AMY1 gene copy number correlates with glucose absorption and visceral fat volume, but not with insulin resistance

Thomas M Barber[1,2]**; Ahsan A Bhatti**[2]**; Patrick JD Elder**[2]**; Sarah P Ball**[3]**; Ronan Calvez**[3]**; David B Ramsden**[4]**; Dan J Cuthbertson**[5]**, Andreas F Pfeiffer**[6,7]**, David Burnett**[3]**; Martin O Weickert**[1,2, 6, 7, 8]

1. Division of Biomedical Sciences, Warwick Medical School, University of Warwick, Coventry, UK
2. Warwickshire Institute for the Study of Diabetes, Endocrinology and Metabolism, University Hospitals Coventry and Warwickshire, Clifford Bridge Road, Coventry, CV2 2DX, UK
3. Micropathology Ltd, University of Warwick Science Park, Coventry, UK
4. Institute of Metabolism and Systems Research, Medical School, University of Birmingham, Birmingham, UK.
5. Department of Obesity and Endocrinology; Clinical Sciences Centre, University Hospital Aintree, Liverpool, UK
6. German Institute of Human Nutrition, Department of Clinical Nutrition, 14458 Potsdam-Rehbruecke, Germany
7. Department of Endocrinology, Diabetes and Nutrition, Campus Benjamin Franklin, Charité-University-Medicine Berlin, 12203 Berlin, Germany.
8. Centre of Applied Biological & Exercise Sciences, Faculty of Health & Life Sciences, Coventry University, Coventry, UK

Corresponding author and to whom reprint requests should be addressed:
Prof Martin O Weickert,
Warwickshire Institute for the Study of Diabetes, Endocrinology and Metabolism,
University Hospitals Coventry and Warwickshire,
Clifford Bridge Road,
Coventry,
CV2 2DX,
United Kingdom.
E-mail: Martin.Weickert@uhcw.nhs.uk

1   **Abstract**

2   ***Background:*** The human amylase gene (*AMY1*) has broad copy number (CN)

3   variation that may associate with BMI.

4   ***Methods:*** DNA was extracted from urine (n=74) and serum (n=6) samples

5   ('ProFiMet' cohort), and buccal (n=17) samples (Oral Starch Challenge [OSC]

6   cohort), and assessed for *AMY1* CN by droplet digital PCR. Association of *AMY1* CN

7   with comprehensive markers of metabolic status (ProFiMet cohort) were analysed

8   with Pearson Correlation Coefficients (CC). For the healthy, euglycemic OSC cohort,

9   glycemic response to OSC was analysed with independent-sample t-tests

10   (subgroups: high *AMY1* CN 9-12, n=10; low *AMY1* CN 4-6, n=7).

11   ***Results:*** There were significant inverse correlations of *AMY1* CN with total visceral

12   fat volume (CC -0.33; P=0.004), and positive correlations of *AMY1* CN with oral

13   glucose insulin sensitivity score (OGIS [derived from OGTT], CC 0.26; P=0.02),

14   serum HDL-cholesterol (CC 0.325; P=0.003), and serum adiponectin (CC 0.249;

15   P=0.026). Linear regression multivariate analysis (adiponectin as dependent

16   variable), showed independent association of adiponectin with *AMY1* CN

17   (Beta=0.29; P=0.03). There were no significant associations between *AMY1* CN and

18   clamp-derived M-value, homeostasis model assessment of insulin resistance (IR),

19   hepatic endogenous glucose production, fecal floral signature or macronutrient

20   dietary preference. Delta (mean) change in blood glucose concentration (fasting to

21   30-min post-OSC), was significantly greater in the high vs low *AMY1* CN subgroups

22   (mean 1.7mmol/l [SEM 0.6] vs 0.9mmol/l [SEM 0.9] respectively; p=0.016).

23   ***Conclusions:*** High *AMY1* CN associates with favourable metabolic profile (lower

24   visceral fat volume; higher serum adiponectin; enhanced glucose absorption

25   following oral glucose and OSC), but not with whole-body or hepatic IR.

26 ***Keywords:*** Salivary amylase; Obesity; Metabolic status; Glucose; Amylase Copy

27 Number

28

29 **Abbreviations**

30

31	*AMY1*:	The human salivary amylase gene
32	*AMY2*:	The human pancreatic amylase gene
33	BMI:	Body Mass Index
34	CC:	Correlation Coefficient
35	CN:	Copy Number
36	CNV:	Copy Number Variation
37	CPIR:	Cephalic Phase Insulin Release
38	CRP:	C-reactive protein
39	CV:	Coefficient of Variation
40	ddPCR:	Droplet digital Polymerase Chain Reaction
41	dPCR:	Digital Polymerase Chain Reaction
42	EDTA:	Ethylenediaminetetraacetic acid
43	EGP:	Endogenous Glucose Production
44	FACS:	Fluorescence-activated cell sorting
45	FGF-21:	Fibroblast Growth Factor-21
46	FisH:	Fluorescence in situ Hybridization
47	FPI:	Fasting plasma insulin
48	GI:	Gastrointestinal
49	HEP-IR:	Hepatic Insulin Resistance
50	HOMA IR:	Homeostasis Model Assessment of Insulin Resistance
51	MRI:	Magnetic Resonance Imaging
52	MRS:	Magnetic Resonance Spectroscopy
53	MUFA:	Monounsaturated fatty acid
54	NEFA	Non-esterified fatty acids
55	OGIS:	Oral Glucose Insulin Sensitivity
56	OGTT:	Oral Glucose Tolerance Test
57	OSC:	Oral Starch Challenge
58	PAI-1:	Plasminogen Activator Inhibitor-1
59	PRT:	Paralogue Ratio Test
60	PUFA:	Polyunsaturated fatty acid
61	qPCR:	Quantitative Polymerase Chain Reaction
62	SFA:	Short chain fatty acid
63	T2D:	Type 2 Diabetes Mellitus
64	UHCW:	University Hospitals Coventry and Warwickshire
65	WHO:	World Health Organization

66

67 **Introduction**

68 The World Health Organization (WHO) estimated global prevalence of adult obesity

69 at 13%, and childhood overweight and obesity at 18% in 2016 (1). As a global threat

70 to human health and longevity, obesity underlies >50 weight-related conditions

71 (including type 2 diabetes mellitus [T2D]), and contributes towards a substantive

72 health-economic burden (1). Lifestyle choices (including diet and activity) influence

73 body weight. However, body mass index (BMI) also has a heritability of 70% (2, 3), a

74 small proportion of which (2.7%) associates with >90 genetic loci (2). Non-mutational

75 genetic differences between individuals, including copy number variation (CNV) of

76 some genes such as those encoding amylase (*AMY*), may underlie some of the

77 'missing' heritability for BMI, identified from genome-wide association studies (3).

