Expert Review: Human amylase gene copy number variation as a determinant of metabolic state

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Abbreviations

AMY1:	The human salivary amylase gene
AMY2:	The human pancreatic amylase gene
BMI:	Body Mass Index
CNV:	Copy Number Variation
ddPCR:	Droplet digital Polymerase Chain Reaction
dPCR:	Digital Polymerase Chain Reaction
DM:	Diabetes Mellitus
CPIR:	Cephalic Phase Insulin Release
GWAS:	Genome-wide Association Study
qPCR:	Quantitative Polymerase Chain Reaction
SCFA:	Short chain fatty acids
SNP:	Single Nucleotide Polymorphism
T2D:	Type 2 Diabetes Mellitus
WHO:	World Health Organization

Keywords

Obesity

Insulin resistance

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Salivary amylase; *AMY1* copy number

Pancreatic amylase; *AMY2* copy number

Serum amylase

Copy number variation

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Introduction

Obesity is one of the most important global health problems. The World Health Organization (WHO) estimated that in 2016, around 13% of adults worldwide were obese, and 18% of children and adolescents were overweight or obese [1]. The global prevalence of obesity tripled in epidemical proportions between the years 1975 and 2016 [1, 2, 3]. The global burden of obesity encompasses a broad spectrum of pathology that confers a substantial healthcare economic impact [1, 4].

Although changes in lifestyle (including diet and activity) have contributed towards increases in body weight [1, 5], genetic effects are also important, Body Mass Index (BMI) having a heritability of up to 70% [3, 6, 7, 8, 9]. Genome-wide association studies (GWAS), which attempt to identify genetic associations of certain traits or conditions by testing for a large number of common genetic variants (single nucleotide polymorphisms) in both those affected and control groups, and then comparing the relative frequencies with which the genetic variants appear [9], have identified more than 90 genetic loci which are associated with BMI [3]. Nevertheless, these identified genetic loci only account for a small proportion (around 2.7%) of the population-based variation in BMI [3, 9]. Furthermore, underlying mechanisms linking gene variants with BMI are incompletely understood [3]. It is possible that epigenetic effects may account for some of the heritability of BMI [9]. It is also possible that some genetic loci with very small effects on BMI have not been identified from the GWAS that have been reported to date and require much larger population-based studies to detect. In addition, it is conceivable that non-mutational genetic differences, such as copy number variation (CNV), influence the metabolic phenotype and may account for some of the missing heritability [9].

CNV refers to the phenomenon whereby genes, or parts of genes, are replicated in sequence or deleted within a chromosome [9]. Detection of single nucleotide polymorphisms (SNP) mutations in GWAS [9] may identify some CNVs due to 'tagging' to surrounding SNPs [10, 11], but more complex multi-allelic CNVs are less readily identified through GWAS [10, 12]. One region of particular interest that

displays such multi-allelic complexity is the genetic sequence coding for the amylase enzymes [10]. In humans, the amylase enzyme occurs in two distinct forms: i) salivary (produced by the salivary glands), and; ii) pancreatic (produced by the exocrine pancreas) [13, 14]. These enzymes play a key role in the digestion of starch. Salivary amylase, the most abundant protein in saliva [15, 16], facilitates hydrolysis of α-1,4 glycosidic bonds within the oral cavity [15], producing maltose, isomaltose, oligosaccharides and a small amount of glucose [13, 15, 17]. Further down the alimentary tract within the ileum, pancreatic amylase continues this process of hydrolysis [15, 18]. Resulting products are broken down into absorbable free glucose by enzymes within the brush border of the ileum [18]. Small amounts of amylase are also produced by the liver **[13, 19-21],** nervous system, mammary tissue, uterus and testes [21].

In humans, salivary amylase is encoded by three specific genes: *AMY1A, AMY1B* and *AMY1C*. Conversely, human pancreatic amylase is encoded by two genes: *AMY2A* and *AMY2B* [10, 22]. Together with a pseudogene, *AMYP1*, these genes are all located within a single section of chromosome 1, at the locus 1p21.1 [22]. The three *AMY1* genes are very similar [10, 22], and may be grouped together and collectively referred to as '*AMY1*', while the two pancreatic genes may also be collectively termed '*AMY2*' [10]. It is well established that human salivary and pancreatic amylase genes manifest remarkable CNV **[22-25]**. Within an individual genome, there may be between 1-27 copies of *AMY1* [21], 0-8 copies of *AMY2A* [26, 27] and 2-6 copies of *AMY2B* [27]. However, the potential implications of amylase gene CNV for BMI and metabolic function have only become apparent in recent years.

In this review, we explore in detail the evidence within the current literature that confirms or refutes association between CNV within the human amylase genes and BMI and other metabolic markers. Possible underlying mechanisms to explain such associations are also explored. Finally, the clinical implications for such an association between amylase gene CNV and BMI and metabolic health is discussed, including suggestions for future directions in this fascinating and emergent field.

Evolutionary drive for CNV within *AMY1*

Before exploring the association between CNV within *AMY1* and BMI, and the possible mechanisms involved, let's take a step back and consider why there is so much variation in copy number within *AMY1* within humans, and what has driven such variation. In short, why is there not just one *AMY1* copy in the human genome (as with most other genes), instead of many?

A clue originates from the observation of a positive correlation between CNV within *AMY1* and the concentration and activity of salivary amylase in humans: increased copies of *AMY1* presumably result in increased production and activity of salivary amylase, thereby facilitating initiation of amylase digestion within the saliva [14, 15, 18, 21, 25, 28-31]. Although pancreatic amylase accounts for most of digestion of starch within the diet, the role of salivary amylase in starch digestion should not be under-estimated [32]. A sizable proportion of starch hydrolysis occurs within the oral cavity during mastication of food [32, 33]. Furthermore, swallowing starch-rich foods before mastication, and thus reduced mixing with saliva, leads to a lower post-prandial blood glucose concentration [32, 34], thereby implying reduced effectiveness of starch digestion.

