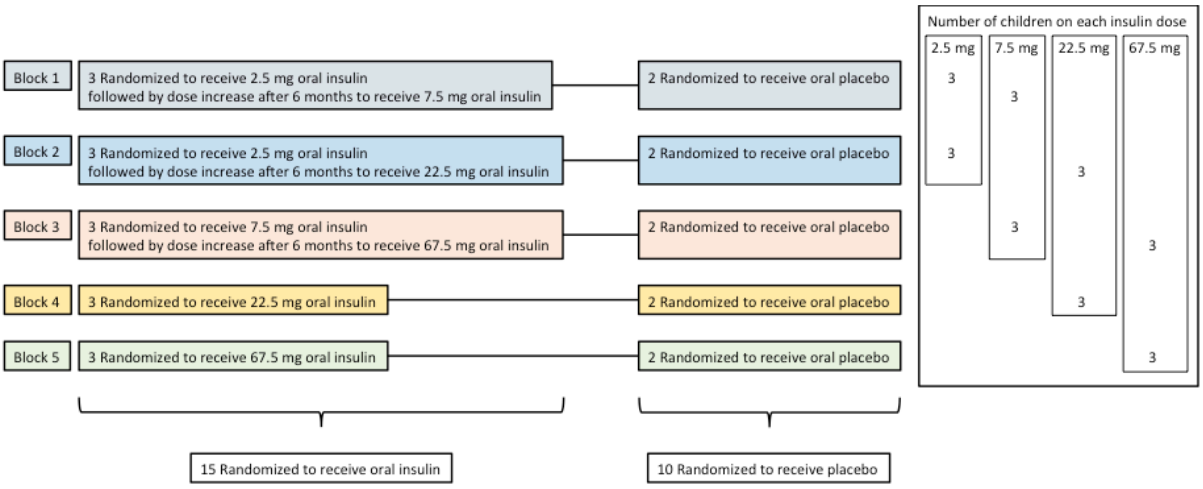
Supplementary Online Content

Bonifacio E, Ziegler A-G, Klingensmith G, et al; Pre-POINT Study Group. Effects of high-dose oral insulin on immune responses in children at high risk for type 1 diabetes: the Pre-POINT randomized clinical trial. *JAMA*.

doi:10.1001/jama.2015.2928

This supplementary material has been provided by the authors to give readers additional information about their work.



eFigure 1. Schematic representation of randomization blocks in the Pre-POINT study. Children in Block 1 received 2.5 mg insulin for 6 months followed by a dose escalation to 7.5 mg insulin for 3–12 months (n=3) or received placebo throughout the study (n=2). Children in Block 2 received 2.5 mg insulin for 6 months followed by a dose escalation to 22.5 mg insulin for 3–12 months (n=3) or received placebo throughout the study (n=2). Children in Block 3 received 7.5 mg insulin for 6 months followed by a dose escalation to 67.5 mg insulin for 3–12 months (n=3) or received placebo throughout the study (n=2). Children in Block 4 received 22.5 mg insulin (n=3) or placebo (n=2) for 3–6 month. Children in Block 5 received 67.5 mg insulin (n=3) or placebo (n=2) for 3–6 months. The number of children that received 2.5 mg, 7.5 mg, 22.5 mg, and 67.5 mg insulin in each Block is shown on the right panel; there were 6 children treated for each dose.

eMethods

Blood tests.

Full blood cell count, plasma urea, electrolytes, and liver function tests were performed at the study site laboratories at baseline, 6 months, and/or the last study visit. Central laboratory measurements were performed for insulin autoantibodies (Bristol, UK for the European sites; Denver, Colorado for the US site); serum IgG- and salivary IgA-binding to insulin (Munich, Germany); $CD4^+$ T cell responses to insulin, and the autoantigens proinsulin and GAD65 (Dresden, Germany).

Parental anxiety.

The parents were asked to complete questionnaires on parental distress associated with study participation before enrolment and at 3 and 9 months after enrolment. The questionnaire included a 6-item short form of the state component of the Spielberger State–Trait Anxiety Inventory to assess the parent's anxiety about their child's risk of developing type 1 diabetes (4-point Likert scale 0= no anxiety, 3= high anxiety; maximum possible score = 18).[1]