78

79 The human salivary amylase (*AMY1*) and pancreatic amylase (*AMY2*) genes are

80 located in a cluster on chromosome 1p21.1 (1, 4). The human genome reference

81 assembly (hg19) shows a structure that includes three copies of the *AMY1* gene

82 repeat (designated *AMY1A*, *AMY1B* and *AMY1C* to define their chromosomal

83 location), and two pancreatic amylase genes (designated *AMY2A* and *AMY2B*). The

84 *AMY1* coding region, and approximately 26.5kb of flanking sequence constitutes the

85 *AMY1* 'copy-variable repeat unit'. Replicates of this repeat unit are 99.9% identical.

86 Although the reference human genome shows only three replicates, in the general

87 human population the *AMY1* region displays remarkable CNV between individuals

88 (5). The CNV of *AMY1* is thought to originate from unequal crossing-over events

89 between the highly homologous components of the gene cluster (6). This CNV

90 appears to post-date the split of modern Humans and Neanderthals (7). Using

91 comparative genome hybridization, an unequal cross-over encompassing *AMY2A*

92   and *AMY1* was shown to be the most common rearrangement event, resulting in

93   *AMY1* CN ranging from 2 to 18 per diploid genome (8).

94

95   Salivary amylase plays a key role in starch digestion during mastication of food (9),

96   through facilitating hydrolysis of 1,4-glycosidic bonds within starch, forming maltose,

97   isomaltose, oligosaccharides and glucose (10, 11). Combined with the actions of

98   pancreatic amylase within the ileum (encoded by the separate *AMY2* amylase gene),

99   ongoing hydrolysis of starch and its intermediate breakdown products, eventually

100   results in the formation of absorbable free glucose (10). Human *AMY1* copy number

101   (CN) associates directly with concentration and activity of salivary amylase (10, 12-

102   15).

103

104   In addition to its role in the production of salivary amylase for breakdown of starch

105   during mastication, there is also evidence for association between *AMY1* CN and

106   BMI. Falchi and colleagues published the first report of an inverse association

107   between human *AMY1* CN and BMI using a quantitative Polymerase Chain Reaction

108   (qPCR) approach (16). Replication in >6,000 participants from cohorts that included

109   those of European and Singaporean Chinese ethnicities, demonstrated that each

110   additional copy of *AMY1* reduced obesity risk by 1.2-fold, and there was an 8-fold

111   greater risk of obesity in individuals with an *AMY1* CN<4, compared to those with an

112   *AMY1* CN>9 (16). Subsequent reported studies on *AMY1* CNV and BMI have shown

113   mixed results. Using a qPCR technique on a population from South Korea, Choi and

114   colleagues showed an association between *AMY1* CN and insulin sensitivity, but not

115   with BMI (17). Using a different approach (digital PCR [dPCR]), Mejia-Benitez and

116   colleagues showed in a cohort of children from Mexico, that high *AMY1* CN

117 appeared to be protective against obesity (18). In a different study reported by

118 Viljakainen and colleagues, that utilized a superior 'droplet digital' PCR (ddPCR)

119 technique (19) in a cohort of Finnish subjects with a history of severe childhood

120 obesity, inverse association between *AMY1* CN and both BMI and percentage body

121 fat in the obese female subjects was shown (20). A further study by Bonnefond and

122 colleagues on a French population of adults and children using ddPCR, also showed

123 an association of higher *AMY1* CN with reduced risk of obesity, particularly in

124 children (21). Nonetheless, other studies using the ddPCR technique, (including one

125 on >4,000 participants (22)), from diverse cohorts have shown no association

126 between *AMY1* CN and BMI (22, 23). Most recently, Shwan and colleagues reported

127 on data from the 1958 Birth Cohort (1,400 subjects and >2,800 subjects from two

128 disease cohorts from the Wellcome Trust Case Control Consortium), using paralogue

129 ratio tests (PRTs) in genomic DNA and array comparative genomic hybridization

130 data, and showed no evidence for association of *AMY1* CN with BMI at any age

131 point (24).

132

133 Despite the controversy in this field based on the current literature outlined here,

134 there does appear to be evidence, at least in some cohorts and particularly in

135 children, for inverse association between *AMY1* CN and BMI. However, the

136 mechanism(s) that underlie this intriguing association remain incompletely

137 understood. Furthermore, to our knowledge there are no published reports to date on

138 possible associations between *AMY1* CN and measures of metabolic status

139 (including euglycemic hyperinsulinaemic clamps, stable isotopic measures of hepatic

140 insulin sensitivity, abdominal magnetic resonance imaging [MRI] and H1 liver

141 spectroscopy), macronutrient (including starch) preference and fecal floral signature.

142   To address this important unanswered question, we explored associations of *AMY1*

143   CN with metabolic status, body composition, macronutrient preference and fecal

144   flora, in the most highly phenotyped cohort reported on to date. We also explored

145   associations of *AMY1* CN with blood glucose excursions following ingestion of

146   glucose and oral starch challenge (OSC).

147

148   **Methods**

149   ***DNA Extraction: Samples and Techniques***

150   *Urine and serum samples from ProFiMet cohort:* For measurement of *AMY1* CN,

151   samples of Caucasian individuals (n=80) of the Protein, Fiber and Metabolic

152   Syndrome ('ProFiMet') data set (clinicaltrials.gov, NCT00579657) were available

153   (urine samples, n=74; serum samples, n=6). Participants of the ProFiMet study were

154   overweight or obese, but non-diabetic. All participants had undergone extensive

155   metabolic phenotyping including standard markers, assessment of dietary intake (5-

156   day food protocols including a weekend day), measurement of whole-body and

157   hepatic Insulin Resistance (IR), assessment of body fat distribution through MRI

158   scans and H1-spectroscopy (25), and assessment of a complete bile acid profile

159   (26). In addition, each participant had assessment of various other biomarkers in

160   blood, urine and feces, including dominant groups of the gut microbiota (27) and

161   amino acid metabolic signatures (28). Details of the study design and participants

162   are described in our core publication (25). The Ethics Committee of the University of

163   Potsdam approved this study (BMBF-FKZ-0313826). All participants provided written

164   informed consent. The Zymo Research *Quick*-DNA Urine Kit and protocol

165   (Cambridge Bioscience, Cambridge, CB23 8SQ UK) was used for extraction of DNA

166   from urine (0.25ml kit elution buffer). Each urine sample (0.5ml) underwent total

167   (cellular and cell-free) DNA extraction. The QIAGEN QIAamp Blood Mini Kit and

168   protocol (Qiagen UK) was used for extraction of DNA from serum (0.05ml kit elution

169   buffer). Each serum sample (0.5ml) underwent DNA extraction. The yield of DNA

170   was determined using a Qubit 3.0 fluorimeter (Life Technologies Ltd).

171

172   *Buccal samples from OSC cohort:* Buccal swabs (Copan Flock Technologies

173   4N6FLOQSwabs) were obtained from ThermoFisher Scientific, UK, Cat. #4479433.

174   Following collection of buccal samples, the swabs were inserted into 2ml tubes with

175   0.3ml of sterile DNAse-free water and agitated. Approximately 0.25ml of fluid was

176   collected and DNA extracted using the Roche High Pure Viral Nucleic Acid Kit,

177   obtained from Roche Diagnostics Ltd, UK (Product No. 11858874001).

