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Gut

1   **Supplementary methods**

2

3   **Abbreviations**

4   ASV – amplicon sequence variant

5   AUC – area under the curve

6   AUROC – area under the receiver-operator curve

7   CMV - cytomegalovirus

8   CRP – C-reactive protein

9   EBV – Epstein-Barr virus

10   ESBL – extended-spectrum beta lactamase

11   FACS – fluorescent-activated cell sorting

12   FMT – fecal microbiota transplantation

13   GAD – glutamate decarboxylase

14   HDLc – high density lipoprotein cholesterol

15   HLA – human leukocyte antigen

16   LDLc – low density lipoprotein cholesterol

17   LMM – linear mixed models analysis

18   LST – lymphocyte stimulation test

19   MMT – mixed meal test

20   MWU – Mann-Whitney U test

21   MRSA **–** methicillin-resistant *Staphylococcus aureus*

22   PBMCs - Peripheral blood mononuclear cells

23   PCR – polymerase chain reaction

24   PPI – preproinsulin

25   Qdot – quantum dot

26   ROC – receiver-operator curve

27   RT qPCR – reverse transcription quantitative PCR

28   T1D – type 1 diabetes

29   TG - triglycerides

30   TT – tetanus toxoid

31   UPLC-MS/MS - ultra high performance liquid chromatography coupled to tandem mass spectrometry

32

33   *Fecal donor recruitment and randomization*

34   Fecal donors completed questionnaires regarding dietary and bowel habits, travel history,

35   comorbidity including family history of diabetes mellitus and medication use. They were screened for

36   the presence of infectious diseases as described previously[1]. Furthermore, donors with 1st or 2nd

37   degree relatives with autoimmune diseases (including Coeliac disease, autoimmune thyroid disease,

38   type 1 diabetes and rheumatoid arthritis) were excluded. Blood was screened for human

39   immunodeficiency virus; human T-lymphotropic virus; Hepatitis A, B, and C; cytomegalovirus (CMV);

40   Epstein–Barr virus (EBV); strongyloides; amoebiasis, and lues. Presence of infection resulted in

41   exclusion, although previous and non-active infections with EBV and CMV were allowed. Donors

42   were also excluded if screening of their feces revealed the presence of pathogenic parasites (e.g.

43   blastocystis hominis, dientamoeba fragilis, giardia lamblia), multiresistent bacteria (*Shigella*,

44   *Campylobacter, Yersinia*, MRSA ,ESBL, *Salmonella, enteropathogenic E. Coli* and *Clostridium difficile*)

45   or viruses (noro-, rota-, astro-, adeno (40/41/52)-, entero-, parecho- and sapovirus) as previously

46   recommended[2]. After an overnight fast, plasma samples were taken for biochemistry and

47   metabolomics and a morning fecal sample was collected.

48   *FMT procedure*

49   Seven healthy lean donors (of whom 3 were used twice) donated for the allogenic gut microbiota

50   transfer to new onset type 1 diabetes (T1D) patients, and the same donor was used for the three

51   consecutive FMT's in an individual T1D patient.

52   After admission, a duodenal tube was placed by gastroscopy or CORTRAK enteral access system. Each

53   patient then underwent complete colon lavage with 2-4L of Klean prep® (macrogol) by duodenal

54   tube until the researcher judged that the bowel was properly lavaged (i.e. no solid excrement, but

55   clear fluid) for approximately 3h. Then, between 200 and 300 grams of feces was processed by

56   dilution in 500 ml of 0.9% saline solution and filtered through unfolded cotton gauzes. The filtrate

57   was used for transplantation two hours after the last administration of Klean prep® by duodenal tube

58   in around 30 minutes using 50cc syringes. After a short observation period the patient was sent

59   home.

