

Glycemic Response after Starch Consumption in Relation to Salivary Amylase Activity and Copy-number Variation of *AMY1* Gene

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Abstract Salivary amylase activity is partially determined by genetic factors and is possibly related with postprandial plasma glucose levels. The aim of this study is to evaluate the association of salivary amylase activity with plasma glucose and insulin levels after consumption of a gelatinized starchy model food (80% amylopectin; 70% gelatinization), as well as to assess the influence of the salivary amylase gene (*AMY1*) copy-number variation in amylase activity and concentration. Our results show a strong and significant relation between copy-number variation of *AMY1* gene measured through qPCR with salivary amylase concentration, with an enhanced correlation with amylase activity when corrected by salivary flow ($r = 0.83$, P -value = 0.003). Subjects with high salivary amylase activity tend to have a higher early increase in plasma insulin concentration and a lower glycemic response after starch ingestion compared to subjects with low salivary amylase activity, although these observations did not achieve statistical significance ($r = 0.41$; P -value = 0.23). In conclusion, we found a strong association between copy-number of *AMY1* gene with salivary amylase activity and concentration. However, we did not find evidences for a major role of salivary amylase activity on glycemic response after starch consumption.

Keywords: amylase, copy number variation, starch, glycemic response

Cite This Article: Gigliola Alberti, Javier Parada, L. Rodrigo Cataldo, Javier Vega, Concepción M. Aguilera, Ana I. Alvarez-Mercado, M. Isabel Hodgson, Alberto López, Isidora Angellotti³, Angel Gil, and José L. Santos, "Glycemic Response after Starch Consumption in Relation to Salivary Amylase Activity and Copy-number Variation of *AMY1* Gene." *Journal of Food and Nutrition Research*, vol. 3, no. 8 (2015): 558-563. doi: 10.12691/jfnr-3-8-11.

1. Introduction

Salivary α -amylase, a calcium metalloenzyme (EC 3.2.1.1), is involved in the digestion of starch in the oral cavity. This enzyme is encoded by the *AMY1* gene, which is known for showing extensive Copy-Number Variation (CNV) [1,2]. Interestingly, a tight and direct correlation has been found between the copy-number of *AMY1* gene and the protein concentration in saliva, suggesting a plausible direct effect of *AMY1* CNV on starch digestion efficiency at oral level [3,4].

There have been several proposed hypotheses regarding the effect of *AMY1* CNV gene on energy and glucose homeostasis [2]. Recently, a controversial link between CNV of *AMY1* gene and obesity was observed in a large epidemiological study [5]. However, methodological limitations regarding the correct measurement of CNV of *AMY1* gene were subsequently reported, indicating a possible lack of genotype-phenotype association [6,7]. Regarding the effect of salivary amylase activity (and

consequently, CNV of *AMY1* gene) on glycemic response after starch consumption, it was described that swallowing food without chewing could be a simple way to reduce postprandial glycaemia after the consumption of high-starch meals [8]. Interestingly, Mandel and Breslin [9] showed that subjects with high endogenous α -amylase levels (carrying high number of copies of *AMY1* gene) showed reduced postprandial glucose levels after starch consumption compared to subjects with low-copy number *AMY1* genes. According to these authors [8], this effect could be attributed to an increased cephalic-phase insulin release in subjects with constitutively high salivary amylase levels. These results are in contrast to the common assumption that the action of salivary α -amylase is not relevant from physiologic point of view, since it is only restricted to oral cavity. Then, it is believed that salivary amylase has a relatively modest effect on starch hydrolysis compared to pancreatic α -amylase or maltase-glucoamylase activities in the small intestine [10]. However, there are also some evidences suggesting a role of salivary α -amylase on starch digestion given that there is a significant passage of salivary α -amylase through

stomach due to an incomplete inactivation by low pH [11]. Moreover, the importance of CNV of *AMY1* gene is illustrated by the apparent selective pressure imposed by dietary starch during human evolution [3].

The aim of this study is to evaluate the association between salivary amylase activity with the glycemic response after consumption of a gelatinized starchy model food (80% amylopectin; 70% gelatinization), as well as to assess the influence of the salivary amylase gene (*AMY1*) copy-number variation in salivary amylase activity or concentration.