178

179   *Whole blood EDTA and muscle samples for validation:* Whole blood EDTA samples

180   (n=43) from patients (both in-patients and out-patients) at University Hospitals

181   Coventry and Warwickshire (UHCW) in the UK were used for DNA extraction. In

182   addition, skeletal muscle biopsy samples from calf muscle from lower limb

183   amputations (n=2) were obtained from the Arden Tissue Bank at UHCW, for the

184   purpose of DNA extraction. A blood sample from a Chimpanzee (*Pan troglodytes)*

185   was also provided by Chester Zoo, Cheshire, UK. The QIAGEN QIAamp Blood Mini

186   Kit and protocol (Qiage UK) was used for extraction of DNA from whole blood. Each

187   whole blood sample had a volume of 5ml.

188

189   *Cultured cell samples for validation:* Validation of *AMY1* CN assay was also provided

190   by DNA that had already been extracted from existing cultured cell samples (n=7).

191   Extracted DNA was obtained from the Coriell Institute (Camden, NJ, USA). These

192   were cell lines NA06993, NA07347, NA10835, NA11930, NA11993, NA12813 and

193   NA18972.

194

195   ***Analysis and Validation of AMY1 CN on Extracted DNA***

196   For all urine (n=74) and serum (n=6) samples from the ProFiMet cohort, and for the

197   buccal samples collected from the OSC cohort, *AMY1* CN was analysed using the

198   'droplet digital PCR' ('ddPCR') technique, a recent practical technology for detection

199   and quantitation of specific nucleic acid sequences (29). Digital PCR relies on

200   dilution of target DNA and subsequent distribution into multiple, discrete replicate

201   PCR reactions. Application of Poisson statistics enables calculation of the absolute

202   measurement of target sequence concentration. We used ddPCR to determine

203   *AMY1* CN because this method allows highly repeatable quantitation of CN, and is

204   less susceptible to PCR inhibition than qPCR. Furthermore ddPCR could be reliably

205   applied to the low yield DNA samples available from the ProFiMet cohort.

206

207   To validate the ddPCR technique, extracted DNA from each whole blood EDTA

208   sample (n=43) or calf muscle (n=2) was analysed for assessment of *AMY1* CN using

209   the same ddPCR technique. For further validation, two separate techniques (PRT

210   and 'microsatellite typing') were used to analyse *AMY1* CN in the whole blood EDTA

211   samples. For the PRT (PRTref12), we used the 'Chromosome 12 Reference

212   Paralogue' to ascertain *AMY1* CN in each sample, as described by Carpenter and

213   colleagues (5). Regarding the 'microsatellite typing', Carpenter and colleagues

214   described a TATC microsatellite region within the *AMY1* gene region (5). Utilization

215   of a PCR amplification of this microsatellite, followed by electrophoresis derived

216   relative peak heights for all the amplification products. Totalling peak height ratios

217   enabled estimation of *AMY1* CN in informative individuals. PCR details were as

218   described for the PRTref12, using TATC assay primers. A flowchart of DNA

219   extractions and analyses, including validation of the ddPCR technique is shown in

220   *figure 1*. To ascertain the precision of the ddPCR assay, two cultured cell samples

221   from the Coriell Institute (Camden, NJ, USA) were assayed on several different days,

222   and the mean *AMY1* CNs and coefficients of variation calculated.

223

224   The yield of DNA from urine and serum samples was much lower than that from

225   blood samples. We therefore assessed the accuracy of ddPCR quantification of

226   *AMY1* CN at low DNA concentrations. DNA extracts from one Coriell sample

227   (NA18972) and ten blood samples were diluted with water to DNA concentrations of

228   0.05-0.28 ng/ul. These diluted extracts were subjected to ddPCR (7.5ul per reaction),

229   and the *AMY1* CN compared with those obtained by analysis of undiluted extracts.

230

231   ***ProFiMet Cohort***

232   ProFiMet remains one of the most well-phenotyped and detailed cohorts of subjects

233   with metabolic syndrome. Details of the ProFiMet cohort have been published (25-

234   28). The ProFiMet cohort consists of overweight and obese participants, with a wide

235   range of BMI from 25Kgm^{-2} to 43Kgm^{-2}. For the purpose of this study, data on body

236   composition, liver fat content, measures of insulin sensitivity and hepatic glucose

237   production, gut microbiota and macronutrient preferences were analysed.

238

239   *Body composition and liver fat content:* Trained staff performed anthropometric

240   measurements using standard methods. Body fat content was assessed using 1.5T

241   MRI (Magnetom Avanto, Siemens Healthcare, Erlangen, Germany) (25). Hepatic

242   lipid content was measured using proton magnetic resonance spectroscopy (^{1}H-

243   MRS) (25, 30).

244

245   *Whole-body insulin resistance (IR) and insulin-induced suppression of hepatic*

246   *glucose production:* Euglycemic–hyperinsulinemic clamps (40 mU/kg/min) were

247   performed in the overnight fasted state at weeks 0, 6, and 18 (n = 15–20 participants

248   per group and study day), to assess whole-body IR (expressed as *M*-value). Tracer

249   experiments were performed in a matched subset of participants (n = 9–11

250   participants per group and study day), to measure (hepatic) endogenous glucose

251   production (EGP), and EGP-suppression by insulin infusion. Calculation of hepatic

252   IR (HEP-IR) was as follows: (EGP x fasting plasma insulin [FPI]) (30). Blood samples

253   were drawn at timed intervals at baseline and throughout the clamp experiments,

254   following 12-h overnight fasting. Samples were immediately chilled and centrifuged,

255   and the supernatant fluid was aliquoted and stored at −80 °C until analysed.

256

257   *Dominant groups of the gut microbiota:* Fecal collection and analysis of gut

258   microbiota from fecal samples are detailed elsewhere (27). Briefly, fecal samples

259   underwent Fluorescence in situ Hybridization (FisH) and bacterial enumeration by

260   flow cytometry (27, 31). Flow cytometry data acquisition was performed with a FACS

261   Calibur flow cytometer (Becton Dickinson) as described previously (32), using the

262   settings of Mueller (27, 31). Changes in gut microbiota were measured for the

263   following major bacterial population groups (respective oligonucleotide probe in

264   brackets): *Clostridium coccoides - Eubacterium rectale* cluster (Erec482), *Roseburia*

265   genus (Rrec584), *Bacteroides-Prevotella* (Bac303), *Clostridium leptum* cluster

266   (Clep866), *Atopobium* cluster (Ato291), *Bifidobacterium* group (Bif164), and

267 clostridial cluster IX (Prop853). *Lactobacillus-Streptococcus* group (Lab158) and

268 *Enterobacteria* (Enter1432) were under the detection limit. (*Roseburia, E. rectale* and

269 *Faecalibacterium* groups are major butyrate producers) (27).