60

61   *Study visits*

62   All study visits were performed at Amsterdam UMC, location AMC. Participants were asked to fill out

63   an online nutritional diary for the duration of one week before each study visit to monitor caloric

64   intake including the amount of dietary carbohydrates, fats, proteins and fibers. During the study

65   visits blood pressure, weight and daily insulin use were documented. Fasting blood samples were

66   taken at each visit and upon centrifugation stored at -80°C for subsequent analyses. Whole blood

67   sodium heparin tubes were kept on room temperature and processed within 24 hours for

68   immunological analyses (described under immunology).

69

70   *Description per study visit*

71   All visits took place after an overnight fast with subjects taking no long acting insulin the night before

72   as previously described (Moran et al., 2013). At each visit blood, fecal and urine sampling and

73   biometric measurements took place. At baseline all patients first underwent gastroduodenoscopy. A

74   small dose of midazolam (2.5 or 5mg) was administered for patient's comfort. Duodenal biopsies

75   were immediately collected in sterile tubes, snap-frozen in liquid nitrogen and stored at -80°C,

76   followed by nasoduodenal tube placement. Then at least 2 hours later, a standardized 2h mixed meal

77   test (MMT)(Nestlé sustacal boost®) was performed as previously described[3] to study residual Beta-

78   cell function. At 2, 9 and 12 months, patients again underwent a mixed-meal test for residual Beta-

79   cell C-peptide secretion. After the 2 hour MMT, a duodenal tube was placed by means of CORTRAK

80   enteral access, bowel cleansing for 6 hours was performed and the fecal transplant procedures were

81   repeated. At 6 months, patients underwent gastroduodenoscopy and biopsies were taken from the

82   duodenum and again thereafter, the mixed-meal test was performed. Of note, the similar daily

83   schedule was used in all patients to minimize variation in measurements between subjects.

84

85   *Mixed meal test*

86   Starting the evening before each mixed meal test, T1D patients interrupted their long-acting insulin

87   injections as previously published [3]. After an overnight fast and without taking their short-acting

88   morning insulin dose, a mixed meal test was performed with Boost High Protein (Nestlé Nutrition,

89   Vervey, Switzerland) at 6 ml/kg body weight with a maximum of 360 ml per person as previously

90   described[4]. Subsequent blood sampling for stimulated C-peptide was performed at -10, 0, 15, 30,

91   45, 60, 90 and 120 minutes. Area under the curve (AUC) was derived according to the trapezoidal

92   rule.

93

94   *Adaptive T-cell Immunity*

95   Whole blood samples were processed within 24 hours after sampling. Peripheral blood mononuclear

96   cells (PBMC's) were used for measurement of immune response. Granulocytes were isolated for

97   DNA-extraction and human leukocyte antigen (HLA) typing.

98

99   *Isolation of Peripheral blood mononuclear cells (PBMC's)*

100 PBMC's were isolated using Ficoll-density gradient centrifugation (ficoll 5.7%, amidotrizoaat 9%,

101 *Pharmacy Leiden University Medical Centre*). After centrifuging, the interphase containing PBMC's

102 was harvest and washed 3 times using PBS. PBMC's were suspended in 2 ml Iscove's modified

103   Dulbecco's Medium (IMDM, *Lonza*) supplemented with L-glutamine, penicillin-streptomycin (Pen

104   Strep) and 15% Human serum and counted.

105

106   *Lymphocyte Stimulation Test (LST)*

107   T-cell proliferation in response to antigenic stimulation was performed as described previously

108   (Kracht, Nature Medicine 2017). Cells were incubated in conditioned medium alone or in the

109   presence of autoantigen proteins glutamate decarboxylase (GAD65), preproinsulin (PPI), insulinoma

110   antigen-1 (IA-2) and a defective ribosomal product of proinsulin mRNA (DRiP) generated by stressed

111   Beta cells[5]. For controls, cells were stimulated with Interleukin-2 (IL-2) or cultured with tetanus

112   toxoid (TT). Cells were incubated for 5 days, after which ^{3}H-thymidine (50µl, 10 µCi/ml) was added

113   for the last 18 hours of the culture.

