COMPLEX COPY NUMBER VARIATION OF *AMY1* DOES NOT ASSOCIATE WITH OBESITY IN TWO EAST ASIAN COHORTS

Rita Y.Y. Yong[1,2], Su'Aidah B. Mustaffa[1,3], Pavandip S. Wasan[1,2], Liang Sheng[5], Christian R. Marshall[6], Stephen W. Scherer[6,7], Yik-Ying Teo[2,4], Eric P.H. Yap[1,2,3,*]

[1] Defence Medical and Environmental Research Institute, DSO National Laboratories, Singapore,

[2] Saw Swee Hock School of Public Health, National University of Singapore, Singapore,

[3] Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore,

[4] Department of Statistics and Applied Probability, Faculty of Science, National University of Singapore, Singapore.

[5] Unit of Biostatistics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore,

[6] The Centre for Applied Genomics, Genetics and Genome Biology, The Hospital for Sick Children, Toronto, ON, Canada.

[7] Department of Molecular Genetics and McLaughlin Centre, University of Toronto, Toronto, ON, Canada.

* Corresponding Author

Abstract

The human amylase gene locus at chromosome 1p21.1 is structurally complex. This region contains two pancreatic amylase genes, *AMY2B*, *AMY2A*, and a salivary gene *AMY1*. The *AMY1* gene harbours extensive copy number variation (CNV), and recent studies have implicated this variation in adaptation to starch-rich diets and in association to obesity for European and Asian populations. In this study, we showed that by combining quantitative PCR and digital PCR, coupled with careful experimental design and calibration, we can improve the resolution of genotyping CNV with high copy numbers (CN). In two East Asian populations of Chinese and Malay ethnicitiy studied, we observed a unique non-normal distribution of *AMY1* diploid CN genotypes with even:odd CN ratio of 4.5 (3.3-4.7), and an association between the common *AMY2A* CN=2 genotype and odd CN of *AMY1*, that could be explained by the underlying haplotypic structure. In two further case-control cohorts (n = 932 & 145, for Chinese and Malays, respectively), we did not observe the previously reported association between *AMY1* and obesity or body mass index (BMI). Improved methods for accurately genotyping multiallelic CNV loci and understanding the haplotype complexity at the *AMY1* locus are necessary for population genetics and association studies.

KEYWORDS

Amylase 1 gene, Amylase 2A gene, Copy Number Variation, multiallelic, Chinese and Malay populations, obesity, quantitative PCR, digital PCR.

Introduction

Human salivary amylase is encoded by the *AMY1A, AMY1B and AMY1C* genes (MIM*104700, *104701, *104702), while pancreatic amylase is encoded by two genes, *AMY2A* (MIM*104650) and *AMY2B* (MIM*104660). The *AMY1* and *AMY2* gene families share homology of 92-94%, both in coding sequences and exon-intron structure, The three copies of *AMY1* genes share > 99.9% sequence identity, and the similarity extends over a region ~27 kb around each copy, suggesting a relatively recent origin of *AMY1* gene duplication [Carpenter, et al., 2015]. In addition, two foreign elements have been identified in the promoter regions of the amylase genes, one of which is responsible for the tissue-specific expression of the salivary amylase [Meisler and Ting, 1993; Perry, et al., 2007]. These genes and a pseudogene *AMYP1* are located as a cluster on chromosome 1p21.1, in a complex genomic locus characterised by inversions, deletions, and tandem duplications (Suppl. Fig. S1a). Though the chromosomal organisation of the amylase gene cluster was first reported 25 years ago through analysis of restriction mapping in pedigrees [Groot, et al., 1989], its implications for Copy Number Variation (CNV) allele structures have not been fully appreciated.

The human amylase genes cluster region is known to be copy number variable [Iafrate, et al., 2004; Sudmant, et al., 2010] and one of the most variable CNV among humans [Sudmant, et al., 2010]. Our initial work in three East Asian populations using standard CNV microarray genotyping methods correctly identified CNVs in the amylase locus but grossly underestimated the extent of CN variation (Supp. Fig. S1b). Studies using qPCR have reported *AMY1* diploid copy number ranging from 2 to 20, with an approximately normal distribution pattern of copy number frequency in several populations [Perry, et al., 2007;

Santos, et al., 2012; Falchi, et al., 2014]. *AMY2A/2B* genes also exhibit CNV, but to a lesser extent than *AMY1* [Groot, et al., 1991; Sudmant, et al., 2010; Falchi, et al., 2014].

Recent studies suggest that these CNVs may have functional significance. *AMY1* has undergone gene number expansion in the human lineage as chimpanzee harbours only 2 copies in its genome. It was argued that this may be related to low dietary starch intake by chimpanzee [Perry, et al., 2007]. In addition to the cross-species differences, the copy number of *AMY1* is highly variable among humans, and this variation correlates positively with the expression of transcript and protein [Perry, et al., 2007; Mandel, et al., 2010; Falchi, et al., 2014]. Significantly higher *AMY1* CN were found in populations with high-starch diets (eg. Japanese & Hadza) compared to those with traditional low-starch diets (eg. Yakut & Biaka), irrespective of their geographical or ethnic origin [Perry, et al., 2007]. These studies suggest adaptive change in the *AMY1* CNV in response to adoption of agriculture and high-starch diet as a selective pressure.

AMY1 CNV has also been linked to human disease with a reduced copy number of *AMY1* associated with increased BMI and predisposing to obesity [Falchi, et al., 2014]. The association was initially identified as gene dosage effect attributed to CNV at amylase genes locus by using microarray combining transcriptomics and GWAS analysis in Swedish sibling-pairs discordant for obesity. The association of *AMY1* copy number to BMI, but not *AMY2A* copy number, was validated by qPCR in the same population, and was replicated in several European populations and one Chinese cohort from Singapore. Furthermore, the same group reported an association with similar effect size of *AMY1* copy number and obesity risk in Mexican children [Mejia-Benitez, et al., 2015]. However, the association reported by Mejia-Benitez et al. [2015] was driven by *AMY1* high CN outliers among the controls, while

the association in Falchi et al [2014] was driven by the bulk of samples producing a shift of the overall CN distribution. This suggests two different association findings rather than a replication. More recently, a negative association finding was reported in two European cohorts [Usher et al. 2015]. In addition, Usher et al. reported a differential even:odd *AMY1* copy number frequency distribution, in contrast to the approximately normal distribution reported in Falchi et al. This differential even:odd pattern was corroborated by Carpenter et al. [2015], and by using different experimental approaches, both groups reported a *AMY1* CN frequency distribution that closely resembled each other for HapMap European samples [Carpenter, et al., 2015; Usher, et al., 2015].

The association reported by Falchi et al. is the first genetic link between carbohydrate metabolism and obesity, an intriguing finding that potentially provides new insight into novel biological mechanisms underlying obesity. However, we also recognised the technical challenge of accurately genotyping multiallelic CNV in a large-scale well-powered association study, due to the large dynamic copy range of a multiallelic CNV such as *AMY1*. This technical difficulty could be the reason why the association of *AMY1* with obesity was not detected in earlier GWAS studies, since studies carried out with array-CGH or SNP array have limitations in detecting copy number >5. Conversely, several multiallelic CNV association studies done with qPCR were fraught with reproducibility issues [Cantsilieris and White, 2013; Cantsilieris, et al., 2014]. Recently, digital PCR has been used for quantifying gene dosage by digital counts of single molecule amplifications and is considered more accurate because of its arithmetic linearity compared to the exponential nature of qPCR [Weaver, et al., 2010; Baker, 2012; Hindson, et al., 2013]. *AMY* CNV genotyping by digital PCR has been reported using emulsion droplets [Usher, et al., 2015] or by high density arrays

of microwells as reported here; both methods require costly custom equipment and/or consumables.

In this study, we developed a novel calibration method combining quantitative real-time PCR (qPCR) and array-based digital-PCR (dPCR), and used this combined method in an attempt to validate the previously reported association of *AMY1* copy number with obesity in two East Asian populations. We also tested the association of obesity with *AMY2A* copy number. *AMY2B* was not studied because it was reported to be largely invariant in Asian populations [Sudmant, et al., 2010; Handsaker, et al., 2015]. We also report here the ~~unexpected~~ finding of an even-odd imbalance in CN genotypes in these populations, and study the underlying haplotype structure.

Materials and Methods

Study sample set

The study sample set was a legacy collection from a previous obesity project, which was granted ethics approval by the DSO IRB in March 2004 (ref no.: DMERI-20030260-R2). Samples were collected from volunteers with written consent. Non-obese control subjects had Body Mass Index (BMI) less than 23 kg/m^2, while obese subjects had BMI of at least 28 kg/m^2, and severely obese subjects had BMI $\geq$ 32 kg/m^2. All volunteers were male, of age ranged from 18 to 21, and of self-reported ethnicity either Chinese or Malay, with all four grandparents of the same race as the volunteer. This study sample set comprised of 519 Chinese controls, 413 Chinese obese, 30 Malay controls and 115 Malay obese. The demographic attributes of these samples are summarised in Table 1. Genomic DNA was

extracted from the buffy coat of whole blood using QIAamp DNA Mini Kit (Qiagen, Germany) and quantification by Nanodrop Spectrophotometer (Thermo Scientific, USA).

Sample Randomisation to avoid Differential Bias

All samples, both cases and controls, were subjected to identical work processes throughout this study. Samples were randomised in all laboratory processes, starting from sample collection, processing, DNA extraction, and qPCR, in order to avoid systematic bias, differential errors and batch effects.

Copy-number estimation for *AMY1* and *AMY2A* by quantitative real-time PCR

The copy numbers of *AMY1* and *AMY2A* genes were estimated by duplex quantitative real-time PCR (qPCR) on a QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific Inc., USA) with 384-well plate. The analysis software was QuantStudio™ 12K Flex Software version 1.1.2. The duplex reactions consisted of two assays, each with two primers and a TaqMan® probe (Thermo Fisher Scientific Inc., USA); one specific for the target, *AMY1* (Hs07226362_cn) or *AMY2A* (Hs04204136_cn), and one specific for the reference *RNaseP* (P/N4403328, Thermo Fisher Scientific, USA). These assays are similar as those used in Falchi et al. (2014) and hence facilitated direct comparison of results. The primers and probe of the Taqman qPCR assay employed for *AMY1* specifically target a region within exon 1 of the *AMY1* gene which is absent in the *AMYP1* pseudogene. This strategy, coupled with primer sequence specificity, ensured specificity of the qPCR assay for *AMY1*. Details of these Taqman assays are summarized in Supplementary Table S1. Detail protocols of qPCR and dPCR can be found in the Supplementary materials.

Each qPCR was carried out in replicates of four. For assay targeting *AMY1*, a final volume of 10 µl per aliquot was used in order to achieve better precision to accommodate the high copy variation in *AMY1*, while a final volume of 4 µl was used in assay for *AMY2A*. Real-time PCR data was analysed with QuantStudio™ 12K Flex Software version 1.1.2, results were then exported to CopyCaller v2.0 software (Thermo Fisher Scientific Inc., USA) for copy number calculation. All analysis settings selected were as recommended in the user guides. Samples with standard deviation of ΔCt >0.25, or with z-score >1.75 were removed and repeated. Diploid copy numbers were estimated by ΔΔCt method using the calibrator DNA sample NA10851 (Coriell Cell Repositories, USA) which carries 6 copies of the *AMY1* gene and 2 copies of *AMY2A*.

The sample NA10851 was selected as a reference sample for several reasons. It is a common reference sample used in many CNV studies carried out globally using microarrays, and it was the calibrator used in our previous qPCR validation study for CNV discovered in-house. It has been compared and calibrated with several reference samples using both qPCR and digital-PCR, and has consistently shown good concordance and reproducibility. Prior to its selection, a few reference samples had been studied as a potential calibrators because their copy numbers had been independently measured and reported in several published studies using methods including fiber-FISH and qPCR. These included NA18956 which was reported to carry 6 copies of *AMY1* and 2 copies of *AMY2A* [Falchi, et al., 2014]; NA18972 with 14 copies of *AMY1* [Perry, et al., 2007; Falchi, et al., 2014]; and NA10472 with 6 copies of *AMY1* [Perry, et al., 2007]. However, the 14 copies of *AMY1* for NA18972 was disputed by a recent study [Carpenter, et al., 2015] and also this study (Supp. Table S2, Supp. Figure S4). The samples NA18956 and NA10851 have consistently showed 6 copies for *AMY1* in our qPCR results. But NA18956 has always displayed slightly lower copy number ratio than

the NA10851, possibly due to mosaicism in this cell line sample. NA18956 was subsequently typed as CN5 by digital PCR. On the other hand, NA10472 had displayed 6 copies of *AMY1* in fiber-FISH [Perry, et al., 2007] and has been typed as CN6 by dPCR. Because NA10851 and NA10472 both consistently typed as CN6 in dPCR, we considered NA10851 calibrated against NA10472, and NA10851 was elected as the calibrator in this study.

The ΔΔCt method assumes equal amplification efficiency for the target and reference genes. PCR efficiency for the *AMY1* target assay and its reference (*RNase P*) assay was 92.76% and 97.44%, respectively, and for *AMY2A* target assay and its reference assay was 96.56% and 98.59%, respectively (Supp. Figs. 2a and 2b). The efficiency between target and reference loci differ by less than 5% is considered sufficiently similar [Fernandez-Jimenez, et al., 2011].

Copy number estimation of *AMY1* by Digital PCR

Digital PCR was performed on Quantstudio™ 3D Digital PCR System (Thermo Fisher Scientific) using Digital PCR 20K Chip which contains 20,000 reaction wells per chip. Thermal cycling was carried out in a Dual Flat Block GeneAmp® PCR System 9700 and analysis with Quantstudio™ 3D AnalysisSuite software. The human genomic DNA was first digested with an appropriate restriction enzyme to break up the tandemly repeated CNV copies. A final concentration of 2.5 ng/µl genomic DNA input into the dPCR chip is equivalent to approximately 750 copies/µl, which is within the recommended target sequence concentrations of between 200 to 2,000 copies/µl for accurate dPCR analysis (Thermo Fisher Scientific Inc., USA). As the *AMY1* copy number increases in some samples, the input DNA was reduced accordingly so to avoid reaction chamber saturation, and to get both target and reference molecules concentration fall around 200 to 2,000 copies/µl in order to achieve

precise dPCR analysis. Each sample was genotyped in duplicate at least, and the number of replicates were increased accordingly for samples with higher copy numbers or showed higher variation between chips.