270

271 ***OSC Cohort***

272 For the OSC cohort, healthy participants (n=17) with no known history of Diabetes

273 Mellitus or obesity were recruited from the community, for the sole purpose of this

274 study. All participants within the OSC cohort were white and of British/Irish ethnicity.

275 Each participant underwent a buccal swab as described, for determination of *AMY1*

276 CN. The OSC cohort were divided into two subgroups, according to *AMY1* CN. The

277 high *AMY1* CN subgroup (n=10) had a range of *AMY1* CNs between 9 and 12 (mean

278 CN 10). The low *AMY1* CN subgroup (n=7) had a range of *AMY1* CNs between 4

279 and 6 (mean CN 5). Each participant was invited to attend for a fasting blood test

280 (following a 12-hour fast) at 9am, followed by a standard OSC. The OSC consisted

281 of 88g (dry weight) of basmati white rice (26.8g carbohydrate per 100g rice), cooked

282 in tap water. Blood glucose measurements were based on finger-prick testing, using

283 a Roche Accu-Chek Performa Nano Blood Glucose System. In addition to fasting

284 tests, blood glucose measurements were also taken post-OSC at 30-min, 60-min,

285 90-min and 120-min.

286

287

288

289

290

291

292 ***Statistical analyses***

293 Statistical analyses were conducted in SPSS (version 26 for Windows; SPSS Inc.,

294 Chicago, IL). For the ProFiMet cohort data, initial analyses involved derivation of

295 Pearson correlation coefficients between *AMY1* CN and the following indices of

296 metabolic status: total visceral fat volume, measures of body composition, hepatic

297 lipid content, whole-body insulin resistance, hepatic EGP, OGTT-derived OGIS

298 score, macronutrient food preferences and fecal flora profiles. Detailed power

299 calculations regarding the ProFiMet cohort have been documented previously (25).

300 To explore independent associations of *AMY1* CN with key markers of metabolic

301 status, we performed linear regression multivariate analysis with serum adiponectin

302 as a dependent variable. Independent variables included other, carefully selected

303 outcome measures from the ProFiMet cohort to provide a detailed assessment of

304 metabolic status, with addition of *AMY1* CN. To avoid wide, multiple testing, we

305 chose a minimalistic range of independent variables, to capture known key aspects

306 of metabolic status. This represented only a small selection of the several hundred

307 metabolic markers within the ProFiMet cohort. Therefore, we did not perform any

308 corrections for multiple testing. For the OSC cohort data, we used independent

309 sample t-tests for comparison between the low and high CN subgroups, including

310 delta change in blood glucose readings from fasting to 30-min post-OSC time-points.

311 A P-value <0.05 was considered significant.

312

313

314

315

316

317 **Results**

318 *DNA Extraction Success*

319 We define successful DNA extraction by sufficient DNA yield to perform a ddPCR

320 assay for *AMY1* CN. For the ProFiMet cohort, of the 98 urine samples available,

321 DNA extraction was successful in 74 samples. For the remaining subjects (n=24)

322 where DNA extraction from urine was unsuccessful, DNA extraction was attempted

323 from serum samples from the same subjects. Of these, DNA was extracted

324 successfully from serum samples (n=6), providing a total of n=80 DNA samples from

325 the ProFiMet cohort, from which the DNA recovered was within the range 0.02-

326 16.7ng/µl; median 0.15ng/µl. Given that all of the urine and serum samples from the

327 ProFiMet cohort had already undergone centrifugation, there was limited cellular

328 material available for DNA extraction in each sample. For this reason, it was not

329 possible to obtain any DNA from some of the urine (n=24) and serum (n=18)

330 samples from the ProFiMet cohort. DNA extraction was successful in all of the whole

331 blood EDTA (n=43) and calf muscle (n=2) samples, and for all of the buccal (n=17)

332 samples in the OSC cohort.

333

334 *Validation and Precision of ddPCR Technique for AMY1 CN Analysis*

335 For all of the reference DNA materials (seven cell lines), the *AMY1* CN assessed by

336 all three methods (PRT, microsatellite and ddPCR) was equivalent to values reported

337 previously (5). We subsequently applied these three methods for *AMY1* CN

338 assessment to anonymised DNA, extracted from 45 samples (EDTA whole blood

339 [n=43] and skeletal muscle [n=2] samples), obtained from the Arden Tissue Bank at

340 UHCW. Statistically, there was no significant difference between *AMY1* CN

341 determined by ddPCR and PRTref12 (Mann Whitney U test p-value=0.96).

342   Microsatellite profiling was uninformative for six individuals due to insufficient allele

343   length variation. In a further eight individuals, the microsatellite profile calculated

344   using the most parsimonious ratio indicated a CN that was half the number

345   estimated by ddPCR and PRT. Application of an alternate CN profile in these cases

346   resulted in *AMY1* CN evaluation consistent with data derived from the other

347   techniques.

348

349   In view of the fact that the paralogue and microsatellite assays were human-specific,

350   we could only apply the *AMY1* ddPCR assay to the DNA extracted from an EDTA

351   whole blood sample, taken from a chimpanzee (Chester Zoo, UK). This revealed a

352   CN of 2 (ie. one copy per chromosome), as expected (7). Assessment of the

353   accuracy of ddPCR to determine *AMY1* CN in low yield DNA samples resulted in

354   good correlations at low CN, but with less accuracy at high copy number (Mann

355   Whitney U test p-value=0.91). Regarding ddPCR precision, separate analyses on

356   different days for Coriell sample NA 11930 (n=7; nominal CN 2) showed a mean CN

357   of 1.9 (CV 1.9%), and for Coriell sample NA 07347 (n=6; nominal CN 10) showed a

358   mean CN of 10.4 (CV 5.8%). Therefore, we believe that the ddPCR assay used in

359   our study is a robust and reliable method for assessment of human *AMY1* CN, even

360   for samples with a low yield of DNA.

361

362   ***Distribution of AMY1 CN for the ProfiMet and OSC Cohorts***

363   A histogram showing the distribution of *AMY1* CN across the ProFiMet cohort is

364   shown in *figure 2*. The CN for the *AMY1* gene in the ProFiMet individuals ranged

365   from 2 to 10**,** compared with a range of 4 to 12 in the OSC cohort. The majority of the

366   ProFiMet cohort (n=69, 86.25%) had between 3 and 8 copies of the *AMY1* gene. The

367 remainder (n=11, 13.75%) had extreme CNs of the *AMY1* gene, either 2 copies or >8

368 copies. Descriptive statistics for the *AMY1* CN between the ProFiMet and OSC

369 cohorts revealed mean 4.7 (SD 1.9) and 7.9 (SD 2.9) respectively.