It has been hypothesised that CNV within AMY1 represents an evolutionary adaptation to increasing levels of starch in the human diet. There is some evidence to support such a hypothesis. First of all, studies have shown that humans and rodents both obtained the expression of salivary amylase independently by a retroviral insertion into the amylase gene region; the fact that it has persisted in both evolutionary lines suggests that the expression of salivary amylase confers a selective advantage [14, 35, 36]. Next, obligate carnivores do not produce salivary amylase [14, 37], whereas animals which feed on starch-rich plant matter, such as fruits and seeds, display salivary amylase activity [14]. Although domesticated dogs do not produce salivary amylase [14, 38, 39, 40], they do display CNV of AMY2B, which is correlated with serum amylase activity [40]. Wolves, on the other hand, follow a carnivorous diet and do not exhibit AMY2B CNV [39]. It has therefore been suggested that dogs acquired AMY2B CNV in order to adapt to a starch-rich diet [14, 39, 40], which represented an important step in their domestication [39].

Furthermore, when compared to other primates which consume lower amounts of starch, humans appear to possess higher *AMY1* copy number [21, 25, 41] and express higher levels of salivary amylase [25]: for example, chimpanzees do not demonstrate *AMY1* CNV at all, possessing only one copy per chromosome [25, 42], and the quantity of salivary amylase produced by humans is around 6-8 times greater [25, 43]. Although gorillas and bonobos do exhibit CNV [21, 25, 38], humans have a higher copy number than the former [38] and the latter's *AMY1* copies may not be functional [25]. This difference in CNV largely reflects their respective diets: while gorillas may have a high starch intake [44], chimpanzees consume very little compared to the majority of human populations [25, 45]. The fact that humans seem to have higher *AMY1* copy numbers than other nonhuman primates, and also have a higher dietary starch intake, supports the idea that *AMY1* CNV, and the increased salivary amylase levels that it engenders, represents an evolutionary adaptation to higher starch consumption. However, it must be acknowledged that *AMY1* and *AMY2* CNV have not yet been systematically studied in these nonhuman primates in any detail, and existing studies have used small sample sizes [21]. In addition, the correlation between *AMY1* copy number and salivary amylase activity has yet to be studied in any species other than humans [21]. This makes it difficult to derive any definitive conclusion from these findings.

Nevertheless, among humans alone, Perry and colleagues found that populations with a diet traditionally high in starch have higher copy numbers of the *AMY1* gene compared with those populations that consume less starch [25], which lends further weight to the hypothesis described above. But if *AMY1* CNV does represent an adaptation to increased levels of starch in the human diet, at what point in evolutionary history did it occur, and why did it occur at that time? It has been suggested that it accompanied the development of agriculture [21, 22, 25, 32, 46, 47], which occurred around 10,000 BC, but it is unlikely that such a major change in the genome could have occurred in such a short space of time. Furthermore, *AMY1* CNV has been found in ancient hunter-gatherers, predating the introduction of agriculture [48, 49].

Hardy and colleagues proposed that *AMY1* CNV may have occurred during the Middle Pleistocene era [30, 31], before humans diverged from Neanderthals, and was therefore present in the first *Homo sapiens*. From this era onwards, there was a significant growth in brain size in human ancestors [30, 31], which would have necessitated a greater supply of preformed glucose. The authors argued that the widespread introduction among hominins of cooking, together with the development of *AMY1* copy numbers and the increased salivary amylase activity it generated, were responsible for this increased preformed glucose supply, and therefore made this neurological development possible [30, 31]. Indeed, once cooking was developed, starch digestion would have become the rate-limiting step in its utilisation [30]. This hypothesis suggests that the development of *AMY1* CNV 'coevolved' with the development of cooking, and provided a selective advantage due to its provision of increased glucose to the developing brain.

However, in analyses performed on a Neandertal and a Denisovan (a member of another distinct archaic hominin population), it was estimated that they had only one *AMY1* copy per chromosome and therefore, like the chimpanzee, did not display CNV [42]; this could indicate that the development of *AMY1* CNV occurred in human ancestors *after* their evolutionary split from the Neandertals and Denisovans around 550-590,000 years ago [42]. A recent study which analysed genetic variation around the amylase gene locus in a global set of human populations reinforced this finding [31]. The authors determined that *AMY1* copy numbers developed in the late Middle Pleistocene period, after humans had diverged from Neanderthals and long after the development of cooking and the increase in hominin brain size had already taken place [21, 31].

Of course, the hypotheses described above are ultimately speculative. In the populations studied by Perry and colleagues [25], limited information on their prehistoric diets is available, and they did not use standard dietary intake assessment methods [21]. Their assertion that *AMY1* copy number is higher in populations that have traditionally consumed a greater amount of dietary starch may not therefore be wholly accurate [21]. Furthermore, we cannot accurately identify the exact temporal origin of *AMY1* CNV, nor when cooking was widely

adopted by human ancestors [30]. However, if the origin of *AMY1* CNV predates the development of agriculture and postdates the widespread introduction of cooking, and may have been present in the first humans, it does not necessarily mean that it has conferred no selective advantage in modern human history [47]. It is still possible that high numbers of *AMY1* copies were favoured in populations with starch-rich diets, and have been retained. This could at least partially explain why some present-day humans do not possess increased *AMY1* copy numbers, as this may represent a secondary loss that arose with a change in diet that no longer favoured increased starch digestion [31]. Indeed, it has been shown that Northeast Siberian populations, who have traditionally followed a low-starch diet, have a high frequency of *AMY2A* deletion and associated low *AMY1* copy number [31]. This hypothesis may also help to explain why there is such great *AMY1* copy number heterogeneity in human populations.

