Islet transplantation stabilizes hemostatic abnormalities and cerebral metabolism in individuals with type 1 diabetes

Francesca D’Addio MD, PhD1,2, Paola Maffi MD2, Paolo Vezzulli MD3, Andrea Vergani MD1, Alessandra Mello MD4, Roberto Bassi MD1, Rita Nano5, Monica Falautano BS6, Elisabetta Coppi MD6, Giovanna Finzi7, Armando D’Angelo MD8, Isabella Fermo8, Fabio Pellegatta PhD9, Stefano La Rosa MD7, Giuseppe Magnani MD9, Lorenzo Piemonti MD5, Andrea Falini MD3, Franco Folli MD, PhD10, Antonio Secchi MD3,11 and Paolo Fiorina MD, PhD1,2

1Nephrology Division, Boston Children’s Hospital, Harvard Medical School, Boston, MA; 2Medicine, 3Neuroradiology, 4Anesthesiology, 5Diabetes Research Institute and 6Neurology Department, San Raffaele Hospital, Milan, Italy; 7Pathology Department, Ospedale di Circolo, Varese, Italy; 8Chromatographic Techniques Service, San Raffaele Hospital, Milan, Italy, 9Centre for the Study of Atherosclerosis, Bassini Hospital, Cinisello Balsamo, Italy, 10University of Texas at San Antonio, San Antonio, TX; 11Universita’ Vita-Salute, Milan, Italy.

Abstract word count: 204
Text word count: 4228
Short running title: Effect of islet transplantation alone on hemostasis and brain metabolism in type 1 diabetes
Tables=1; Figures=3; Supplemental Table=2 Supplemental Figures=2

Address for correspondence:

Paolo Fiorina, MD PhD
Nephrology Division
Boston Children’s Hospital, Harvard Medical School,
300 Longwood Avenue, Enders Building EN511
Boston, MA, USA
Tel: 617-919-2624
Fax: 617-732-5254
E-mail: paolo.fiorina@childrens.harvard.edu

Or

Antonio Secchi, MD
Transplantation Medicine
Ospedale San Raffaele
Via Olgettina 60
Milan, Italy
Tel: +39-02-26432575
Fax: +39-02-26433790
E-mail: antonio.sechci@hsr.it
ABSTRACT

Objective. Islet after kidney transplantation has been shown to positively affect the quality of life for individuals with type 1 diabetes (T1D) by reducing the burden of diabetic complications, but fewer data are available for Islet transplantation alone (ITA). The aim of this study was to assess whether ITA has a positive impact on hemostatic and cerebral abnormalities in individuals with T1D.

Research Design and Methods. Pro-thrombotic factors, platelet function/ultrastructure, cerebral morphology, metabolism and function have been investigated over a 15-month follow-up period, with ELISA/electron microscopy and magnetic resonance imaging, nuclear magnetic resonance spectroscopy (¹H-MRS) and neuropsychological evaluation (POMS and PASAT tests), in 22 individuals with T1D who underwent islet transplantation alone (n=12) or remained on the waiting list (n=10). Patients were homogeneous at the time of enrolment on the waiting list with regard to metabolic criteria, hemostatic parameters and cerebral morphology/metabolism/function.

Results. At 15 months follow-up, the Islet transplantation alone group, but not individuals with T1D remaining on the waiting list, showed: i) improved glucose metabolism; ii) near-normal platelet activation and pro-thrombotic factor levels; iii) near-normal cerebral metabolism and function; and iv) a near-normal neuropsychological test.

Conclusions. Islet transplantation alone, despite immunosuppressive therapy, is associated with a near-normalization of hemostatic and cerebral abnormalities.

Keywords: Type 1 diabetes, islet transplantation, brain metabolism, neuropsychological analysis, cerebrovascular diseases, and hemostasis.
INTRODUCTION

Type 1 diabetes (T1D) is associated with microvascular (i.e. retinopathy, nephropathy and neuropathy) or macrovascular (i.e. cardiovascular, cerebrovascular and peripheral vascular disease) complications, which cause an increase in morbidity and mortality and negatively affect patient quality of life (1, 2). Poor glycemic control is the primary contributor to the onset of chronic complications (3).

Islet transplantation, by replenishing destroyed pancreatic islets, leads to improved glycometabolic control, if not to insulin independence, and reduces the frequency of hypoglycemic episodes, thus delaying diabetic complications and improving patient quality of life (4-6). However, islet transplantation requires the chronic administration of immunosuppressive agents (7), which increase cardiovascular disease (8), atherosclerosis (9) dyslipidemia (10) and are also toxic for β-cells, thus shortening the lifespan of the transplanted islets (11, 12). While in the Islet after kidney transplantation (IAK) group, kidney-transplanted patients receive immunosuppressive therapy prior to transplantation of the islet-graft; individuals with T1D receiving an islet allograft need to begin an immunosuppression regimen at the time of the transplant. It is therefore necessary to demonstrate that the benefit obtained from improvement in glycometabolic control, overcomes the deleterious effect of immunosuppression. Indeed, following IAK, an overall beneficial effect in halting the progression of several major diabetic complications (13) such as nephropathy (14, 15) and cardiovascular disease (16) has been demonstrated, yet fewer data are available on the effect of ITA on diabetic complications (17, 18).