2. Subjects and Methods

2.1. Study Design and Subjects

Using a crossover design, $n = 10$ male adult volunteers, 20.6 ± 0.97 years old with normal-weight (Body Mass Index: 22.74 ± 1.36 kg/m²) were scheduled to visit our clinic early in the morning after an overnight fast (8-12 h.) in two occasions one week apart. In the first visit, participants carried out an Oral Glucose Tolerance Test (OGTT) with 50 grams of glucose. In the second visit, an Oral Meal Test (OMT) with 50 grams of gelatinized starch food was carried out. This study was approved by the Ethics Committee of the School of Medicine (Pontificia Universidad Católica de Chile).

2.2. Serum Glucose and Insulin

Venous blood samples were collected before and after the oral consumption of glucose/starch (basal and 15, 30, 45, 60, 90 and 120 minutes after oral ingestion). Blood samples were centrifuged 20 minutes after blood extraction to allow clotting, and serum was collected and stored at -80°C prior to analysis. Serum insulin levels were measured by radioimmunoassay using the Coat-A-Count insulin RIA Kit (DPC, Los Angeles, USA). Serum glucose levels were measured using the glucose-oxidase GOD-PAP colorimetric method.

2.3. Biochemical Measurements in Saliva

Immediately before the oral glucose or starch tests, a sample of unstimulated whole saliva was collected by passive drool was processed, centrifuged and stored at -80°C until analysis [12]. The salivary flow was determined as a function of the amount of saliva generated during three minutes of passive drool. We quantified α -amylase activity in saliva using a colorimetric assay (<http://www.salimetrics.com>) (1-1902 5PK 1-1902-5) according to manufacturer instructions. The amount of α -amylase protein was measured with western-blot techniques [3] using anti- α -amylase antibodies (Sigma A8273), a secondary antibody (Sigma A0545) and a standard of human amylase (Sigma A1031). Quantification of protein bands after luminol incubation was carried out using the ImageJ software (<http://www.rsbweb.nih.gov/ij>). An ELISA test for the detection of transferrin was carried out (1-1302 5PK 1-1302-5) in order to rule out a possible contamination with blood. This test yielded a mean of 0.9 ± 0.5 mg/dL, indicating that there is no significant blood contamination that could affect salivary amylase activity and concentration measurements.

2.4. Determination of Copy-number Variation of *AMY1* Gene

Copy-number variation of *AMY1* gene was measured following the procedure described by Perry et al. [3] and Santos et al. [2]. DNA was extracted from buffy coat with the QIAamp DNA kit (QIAGEN 51106). Genomic DNA concentration was fluorimetrically quantified (Qubit quantitation platform, Invitrogen) while DNA integrity was visualized in 1% agarose gels. Quantitative Polymerase Chain Reaction (qPCR) was used to estimate copy-number of *AMY1* gene in a Real-time Thermal-cycler Agilent Stratagene MX3000P (Agilent). We used specific primers to amplify human *AMY1* (target gene) and TP53 (control gene) in a PCR mixture containing SYBR Green I as DNA binding dye in a final volume of 15 μ L. A human DNA sample NA18972 (Coriell Institute; <https://ccr.coriell.org/>) having 14 *AMY1* copies was used as a calibrator [3]. The calculation of threshold cycles in DNA samples (*AMY1* and TP53 gene) allowed the estimation of the absolute copy-number of *AMY1* gene [2]. Measurements were carried out in triplicate both for *AMY1* and TP53 genes. Threshold cycles of qPCR showed very high levels of intra-assay repeatability both in *AMY1* and TP53 genes (CV = 0.33% and 0.83% respectively). Estimated PCR efficiencies using *AMY1* and TP53 primers ranged from 1.84 to 1.99 ($R^2 > 0.99$).

2.5. Preparation of the Starchy Model Food

First, 200 ml of distilled water was equilibrated in a bath at $58.4 \pm 2^\circ\text{C}$. Then, A total of 50 grams of potato starch (20% amylose and 80% amylopectin) were suspended in 50 ml of distilled water at room temperature. Next, the suspension of starch was added to 200 ml of distilled water previously equilibrated in a bath at $58.4 \pm 2^\circ\text{C}$, heating and stirring carefully to reach 58.4°C and keeping the mixture at this temperature for 15 minutes. This procedure yields a degree of gelatinization of 70% measured by Differential Scanning Calorimetry (DSC) and Polarized Light Microscopy (PLM), according to the protocol described by Parada and Aguilera [13]. Finally, the gelatinized starch was taken to a water bath at room temperature until cooled to 30°C , which was achieved in about 10 minutes. Then, samples were cooled to 4°C during 12 hours before serving.

