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Salivary *AMY1* Copy Number Variation Modifies Age-Related Type 2 Diabetes Risk

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BACKGROUND: Copy number variation (CNV) in the salivary amylase gene (*AMY1*) modulates salivary α-amylase levels and is associated with postprandial glycemic traits. Whether *AMY1*-CNV plays a role in age-mediated change in insulin resistance (IR) is uncertain.

METHODS: We measured *AMY1*-CNV using duplex quantitative real-time polymerase chain reaction in two studies, the Boston Puerto Rican Health Study (BPRHS, n = 749) and the Genetics of Lipid-Lowering Drug and Diet Network study (GOLDN, n = 980), and plasma metabolomic profiles in the BPRHS. We examined the interaction between *AMY1*-CNV and age by assessing the relationship between age with glycemic traits and type 2 diabetes (T2D) according to high or low copy numbers of the *AMY1* gene. Furthermore, we investigated associations between metabolites and interacting effects of *AMY1*-CNV and age on T2D risk.

RESULTS: We found positive associations of IR with age among subjects with low *AMY1*-copy-numbers in both studies. T2D was marginally correlated with age in participants with low *AMY1*-copy-numbers but not with high *AMY1*-copy-numbers in the BPRHS. Metabolic pathway enrichment analysis identified the pentose metabolic pathway based on metabolites that were associated with both IR and the interactions between *AMY1*-CNV and age. Moreover, in older participants, high *AMY1*-copy-numbers tended to be associated with lower levels of ribonic acid, erythronic acid, and arabinonic acid, all of which were positively associated with IR.

CONCLUSIONS: We found evidence supporting a role of *AMY1*-CNV in modifying the relationship between age and IR. Individuals with low *AMY1*-copy-numbers tend to have increased IR with advancing age.

Introduction

Starch, as a primary source of carbohydrates, contributes substantially to dietary energy intake in the United States and around the world. The enzymatic digestion of starch into smaller carbohydrate molecules such as glucose and maltose begins in the mouth through the action of salivary α-amylase. This enzyme is encoded by the three related amylase genes (*AMY1A*, *AMY1B*, and *AMY1C*) clustered as the *AMY1* locus *(1)*. Extensive variation in copy number of the constituent genes has been described, ranging from 2 to 16 copies *(2)*. Reports showed that individuals with low copy numbers of *AMY1* tended to have decreased levels of salivary α-amylase *(1, 3)* and a decreased capacity to digest starch. Hence, a positive association between *AMY1* copy numbers and improved postprandial glycemic homeostasis would be expected. Moreover, another study reported that individuals with high salivary *AMY1* copy numbers had significantly lower postprandial blood glucose

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concentration following starch ingestion and, thus, a predicted lower risk for type 2 diabetes *(4)*. This was a small study of 14 participants; thus, this observation may not be generalizable to other populations.

Insulin resistance is associated with aging *(5–8)*. Studies using glucose clamp techniques in the early 1980s showed impaired insulin-mediated glucose uptake (IMGU) in peripheral tissues as a part of the aging process *(9, 10)*. Age-related changes in body fat (inflammatory phenotypes) and environmental factors (changes in diet and physical activity) have been associated with insulin resistance *(5, 11)*. Therefore, a decline in dietary carbohydrate tolerance is a common finding in older adult populations *(12, 13)*.

As copy number variation (CNV) at the *AMY1* locus has been shown to modify postprandial glycemic homeostasis *(4)*, we hypothesized that the *AMY1* CNV would modify age-related glycemic traits, including glucose, insulin, and HOMA-IR (homeostatic model assessment of insulin resistance). For this purpose, we used the Boston Puerto Rican Health Study (BPRHS) and the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study to compare correlations between age and glycemic traits in participants carrying either low *AMY1* or high *AMY1* copy numbers. We then determined whether the interaction between *AMY1* copy number and age contributed to specific metabolic pathways on insulin resistance. Our results enhance understanding of *AMY1* CNV links between aging and insulin resistance.

RESEARCH DESIGN AND METHODS

Boston Puerto Rican Health Study (BPRHS) The BPRHS is a longitudinal cohort study to examine and characterize relationships between nutrition, genetics, and health disparities in Puerto Rican adults *(14)*. The population comprises 1500 individuals, self-identified as Puerto Rican, with baseline age ranging from 45 to 75 years *(14)*. Participants completed a comprehensive set of dietary and health assessment questionnaires. Blood, urine, and saliva samples were collected for biomarker and genetic analysis. In this study, we evaluated blood lipids, diabetes-related traits [fasting glucose, fasting insulin and HOMA-IR (calculated according to the formula: fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5) *(15)*], plasma metabolites and salivary *AMY1* copy numbers among participants (n = 749), with or without diabetes at the baseline (Supplemental Fig. 1).

Genetics of lipid lowering drugs and diet network study (GOLDN) GOLDN is part of the PROGENI (PROgram for GENetic Interaction) Network, a group of family intervention studies focusing on gene-environment interactions. The GOLDN study recruited families with at least two siblings from two National Heart, Lung, and Blood Institute Family Heart Study field centers: Minneapolis, MN, and Salt Lake City, UT *(16)*. For the current study, we tested blood lipids, diabetes-related traits (fasting glucose, fasting insulin, and HOMA-IR) and salivary *AMY1* copy numbers among participants (n = 980) at baseline (Supplemental Fig. 1).