114

115   *Fluorescent-activated cell sorting (FACS) analyses and Quantum dot (Qdot)*

116   For phenotyping and quantification of autoreactive CD8+ T-cell s, PBMC were stained with

117   fluorescent antibodies according to a standard, independently validated protocol as described

118   previously [6]. Stained cells were measured using FACS-Canto (phenotyping) and LSR-II (Q-dot)

119   machines (Becton&Dickinson). Phenotyping data were analyzed using FlowJo software (TreeStar)

120   using the gating strategy (supplementary figure 1) or as described previously for Qdot analyses [6].

121

122   *Plasma metabolites*

123   Fasting plasma targeted metabolite measurements were done by Metabolon (Durham, NC), using

124   ultra high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-

125   MS/MS), as previously described [7]. Raw data was normalized to account for inter-day differences.

126   Then, the levels of each metabolite were rescaled to set the median equal to 1 across all samples.

127   Missing values, generally due to the sample measurement falling below the limit of detection, were

128   then imputed with the minimum observed value for the respective metabolite.

129

Biochemistry

131   Glucose and C-reactive protein (CRP, Roche, Switzerland) were determined in fasted plasma samples.

132   C-peptide was measured by radioimmunoassay (Millipore, Amsterdam, The Netherlands). Total

133   cholesterol, high density lipoprotein cholesterol (HDLc), and triglycerides (TG) were determined in

134   EDTA-containing plasma using commercially available enzymatic assays (Randox, Antrim, UK and

135   DiaSys, Germany). All analyses were performed using a Selectra autoanalyzer (Sopachem, The

136   Netherlands). Low density lipoprotein cholesterol (LDLc) was calculated using the Friedewald formula.

137   Calprotectin was determined in feces using a commercial ELISA (Bühlmann, Switzerland). Hba1c was

138   measured by HPLC (Tosoh G8, Tosoh Bioscience)

139

Fecal sample shotgun sequencing and metagenomic pipeline

141   Fecal microbiota were analysed using shotgun sequencing on donor and patient samples taken at 0,

142   6 and 12 months after initiation of study. DNA extraction from fecal samples for shotgun

143   metagenomics was performed as previously described[8]. Subsequently, shotgun metagenomic

144   sequencing was performed (Clinical Microbiomics, Copenhagen, Denmark). Before sequencing, the

145   quality of the DNA samples was evaluated using agarose gel electrophoresis, NanoDrop 2000

146   spectrophotometry and Qubit 2.0 fluorometer quantitation. The genomic DNA was randomly

147   sheared into fragments of around 350 bp. The fragmented DNA was used for library construction

148   using NEBNext Ultra Library Prep Kit for Illumina (New England Biolabs). The prepared DNA libraries

149   were evaluated using Qubit 2.0 fluorometer quantitation and Agilent 2100 Bioanalyzer for the

150   fragment size distribution. Real time quantitative PCR (qPCR) was used to determine the

151   concentration of the final library before sequencing. The library was sequenced on an Illumina HiSeq

152   platform to produce 2 x 150 bp paired-end reads. Raw reads were quality filtered using Trimmomatic

153   (v0.38), removing adapters, trimming the first 5 bp, and then quality trimming reads using a sliding

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154   window of 4 bp and a minimum Q-score of 15. Reads that were shorter than 70 bp after trimming

155   were discarded. Surviving paired reads were mapped against the human genome (GRCh37_hg19)

156   with bowtie2 (v2.3.4.3) in order to remove human reads. Finally, the remaining quality filtered, non-

157   human reads were sub-sampled to 20 million reads per sample and processed using Metaphlan2[9]

158   (v2.7.7) to infer metagenomic microbial species composition and Humann2[10] (v0.11.2) to extract

159   gene counts and functional pathways. In brief, reads were mapped using bowtie2 against microbial

160   pangenomes; unmapped reads were translated and mapped against the full Uniref90 protein

161   database using diamond (v0.8.38). Pathway collection was performed using the MetaCyc database.