Salivary IgA-IAA

Saliva samples (20 µl) were incubated in duplicate for 72 h at 4°C in TBT buffer (50 mM Tris, 1% Tween-20, pH 8.0) in the presence of 7.85 fmol of human Tyr14A [^{125}I]-insulin (Aventis, Frankfurt, Germany; specific activity 13.43 GBq/mg) in a final volume of 55 µl. For precipitation of the immune complexes, 25 µl of anti-human IgA-agarose (Sigma-Aldrich, Steinheim, Germany) re-suspended in 25 µl TBT buffer was added to the tubes, and the tubes were incubated for 1 h at 4°C with shaking, and washing, as previously described for the IAA assay.[2] Bound [^{125}I]-insulin was measured using a gamma counter (Packard Instrument Co., Meriden, CT), and the results were expressed as the counts per minute (cpm). There was no prior threshold definition for saliva IgA binding to insulin. A saliva sample from a control subject was used to define the background cpm in the saliva IgA assay. As positive reference samples, the same saliva was spiked with patient serum containing IgA-insulin antibodies. Follow-up samples were considered positive if their cpm values were both >3 times greater than the cpm in the control saliva sample and > 3 times greater than the cpm in the child's baseline sample and > mean plus 5SD of untreated control children.

Flow Analyses

Blood samples used for lymphocyte analyses were collected at or sent to the Munich central laboratory for European sites or the Denver central laboratory for the US site. Peripheral blood mononuclear cells (PBMCs) were prepared using Ficoll gradients (Lymphoprep, Axis-Shield, Oslo Norway). Fresh PBMCs were used for memory and Treg subset analyses using Flow (eMethods). The remainder was frozen in DMSO/human type AB serum (PAA,).

Two staining tubes were used for fluorescent-activated cell sorting. The first was for memory and activated T lymphocytes, and included the following mouse anti-human monoclonal antibodies: anti-CD3 APC-Cy7 (SK7), anti-CD4 Pacific Blue (RPA-T4), anti-CD8 PerCP (SK1), anti-CD25 PE (M-A251), anti-CD45RA APC (HI100), anti-CCR7 PE-Cy7 (3D12), and anti-CD69 FITC (FN50; all BD Bioscience, San Jose, CA). The second was for T regulatory cells, and included the following mouse anti-human monoclonal antibodies: anti-CD4 PerCP (SK3; BD Bioscience), anti-CD25 PE (M-A251; BD Bioscience), anti-CD45RA APC (HI100; BD Bioscience), anti-CD127 eFluor450 (eBioRDR5; eBioscience), and anti-FOXP3 Alexa Fluor 488 (259D; BioLegend, San Diego, CA). To detect intracellular protein expression, cells were fixed and permeabilized after surface staining using a FOXP3 Fix/Perm buffer set (BioLegend). After processing, the cells were analyzed in a Becton Dickinson LSR II flow cytometer with FACS DIVA™ (Version 7.0; BD Bioscience) acquisition software, and processed using FlowJo software (Version 7.6.5; TreeStar Inc., Ashland, OR).

$CD4^+$ T Cell Response Assay

Peripheral blood mononuclear cells (PBMCs) were thawed with pre-warmed Dulbecco's modified Eagle's medium (4.5 g/l glucose, L-glutamine-free; Lonza, Basel, Switzerland) supplemented with Benzonase® (25 units/ml; 99% purity; Novagen). Cell viability was determined using a trypan blue assay, and only samples with >5 million viable PBMCs were subsequently measured in proliferation assays. PBMCs were washed with phosphate-buffered saline (PBS) and labeled with Cell Proliferation Dye eFluor® 670 (5 µM in PBS for 10 min at 37°C; eBioscience, San Diego, CA). Staining was terminated by adding RPMI1640 (Invitrogen, Carlsbad, CA) containing 5% human serum AB (PAA), 2 mM glutamine (Lonza) and 100

units/ml penicillin/streptomycin (Lonza) at 4°C. Next, 2×10^5 eFluor® 670-stained PBMCs were added to each well of a round-bottom 96-well microtiter plate, and stimulated without an antigen (median, 2 wells per sample) or with insulin (50 µg/ml, Lilly; median, 8 wells per sample), tetanus toxoid (1 µl/ml; Sanofi Pasteur MSD), GAD65 (10 µg/ml; Diamyd Medical, Stockholm, Sweden), or proinsulin (10 µg/ml; Lilly) as antigens. After 5 days, the cells were harvested and stained with the following monoclonal antibodies: anti-CD4 PB (clone RPA-T4; BD Biosciences), anti-CD25 PE (clone M-A251; BD Biosciences), anti-CD8 APC-Cy7 (clone SK1; BD Biosciences), anti-CD45RO PE-Cy7 (clone UCHL1; BD Biosciences), or anti-CD45 RA FITC (clone ALB 11, Beckman Coulter, Inc., Brea, CA). 7-Aminoactinomycin D (BD Biosciences) was used to exclude dead cells. Cells were washed twice with PBS/1% human serum, acquired on a Becton Dickinson FACS Aria II flow cytometer with FACS Diva software, and were analyzed using FlowJo software version 7.6.5 (TreeStar Inc.). $CD4^+$ T cells, which had proliferated as determined by eFluor® 670 dilution and displayed CD25 upregulation, were identified as responding cells and were single-cell-sorted directly into 96-well microplates containing 5 µl PBS (prepared with diethylpyrocarbonate-treated water). A stimulation index was calculated for each antigen using the formula: number of responsive cells per 50,000 acquired live $CD4^+$ T cells in antigen wells/number of responsive cells per 50,000 acquired live $CD4^+$ T cells in wells containing medium only.