AMY1 copy number estimation by quantitative real-time PCR The copy number of the *AMY1* locus was determined by duplex quantitative real-time polymerase chain reaction (qRT-PCR) using DNA isolated with QIAamp DNA Blood Mini kit (Qiagen) from the buffy coat of the blood samples *(3)*. DNA concentration of each sample was determined using NanoDrop 10003.7.1 (Thermo Scientific). Briefly, 10 ng DNA (1 μL) from each sample (together with 5 μL TaqMan genotype mix and 2 μL water) was used to create duplex PCR in 10 μL reaction with two sets of primers (0.5 μL each): one for *AMY1* gene (Hs07226362, Life Technologies Inc.) and one for the reference gene - *RNase P* (RNASEP, Life Technologies Inc.). qRT-PCR was performed in 364-well plates with Applied Biosystem QuanStudio 6 Flex system (Life Technologies Inc.) in the following conditions: starter 95°C 10 min, 40 cycles of 95°C 20 s, and 58.5°C 1 min. In each plate, five dilutions (36.7 ng/μL, 12.2 ng/uL, 4.1 ng/μL, 1.4 ng/μL, 0.5 ng/μL) of the DNA sample NA18972 with a known 14-copy number of *AMY1* locus used as a calibrator (Coriell Cell Repositories) for a standard curve were included with each in 5 replicates together with one no-DNA control (water) and tested DNA samples (10 ng/μl) with each in 3 replicates. Ct values were extracted following analysis with the QuantStudio Real-time PCR software version 1.1 using analysis settings selected as recommended in the user manual. The copy number of the *AMY1* gene was analyzed using CopyCaller 2.1 and estimated by delta delta Ct method: $14 \times 2^{\{\Delta Ct_{AMY1} - \Delta Ct_{calibrator} - 1\}}$, where ΔCt_{AMY1} = average of ($Ct_{AMY1} - Ct_{ref}$) over three replicates, Ct_{AMY1} is the cycle number of *AMY1* that reaches the threshold, Ct_{ref} is the Ct of the reference gene (*RNase P*) that reached the threshold; and $\Delta Ct_{calibrator}$ = average of [$Ct_{AMY1(NA18972)} - Ct_{ref(RNaseP)}$] over 5 dilutions of the NA18972 DNA used to generate the standard curve of each plate. In essence, $Ct_{calibrator}$ was used as a calibrator to normalize the variation across plates.

Metabolomic profile Metabolomic analysis was conducted using plasma samples from 749 participants of the BPRHS by Metabolon, Inc. *(17)*. In brief, plasma samples were shipped on dry ice to Metabolon, and

stored at -80 °C until analysis. The metabolomic analysis was performed using ultrahigh performance liquid chromatography-tandem mass spectroscopy after removing proteins with methanol. Individual metabolites were identified and quantified by estimating the AUC of the peaks with reference to a library of over 4500 purified standards for retention time/index, mass-to-charge ratio, and chromatographic data. After quality control, 526 metabolites were identified and assigned to pathway groups.

STATISTICAL METHODS

Correlations between age and glycemic traits, by high and low AMY1 copy number To test if aging modifies the association between *AMY1* CNV and type 2 diabetes (T2D) risk, we examined the interaction between *AMY1* CNV and age by examining the association between age and glycemic traits according to high and low *AMY1* copy number in both cohorts. In the BPRHS, a linear regression model was implemented with log-transformed metabolic traits as a dependent variable and age as the predictor in R (Version 3.5.3) among participants who were not using antidiabetes medication, adjusting for sex, ancestry, alcohol consumption, current smoking status, physical activity, and daily total energy intake. We fitted a similar log-linear model using GENMOD Procedure (SAS) in the GOLDN study, adjusting for sex, alcohol consumption, current smoking status, physical activity, daily total energy intake, and family relationship. To control for the effect of body mass index (BMI) on diabetes-related traits, we further conducted a similar association study in both cohorts adjusting for BMI.

Association between age and type 2 diabetes prevalence by high and low AMY1 copy number We also tested associations between age and T2D by high and low *AMY1* copy number in both cohorts. In the BPRHS, a logistic regression model was implemented with T2D (selection criteria: fasting glucose $\geq$ 126 mg/dL, or use of antidiabetes medication) as the dependent variable (the outcome) and age as the predictor in R, while adjusting for BMI, sex, ancestry, alcohol consumption, current smoking status, physical activity, and daily total energy intake. In GOLDN, a similar logistic regression model was implemented using the logit link function in the GENMOD procedure in SAS, adjusting for BMI, sex, alcohol consumption, field center, current smoking status, physical activity, daily total energy intake, and family relationship.

Modification of HOMA-IR associated metabolites by AMY1 copy number and age interaction To uncover the connection among HOMA-IR associated metabolites, *AMY1* copy number and age, we first selected plasma metabolites that were associated with HOMA-IR using a linear regression model (P-value < 0.05) in participants who were not using antidiabetes medication from the BPRHS. We then linked those selected metabolites to the metabolites that were associated with the interaction between *AMY1* copy number and age using linear regression in R.