In this study, we examined the effect of islet transplantation alone on hemostatic and cerebrovascular abnormalities in individuals with T1D. Hemostatic abnormalities are evident in T1D (19) and may contribute to the onset of accelerated atherosclerosis and microangiopathy of different vascular districts including the cerebral vasculature, thus leading to morphological, metabolic and functional cerebral abnormalities (20). Features of hemostatic abnormalities include enhanced activation of the clotting system and severe platelet function, which lead to the prothrombotic state, vascular dysfunction/early
atherosclerosis observed in several organs, particularly in the cerebral district in individuals with T1D (20). The resulting diabetic encephalopathy is characterized by a reduction in the volume of the cerebral cortex, a significant loss in neocortical neurons (21), a decrease in mnemonic and abstract reasoning, problem-solving and hand-eye coordination abilities, and an increased risk of Alzheimer’s disease (22). We hypothesize that improvement of glycometabolic control (if not full restoration of normoglycemia) obtained with islet transplantation, may re-establish the physiology of the hemostatic system, thus halting the progression of cerebral abnormalities. The aim of this study is to investigate the relatively short-term effects of islet transplantation on hemostatic profile, platelet function and cerebral morphology (brain volume), metabolism (neuronal/axonal metabolism) and function (cognitive and neuropsychological function).
RESEARCH DESIGN AND METHODS

Patients

This not-randomized pilot study considered 22 individuals with T1D actively enrolled on the islet transplantation waiting list. Twelve out of 22 individuals with T1D underwent ITA, while 10 remained on the waiting list (Supplemental Figure S1). Eligibility criteria for patients and controls are reported below. Briefly, individuals with T1D were retrospectively enrolled in a 24-month period according to the major criteria applied to enter the islet transplantation program as published elsewhere (4, 7). The baseline and demographic characteristics of the 2 groups of individuals with T1D were comparable (Supplemental Table S1) and were re-examined at 15 months follow-up (15.5±2.5 months). Glycometabolic parameters were determined at enrolment time and during the follow-up on single determination, thus are only partially representative of the individual glycometabolic control. Ten healthy volunteers matched for age and gender (CTRL) were studied as well. Patients with a history of cerebrovascular disease (transient ischemic attack/stroke) and/or who were taking an oral anticoagulant agent were excluded. All subjects provided informed consent before study enrolment. Studies not included in the routine clinical follow-up were covered by an appropriate Institutional Review Board approval. ITA is associated with different adverse events due to the procedure and to immunosuppressive treatments. According to our experience, acute complications are represented by thrombosis of a peripheral branch of the portal vein (9%) and bleeding (36%); while chronic complications may include decreased renal function (15%), viral myocarditis (6%), reactivation of CMV infections (6%) (23).

Immunosuppression and Concomitant Therapy

Individuals who underwent islet transplantation, received Daclizumab (Zenapax, Roche; 1 mg/Kg/2 weeks, intravenously 5 doses) as induction therapy. As maintenance, Tacrolimus (Prograf, Fujisawa, serum levels of 3–6 ng/mL) and Sirolimus (Rapamune, Wyeth-Ayerst, range level of 12–15 ng/mL for 90 days and 7–10 ng/mL thereafter) were used. Four individuals were switched to a different
immunosuppressive regimen because of side effects: Tacrolimus plus Mycophenolate Mofetil (MMF, n=2), Sirolimus plus MMF (n=1), Cyclosporine plus MMF (n=1). 4/12 individuals in ITA group and 4/10 individuals with T1D received angiotensin-converting-enzyme inhibitors (ACE-I). 1/12 individual of ITA group received statins during follow up. No drugs were administered to controls.

In order to understand whether immunosuppression may represent a potential bias in our study and may affect coagulatory/inflammatory markers, we additionally considered 10 individuals with end stage renal disease (ESRD) and 10 individuals with ESRD who subsequently received a kidney transplant studied at 15 months of follow up and treated with Thymoglobulin as induction therapy and Tacrolimus plus Sirolimus (or MMF in few cases) as maintenance therapy. Individuals with ESRD were all on hemodialysis treatment at our Hospital and received ACE-I (7/10), beta-blockade agents (3/10), calcium-phosphate binders (10/10), 1-OH vitamin D (8/10) as concomitant therapy. Finally, with the same purpose of understanding whether immunosuppression may affect coagulatory/inflammatory markers in a pure T1D population, we considered 5 individuals with T1D receiving Sirolimus monotherapy (0.1 mg/kg; target levels 8-10 ng/ml; 37-197 days) and atorvastatin (10 mg; 44-279 days) as pre-conditioning for islet transplantation (at least 30 days).

\textit{Laboratory analysis}

Platelet-poor plasma was obtained by centrifugation at room temperature (10 min, 1500g). Determinations of prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen (Fg), antithrombin (AT), fasting homocysteine d-dimer fragments (D-dimer), levels of prothrombin fragments 1+2 (F1+2), protein C (PC), and protein S (PS) were analyzed on fresh plasma samples (24).

\textit{Intracellular calcium in platelets}

Platelet-rich plasma (PRP) was obtained by centrifugation of blood with 1 ml of ACD solution and collection of supernatant. Platelet [Ca2+]i was evaluated under resting conditions as previously described (25).

\textit{Electron microscopy}
Aliquots of PRP were fixed and ultrastructural evaluation of 4 cases for each group included platelet size, morphology, and granule content and areas were performed using the “measure arbitrary area” tool of Analysis Image Processing 3.0 software (Soft Imaging System, Münster, Germany).

**Magnetic Resonance Imaging (MRI) and MR Spectroscopy (MRS) protocols**

MRI and MRS of the proton ($^1$H-MRS) were performed using a 1.5 T scanner (Vision; Siemens, Erlangen, Germany), with a head-dedicated coil for the scanning. Acquired images (T2, T1, axial FLAIR and Coronal Spin) were analyzed to assess micro and macro-angiopathy during the clinical course of diabetes as previously reported (26). A diagnosis of cerebrovascular disease was formulated when lesions were hyperintense in T2-weighted images and FLAIR and hypointense on T1-weighted images. T1-axial images of each participant were transferred to an offline workstation (Sun Sparcstation; Sun Microsystems, Mountain View, CA, USA) for brain volume assessment (27). Single voxel MRS was performed at least 3 times for each individual (28). The peaks of Choline (Cho), Creatine (Cr) and N-acetylaspartate (NAA) were calculated, and their integral function was solved by automatic and manual measurement. Results were expressed as ratios: NAA/Cr, Cho/Cr, and NAA/Cho. Cr was considered an internal standard, as its levels are relatively uniform even in pathological conditions (29). All measurement were performed by the same operator; $WB_{NAA}$ MRS was analyzed as previously described (28).