Gene Expression Profile of Single Cells

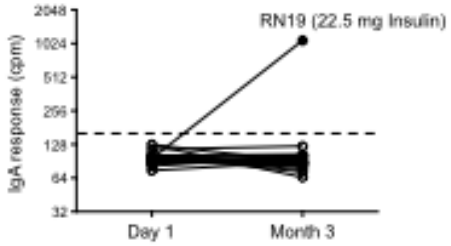
cDNA was synthesized directly from cells using qScript™ cDNA Supermix (Quanta Biosciences, Gaithersburg, MD). Total cDNA was preamplified for 16 cycles, with one cycle at 95°C for 8 and 16 cycles of 95°C for 45 s, 49°C with and increment of 0.3°C/cycle for 1 min each, and 72°C for 1.5, followed by one cycle of 72°C for 7 min, with the TATAA GrandMaster Mix (TATAA Biocenter, Göteborg, Sweden) in the presence of 34 primer pairs in a final volume of 35 µl. Then, 10 µl of preamplified DNA was treated with 1.2 units of exonuclease I. To quantify gene expression, real-time PCR was performed using the BioMark™ HD System (Fluidigm Corporation, South San Francisco, CA) using the 96.96 Dynamic Array IFC according to the GE 96×96 Fast PCR+ Melt protocol with SsoFast EvaGreen Supermix containing Low ROX (Bio-Rad, Hercules, CA) and 5 µM of primers in each assay. The following target genes were analyzed *FOXP3*, *CD127*, *IFNG*, *IL21*, *IL4*, *IL9*, *IL10*, *IL17A*, *IL17F*, *IL22*, *GMCSF*, *TNFA*, *TGFB*, *IL18RAP*, *CCR3*, *CCR4*, *CCR5*, *CCR6*, *CCR7*, *CXCR5*, *TBET*, *CMAF*, *EGR2*, *AHR*, *HELIOS*, *GATA3*, *LAG3*, *ICOS*, *CD40*, and *SRP14* (as a housekeeping gene). Raw data were analyzed using Fluidigm Real-Time PCR analysis software and GenEx Pro 5.3.6 Software (MultiD, Göteborg, Sweden). Additional data analysis was done using KNIME 2.5.2 software.[3] Analysis of multivariate gene expression patterns was performed by t-distributed Stochastic Neighbor Embedding (t-SNE)[4] on the pre-processed Ct values. For pre-processing, a linear model was used to correct for potential confounding effects, which can mask relevant biological variability.[5] In brief, batch effects (dummy coding for each plate/batch) were modeled jointly with dose effects by regressing out the effect of plates on each individual gene while controlling for dose in order to obtain a corrected gene expression dataset.

Islet Autoantibody Positive Children Used for the Analysis of T cell Gene Expression Profiles