Calibration Curve of qPCR with dPCR and Allele Binning

A calibration curve was constructed to calibrate qPCR data to those of dPCR. A total of 31 samples (3 reference samples from Corielle Repository and 28 in-house study samples) were genotyped in both qPCR and dPCR. These 28 in-house samples used to construct the calibration curve have qPCR results covering the full range from CN2 to 62, and with representative sample(s) from respective clusters. The number of dPCR replicates genotyped increased accordingly with the *AMY1* CN of the sample (Supp. Table S2). Instead of using calculated copy numbers, the raw qPCR signal intensity delta-delta Ct ($\Delta\Delta C_T$) was plotted against the logarithm of copy numbers obtained from dPCR. By taking ΔC_T of calibrator into consideration, this allowed the normalization of ΔC_T across inter-plate experimental runs.

Subsequently, these calibrated *AMY1* CN values were binned into copy number classes on the basis of pre-defined thresholds at 0.5, and binned to the nearest integer.

Results

Genotyping of *AMY2A* and *AMY1* copy number by qPCR and dPCR

The technical challenge of genotyping multiallelic CNV increased with the dynamic range of copy numbers. This is demonstrated by the CN genotyping of *AMY2A* and *AMY1* initially carried out by the qPCR method alone. The distribution of diploid CN in this combined population of 1077 individuals was 1 to 4 copies for *AMY2A*, and 2 to 24 copies for *AMY1*,

respectively. The histogram of raw *AMY2A* CN computed from delta C_T showed four CN genotypes clustered discretely (Fig. 1a, Supp. Fig. S3). CN variation at *AMY2A* is low with most individuals having 2 diploid copies, and about 6.5% of Chinese and 7.6% of Malays with *AMY2A* CN that were not 2.

The large range of *AMY1* CN presented a challenge to calling genotypes. Although the raw CN computed from qPCR delta Ct showed clustering, bins were unclear for CN>4, the frequency peaks were not integers, and apparent inflation at higher CN (with outliers of 35 and 62 far beyond what had been previously reported) were noted (Fig. 1b). These were similarly observed in the lower CN of *AMY2A* (Fig. 1a), suggesting a methodological rather than locus-specific artefact. In order to obtain a more accurate CN assignment of these clusters and to assess the reliability of the qPCR results, three reference samples and a set of 28 samples representative of the CN range of *AMY1* were genotyped with digital PCR (dPCR) and their results compared directly with the qPCR data (Supp. Table S2).

While there was a high correlation between CN deduced from dPCR and qPCR (Spearman r=0.988), the relationship was non-linear, with qPCR giving a higher CN estimate compared to dPCR (Fig. 1c). Assuming dPCR gives more accurate CN quantitation, the polynomial trend line from this dPCR calibration set of 31 samples was used to adjust and calibrate the qPCR CN calls for all samples in the study. The histogram of the calibrated qPCR CN for the 1077 samples showed a range of distribution from 2 to 24 diploid copies (Fig. 1d).

While this calibration did not discretise bins above CN4, the peaks fell at integer values, and allowed genotype calling using constant bin sizes and rounding qPCR calibrated CN to the nearest integer (Fig. 1d, 1e). While rounding off CN is routinely used in CNV association

studies [Gonzalez, et al., 2005; Aitman, et al., 2006; Fellermann, et al., 2006; Barnes, et al., 2008], in our work, we demonstrate downstream association analysis using 3 results - integer CN, calibrated CN without binning as well as the non-adjusted continuous qPCR signal intensity (delta-delta C_T).

AMY1 and _AMY2A_ copy number and haplotype frequency distribution in East Asian populations

Interestingly, it was noted that the major clusters of *AMY1* were of even number with diploid CN 4, 6, 8, 10, and 12, and odd CN clusters formed smaller peaks in between (Fig. 1d). The ratio of even CN to odd CN was 4.7, 4.5, 3.3, 4.0 and 4.5 for Chinese control, Chinese obese, Malay control, Malay obese and combined samples, respectively. Repeated dPCR genotyping on selected samples per cluster produced consistent results (Supp. Table S2, Supp. Fig. S4).

This ~~novel~~ observation of differential even:odd diploid CN (Fig. 2) was further investigated by analysis of haplotype structures at the *AMY1* locus. As both qPCR and dPCR provide only the diploid CN counts, frequencies of each haplotype were estimated computationally using the expectation-maximization algorithm (CoNVEM, http://apps.biocompute.org.uk/convem/) which assumes Hardy-Weinberg equilibrium [Gaunt, et al., 2010]. The distribution of haplotype frequencies for *AMY1* and *AMY2A* CNVs are similar between the Chinese and Malay populations (Figs. 3a & 3b). There are 2 common haplotypes for *AMY1*, CN3 and CN5, accounting for ~45% and ~32%, respectively (Fig. 3a) and odd haploid CN occur more frequently than even ones. In *AMY2A* one haplotype containing a single copy of the gene accounts for ~95% in each population (Fig. 3b).

Linkage dysequilibrium (LD) between *AMY2A* and *AMY1* was studied, by correlating diploid CN at these two adjacent loci. Individuals with the major *AMY2A* CN allele of 2, almost always have an even *AMY1* CN, while the non-2 *AMY2A* CN are associated with odd *AMY1* CN in both Chinese (Supp. Table S5) and Malay samples (data not shown). The same pattern of LD was also seen when CN were analysed as integer calls, where it reached statistical significance in all 3 groups, except the Malay control because of small sample n=30 (Supp. Table S3). Similar correlation was also reported by Usher et al. and Carpenter et al. [Carpenter, et al., 2015; Usher, et al., 2015].

Association testing of Obesity with *AMY1* and *AMY2A* copy number

~~In both populations, the modal copy number appeared lower in obese than in lean (Chinese: CN6 vs CN8, Malay CN8 vs CN10) (Fig. 4a).~~ In this study of *AMY1* CN-obesity association testing in two populations, we are unable to replicate the association, using multiple analyses and with sufficient statistical power to detect half the published effect size. The CN differences did not reach statistical significance in univariate analysis carried out for the four pair-wise comparisons; controls versus obese, and controls versus extreme obese, in Chinese and Malay populations separately. The mean difference in CN between each case-control set was small, as was the 95% confidence interval of mean difference (Table 2a). Both T-test and Mann-Whitney tests showed no significant association in all 4 groups of comparison (Table 2a). Logistic regression similarly indicated no significant association in all four case-control sets (Table 2a). *AMY1* copy numbers ≥13 (all CN classes with frequency <3%) were collapsed together into one single category but no significance difference could be detected either. Linear regression was done to test if *AMY1* copy number predicts BMI changes, and results were also negative (results not shown). Results remained not significant when analysis was done using calibrated copy number without rounding to integer. Statistical analysis also

was carried out on direct raw qPCR signal intensity ($\Delta\Delta C_T$) as a continuous measurement, but no association could be detected (Mann-Whitney test, $p>0.05$) (Figs. 4b & 4c). The average BMI at various *AMY1* copy numbers did not show any significant trend, neither in the obese nor the controls of both Chinese and Malays (Suppl. Figs. S5a & S5b).

We calculated if this study had enough statistical power to detect association as in Falchi et al. (2014). It was estimated that the CN mean difference between case-control samples in one of their study cohorts (DESIR cohort, Supplementary table 9 in Falchi et al. 2014) was 0.95 with standard deviation of ~2.0. With these parameters, a sample size of 95 per arm would have 90% power. Assuming a smaller mean difference at 0.5 and standard deviation at 2.0, a sample size of 338 per arm would have 90% power. Hence the Chinese sample set in this study should have 90% power to detect even half the *AMY1* genetic effect size seen in the previous study.

CNV-association testing was similarly carried out for *AMY2A*. The distribution of *AMY2A* copy number in controls and obese samples in Chinese and Malay cohorts were summarized in Fig. 4d. No association between *AMY2A* copy number and obesity was detected by T-test or logistic regression (Table 2b). Mann-Whitney test using $\Delta\Delta C_T$ as a continuous measurement also showed no association in all 4 case-control sample sets (Figs. 4e & 4f).

Discussion

The technical challenge in genotyping multiallelic CNV is exemplified by the *AMY1* and *AMY2A* locus. While genotyping low copy number CNV such as *AMY2A* (diploid CN 1-4) is straightforward, genotyping high copy number CNV like *AMY1* (diploid CN 2-24) is

challenging, as the relative difference between alleles becomes smaller as CN increases and higher measurement precision is needed to quantify high CN. In our *AMY1* genotyping data, CN classes were discrete till CN4 and C_T-based CN overlapped above CN5 (Figs. 1d & 1e). To increase precision at higher copy number, one approach is to increase the number of technical replicates for samples that harbour high copy numbers. However, this does not overcome the non-linear relationship of C_T with CN.

The large number of replicates and linear arithmetic nature of dPCR quantitation provides a solution to these issues. Digital PCR achieves absolute quantification by counting end-point results of single-molecule amplification across a large number of PCR replicates, and its merits for CNV quantitation have been considered elsewhere [Baker M, 2012; [Weaver, et al., 2010; Whale, et al., 2012]. Two main factors influence the reliability of dPCR measurements: the number of replicates analysed and the number of template molecules in the assay [Weaver, et al., 2010; Pinheiro, et al., 2012; Whale, et al., 2012]. If the reference template (2 copies per genome) is loaded at an optimal 0.6 molecules per reaction well (λr=0.6), the number of reaction wells required to achieve 1.1 fold discrimination (ie CN10 vs 11) is approximately 5500 to 8000 [Weaver, et al., 2010; Whale, et al., 2012]. This number is easily achieved within the 20,000 microwell array dPCR platform used in this study. Its limitation is cost and sample throughput, with a maximum 24 samples per run. Hence we used dPCR to validate and calibrate a subset of samples representing the CN clusters identified by qPCR. While the correlation between qPCR and dPCR was high, the non-linear calibration curve allowed the adjustment of high CN qPCR results and assignment of bins (Fig. 1c).

This study has shown that despite the higher variability of qPCR measurement at high copy numbers, it still could retain the relative pattern of the overall results, correctly identifying the even:odd frequency distribution pattern in *AMY1*. In addition, qPCR offers an attractive option for large-scale CNV genotyping owing to its high throughput capability, widespread availability of instrumentation and possibly lower costs. In this study, care has been taken to minimize systematic bias and batch effects. All samples have been subjected to identical laboratory processes, sample randomization was done during genotyping, and four technical replicates were included per sample in qPCR. Samples with high variability between the 4 replicates were removed and re-genotyped. Also, appropriate reference samples with known and validated copy-numbers were used as internal controls. The 2 controls used in Falchi et al. (2014), NA18596 and NA18972, were replaced in this study. NA18596 was found to contain mosaicism and typed by dPCR to be *AMY1* CN5, while NA18972 has been found to contain CN16 by our dPCR assay rather than the reported CN14 or CN18 in other studies (Supp. Table S2, Supp. Fig. S4.) [Perry, et al., 2007; Falchi, et al., 2014; Carpenter, et al., 2015]. Instead, we used another known reference, NA10472 with *AMY1* CN6 [Perry, et al., 2007], to calibrate a common CNV microarray standard NA10851 and both consistently showed *AMY1* CN6 in dPCR (Supp. Fig. S4, Supp. Table S2). NA10851 containing *AMY1* CN 6 was corroborated with Usher et al. (Supplementary table 3 of Usher et al. 2015).

In our study of South East Asian populations comprising ethnic Chinese and Malay we found a ~~novel~~ pattern of differential even:odd *AMY1* frequency distribution (Fig. 2), which could be explained by the underlying haplotype structure. However the evidence of specific *AMY* haplotype can be traced to studies conducted a quarter century ago [[Groot, et al., 1989; Groot, et al., 1990]. Using segregation analysis of Southern blot hybridization patterns in pedigrees, Groot defined three common *AMY* haplotypes [Groot, et al., 1990; Groot, et al.,

1991], and proposed that a general designation *2B-2A-(1A-1B-P1)n-1C* could describe the majority of the *AMY* haplotypes (Fig. 3c). The repeat sequence of about 100kb in size and encompassing the genes *AMY1A-AMY1B-AMYP1* are flanked by segmental duplications (designated as SD1 in Sppl. Fig. S1a), could mediate chromosomal rearrangement via Non-allelic Homologous Recombination (NAHR). This haplotype structure corroborates a recent analysis of sequencing read depths which reported that the salivary genes *AMY1A*, *AMY1B* and the pseudogene *AMYP*1 were much more variable in copy number than the pancreatic genes *AMY2A* and *AMY2B*, while *AMY1C* appeared to be largely invariant [Sudmant, et al., 2010].

Two recent studies independently expanded the range of *AMY* haplotypes and reported similar even:odd distribution pattern for *AMY1* in three HapMap population groups, Chinese cum Japanese, European and Yoruba [Carpenter, et al., 2015; Usher, et al., 2015]. Carpenter et al. used a combination of four methods including Paralogous Ratio Test (PRT), Microsatellite analysis, sequence read depth and fiber-FISH in CNV genotyping. On the other hand, Usher et al adopted whole-genome-sequencing read depth analysis, droplet digital PCR (ddPCR) and genome mapping. The frequency distribution of *AMY1* diploid copy number reported for HapMap CEU closely parallel each other [Carpenter, et al., 2015; Usher, et al., 2015]. While the frequency distribution reported for HapMap CHB+JPT [Usher, et al., 2015] closely resembled the results of this study, with similar modes at CN6 & CN8 (Fig. 2). The similar even:odd pattern was found in all populations in these 3 studies, and the even:odd differential was more pronounced in East Asian populations (Chinese & Malays in this study, and HapMap CHB+JPT) compared to CEU.

Furthermore, the common *AMY* haplotypes deciphered from both studies corroborated with Groot et al. [Groot, et al., 1989; Groot, et al., 1990; Carpenter, et al., 2015; Usher, et al., 2015]. The common haplotypes; AMY*H0, AMY*H1, and AMY*H2 defined by Groot et al. (Fig. 3c), or designated as AH1, AH3, and AH5 in Usher et al. (2015), should be equivalent to *AMY1* haploid copy number 1, 3 and 5 in this study (Fig. 3a). The haplotype frequencies determined experimentally by Usher et al. for the 4 most common *AMY* haplotypes (with *AMY1* haploid CNs 1, 3, 5 & 7) in Europeans closely resembled those determined for Singapore populations by CoNVEM, with AMY*H1 (*AMY1* haploid CN3) being the most common with frequency >40%, and AMY*H2 (*AMY1* haploid CN5) as second with frequency >30% (Figs. 3a & 3c). Importantly, the four most common *AMY1* haplotypes, all with odd copy number of *AMY1* (1, 3, 5 and 7 copies), constituted a total of 90% of all *AMY* haplotypes in European population [Usher, et al., 2015]. Similarly, the 4 odd copy number *AMY1* haplotypes constituted a total of >88% in Chinese or Malays as determined by CoNVEM (Fig. 3a). Therefore, this would explain why majority of the samples have an even *AMY1* diploid copy number in both this study and the two recent European studies [Carpenter, et al., 2015; Usher, et al., 2015].