**Psychological and neuropsychological tests**

Psychological assessment evaluated emotional patterns and quality of life (QoL) of patients. The profile of mood state (POMS) was used for measuring present mood states and disturbance symptoms, dissecting 6 dimensions of mood: tension-anxiety, depression-dejection, anger-hostility, vigor-activity, fatigue-inertia and confusion-bewilderment (POMS-T; -D; -A; -V; -S; -C, respectively) (30). To assess the QoL of patients, the LEIPAD test was administered as reported by our group (28, 31). A neuropsychologist performed neuropsychological evaluation in 1 session by analyzing childhood intellectual failures, global cognitive efficiency, language, attention, reasoning and verbal/visual-spatial
memory as previously reported (28). Mini-mental state examination (MMSE) was employed for examining patients’ global (intellectual) cognitive abilities. Language functioning (oral comprehension and access to internal lexicon) was evaluated with token test and phonemic and semantic word fluency test (32). Attention abilities were analyzed using the Stroop test (28), and the paced auditory serial addition test (PASAT), which evaluated sustained attention and information processing speed (33). Verbal and visual short-term memory function was evaluated with the verbal span and Corsi span test, while verbal long-term memory was assessed with short story recall. Finally, abstract reasoning, through visuo-spatial stimuli, was evaluated with the Wisconsin card sorting test (WCST) (48 cards form) (34). Evaluations have been performed three times after an extensive internal validation).

**Statistical analysis**

Continuous variables are presented as mean±standard deviation (SD) and compared with 2-tail Student t-test for unpaired data (2 groups) or the one-way analysis of variance (3 or more groups), while the Mann-Whitney U test (2 groups) or the Kruskall-Wallis test (3 or more groups) were used if no normal distribution was evident. Categorical variables are presented as proportions with 95% confidence intervals (95% CI) in parentheses and compared using the Chi-square test if the number of observations in each cell is ≥5, or the Fisher’s exact test if it is <5. A p value <0.05 was considered statistically significant. Confidence intervals (CI) were estimated using the binomial exact. Analysis was performed using Stata, version 11.1 (StataCorp. College Station, TX, USA).
RESULTS

Baseline patient characteristics

Individuals with T1D and healthy controls did not differ in any major demographic characteristics at baseline, when considering individuals with T1D who remained on the waiting list (T1D-WL) or those who underwent ITA (T1D-ITA), (Supplemental Table S1). The mean age, duration of diabetes, creatinine level, total cholesterol, HDL cholesterol, exogenous insulin requirement (EIR) and mean arterial pressure (MAP) were similar in the 3 groups of subjects examined (Supplemental Table S1). Significant differences were observed in the major parameters accounting for glycometabolic control between individuals with T1D (T1D-WL and T1D-ITA) and controls, including glycated hemoglobin (HbA1C), serum glucose (S-Glucose) and C-peptide, while no differences were found when comparing T1D-WL vs. T1D-ITA values (Supplemental Table S1). All individuals with T1D (T1D-WL and T1D-ITA) were homogenous at baseline for platelet function and ultrastructural characteristics, prothrombotic factors levels and cerebral morphology/metabolism/function assessment with very consistent results in all the parameters analyzed.

Islet transplantation alone nearly normalizes glycometabolic control

Mean duration of follow-up was 15.5±2.5 months. Renal function (creatinine: T1D-WL=0.8±0.1 vs. ITA=1.0±0.2 mg/dl, p=ns), total cholesterol (T1D-WL=172.5±11.0 vs. ITA=183.5±12.5 mg/dl; ns), HDL cholesterol (T1D-WL=54.3±10.5 vs. ITA=58.9±9.9 mg/dl; ns), triglycerides (T1D-WL=97.0±37.7 vs. ITA=64.1±18.8 mg/dl; ns) and MAP values (T1D-WL=98.6±8.1 vs. ITA=103.8±1.8 mmHg; ns) were similar at 15 months of follow-up in individuals with T1D and ITA patients. Patients did not show any sign of cardiovascular disease (absence of any electrocardiographic signs of myocardial ischemia, and of any echocardiography signs of systolic/diastolic dysfunction). A significant improvement in glycometabolic control was evident in ITA patients as compared to individuals with T1D that remained on the waiting list. S-glucose, C-peptide, HbA1C and EIR values
were all improved in ITA patients (Figures 1A-1D). Islet transplantation restored near-normal glycometabolic control.

**Islet transplantation alone is associated with near-normal platelet morphology, aggregation and calcium platelet homeostasis**

We examined an average of 10 platelets per patient to evaluate platelet area, calcium platelet homeostasis, number of granules, and 10 granules to evaluate granule area (Figures 1E-F). Platelet size at 15 months of follow-up was higher in individuals with T1D compared to controls and ITA patients (T1D-WL=3.860±0.288x10^6, CTRL=3.076±0.197x10^6 and ITA=3.199±0.287x10^6 nm^2, T1D-WL vs. CTRL p=0.02), while ITA patients and controls showed similar platelet size (p=ns). Moreover, platelets in individuals with T1D showed the tendency to aggregate, exhibited a more electron-dense cytoplasm, and displayed more numerous and larger granules than platelets in controls and ITA patients (Figure 1G). This suggests that platelet morphology is altered in individuals with T1D and that islet transplantation is associated with near-normalization of platelet size, aggregation, and platelet granule size and number. The levels of resting [Ca2+]i, analyzed at 15 months of follow-up, were higher in individuals with T1D, but not in ITA patients compared to controls (CTRL=72.2±15.0, T1D-WL=107.7±40.0, ITA=87.6±18.8 nmol, p=0.01; Figure 1F). This suggests that T1D is associated with aberrant calcium platelet homeostasis and that islet transplantation promotes near-normalization of both platelet signalling and the cytosolic calcium pathway.