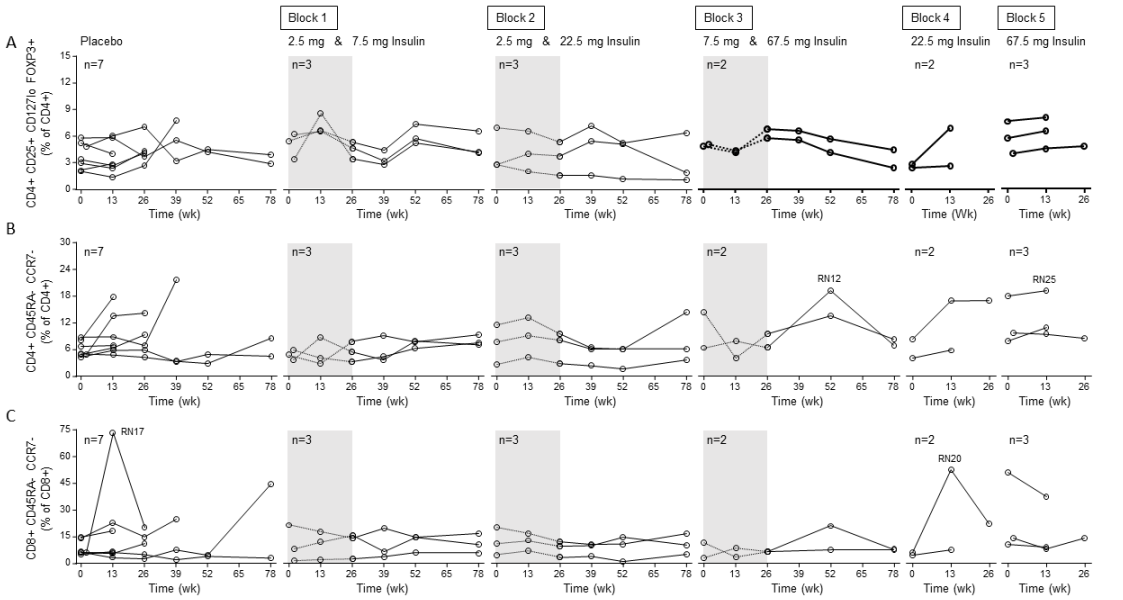
Children from the BABYDIET study[6] were analyzed. The characteristics of the children and the samples used are summarized in eTable 1.

eReferences

1. Spielberger CD, Gorsuch RI, Lushene R. *Test Manual for the State-Trait Anxiety Inventory*. Palo Alto, CA, Consulting Psychologists Press, 1970
2. Naserke HE, Bonifacio E, Ziegler AG. Immunoglobulin G insulin autoantibodies in BABYDIAB offspring appear postnatally: sensitive early detection using a protein A/G-based radiobinding assay. *J Clin Endocrinol Metab*. 1999;84(4):1239-1243.
3. Berthold M, Cebron N, Dill F, et al. KNIME: The Konstanz Information Miner 2007.
4. Van der Maaten L, & Hinton G. Visualizing data using t-SNE. *J Mach Learn Res.* 2008;9:2579-2605.
5. Buettner F, Natarajan KN, Casale FP, et al. Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells. *Nat Biotechnol* 2015; doi: 10.1038/nbt.3102.
6. Hummel S, Pflüger M, Hummel M, Bonifacio E, Ziegler AG. Primary dietary intervention study to reduce the risk of islet autoimmunity in children at increased risk for type 1 diabetes: the BABYDIET study. *Diabetes Care*. 2011;34(6):1301-1305.



eFigure 2. Salivary immunoglobulin A binding to insulin in 25 Pre-POINT children. The child with a positive response is indicated by the randomization number (see eFigure 5 for randomization number sequence). A reference threshold line is indicated at 160 cpm representing the mean cpm response at day 1 plus five standard deviations. Abbreviations: IgA, immunoglobulin A; RN, subject randomization number.



eFigure 3. T lymphocyte phenotyping in peripheral blood of 20 Pre-POINT children on follow-up from baseline (week 0) and during the administration of placebo or insulin. The proportion of $CD4^+$ T cells with a T regulatory cell phenotype (A) and memory phenotype (B), and of $CD8^+$ T cells with an effector memory phenoype (C) are shown.

eFigure 3. T lymphocyte phenotyping in peripheral blood of 20 Pre-POINT children on follow-up from baseline (week 0) and during the administration of placebo or insulin. The proportion of $CD4^+$ T cells with a T regulatory cell phenotype (A) and memory phenotype (B), and of $CD8^+$ T cells with an effector memory phenoype (C) are shown. Abbreviations: RN, randomization number.