The fact that this study has independently discovered the unique characteristics of differential frequency distribution between even and odd diploid copy numbers of *AMY1*, and correlation between *AMY2A* and *AMY1* CN genotypes, has lent support to the reliability of this data set. The unusual even:odd distribution of *AMY1* copy number is a ~~novel~~ finding which is markedly different from many previous publications [Perry, et al., 2007; Santos, et al., 2012; Falchi, et al., 2014]. Previous studies of various sample sizes ranging from hundreds to thousands (~6,200 samples in Falchi et al. 2014) showed histograms with an approximately normal distribution. This finding has now been corroborated by two recent studies which

have shown that this non-normal frequency distribution could be explained by the underlying structural haplotypes [Carpenter, et al., 2015; Usher, et al., 2015]. Furthermore, the *AMY1* diploid copy number distribution in Chinese and Malays in this study (Fig. 2) is similar to that of the North Asian CHB+JPT populations [Usher, et al., 2015]. In addition, the calculated *AMY1* haploid allele frequency in Chinese and Malays (~0.4 for CN3 and ~0.3 for CN5) (Fig. 3a) were also similar to those determined in Europeans (Fig. 3c). It is re-assuring to see that different genotyping approaches and population samples are converging on a similar understanding of variation at this locus of *AMY1*.

The initial impetus of this study was to investigate if the reported association of *AMY1* copy number and obesity [Falchi, et al., 2014] could be replicated in an independent Singapore Chinese cohort. The reported association has been tested on 6,200 subjects, comprising four European cohorts and one Singapore Chinese cohort. The obesity risk (Odds Ratio or OR) per copy reduction of *AMY1* was reported to be 1.19 in European (95% confidence interval (CI) = 1.13 - 1.26), and 1.17 in Singapore Chinese cohort (95% CI = 1.05 – 1.29). On the other hand, *AMY2A* copy number was reported to have no association with BMI or fat mass [Falchi, et al., 2014]. However, various statistical analyses in this study failed to replicate any association between *AMY1* copy number and obesity in both Chinese and Malay sample sets. Like Falchi et al. (2014), no association could be detected between *AMY2A* copy number and obesity in the same populations.

The *AMY1* results are discordant between these two studies, with Falchi reporting near normal CN distribution in all three of its cohorts. In our study, significant effort was placed in minimizing systematic bias and batch effects. Both case and control samples were collected

at a single centre by the same research team, samples were randomized into batches for processing, and a novel calibration method for qPCR genotyping.

The same research group also reported an inverse association between *AMY1* copy number with obesity risk in Mexican children, with similar Odd Ratio at 1.19 but a different allelic architecture from the original study for European adults [Mejia-Benitez, et al., 2015]. In the study of Mejia-Benitez et al. [2015], *AMY1* genotyping was carried out in dPCR using Fluidigm chip with each sample typed in 4x 770 reaction chambers but no pre-dPCR enzyme digestion was indicated. As the *AMY1* gene tandem repeat is about 27 kb, adjacent repeats could co-segregate in high molecular weight preparations of genomic DNA, and cause under-estimation of CN by dPCR. In our hands, dPCR quantification without enzyme digestion resulted in a lower CN call, particularly for samples with high copy numbers (Supp. Fig. S6). These technical differences in methodology could result in differences in results.

~~The *AMY2A* result in this study corroborated with Falchi et al. (2014), who suggested that the positive association result for *AMY1* identified in Falchi et al. could not be due to linkage with the neighbouring *AMY2A* gene.~~ We found that *AMY2A* copy number is not associated to obesity, consistent with earlier findings in European cohort [Falchi et al. 2014]. Taking all analyses into consideration, this study concludes that *AMY1* and *AMY2A* diploid copy numbers are not associated with obesity in the Chinese sample (while the Malay sample was too small to be statistically powered). A similar conclusion was reached by Usher et al., whose study involved 3 European cohorts, totalling >3,500 subjects and with 99% power to detect the reported effect size in Falchi et al (2014), but found no association of *AMY1* copy number with obesity or BMI [Usher, et al., 2015].

Acknowledgements

We thank Mahesh Uttamchandani for his comments on this manuscript, all volunteers for their participation and provision of samples, and the Singapore Ministry of Defence for funding support. We also thank the anonymous reviewers for the insightful comments and constructive suggestions. We declare no conflict of interest.

References

Aitman TJ, Dong R, Vyse TJ, Norsworthy PJ, Johnson MD, Smith J, Mangion J, Roberton-Lowe C, Marshall AJ, Petretto E and others. 2006. Copy number polymorphism in Fcgr3 predisposes to glomerulonephritis in rats and humans. Nature 439:851-5.

Baker M. 2012. Structural variation: the genome's hidden architecture. Nature methods 9:133-7.

Barnes C, Plagnol V, Fitzgerald T, Redon R, Marchini J, Clayton D, Hurles ME. 2008. A robust statistical method for case-control association testing with copy number variation. Nature genetics 40:1245-52.

Cantsilieris S, Western PS, Baird PN, White SJ. 2014. Technical considerations for genotyping multi-allelic copy number variation (CNV), in regions of segmental duplication. BMC genomics 15:329.

Cantsilieris S, White SJ. 2013. Correlating multiallelic copy number polymorphisms with disease susceptibility. Human mutation 34:1-13.

Carpenter D, Dhar S, Mitchell LM, Fu B, Tyson J, Shwan NA, Yang F, Thomas MG, Armour JA. 2015. Obesity, starch digestion and amylase: association between copy number variants at human salivary (AMY1) and pancreatic (AMY2) amylase genes. Human molecular genetics 24:3472-80.

Falchi M, El-Sayed Moustafa JS, Takousis P, Pesce F, Bonnefond A, Andersson-Assarsson JC, Sudmant PH, Dorajoo R, Al-Shafai MN, Bottolo L and others. 2014. Low copy number of the salivary amylase gene predisposes to obesity. Nature genetics 46:492-7.

Fellermann K, Stange DE, Schaeffeler E, Schmalzl H, Wehkamp J, Bevins CL, Reinisch W, Teml A, Schwab M, Lichter P and others. 2006. A chromosome 8 gene-cluster polymorphism with low human beta-defensin 2 gene copy number predisposes to Crohn disease of the colon. American journal of human genetics 79:439-48.

Fernandez-Jimenez N, Castellanos-Rubio A, Plaza-Izurieta L, Gutierrez G, Irastorza I, Castano L, Vitoria JC, Bilbao JR. 2011. Accuracy in copy number calling by qPCR and PRT: a matter of DNA. PloS one 6:e28910.

Gaunt TR, Rodriguez S, Guthrie PA, Day IN. 2010. An expectation-maximization program for determining allelic spectrum from CNV data (CoNVEM): insights into population allelic architecture and its mutational history. Human mutation 31:414-20.

Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, Nibbs RJ, Freedman BI, Quinones MP, Bamshad MJ and others. 2005. The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science 307:1434-40.

Groot PC, Bleeker MJ, Pronk JC, Arwert F, Mager WH, Planta RJ, Eriksson AW, Frants RR. 1989. The human alpha-amylase multigene family consists of haplotypes with variable numbers of genes. Genomics 5:29-42.

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

Groot PC, Mager WH, Henriquez NV, Pronk JC, Arwert F, Planta RJ, Eriksson AW, Frants RR. 1990. Evolution of the human alpha-amylase multigene family through unequal, homologous, and inter- and intrachromosomal crossovers. Genomics 8:97-105.

Handsaker RE, Van Doren V, Berman JR, Genovese G, Kashin S, Boettger LM, McCarroll SA. 2015. Large multiallelic copy number variations in humans. Nature genetics 47:296-303.

Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M. 2013. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nature methods 10:1003-5.

Iafrate AJ, Feuk L, Rivera MN, Listewnik ML, Donahoe PK, Qi Y, Scherer SW, Lee C. 2004. Detection of large-scale variation in the human genome. Nature genetics 36:949-51.

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

Meisler MH, Ting CN. 1993. The remarkable evolutionary history of the human amylase genes. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists 4:503-9.

Mejia-Benitez MA, Bonnefond A, Yengo L, Huyvaert M, Dechaume A, Peralta-Romero J, Klunder-Klunder M, Garcia Mena J, El-Sayed Moustafa JS, Falchi M and others. 2015. Beneficial effect of a high number of copies of salivary amylase AMY1 gene on obesity risk in Mexican children. Diabetologia 58:290-4.

Perry GH, Dominy NJ, Claw KG, Lee AS, Fiegler H, Redon R, Werner J, Villanea FA, Mountain JL, Misra R and others. 2007. Diet and the evolution of human amylase gene copy number variation. Nature genetics 39:1256-60.

Pinheiro LB, Coleman VA, Hindson CM, Herrmann J, Hindson BJ, Bhat S, Emslie KR. 2012. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. Analytical chemistry 84:1003-11.

Santos JL, Saus E, Smalley SV, Cataldo LR, Alberti G, Parada J, Gratacos M, Estivill X. 2012. Copy number polymorphism of the salivary amylase gene: implications in human nutrition research. Journal of nutrigenetics and nutrigenomics 5:117-31.

Sudmant PH, Kitzman JO, Antonacci F, Alkan C, Malig M, Tsalenko A, Sampas N, Bruhn L, Shendure J, Eichler EE. 2010. Diversity of human copy number variation and multicopy genes. Science 330:641-6.

Usher CL, Handsaker RE, Esko T, Tuke MA, Weedon MN, Hastie AR, Cao H, Moon JE, Kashin S, Fuchsberger C and others. 2015. Structural forms of the human amylase locus and their relationships to SNPs, haplotypes and obesity. Nature genetics

Weaver S, Dube S, Mir A, Qin J, Sun G, Ramakrishnan R, Jones RC, Livak KJ. 2010. Taking qPCR to a higher level: Analysis of CNV reveals the power of high throughput qPCR to enhance quantitative resolution. Methods 50:271-6.

Whale AS, Huggett JF, Cowen S, Speirs V, Shaw J, Ellison S, Foy CA, Scott DJ. 2012. Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic acids research 40:e82.

Figure Legends

Figure 1. Distribution of the diploid copy numbers for *AMY2A* and *AMY1* CNVs. Calibration and Binning of *AMY1* CNV. Total sample size 1077 comprising controls and obese from both Chinese and Malays populations.

A: Histogram of *AMY2A* diploid copy number, direct from qPCR not rounded to integer. Discrete delineation of *AMY2A* CN into 4 copy classes is visible.

B: Pre-calibrated *AMY1* diploid copy number, direct from qPCR not rounded to integer.

C: Calibration curve correlating qPCR $\Delta\Delta C_T$ to dPCR Log_2(CN) for *AMY1* CNV.

D: Post Calibration *AMY1* CN. Distribution of the calibrated diploid copy numbers for *AMY1* in all 1077 samples. Histogram shows main clusters at even diploid copy numbers and small clusters at odd copy numbers. *AMY1* diploid copy number ranges from CN2 to 24.

E: *AMY1* calibrated CN sorted in order and binned with pre-defined threshold to nearest integer. Two samples with CN19 and CN24 not shown.

Figure 2. *AMY1* calibrated diploid copy number distribution in 519 Chinese controls and 115 Malay obese. Distribution patterns appeared similar in the 413 Chinese obese and combining all 1077 samples across 2 populations. All displayed the unique even:odd pattern. *Frequency distribution for HapMap samples combining Chinese from Beijing and Japanese from Tokyo (Total n=197). Obtained from Supplementary table T2, Usher et al. 2015. §Frequency distribution for HapMap samples combining European and Asians (Total n=209). Obtained from Supplementary table file 1, Carpenter et al. 2015.

Figure 3. Haploid copy number allele frequencies in the 2 Singapore populations. Allele frequencies were estimated through the EM algorithm implemented in CoNVEM using 519 Chinese control and 115 Malay obese samples. **A:** *AMY1* CNV, **B:** *AMY2A* CNV.

C. Structure of the human amylase haplotypes; AMY*H1, AMY*H1, and AMY*H2 defined by Groot et al.. (Modified from Groot et al. 1989). AMY*H1, AMY*H1, and AMY*H2 are equivalent to AH1, AH3, and AH5, as designated in Usher et al. 2015.

Abbreviation; 2B, *AMY2B*; 2A, *AMY2A*; 1A, *AMY1A*; 1B, *AMY1B*; 1C, *AMY1C*; P1, *AMYP1* pseudogene. The arrow indicates a 100kb homologous sequence which is flanked by segmental duplication listed as SD1 in Supplementary Fig. S1A. (Modified from Groot et al. 1990). [a]Haplotype frequency in HapMap European samples in Usher et al. 2015. [b]*AMY1* Haploid CN estimated by CoNEVM in this study as in Fig. 3A.

D. Association of *AMY2A-AMY1* diploid copy number (CN) in the Chinese sample set. Even diploid copy numbers of *AMY1* tends to associated with *AMY2A* with 2 diploid CN, while *AMY1* odd copy numbers associated with *AMY2A* with CN non-2. Copy numbers of *AMY1* were calibrated by the qPCR-dPCR calibration curve and without rounding to integer. Grey - 519 Chinese controls. Black - 413 Chinese obese samples.

Figure 4. Association testing of AMY1 and AMY2A diploid CN with obesity.

A: *AMY1* diploid CN frequency distribution in two Singapore populations. 519 Chinese controls versus 413 obese, and 30 Malay controls versus 115 obese. *AMY1* CN is calibrated CN rounded to integer.

B & C: Mann-Whitney test of ΔΔCT in controls, obese, and extreme obese samples. Low ΔΔCT values correspond to high *AMY1* copy numbers. B: Chinese samples. C: Malay samples.

D: *AMY2A* diploid CN frequency distribution in two Singapore populations. 519 Chinese controls versus 413 obese, and 30 Malay controls versus 115 obese.

E & F: Mann-Whitney test of ΔΔCT in controls, obese, and extreme obese samples. Low ΔΔCT values correspond to high *AMY2A* copy numbers. E: Chinese samples, and F: Malay samples.

Table Legends

Table 1. Summary information of the two study cohorts.

Table 2. Association testing of Amylase genes diploid copy numbers with obesity. Results of T-test and logistic regression are shown. P-values from Mann-Whitney tests are similarly not significant and results not shown. **A:** *AMY1* CN and obesity. Results were calculated using *AMY1* copy numbers calibrated from qPCR-dPCR calibration curve and rounding to integer. **B:** *AMY2A* CN and obesity. Discrete *AMY2A* copy numbers deduced directly from qPCR.

