Effect of the Purinergic Inhibitor Oxidized ATP in a Model of Islet Allograft Rejection

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The lymphocytic ionotropic purinergic P2X receptors (P2X1R-P2X7R, or P2XRs) sense ATP released during cell damage, thus regulating T-cell activation. We aim to define the role of P2XRs during islet allograft rejection and to establish a novel anti-P2XRs strategy to achieve long-term islet allograft function. Our data demonstrate that P2X1R and P2X7R are induced in islet allograft-infiltrating cells, that only P2X7R is increasingly expressed during alloimmune response, and that P2X1R is augmented in both alloreactive and syngeneic patients after islet transplantation. In vivo short-term P2X7R targeting (using periodate-oxidized ATP (oATP)) delays islet allograft rejection, reduces the frequency of Th1/Th17 cells, and induces hyporesponsiveness toward donor antigens. oATP-treated mice displayed preserved islet grafts with reduced Th1 transcripts. P2X7R targeting and rapamycin synergized in inducing long-term islet function in 80% of transplanted mice and resulted in reshaping of the recipient immune system. In vitro P2X7R targeting using oATP reduced T-cell activation and diminished Th1/Th17 cytokine production. Peripheral blood mononuclear cells obtained from long-term islet-transplanted patients showed an increased percentage of P2X7R+CD4+ T cells compared with controls. The beneficial effects of oATP treatment revealed a role for the purinergic system in islet allograft rejection, and the targeting of P2X7R is a novel strategy to induce long-term islet allograft function. Diabetes 62:1665–1675, 2013

The current mainstay of treatment for type 1 diabetes (T1D) is insulin therapy, which has proven to be a lifesaving breakthrough. However, insulin treatment cannot fully prevent the severe complications related to the disease, including kidney failure and coronary heart disease (1,2). Successful islet transplantation cures T1D, improves glycometabolic control, reduces hypoglycemic episodes, and halts diabetes complications (3–5). Unfortunately, the rate of functioning islet allografts at 5 years is well below 20% (6), primarily although not exclusively, due to alloreactive and autoreactive immune responses (7–9). The anti-islet immune response involves a complex interplay between pathogenic and inflammatory immune pathways, which promote rejection, and regulatory or anti-inflammatory immune pathways, which facilitate tolerance toward transplants (10–12); one such pathway is the purinergic system (13).

The purine ATP is a small molecule (14) present at high concentrations within cells and released after cell damage or death (15) and immune cell activation (16,17); it acts as a danger signal and potent chemotactic mediator (15,18). ATP is abundant at inflammation sites and is sensed by ionotropic purinergic P2X receptors (seven receptors named P2X1-P2X7, or P2XRs) (19–21). In leukocytes, P2XRs regulate cytokine production, activation, and apoptosis (thus constituting an “autocrine alerting system”) (13,22–24). In particular, P2X7R (16,21,25,26) has been linked to T-cell activation and function, serving as a signal amplification mechanism for antigen recognition (13). Recent studies have shown that ATP may be a factor that determines the fate of T cells by promoting Th17 differentiation (24), and Th17 cells have been demonstrated to be relevant in islet allograft rejection (27–29). Inhibiting P2X7R halts the delivery of ATP signals and may redirect the immune system from a Th1/Th17 profile to a more tolerogenic state (30). P2X7R inhibitors are available for human use, including periodate-oxidized ATP (oATP) and CE-224535, thereby rendering P2X7 targeting a potential path to be tested in transplantation. Of particular interest is oATP, a small Schiff-base molecule that irreversibly antagonizes P2X7R through the selective modification of lysine residues in the vicinity of the ATP-binding site (31,32).

We explored the role of P2X7R in islet allograft rejection as a means of establishing an anti–P2X7R-based strategy to redirect the immune system toward a more tolerogenic profile and thus achieving stable tolerance to islet allografts.

RESEARCH DESIGN AND METHODS

Patients. Peripheral blood mononuclear cell (PBMC) fractions were isolated by Picoll (GE Healthcare, Piscataway, NJ) from patients with T1D (n = 8) and patients who had received allogeneic islet transplantation <3 (n = 8) or >3 (n = 8) years earlier, as well as healthy control patients (n = 8). Patient characteristics are presented in Table 1.