2.6. Statistical Methods

Intra- e inter-assay coefficients of variation regarding the measurement of copy-number of *AMY1* gene, as well as reproducibility values were computed according to Bland and Altman [14]. Associations among numerical variables were analysed using the non-parametric Spearman correlation coefficient. Based on data of Perry et al [3], a sample size of $n=10$ would provide adequate statistical power (>80%) with a confidence of 95% to find a significant association between copy-number variation of *AMY1* gene with salivary amylase activity/concentration.

The incremental Area Under the Curve (AUC) was calculated for plasma glucose and insulin levels using the trapezoidal method during the OGTT and the OMT. The Glycaemic index was calculated as the ratio of AUC for plasma glucose during the OMT divided by the corresponding AUC during the OGTT. The Insulinogenic

Index at 15 minutes was calculated as the ratio of insulin plasma concentration at 15 min minus fasting insulin divided by the difference of plasma glucose at the same interval time. Statistical analyses were carried out with STATA 9.0 software (<http://www.stata.com>) and MedCalc 14 (<http://www.medcalc.org>).

3. Results

Figure 1 shows the association between CNV of *AMY1* gene and salivary amylase activity. A significant direct correlation was found between copy number of *AMY1* gene with amylase concentration or activity (Figure 1A and Figure 1B). After correcting for salivary flow, the correlation between copy numbers of *AMY1* gen with

amylase activity was even more significant (Figure 1C). Figure 1D shows that, as expected, salivary amylase activity is strongly correlated with its concentration in saliva. These results confirm that salivary amylase activity and concentration is influenced by CNV of *AMY1*. We also evaluated the reproducibility of *AMY1* copy-number determination in two different days (one week apart) using the same DNA samples, the same calibrator (human sample NA18972), as well as same equipment and operator. In this reproducibility study, we achieved an inter-assay coefficient of variation of 7.8%. The reproducibility value, indicating the 95% of observed differences in the copy-number of *AMY1* calculated in two separate days, was estimated in two copies.