Metabolic pathway enrichment analysis All detected metabolites (n = 526) were organized into metabolic pathways based on the annotation database of Metabolon Inc. Only 54 pathways that contained $\geq$3 detected metabolites in a total of 434 metabolites were included in the pathway analysis. The number of metabolites that linked to both HOMA-IR and *AMY1* copy number by age interaction at $P < 0.05$ was counted within each pathway. Metabolic pathway enrichment by *AMY1* copy number and age interaction was determined by Z score calculation for each pathway, as Z score = [r—n (R/N)]/{n (R/N) [1—(R/N)][1 – (n – 1)/(N – 1)]} *(18, 19)*, where N = 434 is the total number of metabolites fitted in the metabolomic profiling that were included in the 54 pathways, R is the total number of metabolites that significantly associated with *AMY1* copy number and age interaction, n is the total number of metabolites measured in a specific pathway, and r is the number of metabolites significantly associated with *AMY1* copy number and age interaction within a specific pathway. P values of Z scores were derived assuming a normal distribution and 2-sided test. Multiple testing was corrected by the Bonferroni test ($P = 0.05/54$, 0.0009).

Results

POPULATION CHARACTERISTICS

Clinical characteristics for all participants in both populations are listed in Supplemental Table 1. All participants were of self-reported European ancestry in GOLDN, while participants in BPRHS had an admixed ancestry, with 59% ancestry of European origin *(20)*. The mean age of BPRHS participants was about 10 years older than that of GOLDN. Further, more than 70% of BPRHS were women, whereas in GOLDN the proportion of men was similar to that of women. Among 749 participants with *AMY1* CNV and metabolome data in BPRHS, 512 of which (68%) were not using antidiabetes medication. In GOLDN, 933 of 980 (95%) participants were not using antidiabetes medication.

Among participants who were not using antidiabetes medication, the general characteristics of both populations according to high (BPRHS $\geq$ 6; GOLDN $\geq$ 5)

or low *AMY1* copy number (BPRHS < 6; GOLDN < 5) are shown in Table 1. Each group was divided into high and low based on the mean *AMY1* copy number (6.6 in BPRHS and 4.8 in GOLDN). *AMY1* copy number distributions across participants from BPRHS and GOLDN are shown in Fig. 1. In BPRHS, groups with high or low *AMY1* copy number did not differ significantly in age, fasting glucose, fasting insulin, or HOMA-IR (*P*-value > 0.05, Table 1). In GOLDN, mean age, fasting glucose, and HOMA-IR were also similar between high and low *AMY1* copy number groups (*P*-value > 0.05, Table 1). However, the mean level of fasting insulin in participants with high *AMY1* copy number was significantly lower than those with low *AMY1* copy number in GOLDN (*P*-value < 0.05, Table 1).

THE ASSOCIATIONS BETWEEN METABOLIC TRAITS AND AGE DIFFER BY HIGH AND LOW AMY1 COPY NUMBER

To examine whether the *AMY1* CNVs are directly associated with T2D risk, we associated the *AMY1* copy number with fasting glycemic traits in both BPRHS and GOLDN, but the associations were not significant in either population (data not shown). Then, we tested if the interaction between *AMY1* CNVs and age modify glycemic traits. Associations between metabolic traits and age based on high or low *AMY1* copy number were assessed in BPRHS and GOLDN participants who were not

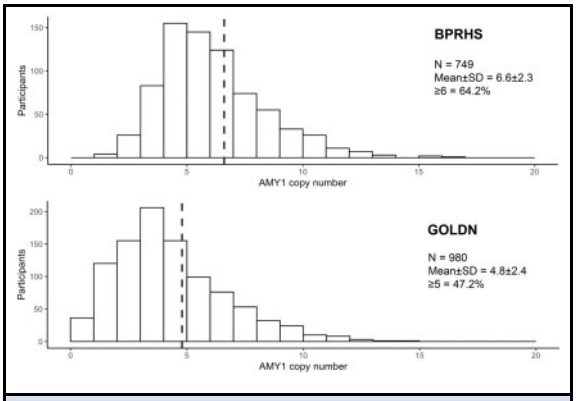

Fig. 1. AMY1 copy number distributions of participants from BPRHS and GOLDN. Dash vertical line depicts the mean of *AMY1* copy number. BPRHS: 64.2% with *AMY1* copy number ≥6. GOLDN: 47.2% with *AMY1* copy number ≥5.

Table 1. Clinical characteristics of participants from BPRHS and GOLDN who were not using antidiabetes medication, according to *AMY1* copy number.