eResults

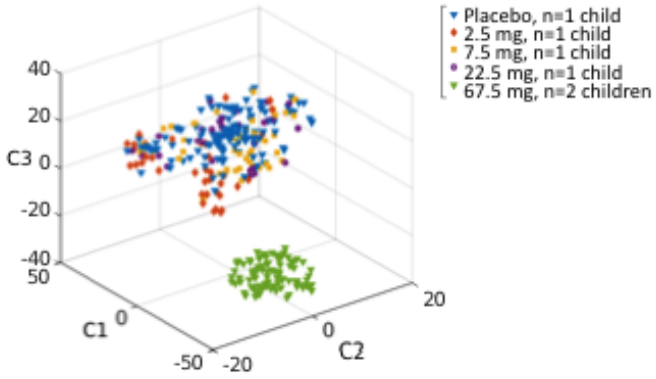
Gene expression of insulin and proinsulin-responsive T cells in Figure 3 from manuscript.
We analyzed the gene expression profiles under insulin and proinsulin stimulated proliferation of single-cell-sorted $CD4^+$ T cells from insulin-treated children (Figure 3). A cluster of insulin-responsive cells expressed *FOXP3* (forkhead box P3) without CD127 or cytokines (*FOXP3* signature cells, 43 [15.2%; 95% CI 11.0%–19.4%] of 282 analyzed cells; Figure 3A). These cells also showed abundant expression of the genes *TGFB* (transforming growth factor-beta) and *CTLA4* (cytotoxic T-lymphocyte-associated protein 4). A second cluster was characterized by *IFNG* (interferon-gamma) gene expression with or without *FOXP3* expression (*IFNG* signature cells; 41 [14.5%; 95% CI 10.4%–18.6%] of 282 analyzed cells). A cluster of cells expressing *IL21* (interleukin-21) (36 [12.8%; 95% CI 8.9%–16.7%] of 282 analyzed cells) was also observed. The remaining cells were mostly negative for the cytokines examined. The ratio of *FOXP3* signature cells to *IFNG* signature cells was 1.05 in samples obtained during oral insulin treatment. Similar profiles were observed for proinsulin-responsive cells (Figure 3A), where *FOXP3* signature cells were increased (15 [18.8%; 95% CI 10.2%–27.4%] of 80 analyzed cells) over *IFNG* signature cells (13 [16.3%; 95% CI 8.2%–24.4%] of 80 analyzed cells) in cells obtained during oral insulin treatment (*FOXP3*/*IFNG* ratio 1.15).
By comparison, proinsulin autoantigen-responsive $CD4^+$ T cells isolated from islet autoantibody positive children enrolled in the BABYDIET study[6] (eTable 2) had a dominance of *IFN* signature cells (104 [29.6%; 95% CI 24.8%–34.4%] of 351 analyzed cells) over FOXP3 signature cells (27 [7.7%; 95% CI 4.9%–10.5%] of 351 analyzed cells; Figure 3B). *IFNG* signature cells (134 [56.5%; 95% CI 50.2%–62.8%] of 237 analyzed cells) were also dominant over *FOXP3* signature cells (5 [2.1%; 95% CI 0.3%–3.9%] of 237 analyzed cells) in tetanus toxoid-responsive cells obtained from islet autoantibody positive children (Figure 3B). Tetanus toxoid-responsive cells also had a prominent component of *IL21*-expressing cells.

Parental anxiety
The mean parental anxiety score (see eMethods) was 9.51 ± 4.1 at enrolment (39 parents), 9.16 ± 3.4 at 3 months (32 parents), and 6.53 ± 4.6 at 9 months (19 parents; $P = .016$).

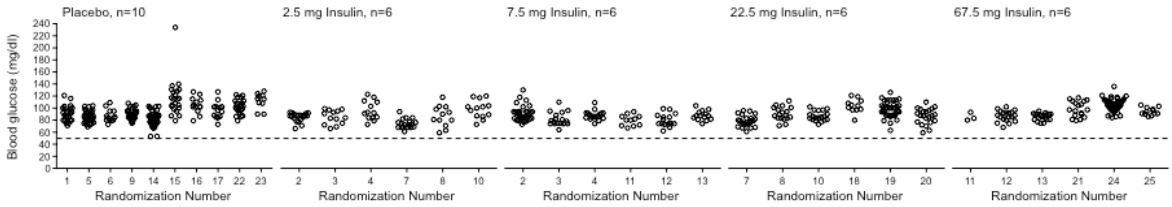
eTable 1. Characteristics of autoantibody positive children from the BABYDIET study[6] included to analyze proinsulin responsive CD4$^+$ T cell gene expression shown in Figure 3C–E

Child	Sex	HLA DR-DQB1	Age at first islet AAb positivity	Age at sample collection	Islet AAbs
7478	F	DR3/DR4-DQB1*0302	4.8 years	4.8 years	Insulin, GAD
9785	M	DR3/DR4-DQB1*0302	2.1 years	2.4–3.2 years	Insulin, GAD
10210	M	DR3/DR4-DQB1*0302	0.7 years	1.8–2.8 years	Insulin, GAD, IA-2
10299	F	DR3/DR4-DQB1*0302	1.3 years	1.3–2 years	Insulin, GAD, IA-2