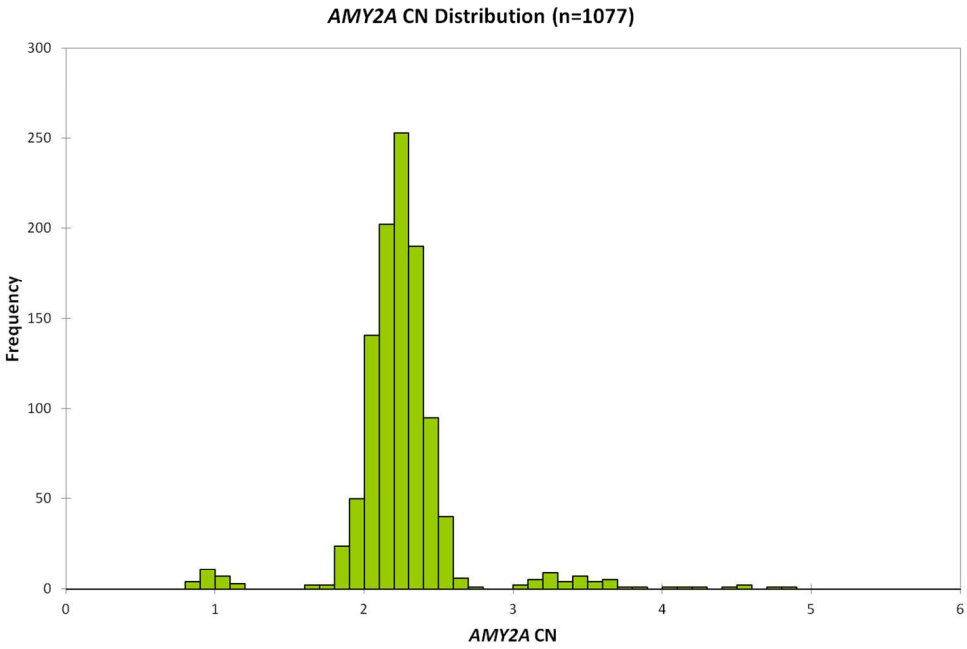


Figure 1. Distribution of the diploid copy numbers for *AMY2A* and *AMY1* CNVs. Calibration and Binning of *AMY1* CNV. Total sample size 1077 comprising controls and obese from both Chinese and Malays populations.
A: Histogram of *AMY2A* diploid copy number, direct from qPCR not rounded to integer. Discrete delineation of *AMY2A* CN into 4 copy classes is visible.

249x168mm (300 x 300 DPI)

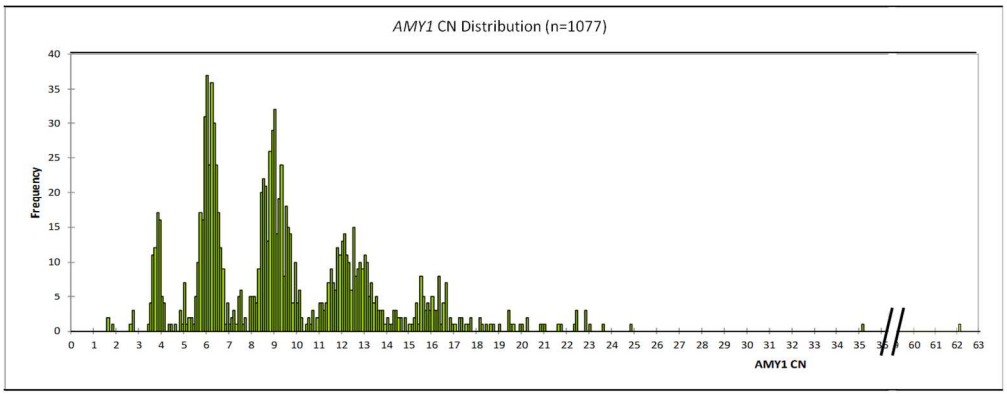


Fig B: Pre-calibrated *AMY1* diploid copy number, direct from qPCR not rounded to integer.
252x97mm (300 x 300 DPI)

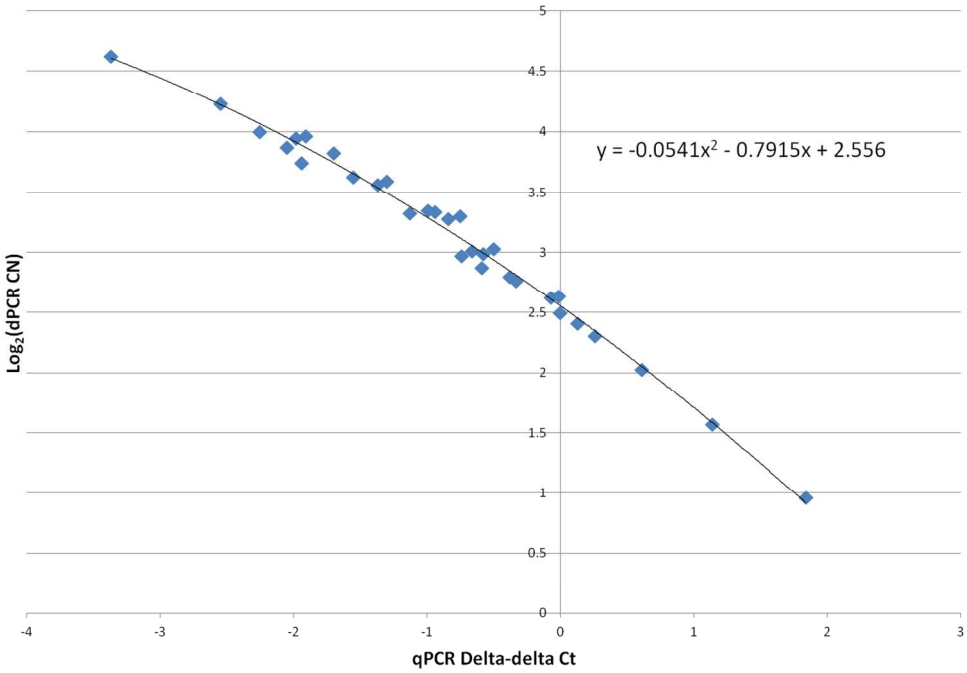


Fig 1C: Calibration curve correlating qPCR ΔΔCT to dPCR Log2(CN) for *AMY1* CNV.
242x168mm (300 x 300 DPI)

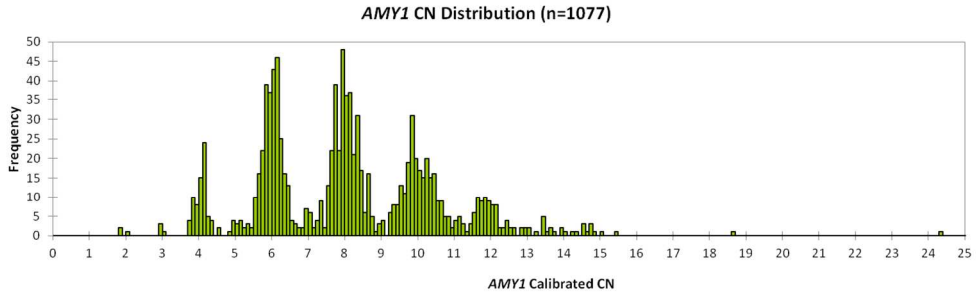


Fig 1D: Post Calibration *AMY1* CN. Distribution of the calibrated diploid copy numbers for *AMY1* in all 1077 samples. Histogram shows main clusters at even diploid copy numbers and small clusters at odd copy numbers. *AMY1* diploid copy number ranges from CN2 to 24.
257x80mm (300 x 300 DPI)

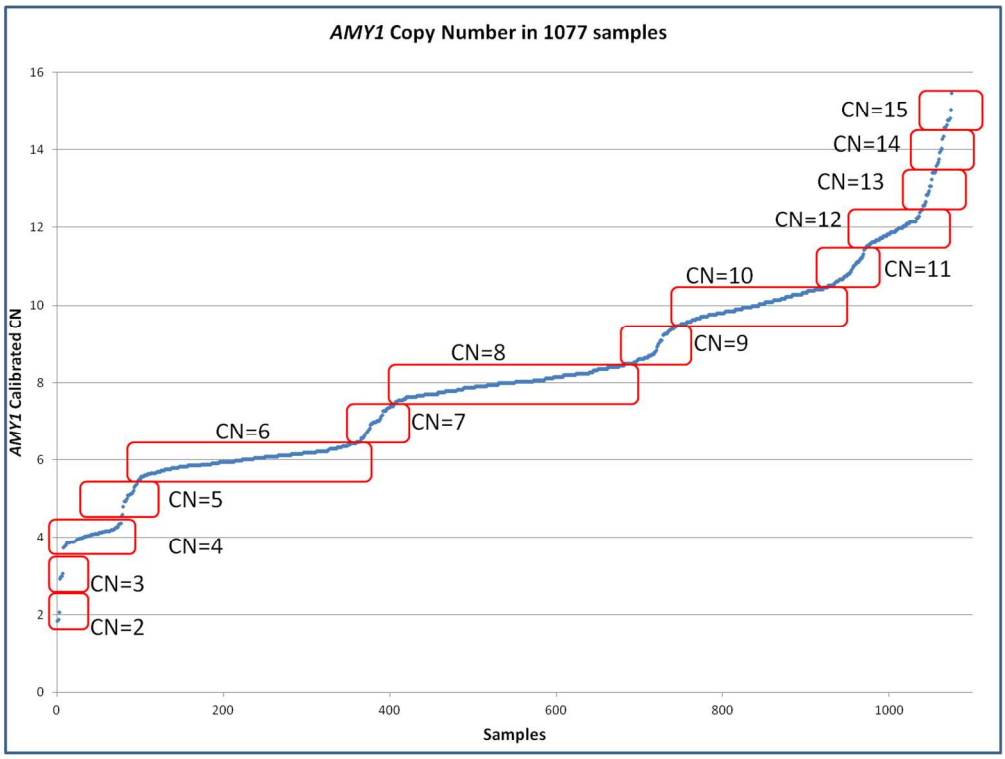
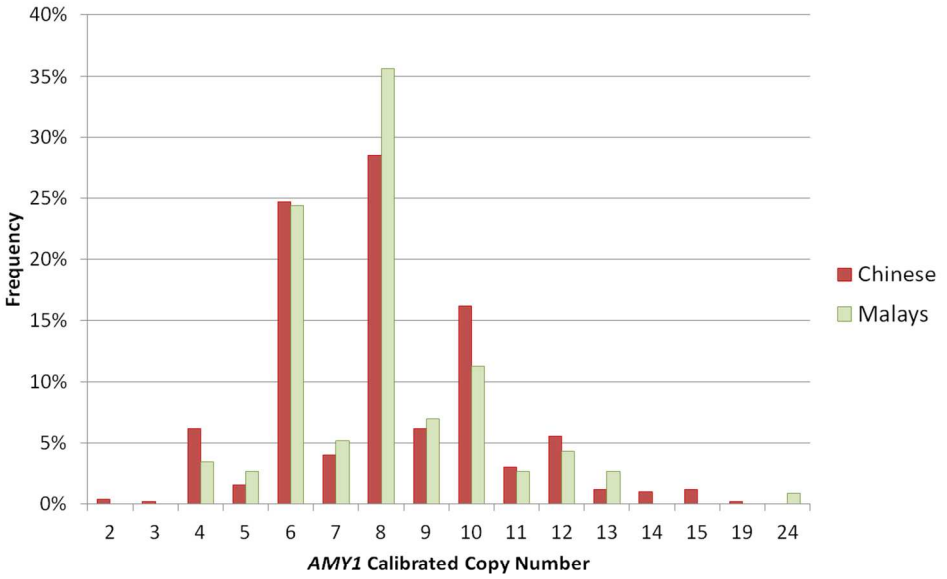


Fig 1E: *AMY1* calibrated CN sorted in order and binned with pre-defined threshold to nearest integer. Two samples with CN19 and CN24 not shown.
255x191mm (300 x 300 DPI)



Populations	*AMY1* Diploid Copy Number (%)															
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	18	19
Singapore Chinese	0.4	0.2	6.2	1.5	24.7	4.0	28.5	6.2	16.2	3.1	5.6	1.2	1.0	1.2	0	0.2
HapMap CHB+JPT*	0	0	5.1	1.0	22.3	4.1	28.4	6.1	18.3	4.1	7.1	1.0	1.0	0.5	0	0
HapMap CEU+CHB+JPT§	1.4	2.4	10.0	9.6	21.5	8.6	18.7	6.2	11.0	3.3	3.8	1.4	1.4	0	0.5	0

Figure 2. *AMY1* calibrated diploid copy number distribution in 519 Chinese controls and 115 Malay obese. Distribution patterns appeared similar in the 413 Chinese obese and combining all 1077 samples across 2 populations. All displayed the unique even:odd pattern. *Frequency distribution for HapMap samples combining Chinese from Beijing and Japanese from Tokyo (Total n=197). Obtained from Supplementary table T2, Usher et al. 2015. §Frequency distribution for HapMap samples combining European and Asians (Total n=209). Obtained from Supplementary table file 1, Carpenter et al. 2015.
226x172mm (300 x 300 DPI)

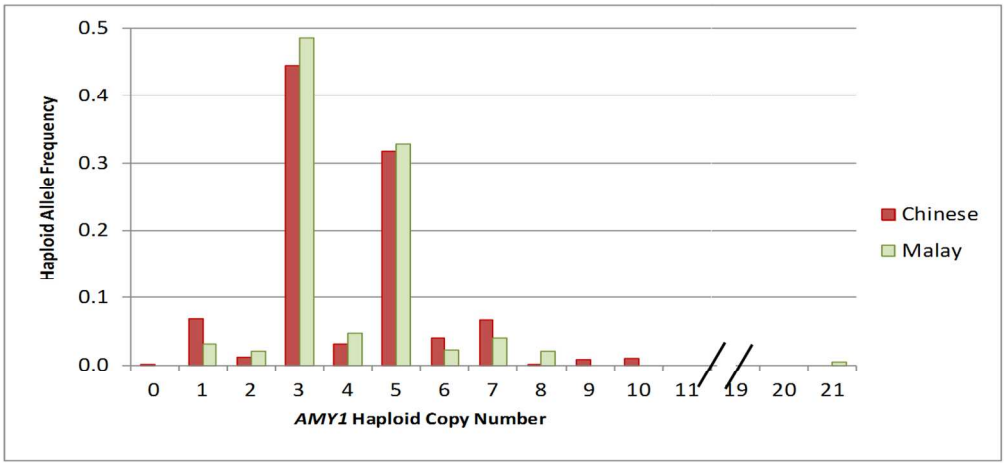


Figure 3. Haploid copy number allele frequencies in the 2 Singapore populations. Allele frequencies were estimated through the EM algorithm implemented in CoNVEM using 519 Chinese control and 115 Malay obese samples.
A: *AMY1* CNV,