	BPRHS[f]		GOLDN[f]	
	AMY1 copy number ≥ 6	*AMY1* copy number < 6	*AMY1* copy number ≥ 5	*AMY1* copy number < 5
N	332	180	444	489
AMY1 copy number	7.8±2.0	4.4±0.8[a]	6.7±1.9	3.0±1.0[a]
Age, years	56.8±7.5	57.1±7.5	48.5±16.7	47.2±16.1
European ancestry	59%	57%	100%	100%
Female (%)	861 (72)	126 (70)[a]	233 (52)	256 (52)
Smoker (%)	176 (53)	95 (53)	119 (27)	142 (29)[a]
Drinker (%)	247 (74)	123 (69)[a]	120 (49)	240 (49)
BMI (kg/m^2)	31.2±7.0	31.6±5.3	28.3±5.5	27.8±5.4
TC (mg/dL)[b]	193±39.4	190±41.7	191±38.6	189±38.1
HDL-C (mg/dL)[c]	47.1±13.1	44.4±11.6[a]	47.1±13.6	47.9±12.6
TG (mg/dL)[d]	154±100	177±168	136±88.2	129±94.8
Glucose (mg/dL)	107±40.3	106±30.5	99.2±10.6	99.0±12.9
Insulin (pmol/L)	111±99.0	115±88.2	97.8±56.2	90.2±48.6[a]
HOMA-IR[e]	4.5±5.6	4.5±4.7	3.5±2.3	3.3±2.3

[f]Data are Mean±SD or N∓ (%).
[a]Statistically significantly different between high and low *AMY1* copy number groups in BPRHS or GOLDN using independent *t*-test, $P < 0.05$.
[b]TC: Total cholesterol, 1 mg/dL TC = 0.0259 mmol/L TC.
[c]HDL-C: High density lipoprotein cholesterol, 1 mg/dL DHL-C = 0.0259 mmol/L HDL-C.
[d]TG: Total triglycerides, 1 mg/dL TG = 0.0113 mmol/L TG.
[e]HOMA-IR: calculated according to the formula [fasting insulin (mU/L) x fasting glucose (mmol/L)/22.5].

using antidiabetes medication, adjusting for sex, ancestry, alcohol consumption, current smoking status, physical activity, and daily total energy intake (Table 2). Fasting glucose was significantly associated with age (β = 2.05E-3, *P*-value = 8.88E-16) in GOLDN participants with high *AMY1* copy number, and was marginally associated (β = 5.15E-3, *P*-value = 0.0450) in BPRHS participants with high *AMY1* copy number. Neither fasting insulin nor HOMA-IR were associated with age in BPRHS participants with high *AMY1* copy number. However, among participants with low *AMY1* copy number, HOMA-IR was significantly associated with age in both BPRHS (β = 0.0269, *P*-value = 0.0102) and GOLDN (β = 0.0103, *P*-value = 1.03E-8). Fasting glucose was associated with age in both populations with low *AMY1* copy number. In summary, we observed associations of fasting glucose with age in both BPRHS and GOLDN participants, regardless of *AMY1* copy number, while HOMA-IR was significantly associated with age only in participants with low *AMY1* copy numbers in both cohorts.

BMI was associated with age in both those with high and low *AMY1* copy number in the GOLDN study (Table 2), which agrees with the well-established positive relationship between BMI and diabetes-related traits *(21–23)*. Hence, to account for the effect of body mass, we further controlled BMI in the association analyses (Table 3). For those participants with low *AMY1* copy numbers, we observed significant associations of HOMA-IR with age in both BPRHS (β = 0.0268, *P*-value = 7.48E-3) and GOLDN (β = 7.75E-3, *P*-value = 1.95E-5) after further adjusting for BMI (Table 3). Using the same model with participants with high *AMY1* copy numbers, HOMA-IR was not associated with age in either cohort (Table 3). We found a similar association of fasting insulin with age (β = 4.03E-3, *P*-value = 2.91E-3, Table 3) in GOLDN participants with low *AMY1* copy numbers. However, the association did not reach significance in the BPRHS (β = 0.0166, *P*-value = 0.056, Table 3).

To further illustrate the interactions between *AMY1* copy number and age on diabetes risk, we assessed the interactions between *AMY1* copy number and age on HOMA-IR by dividing the populations into different numbers of equally-sized bins, (# bins = 2, 3, 4, 5, 6), based on *AMY1* copy number (Supplemental Table 2). In BPRHS, when the population was grouped into 2 or 3 bins, we detected interaction between *AMY1* and age ($P_{interaction}$=0.158 and 0.046, respectively). In GOLDN, 2-bins and 6-bins detected the strongest interaction ($P_{interaction}$= 0.016 and 0.048, respectively). Interestingly, without binning - the original copy number (i.e., ranging from 1-16) showed no advantage in detecting the interaction in both populations ($P_{interaction}$=0.224 and 0.116, in BPRHS and GOLDN, respectively). Hence, to be consistent in both populations, dividing into two bins (i.e., low and high copy number) was justified to detect the interaction between *AMY1* and age on HOMA-IR. When we applied 2-bin method for glucose and insulin (Supplemental Table 2), the interaction between *AMY1* and age was marginally significant (P= 0.0234) for insulin in GOLDN, but the interaction did not reach statistical significance for glucose in GOLDN, neither (*P*-value = 0.3132 and

Table 2. Associations between age and metabolic traits according to *AMY1* copy number in participants who were not using antidiabetes medication, from the BPRHS and GOLDN studies.