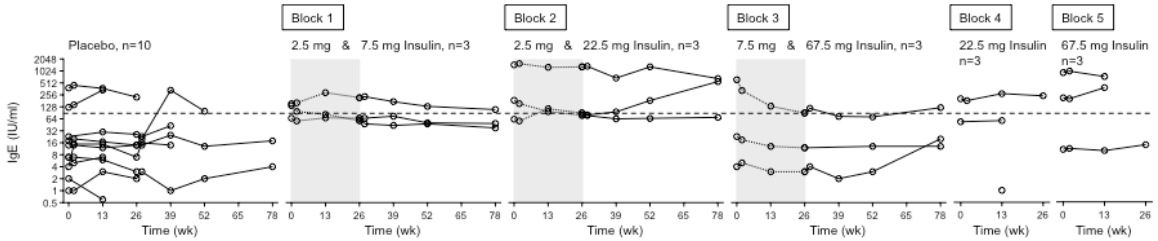
Abbreviations: AAb, autoantibody; F, female; GAD, glutamic acid decarboxylase; HLA, human leukocyte antigen; IA-2, insulinoma-associated antigen-2; M, male.



eFigure 4. Multivariable gene expression analysis of insulin-responsive CD4$^+$ T cells isolated from participants with CD4$^+$ T cell responses to insulin. The data shows t-distributed Stochastic Neighbor Embedding (tSNE) analysis of pre-processed Ct values for all analyzed genes (*FOXP3*, *CD127*, *IFNG*, *IL21*, *IL4*, *IL9*, *IL10*, *IL17A*, *IL17F*, *IL22*, *GMCSF*, *TNFA*, *TGFB*, *IL18RAP*, *CCR3*, *CCR4*, *CCR5*, *CCR6*, *CCR7*, *CXCR5*, *TBET*, *CMAF*, *EGR2*, *AHR*, *HELIOS*, *GATA3*, *LAG3*, *ICOS*, *CD40*, *SRP14*). A linear model was used to correct for confounding effects which can mask relevant biological variability. Batch effects (dummy coding each plate/batch) were modeled jointly with dose effects to obtain a corrected gene expression data set. This resulted in two distinct clusters, with one cluster consisting of cells derived from children receiving 67.5 mg insulin (green symbols) and the second cluster consisting of cells derived from children receiving placebo (blue symbols), 2.5 mg insulin (red symbols), 7.5 mg insulin (yellow symbols), and 22.5 mg insulin (purple symbols). C1, C2, and C3 are component 1, component 2, and component 3 of the tSNE.



eFigure 5. Blood glucose concentrations measured at home, 1 h after taking the study drug. Randomization number refers to the ID of each participant in the trial.



eFigure 6. Total serum immunoglobulin E concentrations at baseline and during medication in study participants. The period of treatment with the lower starting dose, before dose escalation, is indicated with grey shading. An upper reference level is indicated by the broken line at a concentration of 90 IU/ml. Abbreviations: IgE, immunoglobulin E.

eTable 2. Laboratory characteristics of the Pre-POINT study participants at baseline and at the last study visit