Human islet transplantation and immunosuppression. Islet-transplanted patients received the standard triple immunosuppressive regimen: anti-Thymoglobulin (Fresenius, Waltham, MA), followed by FK506 (target blood levels, 6–8 ng/mL; Astellas, Deerfield, IL) and/or cyclosporine (100 ng/mL; Novartis, Basel, Switzerland) and/or rapamycin (8–15 ng/mL; Pfizer, New York, NY) and/or mycophenolate (2 g/day; Roche, Basel, Switzerland) and prednisone (5–10 mg/day; Bruno Farmaceutici, Rome, Italy) (33).

Mice. C57BL/6, BALB/c, and C57BL/6 P2X7−/− mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. All mice were cared for and used in accordance with institutional guidelines approved by the Harvard Medical School.

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and eBioscience. Anti-P2X7R was purchased from Alomone Laboratories (38). V, 7-amino-actinomycin D, and FoxP3 were purchased from BD Biosciences human CD3, CD4, CD45RO, CD45RA, CD8, CD25, CD44, CD62L, IL-17, annexin analyzer (Cellular Technology Ltd, Cleveland, OH). (BD Biosciences, San Jose, CA) (37). Spots were counted using an Immunospot bent spot (ELISPOT) assay was used to measure the number of cells producing technology (Millipore, Billerica, MA).

Interventional studies. Mice were injected intraperitoneally with 1) oATP (Medestea Srl, Turin, Italy) at 250 µg/day for 14 days, 2) rapamycin (LC Laboratories, Woburn, MA) at 0.1 mg/day for 10 days, or 3) oATP-rapamycin at the combination of 1 and 2. oATP, Benzoyl-ATP (Sigma-Aldrich), NF449 (P2X1R antagonist), and NF110 (P2X3R antagonist; Tocris, Minneapolis, MN) was de was measured after 3 days following pulsing with [3H]TdR (Perkin Elmer, Wellesley, MA), and cell counts were quantified using a liquid scintillation counter.

Luminex assays. Serum was collected from treated or control mice and analyzed using the Beadlyte Mouse Multi-Cytokine Beadmaster Kit (Millipore, Billerica, MA).

ATP level determination. Bioluminescent detection of ATP levels was performed in serum samples using the Enlighten ATP assay system (FF2000, Promega Corporation, Madison, WI).

Statistical analysis. Data are expressed as mean ± SEM. Kaplan-Meier analysis was used for analysis of survival. When two groups were compared, a two-sided unpaired Student t test (for parametric data) or a Mann-Whitney test (for nonparametric data) was used. A P value < 0.05 (by two-tailed testing) was considered an indicator of statistical significance. Graphs were generated using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

RESULTS

P2X7R is specifically expressed in islet allograft-infiltrating lymphocytes during the alloimmune response in vivo. We first evaluated the expression of P2X7R mRNA during the alloimmune response in vivo in an islet transplantation model. BALB/c (H-2b) islets were transplanted under the kidney capsule of C57BL/6 (H-2b) mice, rendered diabetic through streptozotocin, and islet allografts and spleens were harvested at day 14, according to the kinetics of islet allograft rejection (39). P2X7R expression levels were compared with baseline values using untransplanted BALB/c islets or naïve C57BL/6 splenocytes. A significantly higher expression of P2X1R (10-fold increase vs. baseline, n = 3; P = 0.0002) and P2X7R (10-fold increase vs. baseline, n = 3; P = 0.01) was evident in the graft (Fig. 1A). No changes were observed in the expression of the remaining P2XR in the graft (Fig. 1A) or in any P2XR in the spleen (data not shown).