370

371 ***Pearson Correlation Coefficients for the ProfiMet Cohort (table 1)***

372 There was a highly significant inverse correlation of *AMY1* CN with total visceral fat

373 volume (correlation coefficient -0.33, P=0.004), and visceral fat volume within the

374 umbilical area (correlation coefficient -0.324, P=0.004). Although there was an

375 inverse correlation of *AMY1* CN with BMI (correlation coefficient -0.214), this failed to

376 reach significance (P=0.057). There was an inverse correlation between *AMY1* CN

377 and lean mass (correlation coefficient -0.216), which just failed to reach significance

378 (P=0.055). There were no significant associations between *AMY1* CN and total fat

379 volume, fat mass, intrahepatic lipid content, umbilical subcutaneous adipose tissue

380 volume, whole-body insulin resistance, HOMA-IR, insulin sensitivity index or hepatic

381 EGP. However, there was a significant direct correlation between *AMY1* CN and

382 (OGTT-derived) OGIS score (correlation coefficient 0.259, P=0.02). Regarding lipid

383 profile, there was a significant correlation between *AMY1* CN and serum HDL-

384 cholesterol (correlation coefficient 0.325, P=0.003), but no significant associations

385 with any of the other components of lipid profile, including triglycerides. There was a

386 significant direct correlation between *AMY1* CN and serum adiponectin (correlation

387 coefficient 0.249, P=0.026). There were no significant correlations with serum leptin,

388 acylated ghrelin, Fibroblast Growth Factor-21 (FGF-21), interleukin 10 or C-reactive

389 protein (CRP). *AMY1* CN showed no significant correlation with any measure of fecal

390 microbiota (FisH analysis). There was no correlation of *AMY1* CN with dietary

391 preferences, including intake of alcohol, total energy, soluble and insoluble fiber,

392 magnesium, protein, meat, fish, milk, legumes, eggs, poultry, carbohydrates,

393 cholesterol, polyunsaturated fatty acids (PUFA), monounsaturated fatty acids

394 (MUFA) and short chain fatty acids (SFA). Associations between *AMY1* CN and total

395 visceral fat volume, umbilical visceral fat volume, OGIS score, serum fasting HDL-

396 Cholesterol and serum adiponectin are shown graphically in *figure 3*.

397

398 ***Linear Regression Multivariate Analyses for the ProFiMet Cohort***

399 Given the central role played by adiponectin as a regulator of metabolic status, and

400 the excellent utility of adiponectin as an indicator of metabolic status, we chose

401 serum adiponectin as a dependent variable for linear regression multivariate

402 analysis. In addition to *AMY1* CN, we used a carefully selected panel of metabolic

403 markers from the ProFiMet cohort as independent variables, to provide a detailed

404 assessment of metabolic status. These included age, BMI, total fat volume,

405 intrahepatic lipid content, whole body insulin resistance, hepatic EGP, fasting

406 glucose, fasting insulin, HbA1C, serum triglycerides, CRP, Plasminogen Activator

407 Inhibitor-1 (PAI-1) and FGF-21. Linear regression multivariate analysis revealed a

408 significant independent association of serum adiponectin with *AMY1* CN (Beta=0.29;

409 P=0.03), and with age (Beta=0.60; P<0.0001). There were no other significant

410 independent associations between serum adiponectin and any other independent

411 variable. Following a repeat linear regression multivariate analysis with serum

412 adiponectin as a dependent variable, and with the addition of gender as an

413 independent variable, revealed significant independent associations of serum

414 adiponectin with *AMY1* CN (Beta=0.26; P=0.046), age (Beta=0.59; P<0.001) and

415 gender (Beta=0.35; P=0.01). Interestingly, one-way ANOVA showed no relationship

416 of *AMY1* CN with gender (P=0.19) or BMI (P=0.4).

417   ***Independent t-test Comparisons for the Oral Starch Challenge Cohort***

418   For the OSC cohort, the high *AMY1* CN subgroup had a lower mean fasting blood

419   glucose concentration than the low *AMY1* CN subgroup (4.9mmol/l [SEM 0.14] vs

420   5.8mmol/l [SEM 2.9] respectively, p=0.011). Delta change (mean) in blood glucose

421   concentration from fasting to 30-min post-OSC was significantly greater in the high

422   *AMY1* CN subgroup compared with the low *AMY1*CN subgroup (mean 1.7mmol/l

423   [SEM 0.6] vs 0.9mmol/l [SEM 0.9] respectively, p = 0.016).

424

425   **Discussion**

426   In this most highly phenotyped ProFiMet cohort, our study is the most

427   comprehensively reported on to date regarding association of human *AMY1* CN with

428   metabolic and dietary parameters. We demonstrate that high *AMY1* CN associates

429   significantly with a favourable metabolic profile, including lower visceral fat volume,

430   higher serum levels of adiponectin and HDL-cholesterol, and enhanced glucose

431   absorption following an oral glucose load. We also demonstrate important negative

432   outcomes, including absence of any association between *AMY1* CN and whole-body

433   insulin resistance, hepatic EGP, macronutrient preference or pattern of fecal flora.

434   Our data reveal pronounced plasma glucose excursions following a glucose load in

435   those with a high *AMY1* CN, without differences in hepatic EGP or measures of

436   insulin sensitivity. Furthermore, high *AMY1* CN associated with a more pronounced

437   blood glucose excursion following OSC in a separate healthy cohort. These data are

438   consistent with co-evolution of high *AMY1* CN (with attendant enhanced salivary

439   digestion of starch to maltose, and thereafter absorbable glucose monomers), with

440   optimized efficiency of glucose absorption across the upper GI tract. Further studies

441   should explore the underlying mechanisms that influence glucose absorption

442   efficiency, and potential role for *AMY1* CN and other (potentially haplotypic) genetic

443   variants in this process.

444

445   The ddPCR is a relatively new technique in which the PCR is executed in a multitude

446   of tiny droplets held in oil suspension, as opposed to conventional PCR which is

447   performed in a single solution. One advantage of ddPCR is that only small amounts

448   of DNA are required. This is evidenced by the fact that in our study, sufficient DNA

449   was extracted from urine and serum samples, in which there were very low yields of

450   DNA. To validate our data, a rigorous procedure involving two totally different

451   methods, microsatellite typing and the PRT were adopted. Agreement was generally

452   excellent amongst data derived from these three separate types of test, with only

453   eight samples giving half the expected values in the strictest interpretation of the

454   microsatellite data. The excellent agreement amongst the data derived from the

455   three methods employed, justifies our decision to use ddPCR in the determination of

456   *AMY1* CN in the ProFiMet samples.

457

458   Although BMI is limited as a reliable indicator of underlying metabolic status, most

459   studies reported to date have focused solely on association between *AMY1* CN and

460   BMI. As discussed earlier, one of the most definitive and highly powered genetic

461   studies in the field, reported on *AMY1* CN in >6,000 subjects and demonstrated a

462   reciprocal association of *AMY1* CN with BMI, and direct correlations between *AMY1*

463   CN, amylase gene expression and serum levels of amylase (16). Our study is unique

464   amongst the reported literature on *AMY1* CNV, in providing a much more detailed

465   and comprehensive assessment of metabolic status than BMI can provide. This

466   includes the first reported measures of visceral fat volume, whole-body insulin

467 resistance and hepatic EGP in the context of *AMY1* CN. Although a trend for

468 reciprocal association between *AMY1* CN and BMI was shown, this just failed to

469 reach statistical significance. A likely explanation is that the ProFiMet cohort only

470 included overweight or obese, without normal weight participants.