		BPRHS					GOLDN			
Traits		N	β[a]	SE	*P*-value		N	β	SE	*P*-value
AMY1 copy number	≥ 6					≥ 5				
BMI (kg/m^2)		332	−2.18E-5	2.26E-3	0.992		444	1.57E-3	6.11E-4	0.0103
Glucose (mg/dL)		332	5.15E-3	2.16E-3	0.0450		444	2.05E-3	2.55E-4	8.88E-16
Insulin (pmol/L)		332	5.42E-3	7.34E-3	0.461		444	1.37E-3	1.58E-3	0.386
HOMA-IR		332	0.0106	8.48E-3	0.214		444	3.31E-3	1.68E-3	0.0490
AMY1 copy number	< 6					< 5				
BMI (kg/m^2)		180	9.51E-4	2.47E-3	0.701		489	2.95E-3	5.90E-4	5.58E-7
Glucose (mg/dL)		180	0.0105	3.26E-3	1.54E-3		489	2.80E-3	4.56E-4	8.33E-10
Insulin (pmol/L)		180	0.0164	8.94E-3	0.0676		489	6.81E-3	1.39E-3	1.00E-6
HOMA-IR		180	0.0269	0.0104	0.0102		489	0.0103	1.80E-3	1.03E-8

[a]*β* according to linear regression model adjusted for sex, ancestry, alcohol consumption, current smoking status, physical activity, and daily total energy intake. Additionally, family relationship and field center were adjusted in GOLDN.

Table 3. Associations between age and diabetes traits, according to *AMY1* copy number in participants who were not using antidiabetes medication in the BPRHS and GOLDN (BMI adjusted).

		BPRHS					GOLDN			
Traits		N	β[a]	SE	*P*-value		N	β	SE	*P*-value
AMY1 copy number	≥ 6					≥ 5				
Glucose (mg/dL)		332	5.34E-3	2.51E-3	0.0342		444	1.82E-3	2.53E-4	6.35E-13
Insulin (pmol/L)		332	5.38E-3	6.62E-3	0.417		444	9.74E-5	1.68E-3	0.954
HOMA-IR		332	0.0107	7.63E-3	0.161		444	2.11E-3	1.90E-3	0.268
AMY1 copy number	< 6					< 5				
Glucose (mg/dL)		180	0.0102	3.15E-3	1.39E-3		489	2.25E-3	4.49E-4	5.42E-7
Insulin (pmol/L)		180	0.0166	8.64E-3	0.0563		489	4.03E-3	1.35E-3	2.91E-3
HOMA-IR		180	0.0268	9.91E-3	7.48E-3		489	7.75E-3	1.82E-3	1.95E-5

[a]*β* according to linear regression model adjusted for sex, ancestry, alcohol consumption, current smoking status, physical activity, daily total energy intake, and BMI. Family relationship and field center were adjusted in GOLDN additionally.

0.2115, respectively) for glucose and insulin in BPRHS. In addition, it is worth mentioning that, after including the interaction in models, the main effect of *AMY1* became significant, especially for 2-bins (i.e., low and high copy number, $P\text{-}_{AMY1}$ =0.007) in GOLDN.

ASSOCIATIONS BETWEEN T2D AND AGE DIFFER BY AMY1 COPY NUMBER

Apart from diabetes-related traits, we investigated associations between T2D prevalence and age among all participants in both cohorts. In the BPRHS, T2D prevalence was associated with age in participants with low *AMY1* copy numbers (β = 0.0533, *P*-value = 6.70E-3), but not with those of high *AMY1* copy numbers (β = 0.0137, *P*-value = 0.315), adjusting for BMI, sex, ancestry, alcohol consumption, current smoking status, physical activity, and daily total energy intak (Table 4). In GOLDN, T2D prevalence was significantly associated with age in both groups of participants with high or low *AMY1* copy numbers using a similar statistical model (Table 4).

HOMA-IR-ASSOCIATED METABOLITES CORRELATED WITH THE INTERACTION BETWEEN AMY1 COPY NUMBER AND AGE

To elucidate potential metabolic pathways through which age and *AMY1* copy number interactions contributed to insulin resistance, we first correlated metabolites with HOMA-IR as a continuous variable in BPRHS participants who were not using antidiabetes medication, using available metabolome data. Two hundred and twenty-one metabolites were found to be correlated (*P*-value < 0.05) with HOMA-IR in BPRHS, and 19 of these were associated with the interaction between *AMY1* copy number and age, controlling for BMI, sex, ancestry, alcohol consumption, current smoking status, physical activity, and daily total energy intake (Table 5). Pathway enrichment analysis, based on the 19 significant metabolites, identified 5 metabolic pathways as overrepresented: pentose (Z-score = 4.93, *P*-value = 8.12E-7), glycolysis/gluconeogenesis/pyruvate (Z-score = 2.73, *P*-value = 6.31E-3), amino-sugar (Z-score = 2.73, *P*-value = 6.31E-3), glutamate (Z-score = 2.98, *P*-value = 2.90E-3) and alanine/aspartate (Z-score = 2.27, *P*-value = 0.0232) Supplemental Table 3).