**Islet transplantation alone is associated with reduced hemostatic abnormalities**

Analysis of hemostatic profile demonstrated high levels of plasmatic Fg, F1+2 and d-dimer in individuals with T1D. Lower levels of Fg (CTRL=262.5±43.0, T1D-WL=328.5±35.0, ITA=367.0±26.0 mg/dL, CTRL vs. all, p<0.005) and F1+2 (CTRL=63±17.8, T1D-WL=113±35.7, ITA=96±45.2 mmol/L, CTRL vs. T1D-WL, p=0.005) were evident in controls as compared to ITA patients and individuals with T1D (Figures 2A-B). Finally d-dimer levels were higher in individuals with T1D as compared to controls and ITA patients (CTRL=0.19±0.02, T1D-WL=1.07±0.80, ITA=0.24±0.02
µg/ml, CTRL vs. all, p=0.01) (Figure 2C). No statistical significant differences were found in fasting homocysteine levels among the three groups of subjects (Figure 2D). Analysis of PT, aPTT and ATIII did not reveal differences among the 3 groups at 15 months of follow-up. Significantly lower levels of protein S and protein C were found in individuals with T1D compared to controls, while in ITA patients, levels were almost normalized at 15 months follow-up (Figures 2E-F). A prothrombotic state is evident in individuals with T1D, while ITA is associated with near-normalization of hemostatic abnormalities.

**Islet transplantation alone does not alter cerebral morphology: a conventional MR imaging study**

Evaluation of vasculopathy with conventional MR imaging revealed that 12 out of 22 individuals with T1D had cerebrovascular disease at baseline, without differences between the 2 groups (T1D-ITA vs. T1D-WL, ns) as compared to 2 out of 9 controls (T1D=54.5% [CI: 36-69%] vs. CTRL=22.2% [CI: 3-6]; p=0.04). No major changes in cerebral morphology were evident at 15 months of follow-up. Mean cerebral volume was similar in individuals with T1D and in ITA patients, while it was significantly lower when compared to controls at 15 months follow-up (CTRL=1.197±98.3, ITA=1.027±90.3, T1D-WL=1.105±67.8, CTRL p<0.03 vs. all), (Figure 3A). Islet transplantation did not modify structural changes in cerebral morphology, which was evident in individuals with T1D at 15 months of follow-up.

**Islet transplantation alone is associated with an improved cerebral metabolism: a MR spectroscopy study**

*Non-localized MRS*: The mean absolute NAA (AbsNAA) (a neuronal marker that indicates neuronal/axonal loss when decreased) (29, 35) showed similar values in the 3 groups of patients at 15 months of follow-up. AbsNAA values, corrected for subject brain volume to adjust for inter-individual variation (NAA concentration [NAA]), failed to reveal any effect in the ITA group (Figure 3B). Likewise, whole brain NAA (WB$_{NAA}$) mean content did not differ among the three groups (CTRL=14.5±1.5; T1D=14.4±1.8; ITA=13.2±1.6; AU, ns), (Figure 3C).
Localized MRS-Single Voxel Spectroscopy: NAA/Cho ratio (an index of neuroaxonal loss/impairment associated with diabetes-related gliosis (36)) was similar in ITA patients and controls, while it was lower in individuals with T1D, suggesting major cerebral tissue damage (CTRL=2.0±0.2, T1D-WL=1.7±0.2, ITA=1.9±0.2 AU; T1D-WL vs. ITA, p=0.02; CTRL vs. T1D-WL, p=0.003), (Figure 3D). The NAA/Cr ratio, an index of neuronal loss/damage when decreased (37), did not show any significant difference in the 3 groups analyzed, although higher values were found in controls as compared to others (CTRL=2.1±0.2, T1D-WL=2.0±0.1, ITA=2.0±0.2 AU, ns), (Figure 3E). Conversely, we documented an increased Cho/Cr ratio (which has been associated with diabetes gliosis (36) when increased) in individuals with T1D, particularly when compared to ITA patients, indicating a near-recovery of the neuronal tissue (T1D-WL=1.1±0.1 and ITA=0.98±0.1, p= 0.01), (Figure 3F). Finally, an inverse linear correlation was found between HbA1c and the NAA/Cho ratio (r=−0.61, p=0.004) (data not shown), thus suggesting that poor glycemic control in individuals with T1D is important in promoting degenerative phenomena and in altering cerebral metabolism.

Islet transplantation alone is associated with improved emotional status: a psychological and neuropsychological assessment

Psychological evaluation was performed by analyzing patients’ emotional axis and quality of life (QoL) at 15 months follow-up using the POMS and LEIPAD test, respectively (30, 31). The POMS test, examining 6 different dimensions of mood (see research design and methods), showed a significant higher score in POMS-D test in individuals with T1D as compared to controls and ITA patients (Table 1). Moreover, the POMS-S test was significantly different among controls, individuals with T1D and ITA patients, with the latter showing less fatigue-inertia (Table 1). Results of the POMS-C test showed higher values in individuals with T1D as compared to ITA patients, while the POMS-A test revealed a significant difference in controls as compared to both individuals with T1D and ITA patients (Table 1). No differences were found in POMS-T and –V tests (Table 1). The analysis of the LEIPAD test did not show any difference in physical function and self care among the 3 groups, while
it revealed a significantly higher score for depression and anxiety in individuals with T1D as compared to ITA patients (Table 1). In addition, life satisfaction score was significantly lower (in which lower score demonstrates improvement) in controls and ITA patients as compared to individuals with T1D (Table 1). Considering both the POMS and LEIPAD tests, despite the fact that some values did not achieve statistical significance, an overall trend demonstrating improvement was evident in ITA patients as compared to individuals with T1D. This was confirmed by neuropsychological assessment, in which ITA patients demonstrated a substantial benefit from transplantation as compared to individuals with T1D in most of the tests (Table 1). Phonemic and semantic fluency also showed higher scores in controls and ITA patients compared to individuals with T1D. Furthermore, the PASAT test revealed significantly higher scores in ITA patients as compared to individuals with T1D and controls (Table 1). The color naming and the word-color interference variants of the Stroop test were higher (indicating a worsened state) in T1D-WL and ITA patients as compared to controls (Table 1). The color-color interference Stroop test revealed lower scores (i.e. worse state) in T1D-WL and ITA patients as compared to controls, although this difference did not reach statistical significance. The number of errors made by the patients during the 3 sections of the Stroop test was significantly lower in controls than in T1D-WL and ITA patients (Table 1). ITA is associated with a near-normalization of psychological and neuropsychological tests.