Ultimately, it will remain difficult to prove whether or not AMY1 copy number expansion conferred a specific evolutionary advantage, and when this occurred. Yet that does not make this phenomenon any less relevant. In the next sections, we examine the metabolic implications for individuals of amylase gene CNV.

Association between CNV within *AMY1* and BMI

The link between CNV within human *AMY1* and BMI was first described by Falchi and colleagues in 2014 [10]. Through a 'gene-centric' and quantitative PCR (qPCR) approach, a strong inverse association between CNV within *AMY1* and BMI and fat mass was shown [10, 50]. This association was first identified in a single cohort, then replicated in >6,000 participants from a number of different cohorts, comprising both European and Singaporean Chinese ethnicities [10]. Each additional copy of the *AMY1* gene reduced the risk of obesity 1.2-fold [10,27]. The risk of obesity in those with CNV<4 was 8-fold higher than in those with CNV>9 [10]. Indeed, this link between *AMY1* and BMI was stronger than that previously identified between obesity and *FTO*, the gene which has been found to have the greatest effect on obesity risk of any of those identified by GWAS [10, 27, 51]. In this study, however, no association between copy numbers of the *AMY2* genes and BMI or fat mass was observed.

Consistent with data from Falchi and colleagues [10], other studies have also shown associations between CNV within human *AMY1* (measured using qPCR) and BMI, obesity and insulin resistance [52], although results have been mixed. In one study on Italian primary school children, although no *overall* association between CNV within *AMY1* and BMI or waist circumference was demonstrated, such an association was present in boys alone [53]. Other authors, using qPCR to assess the relationship between *AMY1* and BMI, failed to establish any correlation. Among these reports is a cross-sectional analysis of an adult South Korean population that showed no association between CNV within *AMY1* and BMI, (although there was association with insulin resistance) [52].

It should be noted that complex CNV is inherently difficult to measure [27, 54], especially for *AMY1* (due to the high number of *AMY1* copies and close proximity of the highly similar *AMY1* and *AMY2* genes) [22, 46]. Unfortunately, qPCR as a technique to measure CNV within *AMY1* has inherent limitations [27, 55, 56]. More accurate techniques, including a combination of qPCR with 'digital PCR' (dPCR), 'droplet digital PCR' (ddPCR), 'sequence read depth techniques' and 'paralogue ratio tests (PRTs) and microsatellite measurements', have shown that the arrangement of the amylase genes is not merely a copy number continuum [46], but categorized into separate haplotypes [22, 27, 46]. Indeed, 8 common haplotypes were shown to account for 98% of the combinations of *AMY1*, *AMY2A* and *AMY2B* copy numbers observed in a European cohort [27]. This is in contrast with data from Falchi and colleagues and other studies using qPCR that showed an approximate normal distribution of *AMY1* copy number [10, 13, 22, 25].

Furthermore, the copy numbers of AMY1 and AMY2A were shown to be significantly correlated in European populations by two studies using accurate CNV measurement techniques: the first used PRTs and microsatellite measurements [46], while the second used a novel method, 'automatic modeling functionality for copy number estimation', or 'AMYCNE', which uses whole genome sequencing data and appears to exhibit a similar level of accuracy to ddPCR [57]. The haplotype arrangements of *AMY1* and *AMY2A* CNV are also correlated. Studies using these

accurate measurement techniques have shown that there is usually parity between the AMY1 and AMY2A copy numbers: in other words, if the copy number of AMY1 is odd, then the copy number of AMY2A is also odd, and vice versa [27, 57]. Moreover, an individual chromosome with an odd AMY1 copy number, will have one copy each of AMY2A and AMY2B, and will therefore not exhibit CNV in the latter two genes; on the other hand, those with an even AMY1 copy number, which is rarer, do exhibit CNV of the AMY2 genes [27, 46, 58]. This means that the copy numbers of AMY1 and AMY2 are dependent on each other. It is possible therefore that the association data outlined above may be influenced by specific *haplotypes* [56, 58], with odd or even haplotypes interacting with the copy numbers in different ways to determine gene expression [58]; or even by *AMY2* copy number [46], rather than simply by copy number of *AMY1*. This insight has mechanistic implications. It is worth noting that one study which examined the relationship between AMY2 copy number and salivary amylase production and activity found no significant correlation between them [58], which suggests that AMY2 copy number has a limited effect on this enzyme. However, this does not necessary exclude a role for AMY2 in the association between CNV with amylase genes and body weight. Rather, it broadens potential mechanisms for this association from those involving solely salivary amylase to those that include pancreatic amylase too. Indeed, although we are not aware of any studies which have directly measured the production and activity of pancreatic amylase in the alimentary tract and compared it to AMY2 copy number, it has been shown to be correlated with levels of pancreatic amylase in the serum [10,58], which indicates that AMY2 copy number influences secretion of this enzyme by the pancreas, just as a role for AMY1 copy number in determining salivary amylase secretion has previously been identified [14, 15, 18, 21, 25, 28-31].

The limitations of qPCR as a method of measuring CNV mean that the results of any study which has used it to measure AMY1 copy number are subject to potential inaccuracy, including those of Falchi and colleagues [27, 56]. It is, however, possible that these studies still identified a true relationship: for example, Falchi et al. reported that their measurements using qPCR were reproducible [10], and reproducible miscalibrated measurements could still have detected a true

association [46]; it has also been shown that qPCR measures could still generate the relative pattern of overall results as produced by more accurate methods [22]. Nevertheless, for the reasons described above, their results must be interpreted with caution.