Discussion

These results suggest an effect of the interaction between age and *AMY1* copy number on insulin resistance. Insulin resistance was associated with advancing age in individuals with low *AMY1* copy number in both cohorts. To our knowledge, our study is the first to report a potential genetic mediator of the effect of amylase activity on age-related glycemic signatures. We demonstrated an association between age and insulin resistance in individuals with low *AMY1* copy number but not those with high *AMY1* copy number. As stated above, numerous studies have shown the correlation of declines in insulin activity with advancing age *(5–9)*. Generally, body fat is believed to be critical in the link between aging and insulin resistance *(24, 25)*. Age-related increases in adiposity would result in higher proinflammatory cytokines that hinder the IRS-PI3K-Akt pathway, causing impaired IMGU in peripheral tissues *(6)*. However, this mechanism fails to explain the associations between aging and insulin resistance in statistical models that account for BMI or body mass *(9, 10)*. In this study, we propose an alternative mechanism to explain the age-

Table 4. Associations between age and T2D prevalence according to *AMY1* copy number in participants from the BPRHS and GOLDN studies (BMI adjusted).

		BPRHS					GOLDN			
Traits		**N**	**β[a]**	**SE**	***P*-value**		**N**	**β**	**SE**	***P*-value**
AMY1 copy number	≥ 6					≥ 5				
T2D		481	0.0137	0.0136	0.315		462	0.0577	0.0129	8.31E-6
AMY1 copy number	< 6					< 5				
T2D		268	0.0533	0.0197	6.70E-3		518	0.0778	0.0140	2.79E-8

[a]*β* according to logistic regression model adjusted for sex, ancestry, alcohol consumption, current smoking status, physical activity, daily total energy intake, and BMI. Family relationship and field center were adjusted in GOLDN additionally.

Table 5. Nineteen metabolites that were associated with both HOMA-IR and the interacting effects between *AMY1* copy number and age (*AMY1**Age), in participants who were not using antidiabetes medication in the BPRHS.

Metabolites	**β (*AMY1**Age)[a]**	**SE (*AMY1**Age)**	***P* (*AMY1**Age)**	**β (HOMA)**	**SE (HOMA)**	***P* (HOMA)**
Ribonic acid	−4.88E-3	1.34E-3	2.89E-4	0.480	0.0950	6.15E-7
Arabinonic acid	−3.02E-3	1.46E-3	0.0393	0.226	0.0988	0.0228
1,5-Anhydroglucitol	4.26E-3	2.16E-3	0.0498	−0.485	0.125	1.14E-4
Erythronic acid	−2.25E-3	8.02E-4	5.23E-3	0.788	0.202	1.09E-4
β-Citrylglutamate	−2.40E-3	1.18E-3	0.0433	0.296	0.100	3.40E-3
γ-Carboxyglutamate	−2.30E-3	9.51E-4	0.0158	0.314	0.155	0.0441
Proline	−1.53E-3	6.75E-4	0.0237	1.31	0.229	1.77E-8
Aspartate	−2.22E-3	9.78E-4	0.0238	4.16	0.699	4.97E-9
N-Acetylleucine	2.85E-3	1.37E-3	0.0381	0.419	0.120	5.11E-4
1-Methyl-4-imidazoleacetate	−2.79E-3	1.26E-3	0.0271	0.215	0.0635	7.44E-4
α-Ketoglutarate	5.00E-3	2.28E-3	0.0287	2.45	0.430	2.06E-8
Deoxycholate	−9.23E-3	4.18E-3	0.0276	0.0707	0.0324	0.0296
2-Linoleoylglycerol	5.69E-3	2.80E-3	0.0427	−0.0702	0.0331	3.41E-3
2-Hydroxypalmitate	1.70E-3	7.86E-4	0.0314	−0.984	0.237	3.93E-5
4-Acetylphenol sulfate	−0.0705	3.05E-3	0.0213	0.0619	0.0305	0.0427
Palmitoyl sphingomyelin	−1.96E-3	9.28E-4	0.0352	−0.317	0.133	0.0172
Stachydrine	−0.0113	4.71E-3	0.0173	0.111	0.0513	0.0309
Quinate	−0.0119	5.69E-3	0.0366	0.0167	8.03E-3	0.0383
Sulfate	−1.60E-3	7.38E-4	0.0302	0.541	0.241	0.0251

[a]*β* (*AMY1**Age) = beta for *AMY1**Age interaction based on a linear regression model adjusted for sex, ancestry, alcohol consumption, current smoking status, physical activity, daily total energy intake, and BMI.

related glycemic dysfunction through genetic-related carbohydrate handling, suggesting that genetic factors on starch metabolism might be involved in the regulation of age-related glycemic traits.

The level of salivary amylase is strongly correlated with the *AMY1* copy number *(2)*. It has also been reported that lower serum amylase levels are associated with susceptibility to T2D *(26)*. To further illustrate the role of *AMY1* copy number on the relation between age and T2D, we investigated associations between T2D prevalence and age among all participants in both cohorts. We did not find any association of T2D with

age in the set of participants with high *AMY1* copy numbers in the BPRHS, as predicted. However, T2D was significantly correlated with age in each group of high or low *AMY1* copy numbers in GOLDN. Further analysis in larger populations or more cohorts is needed to elucidate the effect of *AMY1* copy number on the relation between age and T2D.