249x114mm (300 x 300 DPI)

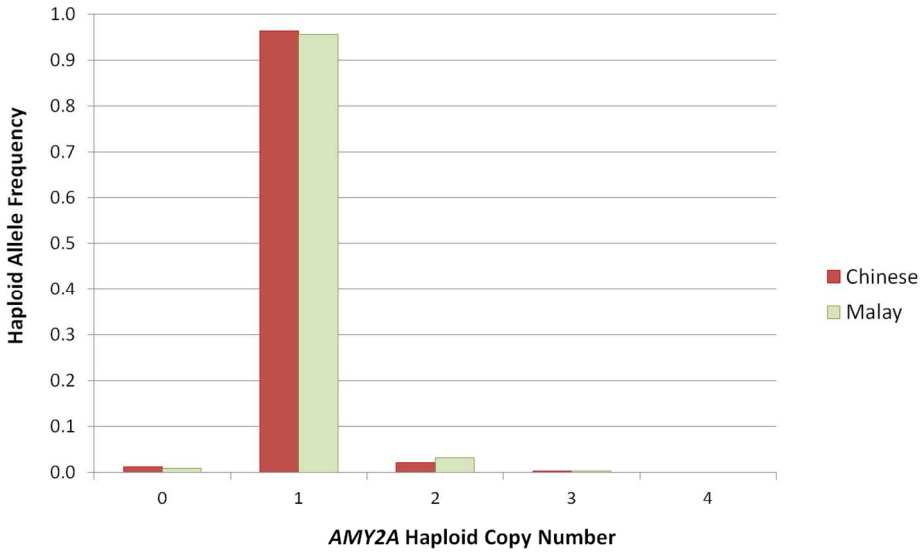


Figure 3. Haploid copy number allele frequencies in the 2 Singapore populations. Allele frequencies were estimated through the EM algorithm implemented in CoNVEM using 519 Chinese control and 115 Malay obese samples.
B: *AMY2A* CNV.

245x152mm (300 x 300 DPI)

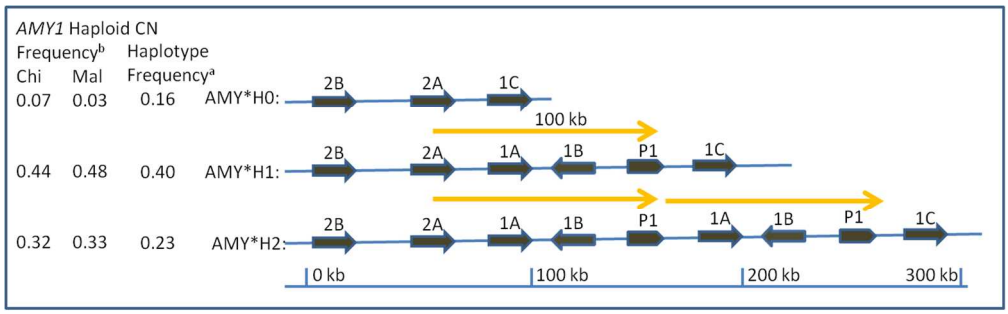


Fig 3C. Structure of the human amylase haplotypes; AMY*H1, AMY*H1, and AMY*H2 defined by Groot et al.. (Modified from Groot et al. 1989). AMY*H1, AMY*H1, and AMY*H2 are equivalent to AH1, AH3, and AH5, as designated in Usher et al. 2015.
Abbreviation; 2B, *AMY2B*; 2A, *AMY2A*; 1A, *AMY1A*; 1B, *AMY1B*; 1C, *AMY1C*; P1, *AMYP1* pseudogene. The arrow indicates a 100kb homologous sequence which is flanked by segmental duplication listed as SD1 in Supplementary Fig. S1A. (Modified from Groot et al. 1990). [a]Haplotype frequency in HapMap European samples in Usher et al. 2015. [b]*AMY1* Haploid CN estimated by CoNEVM in this study as in Fig. 3A.

250x76mm (300 x 300 DPI)

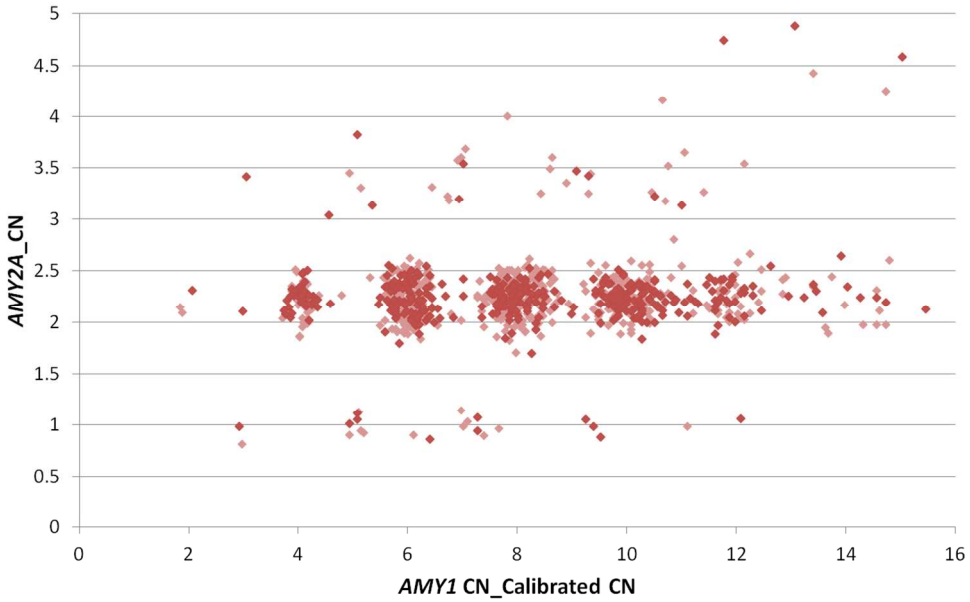


Fig 3D. Association of *AMY2A-AMY1* diploid copy number (CN) in the Chinese sample set. Even diploid copy numbers of *AMY1* tends to associated with *AMY2A* with 2 diploid CN, while *AMY1* odd copy numbers associated with *AMY2A* with CN non-2. Copy numbers of *AMY1* were calibrated by the qPCR-dPCR calibration curve and without rounding to integer. Grey - 519 Chinese controls. Black - 413 Chinese obese samples. 238x149mm (300 x 300 DPI)

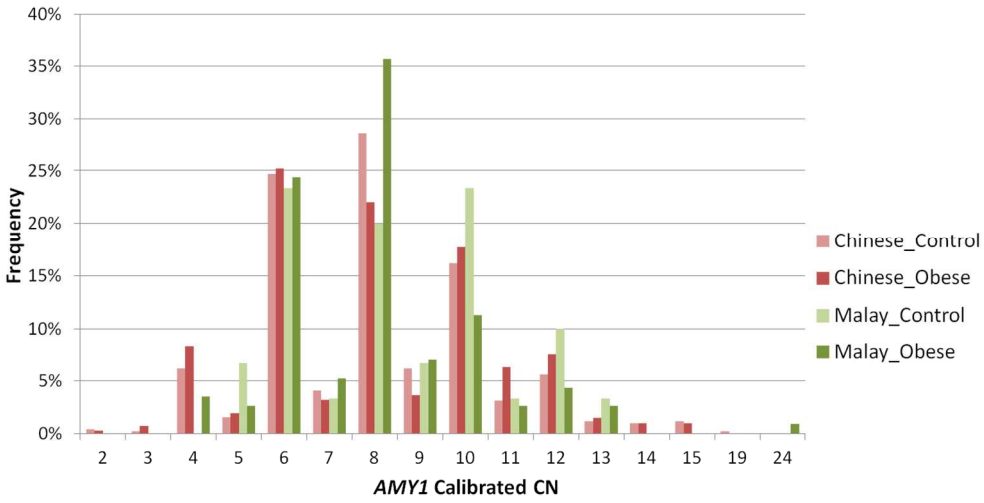


Figure 4. Association testing of *AMY1* and *AMY2A* diploid CN with obesity.
A: *AMY1* diploid CN frequency distribution in two Singapore populations. 519 Chinese controls versus 413 obese, and 30 Malay controls versus 115 obese. *AMY1* CN is calibrated CN rounded to integer.

254x159mm (300 x 300 DPI)

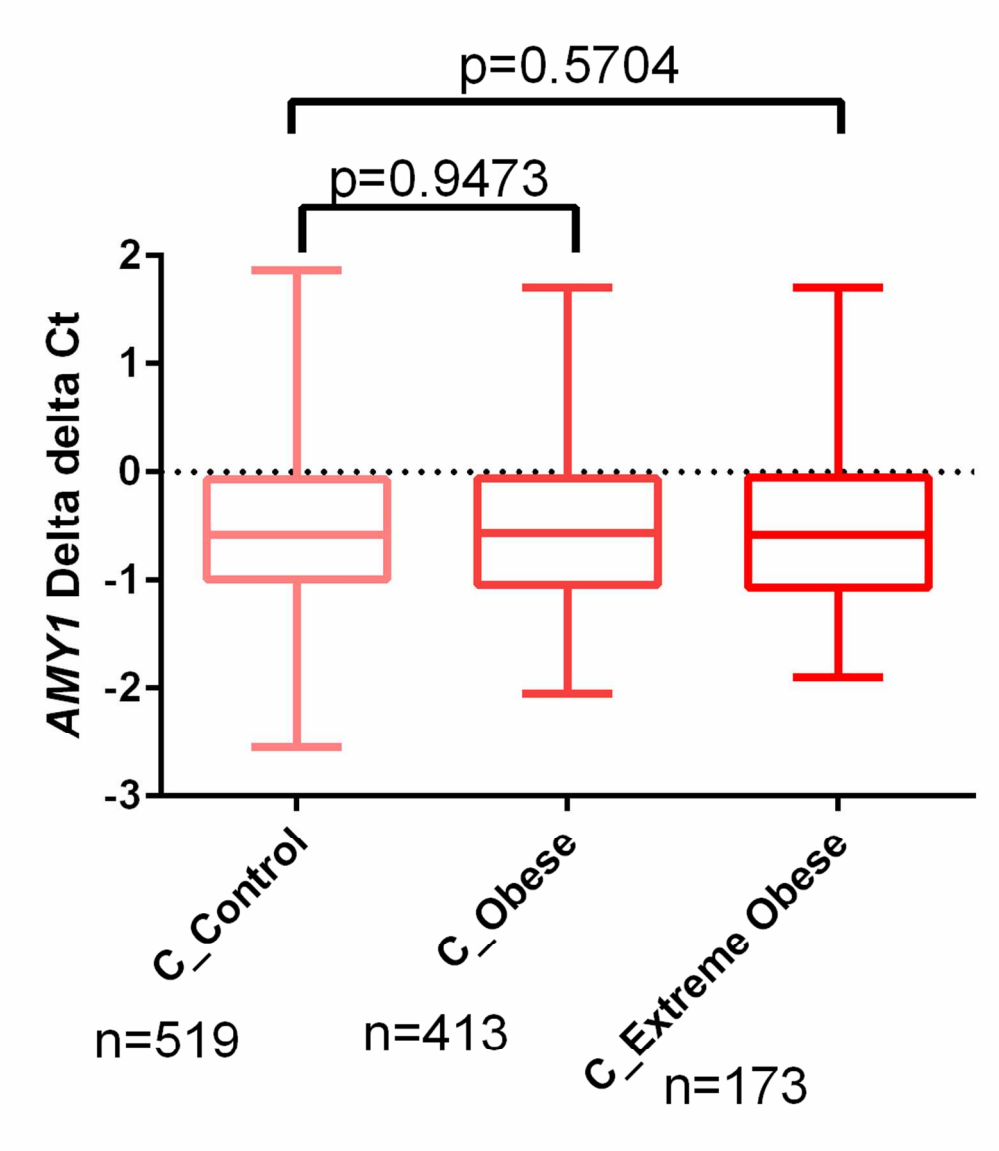


Fig 4B: Mann-Whitney test of ΔΔCT in controls, obese, and extreme obese samples. Low ΔΔCT values correspond to high *AMY1* copy numbers. B: Chinese samples
93x108mm (300 x 300 DPI)

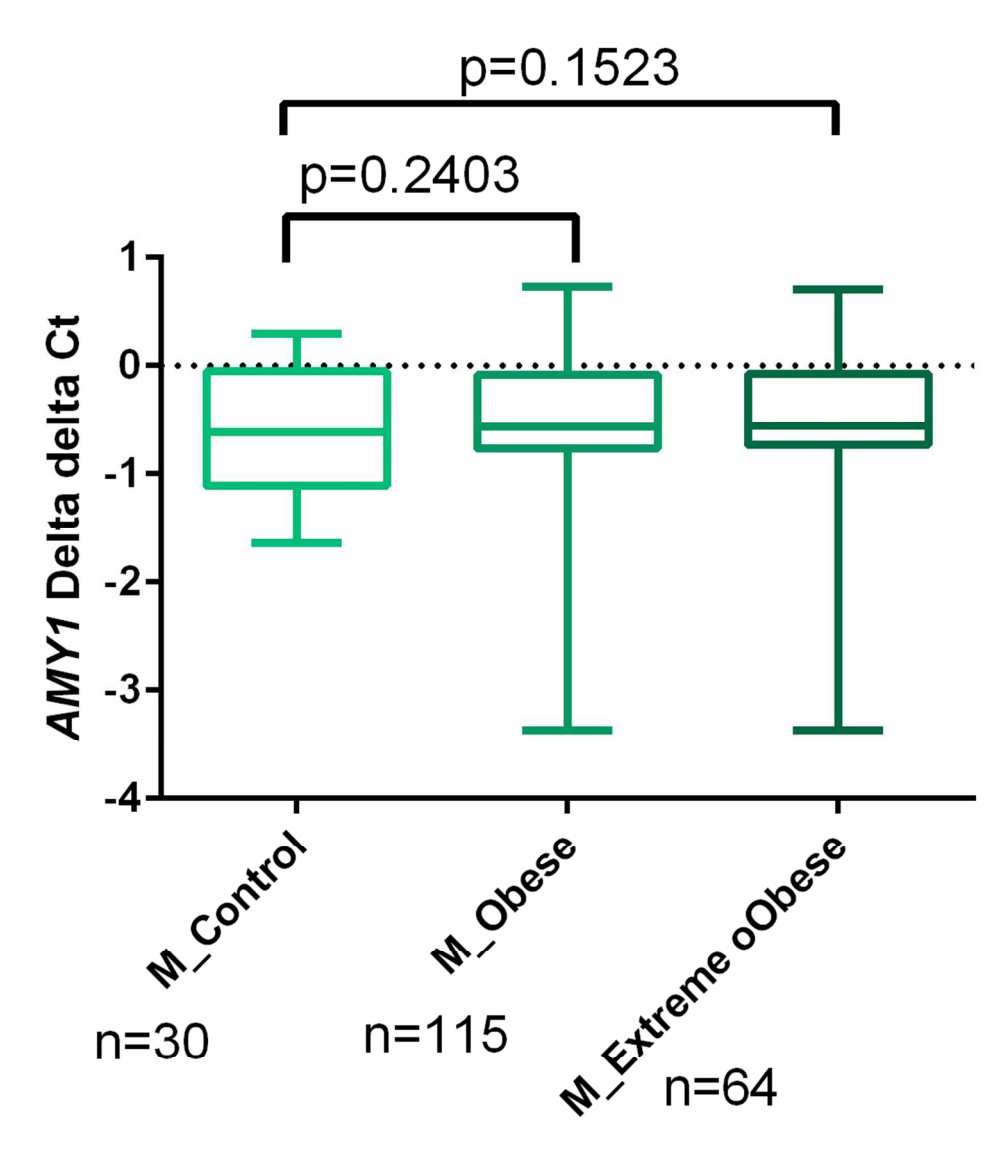


Fig 4C: Mann-Whitney test of ΔΔCT in controls, obese, and extreme obese samples. Low ΔΔCT values correspond to high *AMY1* copy numbers. C: Malay samples.
93x109mm (300 x 300 DPI)