As outlined above, other techniques which are more accurate than qPCR for measuring gene copy number are available. Consistent with data from Falchi and colleagues outlined above [10], employment of such techniques has identified further associations between CNV within *AMY1* and obesity. In one study on Mexican children [59], which used dPCR, this association was apparently influenced by those participants with the highest *AMY1* CNV [27, 59], a high *AMY1* copy number being protective against obesity in this population [59]. No pre-dPCR enzyme digestion was performed on the DNA samples, which may have led to underestimation of *AMY1* CNV and therefore affected the validity of the results of this study [22]. Nevertheless, further evidence to support an association between CNV within *AMY1* comes from two studies which used the ddPCR technique, whose superior accuracy over qPCR has been validated [55]. The first, a study on Finnish participants who had a history of severe childhood obesity showed a significant inverse association between CNV within *AMY1* and both BMI and percentage body fat in obese females [60]. The second conducted two large case-control studies on French middle-aged adults and children [61]. Overall, a higher CNV within *AMY1* was associated with reduced risk for obesity, this association being particularly marked in the child population [61]. It has been hypothesized that genetic factors have a greater effect on BMI in children and adolescents than in adults [61-63], stemming from differences in gene-environment interactions at different ages [61-63]. Interactions between genes influencing BMI with environmental factors (diet and exercise primarily) are likely to be particularly strong during growth and development. This insight may explain the differences in association between CNV within *AMY1* and BMI between the child and adult populations outlined above [61]. Consistent with this hypothesis, other studies have shown isolated associations between CNV within *AMY1* and BMI in young people [53, 59, 61].

As with the qPCR technique, not all studies on the *AMY1* CNV using the ddPCR technique have shown positive results, and there remains some controversy within

the field. In one study reported to have used the ddPCR technique, as well as read depth techniques [21, 27], to measure CNV in three cohorts (over 4,000 participants), no association between CNV within either amylase gene (*AMY1* and *AMY2*) and BMI [27, 56] was shown. Other studies on East Asian and Swedish cohorts, which used a novel method combining qPCR and dPCR, and ddPCR respectively, also showed no such association [22, 64].

What can we conclude from these studies? Although direct comparison between them is difficult due to different techniques and different populations studied, lack of overall uniformity and consistency among data from all reported studies in this field has resulted inevitably in some controversy regarding the veracity of the association between CNV within *AMY1* and BMI. The findings of the first study to identify this association [10] have been contested due to the technical inaccuracies of qPCR techniques used to measure *AMY1* copy number, and the findings of other studies which used similar techniques must inevitably also be questioned [52, 53]. Several high-powered studies which have used more accurate techniques failed to find any association at all, despite having more than adequate power to do so [22, 27, 64]. It is possible (indeed likely) that the many genetic and environmental factors known to influence BMI throughout life have a diluting effect on association between CNV within *AMY1* and BMI. Nevertheless, a recent study which also had significant statistical power did find an overall association between *AMY1* copy number and lower obesity risk which was stronger in children **[61].** This finding was concordant with several other studies, albeit those with potential methodological inaccuracies [53, 59]. It appears that gene effects exerted during growth and development may have a particularly marked effect on the establishment of BMI, and this may well also be true of the association between *AMY1* CNV and obesity. However, it is clear that further high-powered studies are required in order to support this hypothesis, and determine the nature of this relationship in all populations with greater precision.

It should be emphasized that studies reported to date have focused primarily on *associations*. Such data provide little insight on causality or possible mechanisms. It seems reasonable to hypothesize, however, that differences in copy number of *AMY1*

have at least some effect on propensity for weight gain and development of obesity. On first glance, one may assume that such an association between CNV within *AMY1* and obesity is driven by enhanced breakdown of salivary starch and therefore increased absorption of sugar. It is perhaps surprising therefore, that the association data outlined above point in the opposite direction! It appears that low (rather than high) copy number of *AMY1* (and by implication *reduced* amount of salivary amylase, and *reduced* breakdown of starch) is associated with increased risk for obesity. Such an association, which is perhaps contrary to expectation, demands a rational and plausible physiological explanation. To address this question, the role of *AMY1* CNV on postprandial glucose control is explored in the next section.

CNV within *AMY1*, salivary amylase and glucose homeostasis

Differences in the efficiency of starch digestion have been hypothesized to explain the putative association between CNV within *AMY1* and obesity outlined in the last section [59, 61]. Indeed, the relationship between CNV within *AMY1* and metabolic state was initially explored regarding glucose homeostasis.

Mandel and colleagues showed that high salivary amylase concentration and activity (in turn associated with higher CNV within *AMY1*) were associated with enhanced reduction in viscosity of starch (both *in vitro* and within the oral cavity *in vivo*), indicating that salivary amylase has an important effect on starch digestion even before food is swallowed. Furthermore, salivary amylase concentration and activity correlated directly and significantly with *AMY1* CNV [15]. The hypothesis that enhanced oral breakdown of starch in individuals with increased levels of salivary amylase would in turn result in greater glycaemic load from a high-starch meal was refuted by ongoing research from the same group. Those individuals with higher salivary amylase levels in fact had significantly *lower* postprandial blood glucose responses to starch ingestion, and a more pronounced postprandial excursion of insulin within the first 9 minutes following the starch ingestion [32]. Insulin deficiency and resistance in the lower amylase group were eliminated given that both groups exhibited comparable blood glucose and insulin excursions following ingestion of a control glucose solution, absorption of which would not be expected to be influenced by the action of salivary amylase.

Given that blood glucose levels in both (high and low salivary amylase) groups did not start to rise until 15-minutes after starch ingestion, the implication is that the earlier observed rise in plasma insulin levels is 'pre-absorptive', and may therefore relate to 'pre-absorptive' or 'cephalic phase' insulin release (CPIR) [32]. CPIR usually occurs in anticipation of digestion just prior to or during food ingestion [32, 65, 66], in tandem with secretion of gastric acid [32, 65, 67] and pancreatic enzymes [32, 65, 68]. Cephalic phase responses (the anticipatory phase of digestion) that include CPIR prime the body for efficient digestion and assimilation of ingested nutrients and may facilitate prevention of dysglycemia and dyslipidaemia [14]. CPIR is required for normal postprandial glucose tolerance [14, 69] and mediated primarily by autonomic mechanisms triggered by oral sensory stimulation [69]. Absent CPIR results in higher postprandial glucose levels and impaired glucose tolerance in humans [32, 70].