Analyses of serum metabolome data highlight the effect of the *AMY1* copy number-age interaction on those metabolites correlated with insulin resistance in BPRHS. Metabolic enrichment analysis of the significant metabolites suggests that pentose, glycolysis/gluconeogenesis/pyruvate, amino-sugar, glutamate, and alanine/aspartate pathways are associated with the interactions between *AMY1* copy number and age, with pentose metabolism being statistically significant after correction for multiple testing (Supplemental Table 3). In particular, ribonic acid and erythronic acid (Table 5), that were positively associated with HOMA-IR (β = 0.48, 0.788, and *P*-value = 6.15E-7, 1.09E-4, respectively) and had a significant interaction effect between *AMY1* copy number and age (*P*-value = 2.89E-4 and 5.23E-3, respectively), as shown in Supplemental Fig. 2, were predicted to increase less with advancing age in participants with high *AMY1* copy numbers, when compared to that for those with low *AMY1* copy numbers. Erythronic acid is an oxidized product of *N*-acetylglucosamine (GlcNAc) *(27)*. Over the course of some decades, many studies have shown protein modification by O-GlcNAc (O-GlcNAcylation) as a key regulator of the insulin-signaling pathway *(28–31)*. Greater O-GlcNAc or UDP-GlcNAc (the substrate for O-GlcNAcylation) that leads to aberrant O-GlcNAcylation could attenuate insulin signaling by interfering with IRS-1/Akt phosphorylation, blocking insulin-stimulated glucose uptake and therefore contributing to insulin resistance *(29)*. Lower plasma erythronic acid and arabinonic acid levels are suggestive of a decline of O-GlcNAc or UDP-GlcNAc in adipocytes or muscle cells, and this mechanism could underlie our observed interaction between *AMY1* copy number and age. Interestingly, older individuals with high *AMY1* copy number also have increased levels of 1,5-anhydroglucitol, which has been observed to stimulate insulin secretion and suppress postprandial glucose elevation *(32, 33)*. Collectively, these results suggest plausible metabolic pathways through which high *AMY1* copy numbers could improve insulin action in older participants.

There is one limitation worth noting in this study. Specifically, reliance on the outcome variable "HOMA-IR" is a limitation, despite its frequent use as a surrogate estimate for insulin resistance in large population studies. The HOMA-IR estimate is calculated as the product of fasting glucose and fasting insulin, divided by 22.5. It has been documented that both postprandial glucose and insulin levels could be modified by *AMY1* copy numbers after starch ingestion *(4)*. Although we used fasting glucose and fasting insulin in our model, delayed digestion and/or absorption of starch might influence these glycemic traits. Further studies are needed to elucidate whether the differences we reported in HOMA-IR are a consequence of glucose and insulin differences, or whether *AMY1* CNVs have a direct influence on actual measures of insulin resistance. Thus, future studies should use hyperinsulinemic-euglycemic clamps combined with stable isotope techniques *(34)* to accurately measure insulin action in humans after starch meals.

In conclusion, our results suggest a role for *AMY1* copy number in modifying the relationship between age and insulin resistance. Individuals with low *AMY1* copy number have greater likelihood of developing insulin resistance with advancing age, which might be a consequence of the attenuation of insulin signaling transduction by O-GlcNAc. This information contributes to the understanding of genetic mechanisms underlying the relationship between age and insulin resistance.