**Immunosuppression treatment does not exert any beneficial effect on hemostatic abnormalities in islet transplantation**

In order to understand whether concomitant immunosuppressive treatment may have biased our results, we firstly explored the effect of immunosuppression (Tacrolimus and Sirolimus) altered coagulatory/inflammatory markers in individuals with end stage renal disease (ESRD) undergone a kidney transplant (n=10). We finally evaluated whether immunosuppression (Sirolimus) altered coagulatory/inflammatory markers in individuals with T1D (n=5). Immunosuppression did not alter or change the peripheral levels of d-dimers, protein S and C, homocysteine, fibrinogen and F1+2 in
kidney-transplanted individuals compared to individuals with ESRD (Supplemental Figure S2: A-F) or in individuals with T1D (Supplemental Figure S2: G-N). These experiments confirm that immunosuppression has no effect on inflammatory/coagulatory markers, and thus it is not likely involved in the modifications of these markers observed in our study.
DISCUSSION

Insulin, the only treatment so far for individuals with T1D, can only partially prevent long-term diabetic complications, and it is still associated with fatal hypoglycemic episodes (4). Islet transplantation, a therapeutic option available for selected individuals with T1D, has been further examined in the last decade because it can potentially normalize glycometabolic control, at least in the short-term, while providing partial islet function capable of halting the progression of diabetic complications (38, 39). The safety, effectiveness, and minimal invasiveness of islet transplantation combined with kidney transplant have been demonstrated by our group and others (4, 5, 40).

Unfortunately, as with other allogeneic procedures, islet transplantation also requires administration of lifelong immunosuppressive treatment (7). Immunosuppressant (e.g.; Tacrolimus and Sirolimus) increased the incidence of cardiovascular disease due to their detrimental effect on lipid metabolism and blood pressure (11) which may call into question the advantage of islet transplantation alone in truly halting the progression of vascular complications associated with T1D. Our results confirmed that islet transplantation alone is able to reestablish good glycemic control at 15 months of follow-up and to maintain a stable lipid profile and acceptable blood pressure control, despite ongoing immunosuppressive treatment.

Furthermore, our analysis on the hemostasis/thrombosis system demonstrated that patients transplanted with islets alone had normal platelet volume/function and normal levels of pro-thrombotic factors. On the contrary, individuals with T1D, which remained on the waiting list, showed a persistent state of hypercoagulability, which represents a significant risk factor for vascular thrombosis and atherosclerosis in different systems, including the cerebral vascular system. In particular, activation of platelets strictly depends on cytosolic calcium signalling, which tends to be altered in T1D (24).

Platelet activation, together with increased pro-thrombotic factors, leads to persistent activation of the clotting system and facilitates generation of thrombi and vessel occlusion.
We then used $^1$H-MRS to analyze brain metabolism and evaluate whether these hemostatic/platelet alterations resulted in or were associated with any modification of cerebral morphology, metabolism and function (28). Brain content of total creatine (associated with energy metabolism and considered as an internal standard), of choline-containing compounds (playing an important role in the turnover of cellular wall) and of N-acetylaspartate (a marker of neuronal density and functioning), whose relative reductions have been correlated with neuronal/axonal defect in number and function (29, 35) were measured. $^1$H-MRS showed specific alterations in cerebral metabolism, which were otherwise undetectable with standard methods, including signs of atrophy, decrease in brain volume, and reduced axonal metabolism in individuals with T1D as compared to healthy controls. These metabolic abnormalities can be associated with early cognitive decline and possibly senile dementia. Conversely, we observed higher values in NAA/Cho in patients transplanted with islets alone and lower values in the Cho/Cr ratio, indicating a relative sparing of neuronal function from tissue degeneration and loss after islet transplantation alone (36, 37). Finally, we documented a significant improvement in mood profile, depression and anxiety, speed of information processing and attention abilities, with a greater maintenance of neuropsychological attitude in patients transplanted with islets alone as compared to individuals with T1D. The presence of a positive correlation between HbA1C levels and NAA/Cho ratio (a good marker of axonal/neuronal degeneration) demonstrated the importance of good metabolic control in patients transplanted with islets alone (and overall in individuals with T1D) in influencing the preservation of neural structures and cognitive function/metabolism. We acknowledge that long-term immunosuppression is not an option for individuals with T1D, but our study is a proof-of-concept to demonstrate that islet transplant, despite immunosuppression, can prevent the occurrence of major diabetic complications.
LIMITATIONS OF THE STUDY

Our study is not devoid of limitations. First, the sample size is relatively small and a larger study is required to generalize conclusions. Secondly, although the two cohorts of individuals with T1D (T1D-WL and T1D-ITA) were homogeneous at baseline, we cannot completely exclude the presence of biases. In this context, we acknowledge the complexity of our whole clinical assessment, which will require a further validation. Another biased may be represented by concomitant immunosuppression, which may affect coagulatory/inflammatory markers. Indeed, our data in individuals with ESRD who received a kidney transplant and in individuals with T1D treated with Sirolimus, demonstrated that immunosuppression has no effects on coagulatory/inflammatory markers at least in our study and thus should not be a confounding factor. Our results obtained in a relative short period of time, underline the relevance that islet transplantation alone may have in halting diabetic complications in individuals with T1D (Supplemental Table S2). However, the field of islet transplantation needs randomized well-designed and powered studies to confirm in a solid fashion the benefits of this approach.

CONCLUSION

In conclusion, the restoration of near-normal glucose metabolism with islet transplantation alone improves glycometabolic control and hemostatic abnormalities, thereby leading to the near-normalization of cerebral disorders (Supplemental Table S2). Despite the disadvantageous effects of immunosuppressive therapy, islet transplantation may be an important option to limit the progression of major T1D complications, which has to be validated in larger controlled clinical trial.

AUTHOR CONTRIBUTIONS

F.D. designed research, performed research analyzed data, and wrote the paper; P.M. performed research and analyzed data; P.V. performed research; A.V. performed research; A.M. performed research; R.B. performed research; R.N. performed research; M.F. performed research; E.C. performed...
research; G.F. performed research and analyzed data; A.D. performed research; I.F. performed research; S.L. performed research and analyzed data; G.M. edited the paper; L.P. edited the paper; A.F. performed research; F.F. designed research and edited the paper; A.S. designed research and edited the paper; P.F. designed research, wrote and edited the paper. P.F. is the guarantor of the paper.