Although both groups showed CPIR following glucose ingestion, only the high amylase group demonstrated CPIR following starch ingestion [32]. One possible mechanism is that starch breakdown products in the high salivary amylase group are detected in the oral cavity and trigger CPIR [32]. Such a mechanism may implicate carbohydrate activation of lingual taste receptors to release (through autonomic signalling) incretin hormones such as glucagon-like peptide-1 (GLP-1) [32], gastric inhibitory polypeptide (GIP) [13], peptide YY and pancreatic amylase [71, 72]. Other products generated from starch breakdown in the mouth, such as glucose/maltose or short-chain oligosaccharides, may bind to lingual taste and polysaccharide receptors respectively, and activate CPIR even when conscious sweet taste perception does not occur [32]. It should be emphasized that the hypotheses outlined here remain speculative. However, the fast response of CPIR in high salivary amylase individuals implicates a likely autonomic response resulting in incretin effects.

Data from other studies on the relationship between salivary amylase and postprandial glucose response to a starch-rich meal are variable. In one study, correlation between *AMY1* CNV and salivary amylase activity and concentration

was shown. Consistent with data from Mandel and Breslin [32], increased salivary amylase activity correlated with less pronounced postprandial blood glucose excursions and increased insulin levels, although this relationship failed to reach significance [18]. A further study which examined glycaemic response following ingestion of white rice in Asian participants, failed to demonstrate any association between salivary amylase activity (or *AMY1* CNV) and postprandial glycaemic response following a starch-rich meal [29]. Differences in starch preparations, power (based on numbers of participants) and timing of postprandial glucose and insulin measurements between studies may explain some of the apparent heterogeneity of data within the literature [18, 29, 33].

In a study on mice, a novel metabolic pathway for monosaccharides involving taste receptor cells (independent of the type 1 taste receptors 2 and 3 (T1R2/T1R3) which detect sweet taste [73]), which comprises GLUT glucose transporters, sodium glucose cotransporter 1 (SGLT1) and ATP-gated K+ channels (K_{ATP}), was identified [14, 73]. Although salivary amylase in mice is not thought to generate sufficient glucose to activate these novel pathways, membrane-bound disaccharidase enzymes are expressed in taste receptor cells [14, 74]. Locally-produced salivary amylase may generate high levels of oligosaccharides and disaccharides within the vicinity of taste receptors on the tongue, where disaccharidase enzyme activity produces monosaccharides that in turn may be detected by GLUT and SGLT1 transporters [14, 74]. Such a pathway may modulate CPIR in response to lingual sugar detection in mice [14, 75]. Although this pathway has not yet been identified in humans, it is conceivable that might be present.

In humans, salivary amylase may also be implicated in stimulation of the CPIR response and improved glucose tolerance following starch ingestion. Starch breakdown products may be detected in the oral cavity, by either or both the T1R-independent metabolic pathway and the polysaccharide receptor that can bind short-chain oligosaccharides [14, 32, 76]. CPIR triggered by elevated levels of salivary amylase may have provided an evolutionary advantage as starch consumption increased among human populations, thereby promoting *AMY1* CNV expansion within the population [32]. Individuals with lower *AMY1* CNV and

therefore low levels of salivary amylase, may be less well adapted to the digestive demands of a modern high-starch diets, with more pronounced postprandial glycemic excursions, impaired glucose tolerance and subsequent development of insulin resistance [32]. Consistent with this hypothesis, in one study on overweight and obese individuals, a high *AMY1* CNV was associated with lower fasting blood glucose levels [77].

The association between *AMY1* CNV and BMI is likely to be influenced by the amount of starch in the diet. In a large Swedish cohort, BMI actually *increased* significantly with increasing *AMY1* CNV in those with a high starch intake. In those individuals with the lowest *AMY1* CNV, there was a strong inverse association between starch intake and BMI [64]. Those with high *AMY1* CNV may be better adapted to starch digestion, and therefore could absorb a larger amount of glucose from the gastrointestinal tract, thereby contributing to weight-gain [64]. Conversely, those with low *AMY1* CNV are less adapted to digest starch, leading to reduced glucose uptake and increased postprandial satiety (due to undigested starch present in the bowel) [64, 78]. High starch intake in this group may therefore protect against weight-gain [64]. In addition, a further study which compared salivary amylase activity in normal-weight and overweight participants found that it was significantly higher in the overweight group, although there was no significant correlation between salivary amylase activity and BMI [79]; this is contrary to what one might expect from Mandel and Breslin's hypothesis [32]. However, the authors did acknowledge that other factors, including psychological stress and inflammatory status, could significantly affect salivary amylase levels. These studies suggest that the relationship between AMY1 CNV and glucose control is complex.

To complicate our understanding of this relationship to an even greater degree, more recent studies suggest that *AMY1* CNV may have a smaller effect on salivary amylase levels and activity than previously thought [21, 58, 80]. Using more accurate techniques to determine exact *AMY1* CNV than had been used in previous studies [15, 25], Carpenter and colleagues showed that whilst *AMY1* CNV does influence salivary amylase levels and activity, it was difficult to predict levels of

salivary amylase from *AMY1* CNV alone [58]. Given that there is a correlation between AMY1 and AMY2 copy numbers, it would seem logical that AMY2 could also have an effect on salivary amylase, but the same study found no significant correlation between either AMY2A or AMY2B and salivary amylase production and activity [58]. It remains possible that haplotypic structure of the amylase gene region has a more predictable effect on the expression of salivary amylase and, by extension, pancreatic amylase. This requires more research [58].