162

163   *Small intestinal microbiota analyses*

164   Biopsies were added to a bead-beating tube with 300 µl Stool Transport and Recovery (STAR) buffer,

165   0.25 g of sterilized zirconia beads (0.1 mm). 6 µl of Proteinase K (20mg/ml; QIAGEN, Venlo, The

166   Netherlands) was added and incubated for 1hr at 55 °C. The biopsies were then homogenized by

167   bead-beating three times (60 s × 5.5 ms) followed by incubation for 15 min at 95 °C at 1000 rpm.

168   Samples were then centrifuged for 5 min at 4 °C and 14,000 g and supernatants transferred to sterile

169   tubes. Pellets were re-processed using 200 µl STAR buffer and both supernatants were pooled. DNA

170   purification was performed with a customized kit (AS1220; Promega) using 250 µl of the final

171   supernatant pool. DNA was eluted in 50 µl of DNAse- RNAse-free water and its concentration

172   measured using a DS-11 FX+ Spectrophotometer/Fluorometer (DeNovix Inc., Wilmington, USA) with

173   the Qubit™ dsDNA BR Assay kit (Thermo Scientific, Landsmeer, The Netherlands). The V5-V6 region

174   of 16S ribosomal RNA (rRNA) gene was amplified in duplicate PCR reactions for each sample in a total

175   reaction volume of 50 µl. A first step PCR using the 27F and the 1369R primer were used for primary

176   enrichment. 1µl of 10uM primer, 1 µl dNTPs mixture, 0.5 µl Phusion Green Hot Start II High-Fidelity

177   DNA Polymerase (2 U/µl; Thermo Scientific, Landsmeer, The Netherlands), 10 µl 5× Phusion Green HF

178   Buffer, and 36.5 µl DNAse- RNAse-free water. The amplification program included 30 s of initial

179   denaturation step at 98°C, followed by 5 cycles of denaturation at 98 oC for 30 s, annealing at 52 °C

180   for 40 s, elongation at 72 °C for 90 s, and a final extension step at 72 °C for 7 min. On the PCR product

181   a nested PCR was performed using the master mix containing 1 µl of a unique barcoded primer,

182   784F-n and 1064R-n (10 µM each per reaction), 1 µl dNTPs mixture, 0.5 µl Phusion Green Hot Start II

183   High-Fidelity DNA Polymerase (2 U/µl; Thermo Scientific, Landsmeer, The Netherlands), 10 µl 5×

184   Phusion Green HF Buffer, and 36.5 µl DNAse- RNAse-free water. The amplification program included

185   30 s of initial denaturation step at 98°C, followed by 5 cycles of denaturation at 98 °C for 10 s,

186   annealing at 42 °C for 10 s, elongation at 72 °C for 10 s, and a final extension step at 72 °C for 7 min.

187   The PCR product was visualised in 1% agarose gel (~280 bp) and purified with CleanPCR kit (CleanNA,

188   Alphen aan den Rijn, The Netherlands). The concentration of the purified PCR product was measured

189   with Qubit dsDNA BR Assay Kit (Invitrogen, California, USA) and 200 ng of microbial DNA from each

190   sample were pooled for the creation of the final amplicon library which was sequenced (150 bp,

191   paired-end) on the Illumina HiSeq. 2500 platform (GATC Biotech, Constance, Germany).

192   Raw reads were demultiplexed using the Je software suite (v2.0.) allowing no mismatches in the

193   barcodes. After removing the barcodes, linker and primers, reads were mapped against the human

194   genome using bowtie2 in order to remove human reads. Surviving microbial forward and reverse

195   reads were pipelined separately using DADA2[11] (v1.12.1). Amplicon Sequence Variants (ASVs)

196   inferred from the reverse reads were reverse-complemented and matched against ASVs inferred

197   from the forwards reads. Only non-chimeric forward reads ASVs that matched reverse-

198   complemented reverse reads ASVs were kept. ASV sample counts were inferred from the forward

199   reads. ASV taxonomy was assigned using DADA2 and the SILVA (v132) database. The resulting ASV

200   table and taxonomy assignments were integrated using the phyloseq R package (v1.28.0) and

201   rarefied to 60000 counts per sample.