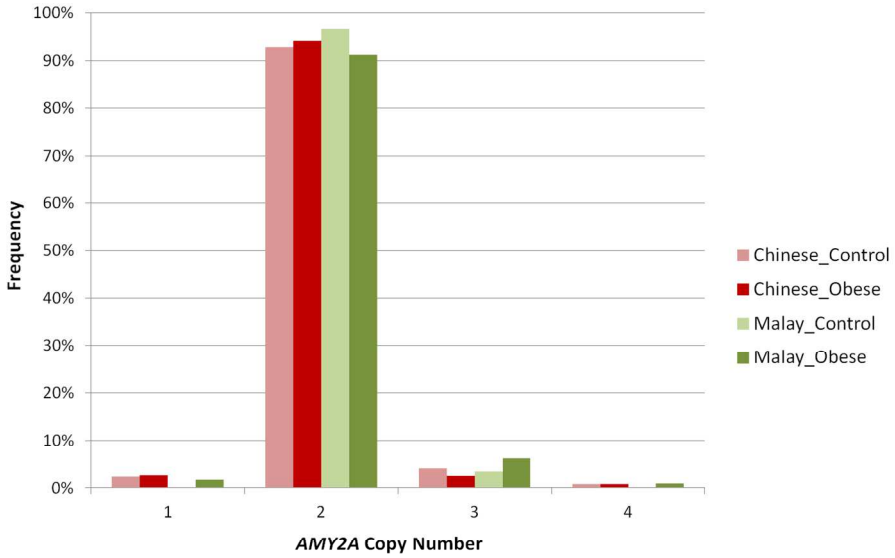


Fig D: *AMY2A* diploid CN frequency distribution in two Singapore populations. 519 Chinese controls versus 413 obese, and 30 Malay controls versus 115 obese.
272x178mm (300 x 300 DPI)

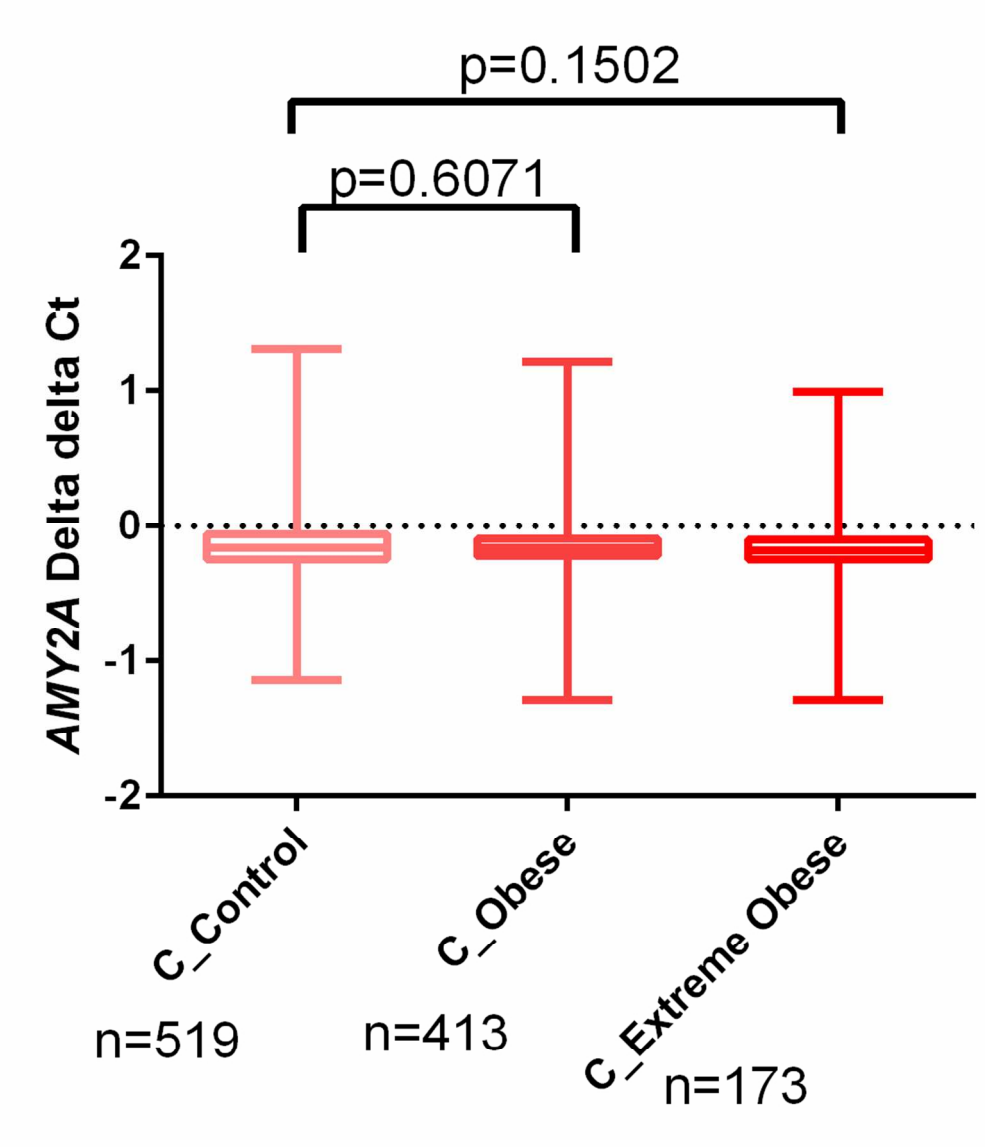


Fig 4E: Mann-Whitney test of ΔΔCT in controls, obese, and extreme obese samples. Low ΔΔCT values correspond to high *AMY2A* copy numbers. E: Chinese samples
93x108mm (300 x 300 DPI)

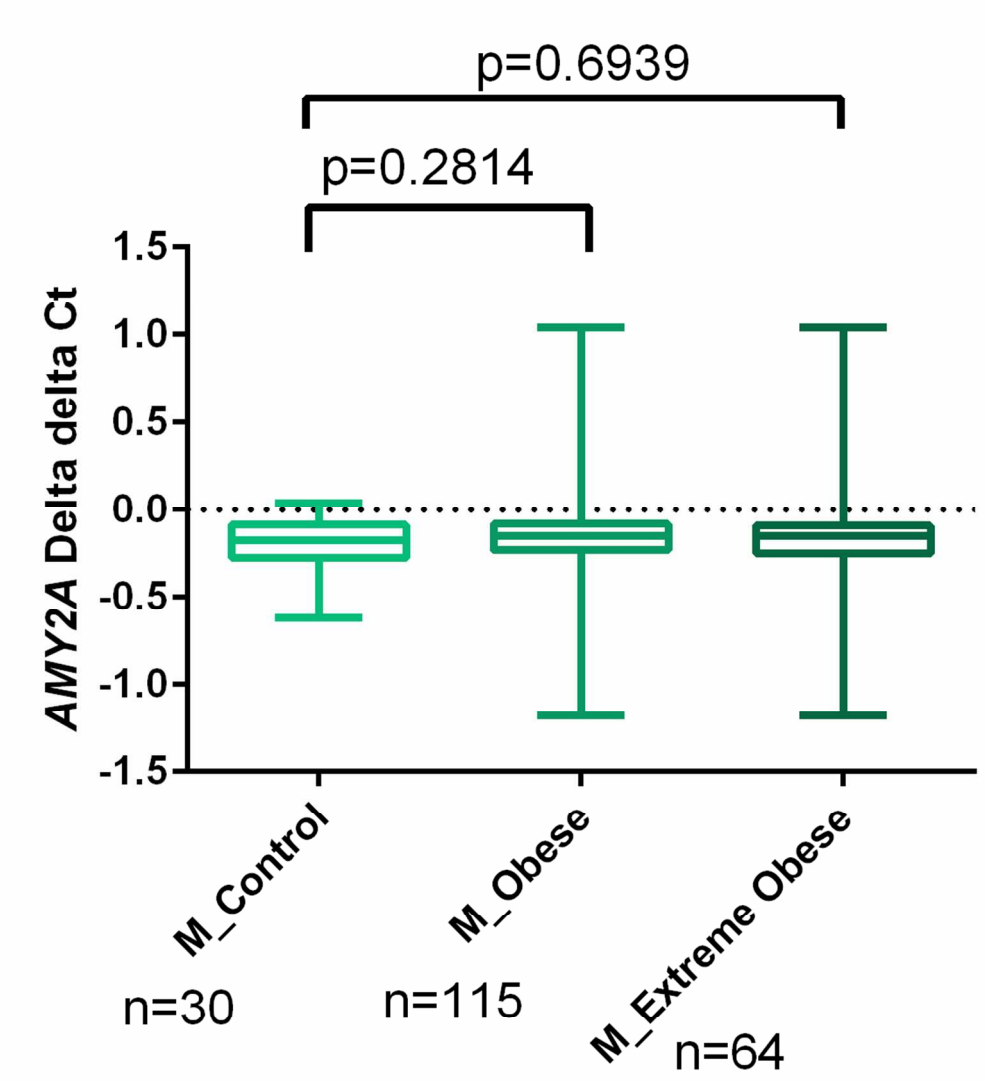


Fig 4F: Mann-Whitney test of ΔΔCT in controls, obese, and extreme obese samples. Low ΔΔCT values correspond to high *AMY2A* copy numbers. F: Malay samples.
97x108mm (300 x 300 DPI)

Sample	Chinese			Malay		
	Control	Obese	Extreme Obese	Control	Obese	Extreme Obese
Number	519	413	173	30	115	64
Average Age (SD)	19.4 (1.1)	19.2 (1.2)	19.3 (1.1)	19.7 (0.9)	19.4 (1.1)	19.3 (1.1)
Average BMI in kg/m^2 (SD)	20.8 (2.0)	34.2 (3.6)	37.5 (2.7)	20.8 (2.2)	36.2 (4.8)	39.3 (4.2)
Min to Max BMI	15.8 - 25.2	28.1 - 49.3	32.5-49.3	17.0-24.7	28.7-51.5	33.8-51.5
Median BMI (1st-3rd quatiles)	21.0 (19.3-22.3)	33.5 (31.4-36.7)	37.2 (35.6-38.6)	20.5 (19.1-22.2)	35.1 (32.6-39.1)	38.8 (35.8-41.1)

Table 1. Summary information of the two study cohorts.

A

		N	T-Test				Logistic Regression		
			CN Mean (SEM)	CN Mean Difference (SEM)	95% CI of Mean Difference	p-values	β (SE)	p-values	OR (95% CI)
Chinese	Controls	519	8.060 (0.105)	0.044 (0.162)	-0.274 to 0.362	0.784	0.007 (0.027)	0.784	1.007 (0.956 - 1.062)
	Obese	413	8.104 (0.126)						
	Controls	519	8.060 (0.105)	0.137 (0.215)	-0.285 to 0.559	0.524	0.023 (0.036)	0.524	1.023 (0.954 - 1.097)
	Extreme Obese	173	8.197 (0.201)						
Malay	Controls	30	8.50 (0.417)	-0.439 (0.502)	-1.432 to 0.554	0.383	-0.068 (0.078)	0.386	0.934 (0.801 - 1.090)
	Obese	115	8.06 (0.232)						
	Controls	30	8.50 (0.417)	-0.469 (0.577)	-1.615 to 0.677	0.419	-0.067 (0.084)	0.422	0.935 (0.794 - 1.102)
	Extreme Obese	64	8.03 (0.343)						

B

		N	T-Test				Logistic Regression		
			CN Mean (SEM)	CN Mean Difference (SEM)	95% CI of Mean Difference	p-values	β (SE)	p-values	OR (95% CI)
Chinese	Controls	519	2.033 (0.013)	-0.021 (0.020)	-0.059 to 0.018	0.290	-0.121 (0.204)	0.552	0.886 (0.594 - 1.321)
	Obese	413	2.012 (0.014)						
	Controls	519	2.033 (0.013)	-0.015 (0.027)	-0.068 to 0.038	0.569	-0.169 (0.296)	0.568	0.845 (0.473 - 1.508)
	Extreme Obese	173	2.017 (0.024)						
Malay	Controls	30	2.033 (0.033)	0.028 (0.063)	-0.097 to 0.152	0.663	0.369 (0.652)	0.571	1.446 (0.403 - 5.192)
	Obese	115	2.061 (0.031)						
	Controls	30	2.033 (0.033)	0.045 (0.078)	-0.111 to 0.201	0.570	0.390 (0.683)	0.568	1.477 (0.387 - 5.635)
	Extreme Obese	64	2.078 (0.051)						

Table 2. Association testing of Amylase genes diploid copy numbers with obesity. Results of T-test and logistic regression are shown. P-values from Mann-Whitney tests are similarly not significant and results not shown. **A:** *AMY1* CN and obesity. Results were calculated using *AMY1* copy numbers calibrated from qPCR-dPCR calibration curve and rounding to integer. **B:** *AMY2A* CN and obesity. Discrete *AMY2A* copy numbers deduced directly from qPCR.