471

472 There remains controversy within the literature regarding association between *AMY1*

473 CN and BMI. Variation in dietary preferences (including dietary starch ingestion)

474 between populations studied may explain some of the differences in strength of

475 association between *AMY1* CN and BMI. In a large Swedish cohort, *AMY1* CN had a

476 significant *direct* association with BMI in those subjects with high starch intake (1,

477 33), likely mediated through enhanced digestion of starch, and absorption of glucose

478 from a starchy diet in those with a higher *AMY1* CN, thereby increasing susceptibility

479 to weight gain (1, 33). In contrast, our data refute any important role for dietary

480 starch, or other macronutrient preference in the mediation of association between

481 *AMY1* CN and metabolic status, including BMI. Our data also refute the notion that

482 *AMY1* CN mediates metabolic influence through changes in colonic undigested

483 starch, as we showed no association of *AMY1* CN with changes in gut microbiota,

484 colonic fermentation rates or levels of colonic SFAs (1, 21, 34).

485

486 In one study, *AMY1* CN correlated directly with a more pronounced excursion of

487 insulin within the early postprandial phase ('pre-absorptive' or 'Cephalic Phase

488 Insulin Response' [CPIR]) following starch ingestion, and lower postprandial glucose

489 responses (9). Oral sensory stimulation triggers CPIR, the 'anticipatory' phase of

490 digestion, mediated by autonomic mechanisms (35). CPIR primes the body for

491 efficient digestion and assimilation of ingested nutrients, and is required for normal

492   postprandial glucose tolerance and prevention of dysglycemia (9, 12). A higher

493   *AMY1* CN may associate with greater metabolic efficiency, particularly with regard to

494   oral starch handling. In a further study, enhanced CPIR associated with salivary

495   amylase levels (9). In people with high levels of salivary amylase, activation of

496   lingual taste receptors may occur through orally released carbohydrate, thereby

497   triggering CPIR through an autonomic process (1, 9).

498

499   We demonstrated an enhanced response of insulin following OGTT in those with

500   high *AMY1* CN in the ProFiMet cohort. However, this rise in the level of serum insulin

501   following oral glucose ingestion occurred in response to enhanced early glucose

502   absorption in those with a high *AMY1* CN. This insulin response to oral glucose

503   ingestion therefore appears to be separate from any *independent* effect of *AMY1* CN

504   on pancreatic insulin response, hepatic EGP or insulin sensitivity, and therefore

505   seems unlikely to be mediated directly through CPIR. It is important to highlight that

506   there was only a *transient* difference in early post-prandial plasma glucose levels

507   following OGTT according to *AMY1* CN in the ProFiMet cohort. Although this

508   transient early postprandial rise in plasma glucose in those participants with a higher

509   *AMY1* CN may confer metabolic risk, we hypothesize that a more likely scenario is

510   that the associated pronounced early postprandial rise in serum insulin in those with

511   a higher *AMY1* CN in the context of equivalent hepatic glucose output and insulin

512   sensitivity (according to *AMY1* CN), would be expected to drive postprandial cellular

513   uptake of glucose and confer other metabolic benefits. Our data are therefore

514   consistent with association of higher *AMY1* CN with a favourable metabolic profile.

515   However, further studies are required to explore and clarify the longer-term metabolic

516   implications of *AMY1* CN, particularly regarding postprandial glucose handling and

517   cellular effects of insulin response.

518

519   Consistent with the data from the ProFiMet cohort, our data from the participants

520   who underwent OSC also showed a more pronounced glucose excursion for the

521   subgroup with a higher *AMY1* CN. In contrast however, data from a study reported

522   by Tan and colleagues on healthy men (n=75) from a multi-ethnic Asian population,

523   showed no significant association between *AMY1* CN and glycemic response to rice

524   ingestion (36). Interestingly, mastication parameters (number of chews and chewing

525   time per mouthful) did associate with glycaemic response to ingested rice (36). A

526   further study was reported by Heianza and colleagues on genetic variants in *AMY1*

527   and *AMY2* CN in >690 overweight and obese participants, randomly assigned to

528   diets varying in macronutrient content (37). In this study, although changes in body

529   weight and waist circumference were significantly different according to *AMY1-AMY2*

530   rs11185098 genotype, at 24-months follow-up there was no significant association

531   between fasting plasma glucose level and *AMY1-AMY2* genotype (37). Further

532   studies are required to explore the association between *AMY1* CN and glucose

533   homeostasis, including fasting glucose and glycemic response to starch ingestion.

534   Future research should focus on the mechanisms whereby *AMY1* CN influences

535   starch digestion, glucose absorption and early insulin release, and how these

536   complex mechanisms contribute to blood glucose excursions following ingestion of

537   carbohydrates, including starch.

538

539   Similar to data from other reported studies, we demonstrated a significant positive

540   association between *AMY1* CN and serum levels of HDL-cholesterol (38) and

541   adiponectin (39). Although speculative, serum amylase may confer cardio-protective,

542   anti-inflammatory and insulin-sensitising properties (40). However, our assessment

543   did not enable any exploration of underlying mechanisms, and it is not possible to

544   infer any causal association between *AMY1* CN and HDL-cholesterol and

545   adiponectin. However, adiponectin and HDL-cholesterol are both known to confer

546   cardio-metabolic benefits (1). Furthermore, we demonstrated an independent and

547   direct association between *AMY1* CN and serum levels of adiponectin. We therefore

548   provide evidence for promotion of an association between high *AMY1* CN and a

549   favourable cardio-metabolic profile.

550

551   Our study does have some limitations including its cross-sectional design, thereby

552   precluding any exploration of association between *AMY1* CN and temporal migration

553   of metabolic indices. Furthermore, a lack of data on either salivary or serum amylase

554   concentrations limited potential insights implicating these factors in the mediation of

555   association between *AMY1* CN and metabolic status. Our ProFiMet cohort only had

556   *AMY1* CN ranging between 2 and 10 copies, rather than the full range of copies

557   (n=2-27) that has been reported in human populations (1). A likely explanation is that

558   the ProFiMet cohort is relatively homogeneous, with Caucasian participants from the

559   German Berlin-Potsdam region, with BMI restricted to the overweight and obese

560   ranges. Furthermore, the OSC cohort had a relatively restricted range of *AMY1* CN

561   ranging between 4 and 12. A likely explanation is the ethnic homogeneity of the OSC

562   cohort, with all OSC participants being white and of British/Irish origin. Finally, we

563   used FisH analyses rather than shotgun metagenomics for gut microbiota

564   assessment. Therefore, our conclusions regarding lack of differences in gut

565   microflora according to *AMY1* CN should be tempered accordingly. Further studies

566   are required across wider cohorts, to explore associations between *AMY1* CN and

567   fecal floral signature, using gold standard methodologies.