To summarize this section, the hypothesis that higher *AMY1* CNV, through increasing salivary amylase levels and activity, improves postprandial glucose control and metabolic status through CPIR is supported by data from both rodent and human studies as outlined above. Furthermore, the effect of *AMY1* CNV on postprandial metabolic status is likely influenced by the amount of starch in the diet. However, there remains some controversy in this field, with lack of uniformity of across all studies, including data on the role of *AMY1* CNV on influencing salivary amylase levels. Further studies are required to elucidate the precise mechanisms that link *AMY1* CNV with postprandial glycaemia status, particularly in relation to starch-content of the diet.

CNV within *AMY1* and obesity

Amylase within the serum (as opposed to amylase within the saliva) is composed of roughly equal proportions of salivary and pancreatic isoforms [10, 14, 81, 82]. Overall levels of serum amylase may therefore be affected by derangements of either one, or both of these isoforms [81]. Low serum amylase levels (resulting from, for example severe chronic pancreatitis [81]), associates with metabolic abnormalities, Diabetes Mellitus (DM) and obesity [14, 81]. As with salivary amylase, levels of serum amylase correlate with CNV of *AMY1* and *AMY2* [10, 17, 60, 61].

A significant inverse correlation between serum amylase levels and BMI was first identified in 1988 [83]. Interestingly, serum amylase levels in obese participants increase following weight-loss. Further studies have replicated inverse correlations between serum amylase levels and BMI [60, 81, 84-89], impaired glucose tolerance

[84], insulin resistance [60, 87, 89], DM [88], waist circumference and percentage body fat [60, 86], and a positive correlation of serum amylase levels with high-density lipoprotein (HDL) cholesterol [84]. Although most of these studies were on Asian populations, others (that have also included assessment of salivary and pancreatic origins for serum amylase levels) have shown comparable results in European populations for each amylase origin [10, 60].

In one study which examined the associations of plasma amylase activity and metabolic traits in a large French population, the former was found to be associated inversely with BMI and plasma fasting glucose levels, and directly with pancreatic ß-cell function [61]. Using both a Mendelian randomization analysis and prospective data collected after 9 years of follow-up, the authors found a causal negative effect of BMI on plasma amylase activity; when they used a linear regression model to investigate the inverse relationship, the effect of plasma amylase activity on change in BMI, over the follow-up period, the salivary component was found to significantly contribute towards increased weight loss. The relationship between plasma amylase activity and BMI may therefore be bi-directional in nature, with each having a negative effect on the other [61].

There may also be sex-differences in the effects of *AMY1* CNV on serum amylase levels and BMI. In the study mentioned above, in which the relationship between AMY1 CNV and BMI was investigated in Finnish participants who had a history of severe childhood obesity, a linear regression was performed to examine the association between *AMY1* CNV and serum amylase concentration. It was found that a single *AMY1* copy had an effect on serum amylase that was almost three times greater in obese women than in obese men, suggesting that AMY1 copies may actually be less functional in obese men. This hypothesis is concordant with the other main finding of this study, that there was a significant inverse association between AMY1 copy number and BMI in obese females alone, and not in obese males or normal-weight controls [60].

It is known that insulin, delivered via a vascular portal route from the pancreatic islet cells directly to the acini [90], regulates pancreatic acinar function and

actually stimulates secretion of pancreatic amylase and other exocrine products [14, 90, 91]. Low serum amylase levels may therefore result from impaired insulin secretion [14, 81, 89] or insulin resistance [89, 92], as occurs in both obesity and type 2 DM [14, 89, 92]. Insulin may also influence release of salivary amylase [14, 93,94], although this mechanism remains incompletely understood [14].

A further mechanism linking serum amylase levels with BMI relates to pancreatic fat deposition. Fatty pancreas is associated with low serum amylase, obesity and markers of metabolic syndrome [95, 96]. It has been suggested that infiltration of the pancreas may cause damage that impairs pancreatic amylase production and release [88, 95]. To lend further weight to this hypothesis, fatty pancreas associates with fatty liver and non-alcoholic fatty liver disease (NAFLD) [96, 97], and an association between NAFLD and low serum amylase levels has also been demonstrated [81, 97, 98]. This suggests that these metabolic processes may all be linked [97]. In addition, it has been shown that pancreatic enzyme deficiency, as defined by low faecal elastase-1 levels, is found in around 50% of patients with type 1 diabetes mellitus and 20% of patients with type 2 diabetes mellitus [87, 99, 100]. These findings provide further evidence of the links between the exocrine and endocrine functions of the pancreas and that these metabolic disease processes cause damage to the pancreas.

As with serum amylase levels and BMI, the relationship between serum amylase levels and lipid profile may be bi-directional. In a study which compared the metabolomic signatures of female participants with lower *AMY1* CNV (four or fewer copies) to those with higher *AMY1* copy numbers (eight or more copies), the low *AMY1* CNV group had higher serum levels of dicarboxylic fatty acids, which indicates up-regulation of ω-oxidation of fatty acids, and lower concentrations of several medium- and long-chain fatty acids [17]. This was indicative of more active uptake and oxidation of fatty acids. Levels of 2-hydroxybutyrate, which is noted to be a biomarker for insulin resistance [101], were also higher those with low AMY1 CN, while serum glucose was also slightly increased. Considered as a whole, these results indicated 'a pattern of reduced cellular glucose uptake, and a consistent metabolic shift toward lipid exploitation' in the women with low *AMY1* CNV [17].

Serum amylase may also function as a cardio-protective factor, in a similar way to the insulin-sensitising, anti-inflammatory and anti-atherogenic functions of adiponectin [85, 86, 102], and has even been shown to correlate with serum adiponectin in Japanese women [86, 103]. In this way, serum amylase may protect against the adverse metabolic features of obesity and T2D [86]. However, this remains speculative, and further studies are required to explore this interesting hypothesis further.