COMPLEX COPY NUMBER VARIATION OF *AMY1* DOES NOT ASSOCIATE WITH OBESITY IN TWO EAST ASIAN COHORTS

Rita YY Yong[1,2], Su'Aidah B. Mustaffa[1,3], Pavandip Singh Wasan[1,2], L Sheng[5], C Marshall[6], S Scherer[6], YY Teo[2,4], Eric PH Yap[2,3,*]

[1] Defence Medical and Environmental Research Institute, DSO National Laboratories, Singapore,

[2] Saw Swee Hock School of Public Health, National University of Singapore, Singapore,

[3] Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore,

[4] Department of Statistics and Applied Probability, Faculty of Science, National University of Singapore, Singapore.

[5] Unit of Biostatistics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore,

[6] The Centre of Applied Genomics, Hospital of the Sick Children, Toronto, Canada

* Corresponding Author

Supplementary Notes for Methods

qPCR & dPCR Protocols

Each qPCR reaction is carried out in a final volume of 10 or 4 µl. Each 10 µl aliquot would contain 10 ng genomic DNA, 0.5 µl each of the Taqman target assay (20x) and the reference assay *RNaseP* (20x), 5 µl of Taqman genotyping master mix (2x), and 3 µl of sterile water. Cycling condition consisted of one cycle of 95ºC 10 min, followed by 40 cycles of 95ºC 15 sec, 60ºC 1 min.

A comparative C_T method was used in estimating diploid copy number. The ΔΔCt method assumes equal amplification efficiency for the target and the reference genes. PCR efficiency was evaluated for both the target assays (*AMY1* & *AMY2A*) and the reference *RNaseP* assay using a standard curve of serial dilutions of a DNA sample of known concentration. Log_{10} of the dilution factor was plotted against Ct mean values for each of the two assays. The slope of the line was then used to calculate PCR efficiency for each assay as follows:

$E = 10^{(-1/slope)}$

PCR Efficiency (%) = (E – 1) x 100

For dPCR protocol, the human genomic DNA was first digested with an appropriate restriction enzyme to break up the tandemly repeated CNV copies. 4 µl of genomic DNA (100ng/µl) was digested with 1µl RsaI (10U/µl) (New England Biolabs, USA), with 1 µl of 10x enzyme buffer in a final volume of 10 µl. Digestion was carried out at 37ºC for 1h followed by enzyme inactivation at 65ºC for 20mins. Digested DNA was diluted 4x to a final volume of 40µl. 4ul of the diluted DNA (10ng/µl) was used in each digital-PCR reaction, which would contain 8µl of the 2x digital-PCR master mix, 0.8µl of 20x target Taqman assay, 0.8µl of 20x reference RNasaP assay, and 2.4µl sterile water to constitute a final volume of 16µl. About 14.5µl of the dPCR reaction product was loaded per

chip. Thermal cycling condition was 1 cycle of 96ºC 10 min, followed by 39 cycles of 60ºC 2 min 98ºC 30sec, and a last cycle of 60ºC 2 min.

Leave-one-out Analysis to test sensitivity of the qPCR-dPCR calibration curve

A leave-one-out analysis was carried out to test the sensitivity of the calibration curve if the number of sample used in constructing the calibration curve is reduced. One sample from the total 31 samples was removed at a time, the calibration curve was re-calculated, and the newly derived calibration curve was used to translate the qPCR CN calls to the calibrated CN calls. Of the total 31 leave-one-out experiments, 6 (19%) showed a reduction of accuracy. In each of these 6 leave-one-out experiments, discordance between dPCR CN and calibrated CN increased by 1.

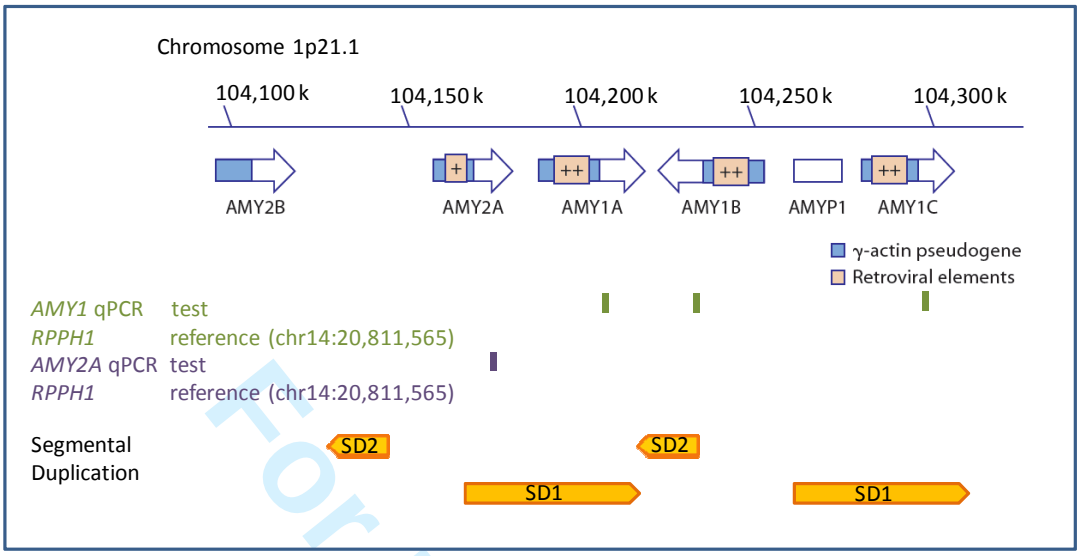
In addition, an early analysis had been carried out using a calibration curve established from 18 samples. The table below contrasts the *AMY1* CN frequency distribution of the 1077 samples using the calibration curves established with 18 samples or 31 samples. There were slight difference in frequency but the difference is not significant (Fisher Exact test two-sided p-value = 0.103). Association testing using the calibrated CN calls derived from the 18 samples calibration curve similarly produced a negative association result.

AMY1 CN	2	3	4	5	6	7	8	9	10	11	12	13	14	15	18	19	24	Total
18 samples Calibration curve	0.3%	0.4%	6.5%	2.5%	24.7%	6.6%	25.3%	6.3%	15.5%	4.5%	4.4%	1.5%	1.2%	0.2%	0.1%		0.1%	100.0%
31 samples Calibration curve	0.3%	0.4%	6.5%	1.9%	24.8%	3.8%	26.6%	5.3%	16.4%	4.3%	6.3%	1.5%	0.8%	0.9%		0.1%	0.1%	100.0%

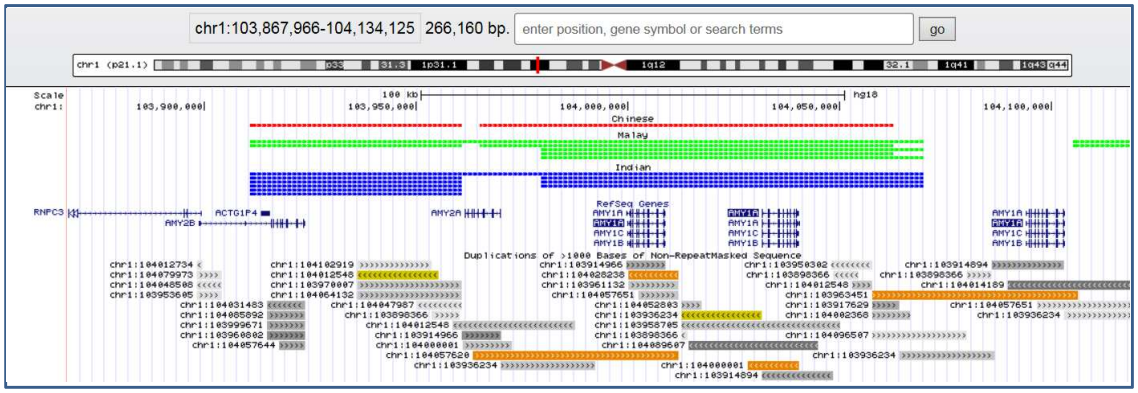
Statistical analysis

Student's t-test, Mann-Whitney test and Correlation test were carried out in GraphPad Prism 6. Fisher's exact test, Cochran-Armitage trend test and power calculation were done in Excel XLSTAT. Logistic and linear regressions were carried out using SPSS v15.0.

Supplementary Figures and Tables

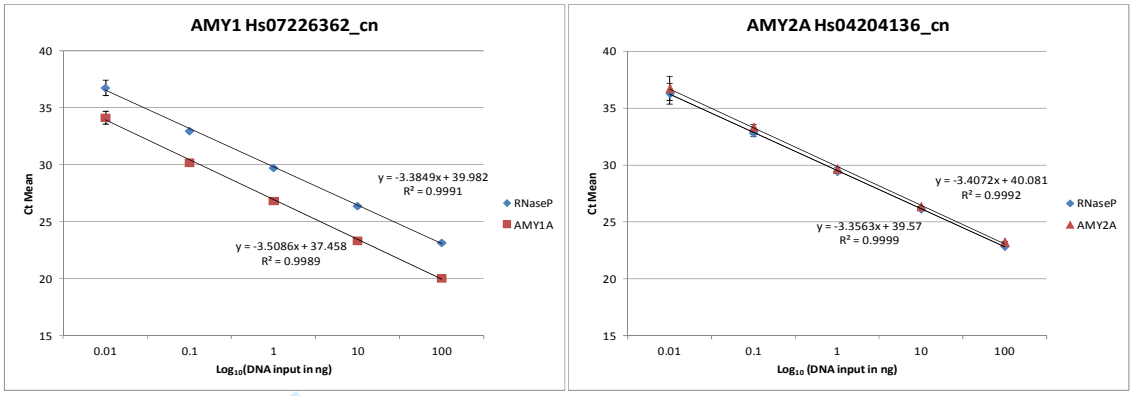


Supp. Figure S1A. Schematic representation of α-amylase gene cluster on chromosome 1.21p.1. Genome co-ordinates according to assembly NCBI37/hg19. (modified from Santos et al. 2012)
A γ-actin processed pseudogene was inserted in 5' position of all *AMY* genes except the pseudogene *AMYP1*. The amount of insertions of the retroviral element is marked with +. Approximate locations of two segmental duplications are indicated, which have implication in generating the more common haplotype structures at this *AMY* locus. The approximate location of the 2 qPCRs for *AMY1* and *AMY2A* are indicated.

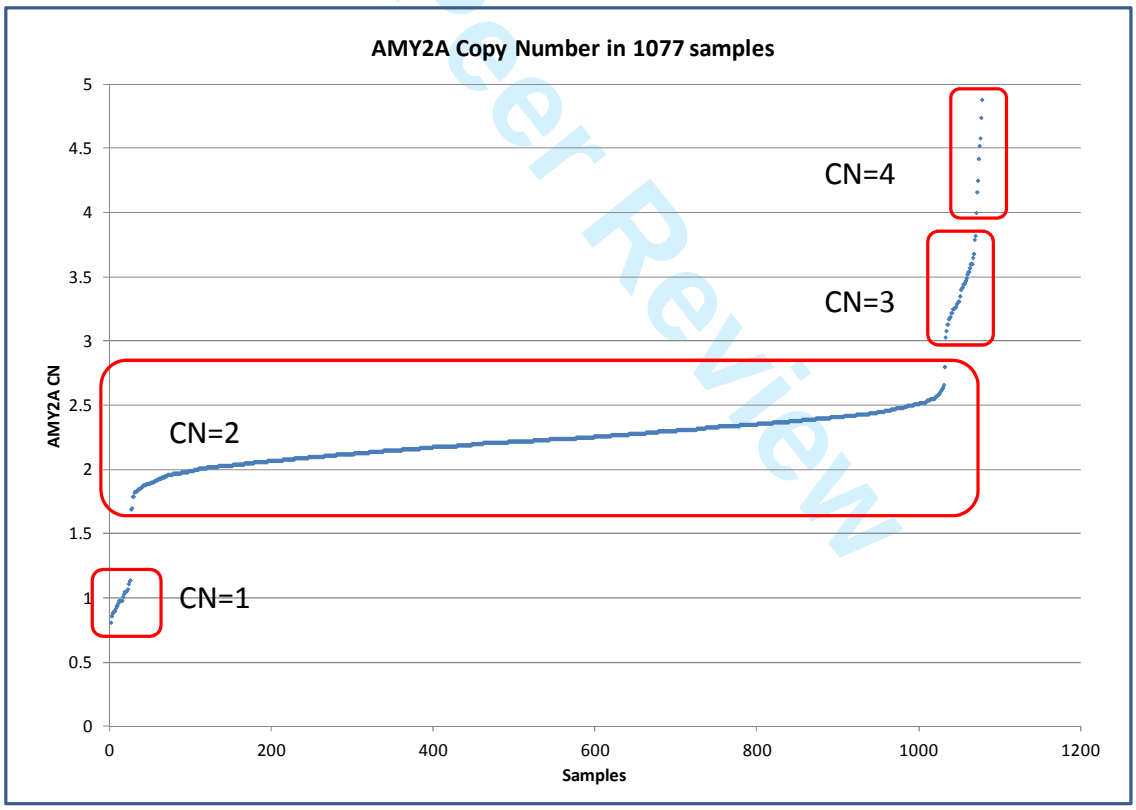


Supp. Figure S1B. Human amylase gene cluster on chromosome 1.21p.1 as displayed in UCSC Genome Browser. Genome assembly is NCBI36/hg18. Positions of *AMY2B, AMY2A, AMY1A, AMY1B, AMYP1* and *AMY1C* are shown. Two groups of CNVs are identified by SNP array in DMERI database, overlapping *AMY2A* and *AMY1*, of sizes 50 kb and 100 kb, respectively. CN2 and CN6 were non-CNV for *AMY2A* and *AMY1*, respectively. Microarray results underestimated the extent of CN variation for both CNVs, especially for *AMY1*. Chinese in red, Malays in green, and Indians in blue lines representing CNV locations, with each line representing one sample. Segmental duplications (SDs) are shown in lowest section. Orange SD, > 99% sequence similarity, the long orange SD corresponds to SD1 of Figure 1 in main paper. Light to dark yellow, 98 - 99% similarity, corresponding to SD2. Light to dark gray, 90 - 98% similarity.