202

203   *Duodenal gene expression*

204   Fresh biopsy samples were snap frozen, stored at −80°C and processed as previously published

205   (Pellegrini et al., 2017). Prior to RNA extraction, biopsies were transferred into 500 µl lysis buffer

206   (mirVana Isolation Kit, Ambion, Austin, TX), homogenized with Tissue Ruptor (Qiagen, Hilden,

207   Germany) and frozen again. Total RNA was extracted with mirVana Kit following manufacturer's

208   instruction and quantified by spectrophotometer lecture (Epoch, Gen5 software; BioTek, Winooski,

209   VT). OD A260/A280 ratio ≥2.0 and *GAPDH* Ct<28 in Taqman single assay identified acceptable quality

210   RNA samples. For reverse transcription PCR, after DNAse treatment (Turbo DNAse, Invitrogen), 5 μg

211   of RNA were retro-transcribed in a 21 μl reaction volume with SuperScript IV RT (Invitrogen)

212   following manufacturer's instructions. Predesigned TaqMan Arrays Human Inflammation Panel and

213   Human Cell Junction Panel (Applied Biosystems, Foster City, CA) were used for gene expression

214   study. A list of genes is reported in supplementary table 1. PCR runs and fluorescence detection were

215   carried out in a 7900 Real-Time PCR System (Applied Biosystems) at the following temperature

216   conditions: 50° C for 2 minutes, 95°C for 10 minutes and 40 cycles of 95° C for 15 seconds and 60° C

217   for 1 minute. Results were expressed as fold changes ($2^{-\Delta Ct}$ method) over a mean of expression of

218   the selected best reference genes: 5 housekeeping (HK) genes for Human Inflammation panel l (β-

219   *actin*, β-*2 Microglobulin*, *GAPDH, RPLP0* and *UBC)* and 4 housekeeping genes for Human Cell Junction

220   Panel (β-*2 Microglobulin*, *GAPDH, RPLP0* and *UBC*).

221

222   *Statistical analysis*

223   For baseline differences between groups, unpaired Student's t-test or the Mann-Whitney U test

224   (MWU) were used dependent on the distribution of the data. Accordingly, data are expressed as

225   mean ± the standard deviation or the median with interquartile range. Post-prandial results (e.g. c-

226   peptide) are described as area under the curves (AUC) for the 2-hour post-prandial follow-up,

227   calculated by using the trapezoidal method. For correlation analyses, Spearman's Rank test was used

228   (as all parameters were non-parametric). For comparison of the primary end point a linear mixed

229   model (LMM) was used (lme4 package in R), where 'allocation' and 'time point' were fixed effects

230   and 'patient entry number' was a random effect. The p value for the interaction between 'allocation'

231   and 'time point' was reported. Additionally, parameters were compared between groups at various

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232   time points using MWU with multiplicity correction. A p-value < 0.05 was considered statistically

233   significant.

234

235   *Missing values*

236   One study participant retracted informed consent after the first visit. This participant was not

237   included in our analyses. All other study participants completed all study visits, therefore missing

238   values are limited. Most missing data points were caused by laboratory problems such as inability to

239   extract DNA or failure to properly process or harvest immune cells. These missing data are

240   considered to be missing completely at random (MCAR). The exception to this is that one subject

241   refused the second gastroduodenoscopy, therefore his duodenal biopsies (small intestinal microbiota

242   and gene expression) after treatment are missing (1 in 20 cases or 5%). This subject has received

243   autologous FMT. We do not assume that having received autologous treatment rather than allogenic

244   (donor) faeces, metabolism or gene expression are in any way related to this person refusing the