Parameter (unit)	**Timepoint**	**Placebo (*n* = 10)**	**Insulin**			
			2.5 mg (*n* = 6)	**7.5 mg (*n* = 6)**	**22.5 mg (*n* = 6)**	**67.5 mg (*n* = 6)**
		Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Erythrocytes[a]	Baseline	4.5 (0.3)	4.5 (0.2)	4.6 (0.4)	4.5 (0.1)	4.8 (0.3)
($\times 10^6$/µL)	End of treatment	4.7 (0.2)	4.6 (0.1)	4.6 (0.3)	4.7 (0.2)	4.7 (0.3)
Leukocytes[b]	Baseline	6939 (2531)	7440 (1183)	6217 (1421)	6750 (2050)	6867 (1730)
($\times 10^3$/µl)	End of treatment	2250 (2250)	7783 (1587)	7900 (2178)	6116 (2704)	6817 (951)
Thrombocytes[c]	Baseline	328 (60)	306 (45)	257 (59)	300 (48)	335 (76)
($\times 10^3$/µL)	End of treatment	303 (58)	299 (40)	327 (58)	286 (52)	314 (60)
Neutrophils[d]	Baseline	3178 (1788)	3338 (926)	2955 (711)	2700 (1245)	3745 (1444)
($\times 10^3$/µl)	End of treatment	3191 (1824)	3432 (1073)	4088 (1412)	2574 (1500)	3262 (743)
Eosinophils[d]	Baseline	218 (131)	500 (450)	233 (182)	392 (301)	278 (185)
($\times 10^3$/µl)	End of treatment	195 (162)	383 (290)	272 (259)	468 (642)	417 (297)
Basophils[d]	Baseline	42 (27)	35 (11)	48 (32)	28 (12)	70 (33)
($\times 10^3$/µl)	End of treatment	46 (29)	60 (29)	150 (93)	58 (35)	40 (16)
Monocytes[d]	Baseline	449 (216)	386 (74)	390 (131)	415 (113)	400 (123)
($\times 10^3$/µl)	End of treatment	416 (143)	453 (138)	284 (142)	458 (157)	358 (59)
Lymphocytes[d]	Baseline	3050 (860)	3182 (809)	2623 (751)	3233 (1468)	2382 (418)
($x10^3$/µl)	End of treatment	2919 (808)	3503 (1225)	3056 (1285)	2570 (1282)	2715 (364)
Hematocrit[e]	Baseline	35 (2)	36 (2)	36 (4)	36 (2)	37 (3)
(%)	End of treatment	36 (2)	37 (2)	36 (3)	38 (2)	37 (2)
Hemoglobin[f]	Baseline	13 (0.7)	12 (0.9)	12 (1.4)	12 (0.7)	13 (1.0)
(g/dL)	End of treatment	13 (0.8)	13 (0.7)	12 (1.0)	13 (0.7)	13 (0.7)
MCH[g]	Baseline	28 (1.1)	28 (1.0)	27 (1.1)	28 (1.1)	27 (1.4)
(pg/cell)	End of treatment	27 (1.1)	28 (1.0)	27 (1.5)	27 (1.6)	27 (1.4)
MCHC[f]	Baseline	36 (1.2)	34 (1.0)	35 (0.5)	35 (0.9)	34 (0.5)
(g/dL)	End of treatment	35 (1.0)	35 (0.6)	34 (0.7)	34 (0.7)	34 (0.5)
MCV[h]	Baseline	78 (2.8)	81 (1.2)	78 (3.1)	81 (4.0)	78 (3.0)
(μm^3)	End of treatment	78 (1.7)	79 (3.3)	79 (3.1)	80 (4.4)	78 (3.6)
Sodium[i]	Baseline	138 (2.2)	138 (0.7)	139 (1.4)	137 (1.4)	138 (2.2)
(mEq/L)	End of	137 (1.6)	139 (0.8)	139 (2.0)	137 (1.0)	138 (2.1)

	treatment					
Potassium[i]	Baseline	4.0 (0.2)	4.1 (0.5)	4.0 (0.2)	3.9 (0.2)	3.9 (0.3)
(mEq/L)	End of treatment	3.9 (0.3)	3.9 (0.2)	3.9 (0.3)	3.9 (0.3)	3.9 (0.2)
Calcium[j]	Baseline	9.6 (0.3)	9.5 (0.4)	9.5 (0.4)	9.6 (0.2)	9.7 (0.3)
(mg/dL)	End of treatment	9.8 (0.4)	9.7 (0.1)	7.9 (3.6)	7.9 (3.5)	9.7 (0.3)
Magnesium[k]	Baseline	1.7 (0.1)	1.9 (0.2)	1.9 (0.1)	1.8 (0.1)	1.8 (0.1)
(mEq/L)	End of treatment	1.8 (0.2)	1.9 (0.1)	1.8 (0.1)	1.8 (0.1)	1.7 (0.1)
Phosphorus[l]	Baseline	4.7 (0.7)	4.4 (0.3)	4.5 (0.6)	4.3 (0.4)	4.1 (0.6)
(mg/dL)	End of treatment	5.0 (0.6)	4.4 (0.5)	4.4 (0.7)	4.3 (0.2)	4.2 (0.4)
Chloride[i]	Baseline	105 (1.9)	107 (1.7)	107 (1.3)	106 (1.5)	105 (2.1)
(mEq/L)	End of treatment	104 (2.1)	107 (1.4)	105 (1.6)	107 (1.3)	105 (1.5)
AST[m]	Baseline	35.9 (4.5)	37.7 (6.0)	38.8 (8.2)	35.0 (2.6)	37.8 (5.4)
(U/L)	End of treatment	36.6 (3.8)	35.0 (5.3)	36.8 (6.2)	38.6 (6.2)	36.8 (9.1)
ALT[m]	Baseline	18.0 (4.8)	19.5 (8.5)	18.2 (2.1)	16.3 (4.3)	19.6 (2.6)
(U/L)	End of treatment	21.1 (4.9)	14.0 (2.4)	18.2 (4.3)	18.4 (7.2)	17.5 (4.8)
GGT[m]	Baseline	10.4 (3.0)	8.5 (0.8)	9.8 (0.7)	11.4 (2.1)	11.0 (1.7)
(U/L)	End of treatment	10.9 (2.8)	9.8 (1.1)	10.0 (1.1)	11.3 (1.1)	11.4 (1.9)
ALP[m]	Baseline	212 (57)	182 (35)	188 (35)	231 (48)	242 (63)
(U/L)	End of treatment	208 (50)	188 (36)	193 (30)	224 (52)	234 (61)
Bilirubin[n]	Baseline	0.5 (0.3)	0.3 (0.1)	0.4 (0.1)	0.6 (0.2)	0.4 (0.2)
(mg/dL)	End of treatment	0.5 (0.3)	0.4 (0.2)	0.3 (0.1)	0.6 (0.2)	0.4 (0.2)
Protein[f]	Baseline	6.8 (0.3)	6.9 (0.2)	6.8 (0.2)	6.7 (0.6)	7.2 (0.2)
(g/dL)	End of treatment	6.9 (0.6)	7.0 (0.2)	7.0 (0.2)	7.0 (0.3)	7.0 (0.3)
Albumin[f]	Baseline	4.2 (0.2)	4.3 (0.1)	4.1 (0.4)	4.1 (0.2)	4.1 (0.2)
(g/dL)	End of treatment	4.2 (0.3)	4.3 (0.2)	3.9 (0.1)	4.3 (0.1)	4.0 (0.3)
Urea[o]	Baseline	16.1 (8.2)	23.3 (6.1)	24.8 (8.7)	22.8 (7.2)	24.8 (8.4)
(mg/dL)	End of treatment	17.9 (8.7)	27.7 (5.0)	25.6 (9.5)	22.2 (5.9)	25.4 (7.7)
Creatinine[p]	Baseline	0.4 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)
(mg/dL)	End of treatment	0.4 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)