To explore the possible effects of ischemia/reperfusion injury and peritransplant inflammation on P2X7R expression levels, we evaluated P2X7R expression in the setting of syngeneic islet transplantation—C57BL/6 islets into hyperglycemic C57BL/6 mice—in which the effects of the alloimmune response are absent. Day 14 after transplantation, P2X1R expression was increased in the syngeneic graft compared with baseline (14-fold increase, n = 3; P = 0.008), similarly to what we observed in allogeneic
We performed immune profiling of islet-transplanted oATP-treated and untreated mice at day 14 after transplantation. When splenocytes harvested from oATP-treated recipients were challenged with donor-derived splenocytes, a reduction was observed at day 14 in the number of cells producing IFN-γ (oATP-treated = 117 ± 9 vs. untreated = 418 ± 26, n = 5; P < 0.0001; Fig. 2B), with no effect on the number of cells producing IL-4 (Fig. 2C). No significant changes in the percentages of peripheral CD4+ T effector cells were observed between groups (CD4+FoxP3+CD62LlowCD4+ T cells; Fig. 2D). Slight reductions were evident in the percentages of CDS+ T effector cells (Fig. 2E) and of Th17 cells (Fig. 2F). No differences were observed in the percentages of regulatory T-cells (CD25+FoxP3+CD4+ T cells, or Tregs; Fig. 2G). The cytokine profile of sera showed that IL-6, a Th17 cytokine, was reduced in oATP-treated recipients (Fig. 2K), whereas only minor changes were observed for all other cytokines examined (Fig. 2H–J, L and M).

**Targeting P2X7R in vivo reduces infiltration and Th1 transcripts in islet allografts.** We next analyzed the pathology of islet allografts 14 days after transplantation. Iset structure was substantially compromised, and insulin or glucagon staining were undetectable in untreated mice (Fig. 3A, E and F, respectively), with diffuse infiltration primarily constituted by CD3+ T cells and MAC2+ macrophages, with very few FoxP3+ cells (Fig. 3A–D). Conversely, islet structure and insulin and glucagon staining were maintained in oATP-treated mice (Fig. 3G, K and L, respectively), with infiltration mainly confined to the islet borders with several FoxP3+ cells (Fig. 3G–J). Semi-quantitative analysis confirmed preserved insulin staining at day 14 in oATP-treated mice but not in the untreated mice (Fig. 3M), with no major differences in the overall infiltrate (Fig. 3N). A reduction in the mRNA expression of
Targeting P2X7R in vitro reduces the severity of the alloimmune response. To confirm the data we obtained in vivo, we evaluated the effect of targeting P2X7R in vitro using alloimmune-relevant assays. Responder splenocytes from C57BL/6 mice were challenged with irradiated stimulator splenocytes from BALB/c mice. The numbers of cells producing IFN-γ and IL-4 were evaluated in an ELISPOT assay in the presence of increasing concentrations of oATP. P2X7R targeting robustly inhibited IFN-γ production (cells producing IFN-γ: oATP 100 μmol/L = 30 ± 2 vs. control = 190 ± 4, n = 5; P < 0.0001; Fig. 4A), but a dose-dependent increase in IL-4 production was observed (Fig. 4B). To confirm in vitro suppression of the alloantigen response, we performed a mixed leukocyte reaction experiment in which alloantigen-mediated cell proliferation appeared to be suppressed (number of proliferating cells as measured by 3H-thymidine incorporation: oATP 100 μmol/L = 136 ± 5 vs. control = 6,199 ± 498, n = 5; P < 0.0001; Fig. 4C). C57BL6 splenocytes were also challenged with alloergic cells in the presence of oATP (100 μmol/L) for 24 h, and apoptosis was assessed by fluorescence-activated cell sorter as the percentage of annexin V−/7AAD− cells; no increase in the percentage of apoptotic cells was detected (Fig. 4D).

We then tested the effect of increasing doses of oATP on Th1/Th17 cytokine levels. A dose-dependent response was evident, in which oATP reduced the number of cells producing Th1 cytokines (IFN-γ and IL-2) and Th17 cytokines (IL-6 and IL-17; Fig. 4E–H). Because it has been reported that oATP may affect other P2XR, we made use of the availability of the selective P2X1R inhibitor NF449 and of the selective P2X3R inhibitor NF110. C57BL6 splenocytes were stimulated with irradiated BALB/c splenocytes, and IFN-γ production was evaluated in the presence of these inhibitors. P2X1R and P2X3R inhibition failed to modulate alloantigen-mediated IFN-γ production (Fig. 4I and J); these data confirmed the marginal role of P2X1R and P2X3R in the alloimmune response, at least in vitro.