ACKNOWLEDGMENTS

This work was supported by Juvenile Diabetes Research Foundation (JDRF Grant: JT01Y01). Francesca D’Addio is a recipient of Italian Scientists and Scholars of North America Foundation (ISSNAF)-Fondazione Marche Fellowship. Paolo Fiorina is the recipient of a JDRF-Career Development Award, an ASN Career Development Award, and an ADA mentor-based fellowship. P.F. is supported by a Translational Research Program (TRP) grant from Boston Children's Hospital. P.F. is recipient of a Minister of Health of Italy grant: ("Staminali"RF-FSR-2008-1213704). An NIH-Research Training Grant to Boston Children’s Hospital in Pediatric Nephrology (T32DK007726-28) supports A.V. Roberto Bassi is supported by an ADA mentor-based fellowship to P.F. Paolo Fiorina 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. Conflict of interest: none.
REFERENCES


**TABLES AND FIGURE LEGENDS**

**Table 1.** Psychological evaluation: profile of mood state analysis of depression-dejection and fatigue-inertia tracts, as well as evaluation of physical function showed better results in ITA compared to individuals with T1D-WL. Neuropsychological evaluation: phonemic word fluency, paced auditory serial addition test and Stroop tests are near-normalized in ITA patients compared to T1D-WL (15 months follow up).

<table>
<thead>
<tr>
<th></th>
<th>CTRL (n=10)</th>
<th>T1D-WL (n=10)</th>
<th>ITA (n=12)</th>
<th><strong>p value</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>POMS-T</td>
<td>10.8±1.3</td>
<td>11.6±6.2</td>
<td>9.6±2.7</td>
<td>0.38*; 0.28§; 0.81§</td>
</tr>
<tr>
<td>POMS-D</td>
<td>8.6±0.9*</td>
<td>9.2±5.6#</td>
<td>2.6±2.4*,#</td>
<td>0.0002*; 0.02#</td>
</tr>
<tr>
<td>POMS-A</td>
<td>10.2±2.5*,§</td>
<td>5.2±4.0 §</td>
<td>5.8±3.5*</td>
<td>0.03*; 0.01§</td>
</tr>
<tr>
<td>POMS-V</td>
<td>14.0±3.1</td>
<td>13.9±7.8</td>
<td>17.6±5.8</td>
<td>0.38*; 0.22§; 0.97§</td>
</tr>
<tr>
<td>POMS-S</td>
<td>9.0±2.2*,§</td>
<td>5.5±3.5§</td>
<td>4.6±2.5*</td>
<td>0.008*; 0.04§;</td>
</tr>
<tr>
<td>POMS-C</td>
<td>4.8±1.1</td>
<td>8.2±3.9 §</td>
<td>4.3±2.0#</td>
<td>0.04#</td>
</tr>
<tr>
<td>Physical function</td>
<td>2.4±1.1</td>
<td>4.3±2.2</td>
<td>2.8±0.4</td>
<td>0.14*; 0.09#; 0.48§</td>
</tr>
<tr>
<td>Self-care</td>
<td>0.2±0.4</td>
<td>0.8±1.7</td>
<td>0.0±0.0</td>
<td>0.33*; 0.64#; 0.14§</td>
</tr>
<tr>
<td>Depression and anxiety</td>
<td>2.0±0.7</td>
<td>3.5±1.9#</td>
<td>1.2±0.8#</td>
<td>0.02#</td>
</tr>
<tr>
<td>Cognitive functioning</td>
<td>2.2±0.4</td>
<td>3.9±2.2</td>
<td>3.0±0.7</td>
<td>0.41*; 0.13#; 0.07§</td>
</tr>
<tr>
<td>Social functioning</td>
<td>2.4±1.1</td>
<td>3.3±2.5</td>
<td>3.8±1.9</td>
<td>0.73*; 0.55#; 0.30§</td>
</tr>
<tr>
<td>Sexual functioning</td>
<td>1.8±0.8</td>
<td>2.2±1.9</td>
<td>2.4±0.8</td>
<td>0.85*; 0.65#; 0.30§</td>
</tr>
<tr>
<td>Life satisfaction</td>
<td>2.4±0.5*,§</td>
<td>7.3±2.3§</td>
<td>5.5±3.2*</td>
<td>0.0006§; 0.05*</td>
</tr>
<tr>
<td>MMSE</td>
<td>29.0±0.7</td>
<td>29.1±1.2</td>
<td>28.8±1.3</td>
<td>0.67*; 0.90#; 0.82§</td>
</tr>
<tr>
<td>Token</td>
<td>33.4±0.8</td>
<td>33.6±1.9</td>
<td>33.0±1.8</td>
<td>0.56*; 0.75#; 0.83§</td>
</tr>
<tr>
<td>Phonemic fluency</td>
<td>38.6±6.5§</td>
<td>30.5±9.9§</td>
<td>33.4±8.8</td>
<td>0.03§</td>
</tr>
<tr>
<td>Semantic fluency</td>
<td>49.2±7.3§</td>
<td>39.1±10.0§</td>
<td>46.8±9.6</td>
<td>0.03§</td>
</tr>
<tr>
<td>Long-term verbal memory</td>
<td>16.8±1.9</td>
<td>14.2±4.6</td>
<td>14.8±4.3</td>
<td>0.81*; 0.22#; 0.37§</td>
</tr>
<tr>
<td>Short-term verbal memory</td>
<td>4.9±0.5</td>
<td>5.3±1.1</td>
<td>5.3±0.7</td>
<td>0.89*; 0.44#; 0.40§</td>
</tr>
<tr>
<td>Corsi</td>
<td>5.1±0.8</td>
<td>4.5±0.6</td>
<td>4.8±0.5</td>
<td>0.42*; 0.17#; 0.44§</td>
</tr>
<tr>
<td>PASAT</td>
<td>41.4±9.1*</td>
<td>36.8±14.8#</td>
<td>49.5±7.2*,</td>
<td>0.04*; 0.05*</td>
</tr>
<tr>
<td>Stroop 1</td>
<td>14.0±3.4*,#</td>
<td>21.7±4.7§</td>
<td>23.0±3.8*</td>
<td>0.02§; 0.01*</td>
</tr>
<tr>
<td>Stroop 2</td>
<td>17.2±5.5*,§</td>
<td>35.7±5.6§</td>
<td>32.5±6.1*</td>
<td>0.0001§; 0.003*;</td>
</tr>
<tr>
<td>Stroop 3</td>
<td>36.8±21.4</td>
<td>46.8±4.4</td>
<td>43.2±2.3</td>
<td>0.16*; 0.24#; 0.55§</td>
</tr>
<tr>
<td>Stroop error</td>
<td>0.6±1.3*,#</td>
<td>3.8±3.4#</td>
<td>4.1±3.4*</td>
<td>0.03; 0.02#</td>
</tr>
<tr>
<td>WCST</td>
<td>6.0±0.0</td>
<td>5.9±0.3</td>
<td>6.0±0.0</td>
<td>0.89*; 0.49#; 0.74§</td>
</tr>
</tbody>
</table>