Abbreviations: MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; AST, aspartate amino transaminase; ALT, alanine aminotransaminase; GGT, γ-glutamyl transferase; ALP, alkaline phosphatase.

[a] To convert erythrocytes to $\times 10^{12}$/L, multiply values by 1.

[b] To convert leukocytes to $\times 10^{9}$/L, multiply values by 0.001.

[c] To convert thrombocytes to $\times 10^{9}$/L, multiply values by 1.

[d] To convert neutrophils, eosinophils, basophiles, monocytes, and lymphocytes to $\times 10^{9}$/L, multiply values by 0.001.

[e] To convert hematocrit to a proportion of 1.0, multiply values by 0.01.

[f] To convert hemoglobin, MCHC, protein, and albumin to g/L, multiply values by 10.

[g] To convert MCH to pg/cell, multiply values by 1.
[h] To convert MCV to fL, multiply values by 1.
[i] To convert sodium, potassium and chloride to mmol/L, multiply values by 1.
[j] To convert calcium to mmol/L, multiply values by 0.25.
[k] To convert magnesium to mmol/L, multiply values by 0.5.
[l] To convert phosphorus to mmol/L, multiply values by 0.323.
[m] To convert AST, ALT, GGT, and ALP to µkat/L, multiply values by 0.0167.
[n] To convert bilirubin to µmol/L, multiply values by 17.104.
[o] To convert urea to mmol/L, multiply values by 0.357.
[p] To convert creatinine to µmol/L, multiply values by 88.4.

eTable 3. Adverse events reported during the study (number of events during treatment)

	Placebo	**Insulin**			
		2.5 mg	**7.5 mg**	**22.5 mg**	**67.5 mg**
No. of children	10	6	6	6	6
Exposure, mo[a]	102	36	54	48	48
Infections	20	12	14	6	12
Common cold	8	9	10	4	10
Viral infection	6	1	1	1	1
Ear infection	4	0	0	0	0
Other	2	2	3	1	1
Gastrointestinal disorders	7	3	2	3	3
Fever – non-infectious or not specified	2	0	0	2	0
Immune system disorders (seasonal, allergy symptoms)	1	0	0	0	1
Injury, poisoning and procedural complications	3	0	0	1[b]	1
Skin disorders	0	1	0	1	1
Surgical procedures	0	0	0	0	1[c]
Other	2	3	0	0	0
Total	35	19	16	13	19

[a]Cumulative duration of exposure in all childrento the indicated study drug/dose.
[b]Serious adverse event not related to the study drug (dislocated fracture of the left ulna and radius).
[c]Serious adverse event not related to the study drug (tonsillectomy).