List of Human Genes: *AMY1A*: amylase alpha 1A; Previous symbol: *AMY1*

AMY1B: amylase alpha 1B; Previous symbol: *AMY1*

AMY1C: amylase alpha 1C; Previous symbol: *AMY1*

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References

1. 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;24:3472–80.
2. 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;39:1256–60.
3. 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;46:492–7.
4. Mandel AL, Breslin P. High endogenous salivary amylase activity is associated with improved glycemic homeostasis following starch ingestion in adults. J Nutr 2012; 142:853–8.
5. Ryan AS. Insulin resistance with aging. Sports Med 2000;30:327–46.
6. Park MH, Kim DH, Lee EK, Kim ND, Im DS, Lee J, et al. Age-related inflammation and insulin resistance: a review of their intricate interdependency. Arch Pharm Res 2014;37:1507–14.
7. Barzilai N, Ferrucci L. Insulin resistance and aging: a cause or a protective response? J Gerontol A Biol Sci Med Sci 2012;67:1329–31.
8. Refaie MR, Sayed-Ahmed NA, Bakr AM, Abdel Aziz MY, El Kannishi MH, Abdel-Gawad SS. Aging is an inevitable risk factor for insulin resistance. J Taibah Univ Med Sci 2006;1:30–41.
9. Rowe JW, Minaker KL, Pallotta JA, Flier JS. Characterization of the insulin resistance of aging. J Clin Invest 1983;71:1581–7.
10. Chen M, Bergman RN, Pacini G, Porte D Jr. Pathogenesis of age-related glucose intolerance in man: insulin resistance and decreased beta-cell function. J Clin Mol Endocrinol 1985;60:13–20.
11. Sepe A, Tchkonia T, Thomou T, Zamboni M, Kirkland JL. Aging and regional differences in fat cell progenitors - a mini-review. Gerontology 2011;57:66–75.
12. Davidson MB. The effect of aging on carbohydrate metabolism: a review of the English literature and a practical approach to the diagnosis of diabetes mellitus in the elderly. Metab Clin Exp 1979;28:688–705.
13. Ahmed T, Haboubi N. Assessment and management of nutrition in older people and its importance to health. Clin Interv Aging 2010;5:207–16.
14. Tucker KL, Mattei J, Noel SE, Collado BM, Mendez J, Nelson J, et al. The Boston Puerto Rican Health Study, a longitudinal cohort study on health disparities in Puerto Rican adults: challenges and opportunities. BMC Public Health 2010;10:107.
15. Bonora E, Formentini G, Calcaterra F, Lombardi S, Marini F, Zenari L, et al. Homa-estimated insulin resistance is an independent predictor of cardiovascular disease in type 2 diabetic subjects. Diabetes Care 2002;25: 1135–41.
16. Corella D, Arnett DK, Tsai MY, Kabagambe EK, Peacock JM, Hixson JE, et al. The -256T>C polymorphism in the apolipoprotein A-II gene promoter is associated with body mass index and food intake in the genetics of lipid lowering drugs and diet network study. Clin Chem 2007;53:1144–52.
17. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. Anal Chem 2009;81: 6656–67.
18. Lai CQ, Smith CE, Parnell LD, Lee YC, Corella D, Hopkins P, et al. Epigenomics and metabolomics reveal the mechanism of the apoA2-saturated fat intake interaction affecting obesity. Am J Clin Nutr 2018;108:188–200.
19. Doniger SW, Salomonis N, Dahlquist KD, Vranizan K, Lawlor SC, Conklin BR. Mappfinder: using gene ontology and genmapp to create a global gene-expression profile from microarray data. Genome Biol 2003;4:R7.
20. Lai C-Q, Tucker KL, Choudhry S, Parnell LD, Mattei J, García-Bailo B, et al. Population admixture associated with disease prevalence in the Boston Puerto Rican Health Study. Hum Genet 2009;125:199–209.
21. Narayan KMV, Boyle JP, Thompson TJ, Gregg EW, Williamson DF. Effect of BMI on lifetime risk for diabetes in the U.S. Diabetes Care 2007;30:1562–6.
22. Ganz ML, Wintfeld N, Li Q, Alas V, Langer J, Hammer M. The association of body mass index with the risk of type 2 diabetes: a case–control study nested in an electronic health records system in the United States. Diabetol Metab Syndr 2014;6:50.
23. Chan JM, Rimm EB, Colditz GA, Stampfer MJ, Willett WC. Obesity, fat distribution, and weight gain as risk factors for clinical diabetes in men. Diabetes Care 1994;17:961–9.
24. Kohrt WM, Kirwan JP, Staten MA, Bourey RE, King DS, Holloszy JO. Insulin resistance in aging is related to abdominal obesity. Diabetes 1993;42:273–81.
25. Coon PJ, Rogus EM, Drinkwater D, Muller DC, Goldberg AP. Role of body fat distribution in the decline in insulin sensitivity and glucose tolerance with age. J Clin Mol Endocrinol 1992;75:1125–32.
26. Nakajima K, Nemoto T, Muneyuki T, Kakei M, Fuchigami H, Munakata H. Low serum amylase in association with metabolic syndrome and diabetes: a community-based study. Cardiovasc Diabetol 2011; 10:34.
27. Jahn M, Baynes JW, Spiteller G. The reaction of hyaluronic acid and its monomers, glucuronic acid and *N*-acetylglucosamine, with reactive oxygen species. Carbohydr Res 1999;321:228–34.
28. Slawson C, Housley MP, Hart GW. O-GlcNAc cycling: how a single sugar post-translational modification is changing the way we think about signaling networks. J Cell Biochem 2006;97:71–83.
29. Yang X, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, et al. Phosphoinositide signalling links O-GlcNAc transferase to insulin resistance. Nature 2008;451: 964–9.
30. Whelan SA, Dias WB, Thiruneelakantapillai L, Lane MD, Hart GW. Regulation of insulin receptor substrate 1 (IRS-1)/AKT kinase-mediated insulin signaling by O-linked beta-*N*-acetylglucosamine in 3T3-L1 adipocytes. J Biol Chem 2010;285:5204–11.
31. Myslicki JP, Belke DD, Shearer J. Role of O-GlcNAcylation in nutritional sensing, insulin resistance and in mediating the benefits of exercise. Appl Physiol Nutr Metab 2014;39:1205–13.
32. Yamanouchi T, Inoue T, Ichiyanagi K, Sakai T, Ogata N. 1,5-anhydroglucitol stimulates insulin release in insulinoma cell lines. Biochim Biophys Acta 2003;1623: 82–7.
33. Nakamura S, Tanabe K, Yoshinaga K, Shimura F, Oku T. Effects of 1,5-anhydroglucitol on postprandial blood glucose and insulin levels and hydrogen excretion in rats and healthy humans. Br J Nutr 2017;118: 81–91.
34. Tam CS, Xie W, Johnson WD, Cefalu WT, Redman LM, Ravussin E. Defining insulin resistance from hyperinsulinemic-euglycemic clamps. Diabetes Care 2012;35:1605–10.