568

569   To summarize, we demonstrate significant direct associations between *AMY1* CN

570   and serum adiponectin, HDL-cholesterol and efficiency of glucose absorption

571   following both oral glucose load and OSC. We also show reciprocal association

572   between *AMY1* CN and visceral fat volume. We exclude any mediating effects of

573   macronutrient food preference, insulin resistance or fecal floral signature on the

574   associations between *AMY1* CN and metabolic status. In those subjects with a

575   higher *AMY1* CN, a more pronounced glucose excursion following an oral glucose

576   load, without concomitant changes in whole-body or hepatic IR, strongly suggests

577   improved efficiency of glucose *absorption* across the upper GI tract. We hypothesize

578   co-evolution of enhanced salivary starch digestion (through increased *AMY1* CN),

579   with genetic determinants of enhanced upper GI glucose absorption, and early

580   postprandial insulin release. As such, postprandial metabolic efficiency through

581   optimized glucose absorption across the upper GI tract, and efficient assimilation of

582   absorbed glucose through early insulin release, would complement early enhanced

583   salivary starch breakdown (in those with higher *AMY1* CN). In short, we hypothesize

584   that key physiological aspects of starch digestion, glucose absorption and

585   assimilation, are connected through genetic and/or other mechanisms. This model

586   should be used as a focus for future research, to gain further insight into the

587   mechanisms that mediate association between human *AMY1* CN and metabolic

588   status.

589

590

591 **Acknowledgements**

594

595 **Statement of Authors' Contributions**

596 This research study protocol was designed by TMB, DB, DBR and MOW. Execution

597 of the study was led by AAB, PJDE and DB, with substantial input from SPB and RC.

598 Data analysis was conducted by TMB, DB and MOW. All authors contributed to the

599 preparation of this manuscript, and write-up was mainly coordinated by TMB with

600 input from DB, DJC, AFP, DBR and MOW. All authors have read and approved this

601 manuscript, including its final version.

References

1.   Elder PJD, Ramsden DB, Burnett D, Weickert MO, Barber TM. Human amylase gene copy number variation as a determinant of metabolic state. Expert Rev Endocrinol Metab. 2018;13(4):193-205.

2.   Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, et al. Genetic studies of body mass index yield new insights for obesity biology. Nature. 2015;518(7538):197-206.

3.   Waalen J. The genetics of human obesity. Transl Res. 2014;164(4):293-301.

4.   Dracopoli NC, Meisler MH. Mapping the human amylase gene cluster on the proximal short arm of chromosome 1 using a highly informative (CA)n repeat. Genomics. 1990;7(1):97-102.

5.   Carpenter D, Dhar S, Mitchell LM, Fu B, Tyson J, Shwan NA, et al. Obesity, starch digestion and amylase: association between copy number variants at human salivary (AMY1) and pancreatic (AMY2) amylase genes. Hum Mol Genet. 2015;24(12):3472-80.

6.   Groot PC, Mager WH, Frants RR. Interpretation of polymorphic DNA patterns in the human alpha-amylase multigene family. Genomics. 1991;10(3):779-85.

7.   Inchley CE, Larbey CD, Shwan NA, Pagani L, Saag L, Antao T, et al. Selective sweep on human amylase genes postdates the split with Neanderthals. Sci Rep. 2016;6:37198.

8.   Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, et al. Detection of large-scale variation in the human genome. Nat Genet. 2004;36(9):949-51.

9.   Mandel AL, Breslin PA. High endogenous salivary amylase activity is associated with improved glycemic homeostasis following starch ingestion in adults. J Nutr. 2012;142(5):853-8.

10.   Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S, Breslin PA. Individual differences in AMY1 gene copy number, salivary alpha-amylase levels, and the perception of oral starch. PLoS One. 2010;5(10):e13352.

11.   Santos JL, Saus E, Smalley SV, Cataldo LR, Alberti G, Parada J, et al. Copy number polymorphism of the salivary amylase gene: implications in human nutrition research. J Nutrigenet Nutrigenomics. 2012;5(3):117-31.

12.   Peyrot des Gachons C, Breslin PA. Salivary Amylase: Digestion and Metabolic Syndrome. Curr Diab Rep. 2016;16(10):102.

13.   Fernandez CI, Wiley AS. Rethinking the starch digestion hypothesis for AMY1 copy number variation in humans. Am J Phys Anthropol. 2017;163(4):645-57.

14.   Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H, Redon R, et al. Diet and the evolution of human amylase gene copy number variation. Nat Genet. 2007;39(10):1256-60.

15.   Yang ZM, Lin J, Chen LH, Zhang M, Chen WW, Yang XR. The roles of AMY1 copies and protein expression in human salivary alpha-amylase activity. Physiol Behav. 2015;138:173-8.

16.   Falchi M, El-Sayed Moustafa JS, Takousis P, Pesce F, Bonnefond A, Andersson-Assarsson JC, et al. Low copy number of the salivary amylase gene predisposes to obesity. Nat Genet. 2014;46(5):492-7.

17.   Choi YJ, Nam YS, Yun JM, Park JH, Cho BL, Son HY, et al. Association between salivary amylase (AMY1) gene copy numbers and insulin resistance in asymptomatic Korean men. Diabet Med. 2015;32(12):1588-95.

18.   Mejia-Benitez MA, Bonnefond A, Yengo L, Huyvaert M, Dechaume A, Peralta-Romero J, et al. Beneficial effect of a high number of copies of salivary amylase AMY1 gene on obesity risk in Mexican children. Diabetologia. 2015;58(2):290-4.

19.   Ooi DS, Tan VM, Ong SG, Chan YH, Heng CK, Lee YS. Differences in AMY1 Gene Copy Numbers Derived from Blood, Buccal Cells and Saliva Using Quantitative and Droplet Digital PCR Methods: Flagging the Pitfall. PLoS One. 2017;12(1):e0170767.

20.   Viljakainen H, Andersson-Assarsson JC, Armenio M, Pekkinen M, Pettersson M, Valta H, et al. Low Copy Number of the AMY1 Locus Is Associated with Early-Onset Female Obesity in Finland. PLoS One. 2015;10(7):e0131883.

21.   Bonnefond A, Yengo L, Dechaume A, Canouil M, Castelain M, Roger E, et al. Relationship between salivary/pancreatic amylase and body mass index: a systems biology approach. BMC Med. 2017;15(1):37.

22.   Usher CL, Handsaker RE, Esko T, Tuke MA, Weedon MN, Hastie AR, et al. Structural forms of the human amylase locus and their relationships to SNPs, haplotypes and obesity. Nat Genet. 2015;47(8):921-5.

23.   Yong RY, Mustaffa SB, Wasan PS, Sheng L, Marshall CR, Scherer SW, et al. Complex Copy Number Variation of AMY1 does not Associate with Obesity in two East Asian Cohorts. Hum Mutat. 2016;37(7):669-78.

24.   Shwan NAA, Armour JAL. No Evidence for Association of BMI with Salivary Amylase Gene Copy Number in the UK 1958 Birth Cohort. Obesity (Silver Spring). 2019;27(9):1533-8.