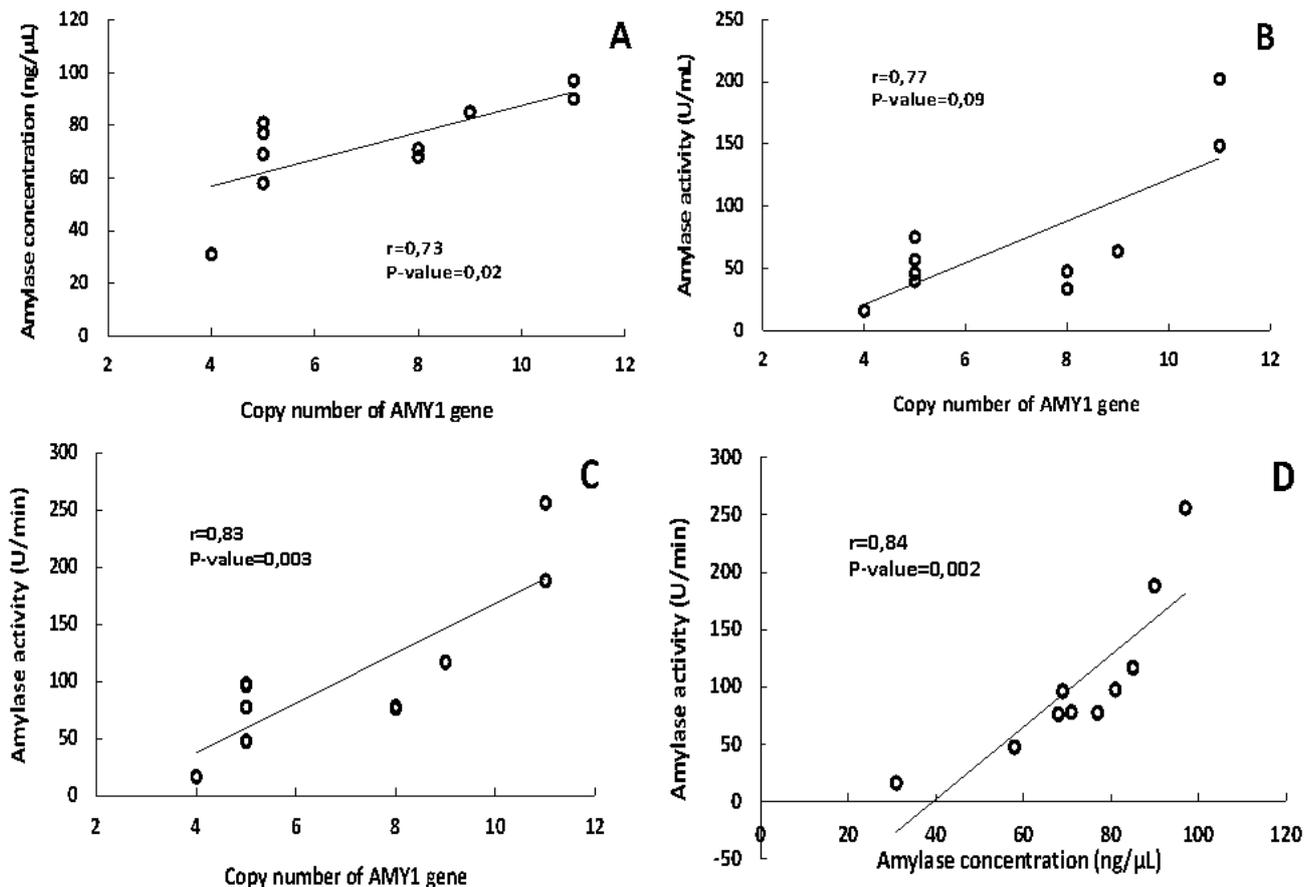


Figure 1. Association between copy-number of *AMY1* gene with salivary amylase activity/concentration: (A) copy-number of *AMY1* gene versus amylase concentration; (B) Copy-number of *AMY1* gene versus amylase activity; (C) Copy-number of *AMY1* gene versus amylase activity corrected by salivary flow; and (D) Association between amylase concentration and activity in saliva

No significant associations were found between copy-number *AMY1* gene with the area under glycaemic curve, the glycaemic index, the Insulinogenic Index at 15 min. or the insulin change from baseline to 15 min after the start of starch consumption. Figure 2 shows the correlation analysis between salivary amylase activities with variables related to postprandial changes of plasma glucose and insulin levels after glucose and starch consumption. Both area under glycaemic curve (Figure 2A) and the glycaemic index (Figure 2B) tended to diminish when amylase activity increased, without achieving statistical significance. Figure 2C and Figure 2D show that changes of amylase activity are not statistically associated with increased postprandial insulin plasma levels measured as

Insulinogenic Index and insulin change from baseline (both calculated at 15 min after start of consumption).

4. Discussion

Starch is a complex carbohydrate naturally occurring in plant food, especially in tubers, legumes, and cereals, and is the one of the main sources of dietary energy for human beings worldwide [14]. Its digestion is initiated by the salivary α -amylase encoded by *AMY1* genes in the oral cavity [10], followed by the action of two pancreatic α -amylases (encoded by *AMY2A* and *AMY2B* genes) acting in the small intestine. The process of starch digestion leading to free glucose is completed by enzymes of the

small intestine brush border. Although salivary α -amylase plays an important role in an early breakdown of starch components, its action on starch digestion is relatively unimportant compared to the effect of the pancreatic α -amylase, due that salivary α -amylase is a short-lived action enzyme that is inhibited by the low pH in the stomach [11]. However, it has been also suggested that salivary α -amylase activity may affect the postprandial glycemic response following mechanisms not related with starch digestion/absorption into gut, but possibly affecting the cephalic phase of insulin secretion [9]. Additionally, salivary amylase levels may be related to different health outcomes such as glycemic response after starch consumption, modulation of the action of α -amylase inhibitors, taste perception, satiety, psychosocial stress and oral health [2].