Genetic targeting of P2X7R slightly prolongs islet graft survival. We also determined the effect of genetic targeting of P2X7R on islet graft survival. P2X7R−/− C57BL6 mice were transplanted with alloergic BALB/c islets, and partial prolongation of graft survival was observed compared with wild-type recipients (MST: P2X7R−/− = 19 days, n = 10; P < 0.0001 vs. wild-type; Fig. 5A). oATP treatment on P2X7R−/− recipients was able to further
prolong graft survival (MST: oATP-treated P2X7R−/− = 26 days, n = 5; P = 0.04 vs. untreated P2X7R−/−; Fig. 5A).

To investigate the purinergic system function in P2X7R−/− mice, we analyzed the effect of benzoyl-ATP (Bz-ATP), a synthetic agonist of the purinergic receptors, in CD4+ and CD8+ T cells. When 1 μmol/L Bz-ATP was added to the cultured cells, IFN-γ production increased in CD4+ (Fig. 5B) and CD8+ T cells (Fig. 5D) isolated from wild-type mice. Bz-ATP similarly stimulated IFN-γ production from CD4+ (Fig. 5C) and CD8+ T cells (Fig. 5E) isolated from P2X7R−/− mice. We hypothesized that compensatory mechanisms may have arisen in P2X7R−/− mice, and in particular, we investigated a possible upregulation of P2X1R, P2X3R, and P2X4R, which also have been associated with the immune response (25). CD4+ and CD8+ T cells were extracted from wild-type and P2X7R−/− mice, and the relative expression of P2XRs was assessed by real-time PCR. Higher levels of P2X1R and P2X4R were found
in P2X7R−/−-derived CD4+ T cells (P2X1R: 2-fold increase vs. wild-type, n = 3, P = 0.02; P2X4R: 1.7-fold increase vs. wild-type, n = 3, P = 0.03; Fig. 5F) and CD8+ T cells (P2X1R: 10-fold increase vs. wild-type, n = 3, P = 0.003; P2X4R: 1.3-fold increase vs. wild-type, n = 3, P = 0.04; Fig. 5G). Promotion of islet graft survival obtained by oATP treatment also in P2X7R−/− recipients demonstrates that the targeting of other purinergic receptors may contribute to the immunomodulatory effects of oATP; however, some caution should be used when analyzing data obtained from P2X7R−/− mice because compensatory mechanisms in the purinergic system are present. **Rapamycin and P2X7R targeting in vivo synergize in reducing the alloimmune response.** To evaluate a potential clinically relevant protocol, we tested oATP combined with a clinical-grade dose of rapamycin (oATP at 250 μg day 0 through day 15 + rapamycin at 0.1 mg/kg day 0 through day 10); five of seven islet-transplanted mice treated with oATP+rapamycin showed long-term graft function at 100 days after transplantation (MST: rapamycin = 18; oATP+rapamycin = indefinite, n = 7; oATP+rapamycin vs. both untreated and rapamycin, P < 0.0001; oATP+rapamycin vs. oATP, P = 0.04; Fig. 6A). Rapamycin was more effective in prolonging islet graft survival in P2X7R−/− compared with wild-type recipients (n = 7; P = 0.04 vs. rapamycin in wild-type; Fig. 6A), confirming the synergistic effect between P2X7R-targeting and rapamycin. Better protection of islet grafts was observed in wild-type recipients treated with oATP+rapamycin than in P2X7R−/− recipients treated with rapamycin alone (Fig. 6A), indicating that the oATP may also target other purinergic receptors. Pathology of islet allografts in long-term protected islet-transplanted oATP+rapamycin-treated mice revealed preserved architecture (Fig. 6B) and maintained insulin (Fig. 6F) and glucagon (Fig. 6G) staining. Islet infiltrate was evident but appeared confined to the border of islet allografts (Figs. 6C and E) At higher magnifications, FoxP3+ cells infiltrate appeared nearly as abundant as the CD3+ T-cell infiltrate, suggesting a high proportion of Tregs (Fig. 6C and D, high-magnification insert). Mice treated with short-course oATP+rapamycin were further protected from the alloimmune response, as demonstrated by a decrease in the responsiveness toward donor alloantigen and measured by the reduction in cells producing IFN-γ during ex vivo challenge with alloantigens (cells producing IFN-γ: oATP+rapamycin-treated at day 100 = 60 ± 8 vs. untreated at day 14, n = 3, P < 0.0001; vs. oATP-treated at day 14, n = 3, P = 0.008; Fig. 6H). A parallel reduction in
the percentage of CD4+ and CD8+ effector cells was observed in oATP+rapamycin-treated mice. The percentages of peripheral CD4+ effector T cells (Fig. 6J) and CD8+ effector T cells (Fig. 6J) were greatly reduced compared with those observed in oATP-treated mice at day 14. Stable levels of Th17 cells (Fig. 6K) and a slight decrease in the number of Tregs were observed in oATP-treated P2X7R−/− mice (Fig. 6L). A better protection of the islet graft structure was observed in rapamycin-treated P2X7R−/− mice (Fig. 6M, Q, and R) at day 14 after transplantation. Immune cell infiltration was reduced in rapamycin-treated P2X7R−/− recipients (Fig. 6T, U, and V) compared with untreated P2X7R−/− recipients (Fig. 6N, O, and P).