From the above it is clear that close links exist between insulin and amylase release. As outlined, insulin may drive both pancreatic and salivary amylase production, thereby preparing the body for digestion. Salivary amylase release may in turn augment insulin release via CPIR in the oral cavity, and may therefore also augment pancreatic amylase release; as far as the authors are aware, there is as yet no significant evidence for any effect of pancreatic amylase on salivary amylase. Serum amylase may have endocrine effects on metabolism by increasing insulin sensitivity. These mechanisms demonstrate the existence of an 'amylase-insulin axis', whereby both affect the secretion of the other. It seems likely that the efficacy of the protective metabolic mechanisms involving the amylase-insulin axis is enhanced by copy number expansion of the amylase genes (or at least the specific haplotype arrangement of the amylase gene region). Ectopic fat deposition in the pancreas (as occurs in T2D and obesity) is likely to impact negatively on the efficient functioning of the amylase-insulin axis. Finally, it should be noted that serum amylase levels are influenced by many other factors that include psychosocial stress [81, 104], diet [18], hydration levels [18], renal function [81, 105], and certain medications used for T2D including dipeptidyl peptidase-4 inhibitors and glucagon-like peptide 1 receptor agonists [81].

It seems logical that the identification of a low serum amylase level has multiple potential clinical applications. It has been suggested that it is helpful in the diagnosis of chronic pancreatitis [106], but given its associations with obesity, impaired glucose tolerance and insulin resistance, it is feasible that it could be a useful tool in the recognition and monitoring of other metabolic abnormalities. The

measurement of serum amylase is an affordable test [86] and low levels could represent a biomarker of pancreatic ß-cell function [89] and be used to identify T2D in asymptomatic patients [86, 88]. However, these potential uses are problematic. First of all, the cut-off point for low serum amylase has yet to be clearly defined because its clinical use has so far been limited and measurement techniques differ [81]. Secondly, as mentioned above, serum amylase levels are affected by other factors: for example, its level is increased in patients with renal dysfunction due to reduced excretion, which means that a low amylase level in a patient with chronic kidney disease could appear normal [81]. Thirdly, as described above, the pathophysiological mechanisms that cause serum amylase to be low are complex and have yet to be fully explained, and further studies are needed to improve our understanding of them [87]. Lastly, as far as we are aware, no studies have yet examined whether it is useful to measure the individual pancreatic and salivary components of serum amylase, or just the total level. More work is therefore required in order to determine whether testing for low serum amylase is an effective means of identifying and monitoring these metabolic diseases [84, 87].

Taken together, it is not surprising that the field of amylase research and its links with metabolic health remain controversial, and studies on the effects of amylase are inherently difficult to execute and interpret due to the complexities highlighted.

Concluding remarks

Amylase is an enzyme that acts primarily within the alimentary tract (mouth and ileum) to digest starch into disaccharides and monosaccharides that can be absorbed and assimilated. It is interesting that amylase is released into the serum from both salivary and pancreatic sources. Contrary to alimentary amylase, the role of *serum* amylase seems enigmatic, although evidence presented in this review reveals an amylase-insulin axis, providing integration between dietary signals (primarily starch content of food), and metabolic status (primarily insulin release). The precise mechanisms involved have yet to be elucidated, although amylase and insulin release appear to be influenced directly by each other.

Furthermore, autonomic signals may provide an 'early warning' for insulin release during and just prior to mastication of food via CPIR, and this process is likely to involve salivary amylase.

It is plausible (although difficult to prove) that the expansion of the CNV within *AMY1* was driven by a need for increased levels of salivary amylase, in turn driven by a need to digest more starch, at a specific time in early human development. As outlined, there appears to be association between CNV within *AMY1* and both postprandial glycaemic control and BMI, although there is lack of uniformity of data among studies. Higher CNV within *AMY1* appears to associate with a more favourable postprandial glycaemic and metabolic status. The underlying mechanism for this association may implicate CPIR and early insulin release following starch ingestion, with this response influenced by salivary amylase level, which in turn is influenced by CNV within *AMY1*. The negative association between CNV within *AMY1* and BMI however is more difficult to explain. Obesity is associated with impaired beta-cell insulin release, insulin resistance and ectopic pancreatic fatty infiltration, each of which would be expected to impact negatively on pancreatic amylase release. This is one explanation for an inverse relationship between serum amylase levels and BMI. However, this does not explain the inverse relationship between CNV within *AMY1* and BMI. It remains possible that serum amylase (in turn influenced by CNV within *AMY1*) have hitherto unknown effects on propensity for weight gain, and this should be a focus for future research.

The potential metabolic effects of human amylase, their interactions and their protective properties are graphically displayed and summarised in Figure 1 and Table 1 respectively.

Expert commentary

The amylase field is complex. Amylase is encoded **by** multiple genes (divided broadly into salivary and pancreatic), each of which has many copies, the precise number being variable within the population. The copy number of each amylase gene likely influences its expression and the levels and activity of amylase in both

the alimentary tract and serum. Production and release of amylase is also likely to be influenced by epigenetic factors, the nature of which is incompletely understood. There may be age- and sex-effects on the relationship between copy numbers of the amylase genes and amylase levels. Amylase is itself influenced by other factors that include insulin, and amylase may also have both direct and indirect (possibly via autonomic signals) effects on insulin release. Associations between both amylase levels and copy number of the amylase genes versus postprandial metabolic status and BMI also likely pertain. These associations are likely affected by diet (primarily by starch content of food). Although the precise role of serum amylase is incompletely understood, it may play a role in the interplay between digestion and metabolic status through the amylase-insulin axis. This may explain why low serum amylase levels are associated with higher BMI and worse metabolic status. The link between low CNV of *AMY1* and obesity however is more difficult to explain.