Regarding the variation of *AMY1* gene copy-numbers, it has been proposed that current *AMY1* copy-number in humans could be the result of a positive selection during evolution, given that *AMY1* copy-number is higher in populations evolved under high-starch diets versus *AMY1* copies in populations evolved under low-starch diets [3]. In Chile, it has been previously described a wide variation in CNV of *AMY1* gene [2]. In our laboratory, we have found an adequate, although imperfect, reproducibility of copy-number of *AMY1* determination when DNA samples were processed in two different days. Interestingly, Falchi et al. [5] recently investigated the effect of *AMY1* gene dosage through qPCR on adiposity, finding that a low copy number of *AMY1* is related with a significant higher risk of obesity. Subsequently, methodological limitations regarding the correct measurement of CNV of *AMY1* gene were reported, indicating a possible lack of CNV-obesity association for this gene [6,7]. In any case, qPCR is still a useful assay in determining CNV of *AMY1* in many contexts, and it has been widely used in the literature for this purpose [2,3,4,5,9], with a demonstrated discriminatory capacity to distinguish subjects with high or low endogenous amylase activity [9]. However, recent techniques such as droplet digital PCR are more accurate when dealing with complex CNVs that are characterized by highly clustered genic structures [7]. As a limitation in our study, we assume some possible bias in our CNV *AMY1* measurements obtained through qPCR, and also report a note of caution regarding additional measurement errors due to lack of complete reproducibility. In this context, although the determination of copy-numbers of *AMY1* by qPCR is probably not completely accurate in complex CNV, it still may serve well to rank categories of subjects according to their copy-number variation of *AMY1* gene, being a useful variable to define groups with different salivary amylase activity. We also have found that *AMY1* gene copy-number shows strong association with amylase activity and concentration, as it was previously described [3,4]. As expected, we also have found that salivary amylase activity and amylase concentration are tightly associated, suggesting that both variables provide essentially the same information in our healthy subjects.

Several factors such as previous diet, psycho-social stress and level of hydration may affect salivary amylase activity or concentration apart from copy-number of *AMY1* gene. In our opinion, salivary amylase activity is the most adequate variable for testing the effect of salivary

amylase on postprandial glycemic response. In this context, salivary amylase is easier to measure than salivary amylase concentration, which is assessed through western-blots (a more laborious test that only provides semi-quantitative outcomes of salivary amylase content). Additionally, salivary amylase activity reflects the current capacity of salivary amylase, better than using *AMY1* copy-numbers, that measure constitutive low or high levels rather than current levels.

Regarding the association between salivary amylase activity and glycemic response, our results discard a major role for salivary amylase activity in determining postprandial glycaemia. However, although not significant, our results are still concordant with the hypothesis of a minor effect of high salivary amylase in increasing serum insulin levels in the first minutes after starch consumption. This early increased insulin secretion might partially explain the lower glycemic response (AUC for glucose) previously observed in subjects with higher endogenous salivary amylase activity [9]. In this context, Figure 2 suggests that subjects carrying high amylase activity tend to have a lower area under 120-minute glycemic curve (Figure 2A), a lower Glycemic Index (Figure 2B), and a higher plasma insulin concentration early after the first 15 minutes starch ingestion (Figure 2C and Figure 2D) compared to subjects with lower salivary amylase activity levels. However, we were unable to confirm or discard such hypothesis given the low sample size of our study. On the other hand, the process of mastication both increases the time of food in the oral cavity and amylase action. In connection with this concept, conflicting results are published regarding the postprandial blood glucose concentration upon thorough mastication of a meal [8,16]. Using a liquid meal containing starch, Mandel & Breslin [9] observed that insulin secretion occurs early after starch consumption, being this early secretion enhanced in subjects with higher salivary amylase activity. These authors also found that subjects with higher amylase activity showed significantly lower area under the postprandial blood glucose curve, lower peak blood glucose concentrations and higher serum insulin concentrations than baseline during the preabsorptive period (<10 minutes after starch ingestion) compared to subjects with low salivary amylase activity. Then, it was proposed that the release of starch digestion products in the oral cavity may signal the body to prepare for incoming starch through vagal activation, resulting in an early insulin release termed preabsorptive insulin or cephalic phase of insulin secretion [9,17,18,19]. Interestingly, Butterworth et al. [20] suggest that sweet receptors in the gastrointestinal tract may detect maltose derived from starch, possibly affecting intestinal peptides release that influence Central Nervous System (CNS)-mediated control of gastric emptying, insulin secretion and appetite. Moreover, it has been observed that starch hydrolysis in the mouth also depends on the initial structure of the food as well as in the breakdown of solid food [10], suggesting that the food microstructure could also affect the cephalic phase of insulin secretion. Additionally, the glycemic response also depends of the type of starch (slowly versus rapid digestible) that is consumed [21,22].