**Abbreviations.** CTRL (control subjects); T1D-WL (type 1 diabetic patients on waiting list); ITA (islet transplanted patients); POMS-T (profile of mood state-tension-anxiety); -D (depression-dejection); -A
(anger-hostility); -V (vigor-activity); -S (fatigue-inertia); -C (confusion-bewilderment); MMSE (mini mental state examination); PASAT (paced auditory serial addition test); Stroop 1 (stroop test colour naming); Stroop 2 (stroop test word-colour interference); Stroop 3 (stroop test colour-colour interference); WCST (Wisconsin card sorting test).

The symbols contained in the table (*#§) are meant to represent the statistical significant tests between paired values, and each of them is recalled in the “p value” column reporting the exact value (*p value CTRL vs. ITA; § CTRL vs. T1D-WL; #p value T1D-WL vs. ITA).
FIGURES

Figure 1. Metabolic parameters, resting calcium and platelet morphology in patients who underwent islet transplant alone (ITA), in individuals with type 1 diabetes (T1D-WL) and in healthy controls (CTRL) at 15 months of follow-up. Serum glucose (A), C-Peptide (B), glycated hemoglobin (HbA1C) (C) and exogenous insulin requirement (D) were improved in ITA patients as compared to individuals with T1D (T1D-WL). Platelet areas were also higher in the T1D-WL as compared to the CTRL group (p=0.02) (E). (F) Resting [Ca2+]i was significantly higher in the T1D-WL group than in ITA and CTRL groups (p=0.01). Electron microscopy revealed that in the CTRL group (G), platelets show secretory granules (primarily alpha-type), glycogen, and several tubules and vesicles. Platelets from individuals with T1D displayed a tendency to aggregate, and their cytoplasm was denser than platelets in the CTRL and ITA groups (G).

Figure 2. Hypercoagulability markers in patients who underwent islet transplant alone (ITA), in individuals with type 1 diabetes (T1D-WL) and in healthy controls (CTRL) at 15 months of follow-up. Fibrinogen (p=0.005; A), F1+2 (p=0.005; B) and D-dimer levels (p=0.01; C) were higher in individuals with T1D (T1D-WL) as compared with patients in the CTRL group (p=0.01; C). No differences were found among groups in fasting homocysteine levels (D). Protein S and C activity were lower in individuals with T1D (T1D-WL) as compared to CTRL, and a near-normalization of their levels was evident in the ITA group (Protein S: CTRL vs. T1D-WL, p=0.03, E; Protein C CTRL vs. T1D-WL, p=0.01, F).

Figure 3. Conventional MRI imaging and 1H-MRS analysis in patients who underwent islet transplant alone (ITA), in individuals with type 1 diabetes (T1D) and in healthy controls (CTRL) at 15 months of follow-up. Brain volume (A) was higher in CTRL as compared to ITA and T1D
groups. No other differences between groups were evident in N-acetylaspartate concentration (NAA) (B), whole brain N-acetylaspartate (WB$_{\text{NAA}}$) (C) and NAA/Creatine (Cr) (E). NAA/Choline (Cho) (D) was significantly lower in individuals with T1D (T1D-WL) compared to controls, while it was near normalized in ITA patients. The Cho/Cr ratio was increased in T1D-WL as compared to both ITA patients and controls, suggesting an increase in degenerative processes (p=0.01) (F). AU (arbitrary unit).
Figure 1
Figure 2
Figure 3
ONLINE-ONLY SUPPLEMENTAL MATERIAL

TABLE AND FIGURE LEGENDS

Tables

Supplemental Table S1. Baseline demographic and metabolic characteristics of the 3 patient groups: healthy subjects (CTRL), individuals with type 1 diabetes (T1D) who remained on the waiting list (T1D-WL), and who underwent islet transplantation alone (T1D-ITA).

Supplemental Table S2. Results overview using the following arbitrary unit: +++ (high improvement); ++ (mild improvement); + (slight improvement); = no improvement; --- (high worsening); -- (mild worsening), - (slight worsening).

Figures

Supplemental Figure S1. Flow chart describing the study design.

Supplemental Figure S2. Coagulatory/inflammatory markers in individuals with end stage renal disease (ESRD), in individuals with ESRD who received a kidney transplant (KA), in individuals with T1D (T1D) and in individuals with T1D treated with Sirolimus (T1D-SRL) and in healthy controls (CTRL) at 15 months of follow-up. Fibrinogen (p=ns; A), F1+2 (p=ns; B), D-dimer levels (p=ns; C), Homocysteine levels (p=ns; D), Protein S (p=ns; E) and Protein C activity (p=ns; F) were similar in individuals with ESRD and in those with ESRD who received a kidney transplant (KA). No differences in the aforementioned markers were evident between individuals with T1D receiving Sirolimus (T1D-SRL) and those who did not (T1D) (G-N).
Screening of individuals with T1D on waiting list for islet transplantation
N=22

### Inclusion Criteria
- age range 18–65;
- diabetes duration >5 years;
- reduced awareness to hypoglycemia/ketoacidosis;
- microalbuminuria <150 mg/L
- creatinine levels <1.5 mg/dL;
- absence of cardiomyopathy, severe diabetic complications, malignancy, chronic infection.

