that SRL treatment in our animals changed insulin sensitivity. Um *et al.* [13] observed a similar effect in their mice. Their S6K1 knock-out mice showed higher insulin sensitivity. They attributed this effect to the absence of the physiological negative feedback mechanism that S6K1 exerts on the IRS. Possibly, SRL treatment in our animals altered this mechanism in a way that might enhance insulin sensitivity.

Furthermore, we could show that patients with de novo SRL-based treatment showed less increase of BMI during the first 2 years after transplantation than patients with a CsA-based regimen. Certainly, these studies were not designed to primarily analyze the influence of mTOR inhibition on body weight; however, they at least show results that resemble the animal data. Diabetic nephropathy is among the leading causes of end-stage renal disease, and metabolic syndrome and insulin resistance are also frequent problems after organ transplantation. Other immunosuppressive substances used in transplantation such as steroids and CNIs further contribute to insulin resistance or β-cell toxicity [26]. Cardiovascular mortality as a possible consequence of metabolic disorders is one of the leading causes of death after kidney transplantation. Therefore, strategies to reduce insulin resistance are urgently needed in this patient population. Less increase of body mass might be part of such a strategy. Therefore, our clinical data, demonstrating less increase of body weight in patients treated with SRL is a valuable hint. However, clinical data elucidating the influence of mTOR inhibition are scarce, and especially long-term data are needed in order to evaluate if the described influence on weight gain associated with mTOR inhibition is harmful or beneficial. These data are certainly not sufficient to advocate the general use of immunosuppressive regimens that are free of CNIs and based on SRL de novo. In spite of promising results of SRL-based and CNI-free immunosuppression after kidney transplantation in single center studies in terms of graft and patient outcome, these benefits could not be confirmed after one or 2 years post-transplantation in larger multicenter trials [27].

In the rat model, mean SRL trough blood concentrations averaged at 39ng/ml. In the two trials in human renal transplant recipients [14,15] SRL trough concentrations (24 h after the daily dose) were targeted to 30 ng/ml for 2 months and 15 ng/ml thereafter. The observed mean troughs in these studies were somewhat higher than the targeted levels. While one cannot strictly compare the SRL concentration-time profiles between the animal and human studies based on this limited information, the SRL whole blood concentrations appear to be approximately the same order of magnitude. It should be pointed out, however, that SRL trough concentrations today are generally targeted to lower values than in those two early SRL-based human therapy trials.

One of the drawbacks of this study consists in the lack of a more sophisticated analysis of the different body compartments. Certainly, waist and impedance measurements as well as analysis of adipose and muscular tissue should be included in future studies.

In conclusion, our study demonstrates that treatment with mTOR inhibitors in otherwise healthy animals alters weight gain, adipocyte diameter and insulin sensitivity. Furthermore, the influence of mTOR inhibition on body weight of transplant patients resembles the observations made in animals.

Authorship

JR: design and performed research, collected and analyzed data and wrote the paper. EMA: performed research, collected data and wrote the paper. JTB and YB: analyzed human data. DMR, EBM, MJRB, AGD, IR and LFQ: collected data. JMC and FD: design research and wrote the paper.

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