245   second gastroscopy, therefore we consider these data to be 'missing at random'(MAR). Key variables

246   fasting C-peptide, C-peptide AUC, A1c and weight are complete (0% missing). The immunological

247   parameters mentioned in the text and figures (main figure 6 and supplementary figure 3) are all

248   based on complete data sets i.e. no missing values (CD4+ CM T cells, CD8+ T cells, CD8+CXCR3+ T

249   cells and CD4+CXCR3+ T cells). Most gene expression data in the manuscript and main and

250   supplemental figures (CCL22, CLDN12, CCL4, CD86, CCL19, CLDN 14, CCR5, CCL18, CD14) is 95%

251   complete (see above). For CCL13 one extra baseline measurement is missing, for CXCL12 one 'after

252   treatment' time point is missing, for CXCL1 two baseline and 1 after treatment time point is missing.

253   Some immunological analyses have suffered from missing data, e.g. the lymphocyte stimulation tests

254   (LST) analyses (1 to 4/20 (5-20%) of cases depending on the parameter). However, these data are not

255   mentioned in the figures (there was no statistically significant difference between the groups). The

256   fecal microbiota dataset is complete (complete case analysis). The missing values in the metabolite

257   data were imputed (see paragraph on metabolite analysis), therefore complete case analysis was

258   performed. No other data have been imputed.

259

260

261

262   *Machine learning and follow-up statistical analyses*

263   This technique was used on duodenal microbial composition (perform RT-qPCR on biopsies), on fecal

264   microbiota composition and metabolic pathway abundance (Shotgun sequencing), on plasma

265   metabolite levels and on duodenal gene expression levels data. To predict treatment groups, we

266   used the relative change (delta) of each parameter between 0 and 12 months. For duodenal

267   microbes and duodenal gene expression, we used delta 0 vs 6 months as no 12 months' time point

268   was available. For prediction of responders vs non-responders baseline values, delta 0 vs 6 months

269   and delta 0 vs 12 months were used. Each analysis produced a ranked list of the top 30 most

270   discriminative features. We selected the top parameters from each analysis that accurately (i.e.,

271   areau under the receiver-operator curve (AUROC)≥0.8) or moderately (AUROC > 0.7) predicted group

272   allocation for closer study, using an arbitrary cut off. This cut off was generally a relative importance

273   of around 30% or higher (for an example of this see figure 2C, from which the top 4 features were

274   selected). Then, we visualized the change in time of the selected parameters (Wilcoxon's signed rank

275   tests) and studied between-group differences (MWU) at each time point and finally, using

276   Spearman's rank test, we correlated these parameters with our primary end point and with other key

277   parameters that were identified in this way. For the most important analyses supplementary figures

278   showing the top 30 selected features are presented.

279

280   *Analysis of responders and non-responders irrespective of treatment group*

281   We investigated whether baseline characteristics of T1D patients can predict response to FMT

282   therapy at 12 months follow-up and which bacterial strains and plasma metabolites were associated

283   with this response. Clinical response was defined as <10% decline in Beta-cell function compared to

284   baseline at 12 months follow-up, which is significantly less than the expected natural 12 months

285   decline of 20% in beta cell function [4,12]. We chose responders at 12 months for our analyses

286   because our primary end point (MMT stimulated C-peptide) was significantly different at 12 (but not

287   at 6) months. At 12 months follow-up, clinical response sustained in 10 subjects of whom 3 had

288   received allogenic and 7 had received autologous FMT (see Figure 4A-B). We next used predictive

289   modelling to determine which parameters (either their baseline values or delta 0-12 month values)

290   were predictors of clinical response to FMT.

291

292   *Patient and public involvement*

293   This research was done without patient involvement. Patients were not invited to comment on the

294   study design and were not consulted to develop patient relevant outcomes or interpret the results.

295   Patients were not invited to contribute to the writing or editing of this document for readability or

296   accuracy.

297

298   **References**

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