**P2X7R expression is increased in CD4+ T cells obtained from islet-transplanted patients.** We then sought to determine the P2X7R expression profile in lymphocytes obtained from islet-transplanted patients to determine the relevance of the P2X7R/ATP system in human T cells and, potentially, in islet rejection in humans. PBMCs from islet-transplanted patients, T1D patients, and healthy control subjects were harvested, and CD4+ T cells, a key population in islet rejection, were analyzed by flow cytometry (Table 1). In healthy controls, the P2X7R+CD4+ T-cell population represented 17% of all PBMCs (Figs. 7A and F), and P2X7R was expressed in almost 50% of peripheral CD4+ T cells (data not shown). The percentage of P2X7R+CD4+ T cells observed in recently well-functioning transplanted patients (<3 years from last islet infusion) were comparable to those of healthy control subjects (Fig. 7A, C, and F). Conversely, the frequency of the P2X7R+CD4+ T-cell population significantly increased in long-term islet-transplanted patients (>3 years; Fig. 7A, D, and F) and in T1D patients (Figs. 7A, B, and F). Among CD4+ T cells, an expansion of P2X7R+CD45RA+ cells (T cells with a mature phenotype or memory T cells) was observed in long-term islet-transplanted patients (Fig. 7G, J, and L) and in T1D patients (Fig. 7G, H, and L) compared with healthy control subjects. Conversely, a reduction in naive P2X7R+CD45RA+ cells (Fig. 7M, N, P, and R) was observed. These data demonstrate a similar P2X7R expression pattern on CD4+ T cells in long-term islet-transplanted patients and in T1D patients, whereas a different pattern is expressed on recently islet-transplanted patients and healthy control subjects.
The specific human P2X7R antagonist CE-224535 inhibits the alloimmune response in vitro. To confirm the immunomodulatory effect of P2X7R inhibition, we challenged PBMCs obtained from healthy control subjects with allogeneic irradiated PBMCs obtained from different donors in an ELISPOT assay. A reduction in the number of cells producing IFN-γ was observed with CE-224535 treatment at 100 μmol/L (29 ± 1 vs. control 51 ± 3, n = 5, P = 0.003; Fig. 7S).

DISCUSSION
Successful islet transplantation can improve metabolic control and can reestablish normoglycemia in subjects with T1D (3). Unfortunately, transplanted islets are subjected to the damaging effects of the alloimmune response and the recurrence of autoimmunity (20% of the patients insulin-free at 5 years) (3,7,8,40). The better outcomes of autologous islet transplantation from patients with chronic pancreatitis (47% insulin-free at 5 years) (41), in whom the allo- and autoimmune responses are absent, confirms the primary role of immune response in islet graft failure,
although multiple factors are ultimately responsible for islet graft loss (3). Considering the potential advantages of islet transplantation in diabetes complications and the relatively low invasiveness of the procedure, additional research is required to develop novel strategies to lessen the need for lifelong immunosuppression (42).

We studied the role of the ionotropic purinergic P2X receptors in islet allograft rejection, particularly in light of recent studies that have demonstrated roles for the ATP/P2X7R system in T-cell activation and in Th1/Th17-cell generation (24–26). P2X7R may represent a crossroads for naïve T cells to become activated during the alloimmune response. Our data show that the expression of P2X1R and P2X7R is higher in the islet allograft during rejection, whereas in the syngeneic setting, only P2X1R has higher expression. These data seem to link P2X7R expression specifically to the alloimmune response, whereas P2X1R expression appears to be more related to ischemia/reperfusion and peritransplant inflammation.