Study enrollment and baseline evaluation
(glycometabolic control, hemostasis status, cerebral morphology and metabolism)

- Patients underwent islet transplantation
  N=12

- Patients remaining on waiting list
  N=10

15 months Follow-up Analysis
(glycometabolic control, hemostasis status, cerebral morphology and metabolism)

Supplemental Figure S1
**Supplemental Table S1.** Baseline demographic and metabolic characteristics of the 3 patient groups: healthy subjects (CTRL), individuals with type 1 diabetes (T1D) who remained on the waiting list (T1D-WL), and who underwent islet transplantation alone (T1D-ITA).

<table>
<thead>
<tr>
<th></th>
<th>CTRL (n=10)</th>
<th>T1D on waiting list (n=10)</th>
<th>T1D before ITA (n=12)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>4/6</td>
<td>2/8</td>
<td>4/8</td>
<td>0.36*; 0.52#; 0.78§</td>
</tr>
<tr>
<td>Age (years)</td>
<td>42.0±5.0</td>
<td>42.0± 12.3</td>
<td>36.9± 7.3</td>
<td>0.29*; 0.17#; 0.22§</td>
</tr>
<tr>
<td>Years of T1D</td>
<td>-</td>
<td>27.1±8.5</td>
<td>23.3± 10.4</td>
<td>0.41§</td>
</tr>
<tr>
<td>Total-C (mg/dl)</td>
<td>174.3±12.5</td>
<td>192.0± 28.0</td>
<td>183.5±12.0</td>
<td>0.12*; 0.56#; 0.55§</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>57.2±5.4*</td>
<td>51.5±6.4*</td>
<td>52.0±8.1</td>
<td>0.29*; 0.56#; 0.72§</td>
</tr>
<tr>
<td>Trig (mg/dl)</td>
<td>87.4±4.3</td>
<td>85.0±3.0</td>
<td>92.5±15.0</td>
<td>0.79*; 0.27#; 0.77§</td>
</tr>
<tr>
<td>HbA1c % (mmol/mol)</td>
<td>5.3±1.0 (34±10.9)*</td>
<td>8.3±1.1 (67±12.0)*</td>
<td>8.2±0.9 (66±9.8)§</td>
<td>0.00002*, 0.0001§</td>
</tr>
<tr>
<td>S-Gluc (mg/dl)</td>
<td>87.4±4.3*</td>
<td>164.0±51.0*</td>
<td>193.8±80.0§</td>
<td>0.0001*, 0.002§</td>
</tr>
<tr>
<td>EIR (UI)</td>
<td>-</td>
<td>39.8±10.0</td>
<td>42.1±5.1</td>
<td>N/A, 0.50§</td>
</tr>
<tr>
<td>C-peptide (ng/ml)</td>
<td>2.4±0.4*</td>
<td>0.17±0.2*</td>
<td>0.18±0.3§</td>
<td>&lt;0.00001*§</td>
</tr>
<tr>
<td>BMI</td>
<td>25.5±0.7</td>
<td>23.0±2.4</td>
<td>23.5± 3.1</td>
<td>0.22*; 0.71#; 0.25§</td>
</tr>
<tr>
<td>S-Creatinine (mg/dl)</td>
<td>0.7±0.4</td>
<td>0.8±0.3</td>
<td>1.07±0.5</td>
<td>0.23*; 0.21#; 0.31§</td>
</tr>
<tr>
<td>Mean Arterial Pressure (mmHg)</td>
<td>98.75±1.8</td>
<td>99.38±1.4</td>
<td>101.8±1.2</td>
<td>0.23*; 0.21#; 0.31§</td>
</tr>
</tbody>
</table>

**Abbreviations.** CTRL (control subjects); T1D (type 1 diabetic patients); ITA (islet transplanted patients); Total-C (total cholesterol); HDL-C (high-density lipoprotein); Trig (triglycerides); HbA1c % (glycated hemoglobin A1c %); S-Gluc (serum glucose); EIR (exogenous insulin requirement). The symbols contained in the table (*#§) are meant to represent the statistical significant tests between paired values, and each of them is recalled in the “p value” column reporting the exact value (*p value CTRL vs. T1D on waiting list; § CTRL vs. T1D before ITA; #p value T1D on waiting list vs. T1D before ITA).
**Supplemental Table S2. Results Overview.** Arbitrary unit: +++ (high improvement); ++ (mild improvement); + (slight improvement); = (no improvement); -- (mild worsening); --- (high worsening).

<table>
<thead>
<tr>
<th>Results</th>
<th>ITA vs. T1D-WL</th>
<th>ITA vs. CTRL</th>
<th>T1D-WL vs. CTRL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose metabolism</td>
<td>+++</td>
<td>=</td>
<td>---</td>
</tr>
<tr>
<td>Renal Function</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Blood Pressure Control</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Coagulation</td>
<td>++</td>
<td>=</td>
<td>---</td>
</tr>
<tr>
<td>Platelet Morphology and aggregation</td>
<td>++</td>
<td>=</td>
<td>---</td>
</tr>
<tr>
<td>Platelets Calcium Signaling</td>
<td>+</td>
<td>=</td>
<td>---</td>
</tr>
<tr>
<td>Brain volume</td>
<td>=</td>
<td>=</td>
<td>-</td>
</tr>
<tr>
<td>NAA/Cho ratio</td>
<td>+++</td>
<td>=</td>
<td>---</td>
</tr>
<tr>
<td>NAA/Cr ratio</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Cho/Cr Ratio</td>
<td>+++</td>
<td>=</td>
<td>-</td>
</tr>
<tr>
<td>NAA/WB NAA</td>
<td>=</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>POMS test</td>
<td>+++</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>LEIPAD test</td>
<td>++</td>
<td>=</td>
<td>-</td>
</tr>
<tr>
<td>PASAT test</td>
<td>+++</td>
<td>=</td>
<td>---</td>
</tr>
<tr>
<td>Stroop test</td>
<td>=</td>
<td>-</td>
<td>--</td>
</tr>
</tbody>
</table>