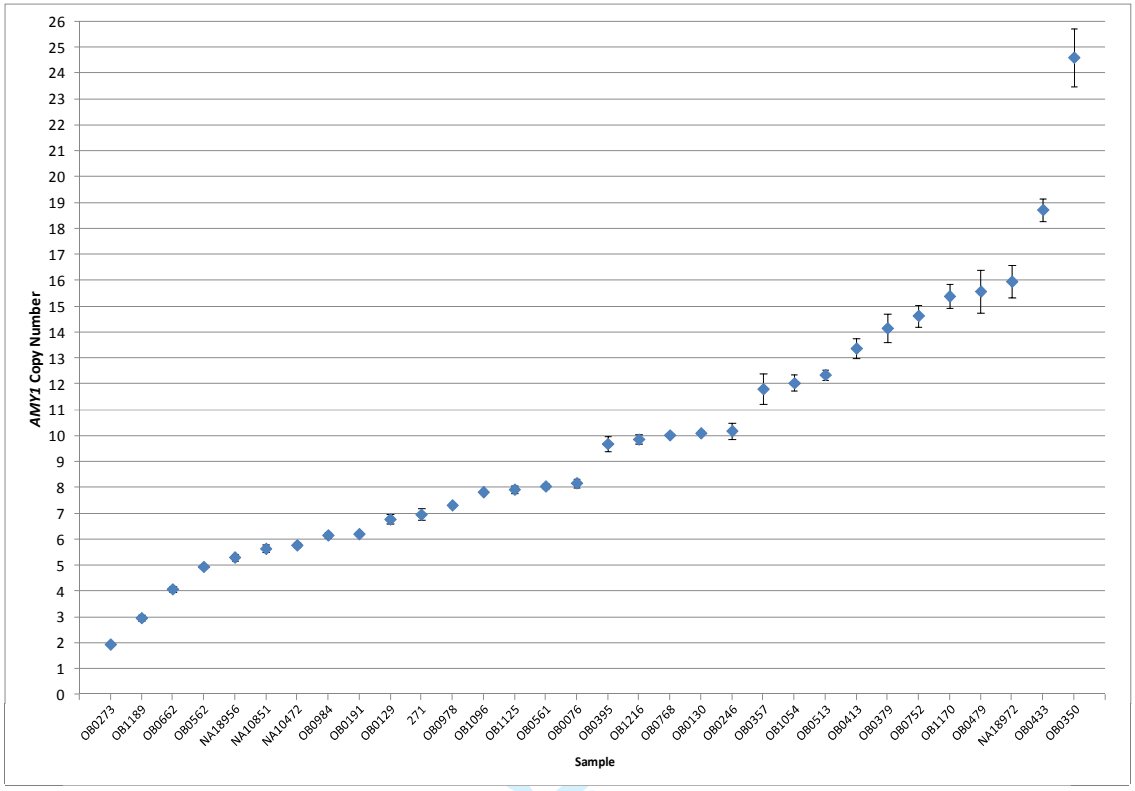
A B



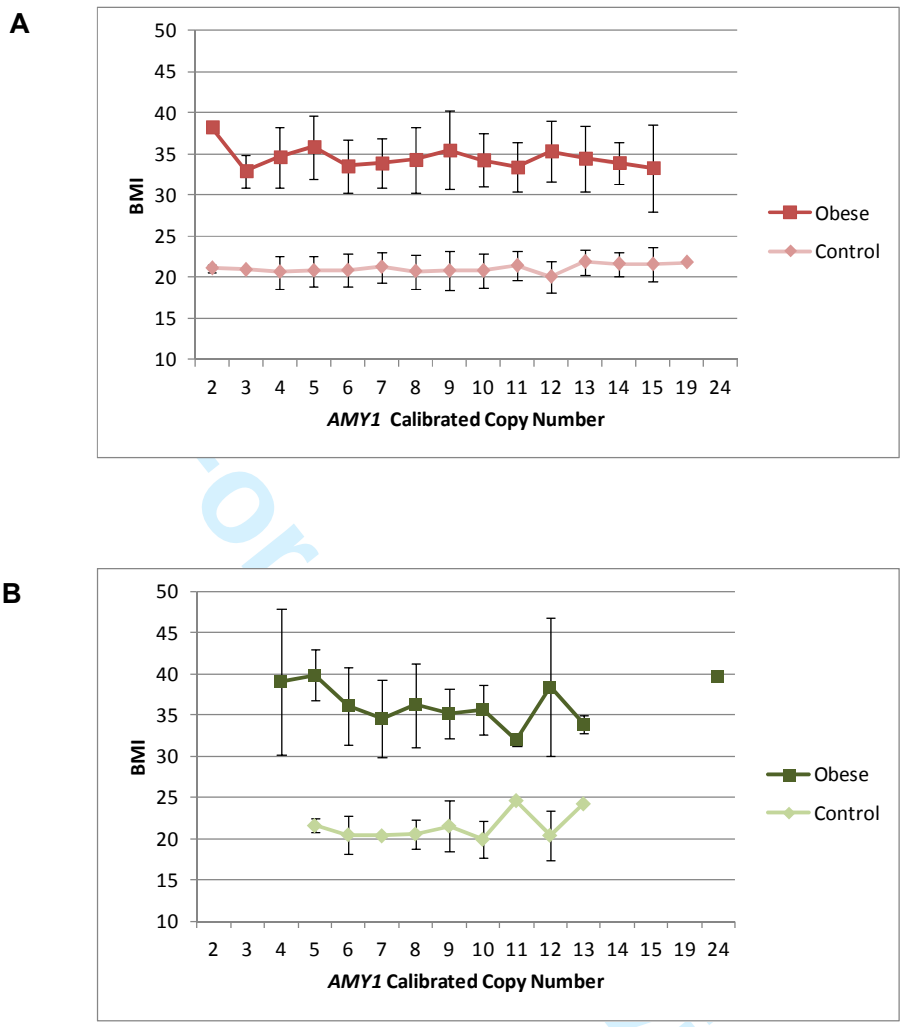
Supp. Figure S2. Real-time PCR standard curve representing PCR efficiency. A: Taqman assay hs07226362_cn targeting *AMY1* and the *RNaseP* reference assay. B: taqman assay hs04204136_cn targeting *AMY2A* and the *RNaseP* reference assay.



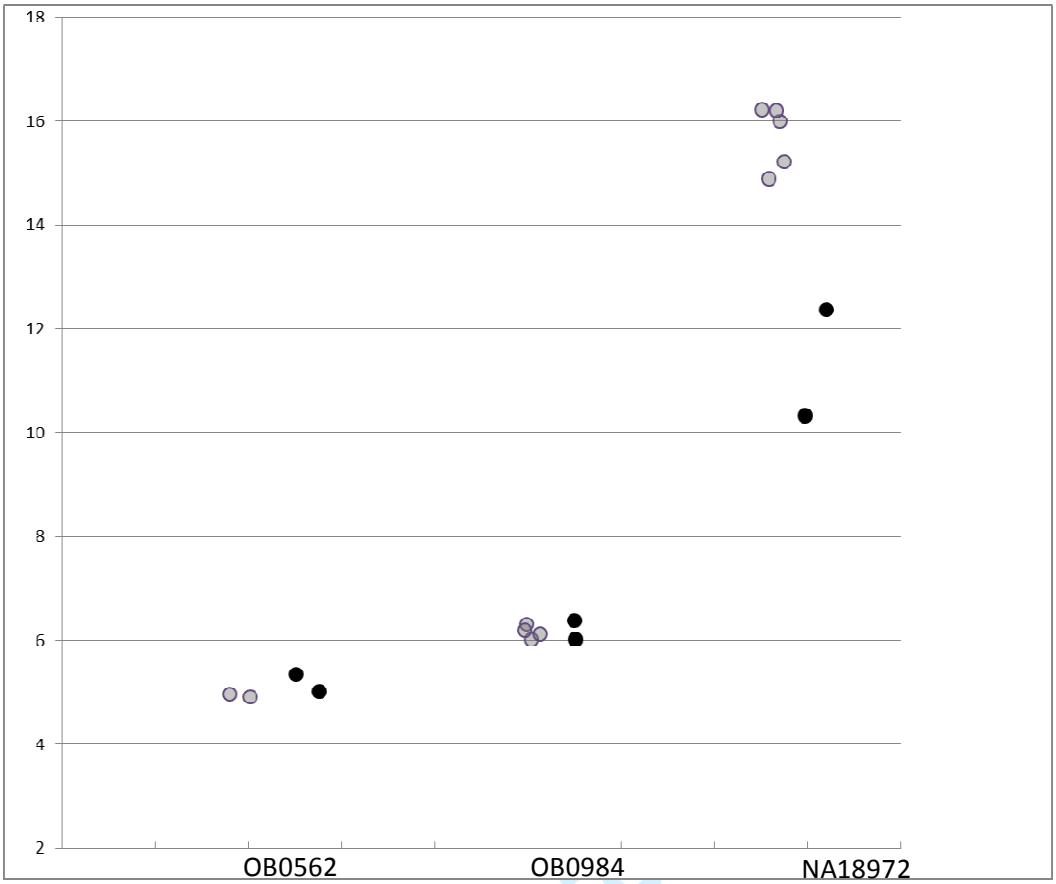
Supp. Figure S3. Distribution of the diploid copy numbers for *AMY2A* CNV. Total sample size 1077 comprising controls and obese from both Chinese and Malays populations. *AMY2A* CN sorted in order to show the discrete delineation of the 4 copy classes. Correspond to Figure 2A in main text.



Supp. Figure S4. AMY1 diploid copy number determined by dPCR for 32 samples, four of which were cell line reference samples from Coriell Repository. Error bar represents standard deviation. Number of replicates per sample as in Supplementary Table 2.



Supp. Figure S5. Average BMI at different *AMY1* diploid copy numbers. Error bar represents standard deviation. Some points with no error bar because they are singletons. **A:** 519 Chinese controls versus 413 obese. **B:** 30 Malay controls versus 115 obese.



Supp. Figure S6. Comparison of dPCR performance with or without restriction enzyme Rsa I digestion. Each dot represents one dPCR run. Grey - With digestion. Black - No digestion.

Gene	Assay ID	Cytogenetic Band	Chromosome	Location on NCBI Genome Assembly GRCh37	Location on Transcript or Gene	Reporter Dye	Quencher	Amplicon size (bp)	Context Sequence
AMY1A, AMY1B, AMY1C	Hs07226362_cn	1p21.1	1	104,198,366, 104,238,841, 104,292,504	Within Exon 1	FAM	NFQ	101	ATGTGTCAGGGCTGAGTGTTCTGAG
AMY2A	Hs04204136_cn	1p21.1	1	104,161,065	Within Intron 2	FAM	NFQ	111	TTAGGTGACTTGTGTCTCCATCCGT
RPPH1		14q11.2	14	20,811,565	within single exon	VIC	TAMRA	87	GAGCTTCCCTCCGCCCTATGGGAAAA

Supp. Table S1. Characteristics of Taqman® copy number assays targeting *AMY1* and *AMY2A* genes. Genome co-ordinate according to NCBI37/hg19.

	dPCR				qPCR			
Sample	Replicates	CN Mean	CN SD	Log_2 (CN)	Replicates	ΔΔCt	ΔΔCt SD	CN Mean
OB0273	3	1.95	0.03	0.96	4	1.84	-0.11	1.68
OB1189	5	2.97	0.10	1.57	4	1.14	0.03	2.71
OB0662	5	4.08	0.11	2.03	4	0.61	0.01	3.93
OB0562	2	4.95	0.03	2.31	4	0.26	0.01	5.01
NA18956	12	5.31	0.13	2.41	48	0.13	0.01	5.50
NA10851	11	5.85	0.14	2.50	48	0.00	0.00	6.00
OB0984	4	6.16	0.12	2.62	4	-0.07	0.00	6.29
OB0191	2	6.21	0.10	2.63	4	-0.01	0.00	6.05
OB0129	2	6.78	0.19	2.76	4	-0.33	0.00	7.52
271	2	6.96	0.22	2.80	48	-0.38	0.01	7.82
OB0978	2	7.32	0.09	2.87	4	-0.59	0.01	9.01
OB1096	2	7.83	0.03	2.97	4	-0.74	0.02	9.99
OB1125	2	7.93	0.15	2.99	4	-0.58	0.02	8.97
OB0561	2	8.06	0.10	3.01	4	-0.66	0.02	9.50
OB0076	2	8.18	0.17	3.03	4	-0.50	0.00	8.50
OB0395	3	9.69	0.30	3.28	4	-0.84	0.06	10.76
OB1216	3	9.87	0.19	3.30	4	-0.75	0.01	10.12
OB0768	2	10.03	0.03	3.33	4	-1.13	0.05	13.16
OB0130	2	10.11	0.07	3.34	4	-0.94	0.05	11.48
OB0246	4	10.19	0.31	3.35	4	-0.99	0.03	11.88
OB0357	4	11.81	0.58	3.56	4	-1.37	0.05	15.50
OB1054	4	12.04	0.31	3.59	4	-1.30	0.01	14.81
OB0513	5	12.36	0.21	3.63	4	-1.55	0.02	17.60
OB0413	4	13.38	0.37	3.74	4	-1.94	0.06	23.07
OB0379	4	14.15	0.55	3.82	4	-1.70	0.02	19.51
OB0752	9	14.64	0.42	3.87	4	-2.05	0.04	24.81
OB1170	9	15.39	0.47	3.94	4	-1.98	0.01	23.64
OB0479	5	15.58	0.82	3.96	4	-1.91	0.04	22.48
NA18972	21	15.96	0.63	4.00	48	-2.25	0.01	28.63
OB0433	10	18.73	0.44	4.23	4	-2.55	0.02	35.13
OB0350	17	24.61	1.13	4.62	4	-3.37	0.06	62.17

Supp. Table S2. Comparison of dPCR and qPCR results for 28 samples and 3 reference specimens.

A

Chinese Controls n=519			
Count	AMY2A CN		
AMY1 CN	2	non2	
even	421	7	428
odd	61	30	91
	482	37	519
Fisher's exact test:			
p-value (Two-tailed)	<0.0001		

B

Chinese Obese n=413			
Count	AMY2A CN		
AMY1 CN	2	non2	
even	334	4	338
odd	55	20	75
	389	24	413
Fisher's exact test:			
p-value (Two-tailed)	<0.0001		

C

Malay Controls n=30			
Count	AMY2A CN		
AMY1 CN	2	non2	
even	23	0	23
odd	6	1	7
	29	1	30
Fisher's exact test:			
p-value (Two-tailed)	= 0.2333		

D

Maly Obese n=115			
Count	AMY2A CN		
AMY1 CN	2	non2	
even	87	5	92
odd	18	5	23
	105	10	115
Fisher's exact test:			
p-value (Two-tailed)	=0.0264		

Supp. Table S3. Association testing of *AMY2A* and *AMY1* diploid copy numbers. a) 519 Chinese controls. b) 413 Chinese obese samples. c) 30 Malay controls. d) 115 Malay obese samples.