In our model of islet allotransplantation, P2X7R targeting with oATP was able to promote long-term graft function in 30% of recipients, and this effect was associated with an extensive reshaping of the immune system, specifically in a reduction of the anti-islet Th1 response, a reduction in CD4+ T effector and Th17 cells, which are well known to promote the alloimmune response (29), and a preservation of Tregs, which, on the contrary, promote graft acceptance (43). Of particular interest in the clinical setting is the synergistic effect observed between rapamycin, which is a pivotal drug in clinical islet transplantation (44), and oATP. This combination treatment resulted in a preponderance of islet-transplanted mice accepting a fully major histo compatibility complex–mismatched allograft long-term, with hyporesponsiveness toward alloantigen and preservation of islet graft morphology and insulin staining.

Although oATP is described as one of the most effective P2X7R inhibitors in vivo (45,46), the targeting of P2X7R has also been associated with the targeting of other purinergic receptors (22). Our data in P2X7R−/− recipients confirmed the presence of additional targets for oATP, and this targeting appears beneficial in the context of allogeneic islet transplantation providing additional immunomodulation. In particular, our data suggest that the effect could be mediated by P2X1R targeting, which is upregulated in the syngeneic and allogeneic graft and in...
the CD4+ and CD8+ T cells obtained from P2X7R−/− mice. However, some caution should be used during analysis of data using P2X7R−/− recipients; in these mice, the immunological function of the other P2XRs may in fact become more relevant, as suggested by the preserved response to Bz-ATP in P2X7R−/−-derived lymphocytes, accompanied by the upregulation of other P2XRs. Further development of specific antagonists for the different P2XRs suitable for in vivo use (22,46) will help us to better dissect the relative contribution of the targeting various P2XRs in islet graft rejection.

In vitro, the targeting of P2X7R with oATP during allostimulation inhibited the production of Th1/Th17 cytokines and T-cell activation, confirming that P2X7R signaling is required to trigger the Th1/Th17 immune response (21,25); on the contrary, the expansion of Tregs is not evident in our model, although this has been reported in other settings (30).

By studying PBMCs obtained from different populations of human subjects, we were able to evaluate the expression of P2X7R in different lymphocyte subsets. The P2X7R+/CD4+ T-cell population appeared to be upregulated in T1D patients and islet-transplanted patients in the long-term, suggesting a link between activation of the immune system, the anti-islet response, and expression of P2X7R. Interestingly, an increase in memory and decrease of naive CD4+ T-cells expressing P2X7R was observed in long-term islet-transplanted recipients. Considering that progressive loss of islet graft function may be associated with a mounting of memory allo- and autoimmune cell responses (40,47), identifying potential new targets for the memory-cell subpopulation is highly desirable. In this sense, the targeting of P2X7R may represent a viable option, particularly in light of the availability of clinical-grade therapeutics, such as the human-specific inhibitor CE-224535.

In conclusion, P2X7R-mediated alloimmunity is an important pathway in the alloimmune response that contributes to priming of Th1/Th17 effector cells. Targeting of P2X7R may thus be beneficial in preventing islet allograft rejection and inducing tolerance, thereby reducing the need for immunosuppressive agents. Indeed, complete tolerance induction may require a specific P2X7R-targeting approach to abrogate Th1/Th17 alloimmunity and to facilitate a Treg sparing/expansion strategy. In view of the novel anti-P2X7R inhibitors available for clinical use (e.g., CE-224535, AZD9056, and GSK1482160), our data may have significant clinical relevance for the field of islet transplantation.

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A.V. designed and performed research, analyzed data, and wrote the paper. C.F. performed research, analyzed data, and wrote the paper. F.D., S.T., M.P., F.Ga., M.C., R.B., R.D.M., D.C., R.G., M.E.F., A.S., and P.M. performed research. F.Gr. edited the paper. C.R. designed research and edited the paper. M.H.S. designed research. A.P. and P.F. designed research and wrote and edited the paper. P.F. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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