More accurate characterization of the amylase gene region has revealed that variation in copy number may be a product of specific *haplotypes*, rather than a continuum in its own right [27, 46, 58]. Association between *AMY1* CNV and BMI may therefore be more related to haplotypes than to copy number *per se* [56]. Whilst no study has yet found an association between *AMY2* CNV and BMI, the correlation between the CNV of *AMY1* and *AMY2* implies that indirect effects of *AMY2* CNV on BMI may exist [46]. Further large-scale studies, measuring amylase gene CNV and identifying specific haplotypes with accurate techniques (including ddPCR) are required to untangle the complex underlying relationships between the amylase gene region, amylase levels, metabolic status and BMI, and how these relationships are influenced by age and sex and other, perhaps environmental and dietary factors.

Five-year view

When one considers how the study of the amylase enzymes and their gene region has evolved over the last five years, and the complexity of the metabolic relationships which form the basis of this field, it seems challenging to predict exactly how it will progress over the next five. Nevertheless, there are several

promising research avenues that have yet to be explored in detail and have the potential to broaden our understanding, including interaction between the amylase gene region and gut microbiota. In a study which measured change in body fat percentage in mice fed a high-fat, high-sucrose diet, a SNP near the amylase gene region was found to be significantly associated with increased body fat growth [10, 59, 61, 107]. This locus also associated with significant enrichment of gram-negative *Enterobacteriaceae* bacteria within the gut [107], previously shown to predominate in the guts of overweight and obese children [10, 61, 108]. Metabolic and digestive effects of amylase levels (influenced by amylase gene copy number) may therefore be mediated via gut bacteria [59, 61]. This could be related to flux of undigested starch into the colon, which may be more likely in those with low amylase gene copy number and resulting low amylase activity, and as such increased fermentation by the gut microbiota with production of short chain fatty acids (SCFA) [109]. Although diet-induced increases of SCFA have been shown to have beneficial metabolic effects in relatively short-term studies mainly in animal models [110], long term diet-induced increases of SCFA appear to significantly contribute to energy intake both in humans and rodents [111, 112], with a contribution of up to 10 percent to energy intake even in humans [113]. Therefore, increased SCFA production related to non-digested starch may contribute to weight gain in the long term, thereby outweighing the short term metabolic benefits of starch-induced increased colonic fermentation. Furthermore, increased plasma salivary amylase activity is associated with higher plasma levels of lactate [61], which is itself produced by fermentation of complex carbohydrates within the gut [61, 114]. A higher lactate-butyrate ratio may reduce the risk of weight gain and obesity through interaction with gut microbiota [61, 114]. Since alterations of gut microbiota may represent a potential future therapeutic strategy for insulin resistance and obesity [59, 115], their interactions with amylase and the metabolic sequelae of such interactions should be a focus for future research.

Other areas for future investigation include the expression of amylase within the liver, increased levels of which have been associated with obesity in mice [19]. Expression of amylase within adipose tissue, in which *AMY1* is one of the most highly expressed genes [10], also merits further investigation. Paracrine effects of

amylase within adipose tissue may shed insight into the links between *AMY1* CNV and propensity for weight-gain. To the best of our knowledge, no study has yet examined the relationship between the amylase genes and other genetic loci which have been associated with obesity and T2D such as *FTO*, *MC4R*, *CDKAL1*, *KCNQ1* and *KLF9* [3, 10, 51, 116, 117]: such research could help us to understand how these genes interact with each other to alter the risk of metabolic disease, and whether their mechanisms are related. Finally, the amylase-insulin axis needs further exploration, particularly regarding the potential for amylase to stimulate pancreatic insulin release (either directly or via CPIR); this research may also help us to understand if, and how, we can use low serum amylase levels in clinical practice. Looking beyond the next five years, such insight could be used to develop future novel therapeutic strategies for T2D, in which pancreatic insulin release is optimized within the postprandial phase, thereby improving overall glycaemic control. Such a therapy could be targeted particularly in patients known to have low CNV within *AMY1*, and low salivary and serum levels of amylase, who may benefit from a targeted approach to improve postprandial insulin release. It could also be complementary to current therapies that target (amongst other things) pancreatic insulin release such as the GLP-1 analogues.

Obesity is the most important threat to our health, and prevention and effective management of weight-gain and obesity should be prioritized. Further study of the amylase genes and the association of their copy numbers with metabolic status and BMI will provide invaluable insight into the genetic determinants of metabolic dysfunction and BMI; and may shed light on some of the missing heritability of the latter. Insight into predisposition to future development of T2D and weight-gain, independent of specific diet and lifestyle, may also be gained from such focused study. In short, future focus on amylase research has much fruit to bear and will hold important insights into the complex interlinks between diet, digestion, metabolic health and BMI that in turn will provide rationale and inspiration for future novel therapeutic developments that may help to fight the obesity epidemic.

Key issues

- In humans, the amylase enzyme occurs in two distinct forms: salivary (produced by the salivary glands) and pancreatic (produced by the exocrine pancreas). These are encoded by *AMY1* and *AMY2* genes respectively.
- Human salivary and pancreatic amylase genes exhibit extensive copy number variation.
- Increased *AMY1* copy number, which could lead to increased salivary amylase levels, has been linked to reduced risk of obesity and insulin resistance.
- Salivary amylase may act to improve glucose tolerance and reduce the risk of insulin resistance by activating early 'cephalic phase' insulin release, thereby reducing post-prandial glycaemia.
- However, copy number variation of the amylase gene region is notoriously difficult to measure and these findings have been questioned. The relationship between the amylase gene region and salivary amylase may be more strongly mediated by haplotype arrangement, rather than absolute copy number.
- Serum amylase is composed of roughly equal proportions of salivary and pancreatic isoforms, whose respective levels are influenced by *AMY1* and *AMY2* copy number and insulin. Low serum amylase levels have been associated with metabolic abnormalities, Diabetes Mellitus and obesity.
- Serum amylase may reduce the risk of metabolic abnormalities by increasing insulin sensitivity.
- Further research is needed in order to further elucidate the functional properties of the amylase gene region, the nature of the amylase-insulin axis, and the way in which they may interact with the gut microbiota. Improving our understanding of this area could lead to future novel therapies for metabolic abnormalities.

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