25.   Weickert MO, Roden M, Isken F, Hoffmann D, Nowotny P, Osterhoff M, et al. Effects of supplemented isoenergetic diets differing in cereal fiber and protein content on insulin sensitivity in overweight humans. Am J Clin Nutr. 2011;94(2):459-71.

26.   Weickert MO, Hattersley JG, Kyrou I, Arafat AM, Rudovich N, Roden M, et al. Effects of supplemented isoenergetic diets varying in cereal fiber and protein content on the bile acid metabolic signature and relation to insulin resistance. Nutr Diabetes. 2018;8(1):11.

27.   Weickert MO, Arafat AM, Blaut M, Alpert C, Becker N, Leupelt V, et al. Changes in dominant groups of the gut microbiota do not explain cereal-fiber induced improvement of whole-body insulin sensitivity. Nutr Metab (Lond). 2011;8:90.

28.   Hattersley JG, Pfeiffer AF, Roden M, Petzke KJ, Hoffmann D, Rudovich NN, et al. Modulation of amino acid metabolic signatures by supplemented isoenergetic diets differing in protein and cereal fiber content. J Clin Endocrinol Metab. 2014;99(12):E2599-609.

29.   Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, et al. The digital MIQE guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clin Chem. 2013;59(6):892-902.

30.   Hattersley JG, Mohlig M, Roden M, Arafat AM, Loeffelholz CV, Nowotny P, et al. Quantifying the improvement of surrogate indices of hepatic insulin resistance using complex measurement techniques. PLoS One. 2012;7(6):e39029.

31.   Mueller S, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, et al. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol. 2006;72(2):1027-33.

32.   Rigottier-Gois L, Bourhis AG, Gramet G, Rochet V, Dore J. Fluorescent hybridisation combined with flow cytometry and hybridisation of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes. FEMS Microbiol Ecol. 2003;43(2):237-45.

33.   Rukh G, Ericson U, Andersson-Assarsson J, Orho-Melander M, Sonestedt E. Dietary starch intake modifies the relation between copy number variation in the salivary amylase gene and BMI. Am J Clin Nutr. 2017;106(1):256-62.

34.   Weickert MO. What dietary modification best improves insulin sensitivity and why? Clin Endocrinol (Oxf). 2012;77(4):508-12.

35.   Ahren B, Holst JJ. The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. Diabetes. 2001;50(5):1030-8.

36.   Tan VM, Ooi DS, Kapur J, Wu T, Chan YH, Henry CJ, et al. The role of digestive factors in determining glycemic response in a multiethnic Asian population. Eur J Nutr. 2016;55(4):1573-81.

37.   Heianza Y, Sun D, Wang T, Huang T, Bray GA, Sacks FM, et al. Starch Digestion-Related Amylase Genetic Variant Affects 2-Year Changes in Adiposity in Response to Weight-Loss Diets: The POUNDS Lost Trial. Diabetes. 2017;66(9):2416-23.

38.   Teff KL, Engelman K. Oral sensory stimulation improves glucose tolerance in humans: effects on insulin, C-peptide, and glucagon. Am J Physiol. 1996;270(6 Pt 2):R1371-9.

39.   Lee JG, Park SW, Cho BM, Lee S, Kim YJ, Jeong DW, et al. Serum amylase and risk of the metabolic syndrome in Korean adults. Clin Chim Acta. 2011;412(19-20):1848-53.

40.   Nakajima K, Muneyuki T, Munakata H, Kakei M. Revisiting the cardiometabolic relevance of serum amylase. BMC Res Notes. 2011;4:419.

Table 1: **Pearson Correlation Coefficients with *AMY1* CN**
(Data from ProFiMet cohort, n=80; P<0.05 is statistically significant, marked with* and highlighted in bold type; NS=non-significant; SIB=stable isotope basal; SISS=stable isotope steady state)

Variable	Correlation Coefficient	P-value
Body composition		
BMI	-0.214	0.057
Fat Mass (BodPod)	-0.085	NS
Lean Mass (BodPod)	-0.216	0.055
Total Fat Volume	-0.070	NS
Subcutaneous adipose tissue (umbilical)	0.025	NS
Visceral and hepatic indices		
Total Visceral Fat Volume	-0.330	**0.004***
Visceral adipose tissue (umbilical)	-0.324	**0.004***
Intrahepatic Lipid Content	-0.094	NS
Hepatic Endogenous Glucose Production (SIB)	-0.047	NS
Hepatic Endogenous Glucose Production (SISS)	-0.095	NS
Biochemical metabolic markers		
Fasting Glucose	0.003	NS
Fasting Insulin	-0.141	NS
OGIS (OGTT-derived)	0.259	**0.02***
Whole-body insulin resistance	0.129	NS
HOMA-IR	-0.128	NS
Insulin Sensitivity Index	0.114	NS
HbA1C	-0.126	NS
Serum Total Cholesterol	0.091	NS
Serum HDL Cholesterol	0.325	**0.003***
Serum triglycerides	-0.110	NS
Serum LDL Cholesterol	0.001	NS
NEFA	0.001	NS
Inflammatory markers and adipokines		
Serum CRP	-0.137	NS
Serum PAI-1	-0.04	NS
Serum Interleukin 10	0.070	NS
Serum FGF-21	-0.028	NS
Serum adiponectin	0.249	**0.026***
Appetite hormones		
Serum ghrelin	0.227	**0.044***
Serum acylated ghrelin	0.158	NS
Serum leptin	0.077	NS
Macronutrient Intake		
Alcohol intake	-0.200	NS
Total energy intake	-0.155	NS
Total fibre intake	0.129	NS
Soluble fibre intake	0.107	NS
Insoluble fibre intake	0.174	NS
Magnesium intake	-0.130	NS
Protein intake	-0.200	NS
Red meat intake	-0.102	NS
Fish intake	0.110	NS
Milk intake	-0.131	NS
Legume intake	0.021	NS
Egg intake	-0.078	NS
Poultry intake	-0.116	NS
Carbohydrate intake	-0.135	NS
Cholesterol intake	-0.110	NS
PUFA intake	0.041	NS
MUFA intake	-0.052	NS
SFA intake	-0.121	NS

Figure Legends

Figure 1: Flowchart of DNA extractions and analyses, including validation of the ddPCR technique
OSC=oral starch challenge; mus=muscle; PRT=Paralogue Ratio Test; ddPCR=droplet digital polymerase chain reaction; AMY1= human salivary amylase gene; CN=copy number; EDTA=Ethylenediaminetetraacetic acid

Figure 2: Histogram showing the distribution of *AMY1* CN amongst the ProFiMet Cohort

Figure 3: Associations between *AMY1* CN and total visceral fat volume, umbilical visceral fat volume, OGIS score, serum fasting HDL-Cholesterol and serum adiponectin amongst the ProFiMet Cohort (mean values and standard error of the mean [SEM])
AMY1: The human salivary amylase gene
CN: Copy Number
HDL: High Density Lipoprotein
OGIS: Oral Glucose Insulin Sensitivity