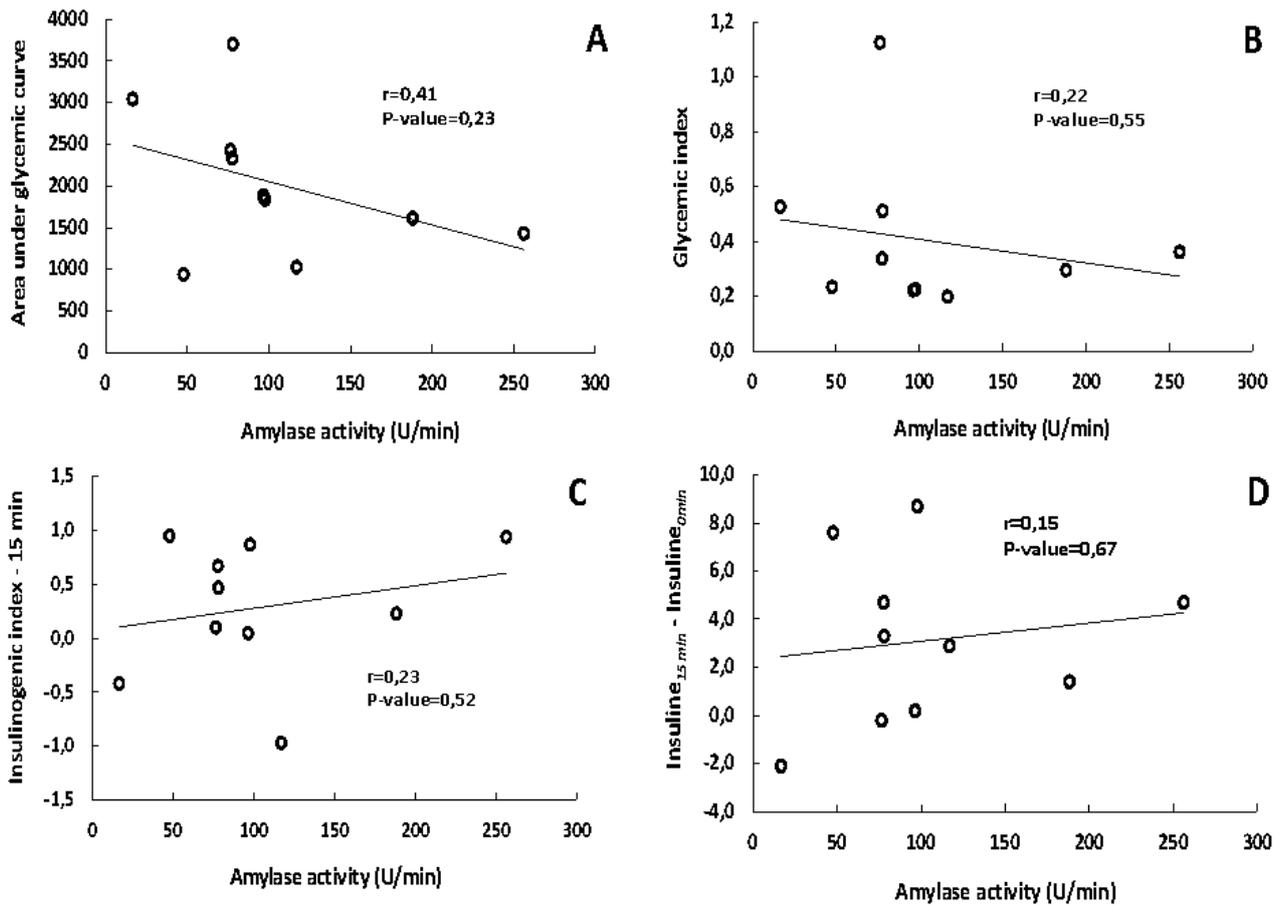


Figure 2. Association between salivary amylase activity on glycemic response after starch consumption: (A) Amylase activity versus postprandial area under glycemic curve; (B) Amylase activity versus glycemic index; (C) Amylase activity versus Insulinogenic Index (at 15 min after consumption starting); and (D) Serum insulin levels change at 15 min after starch consumption

Our study has several limitations: first, we have not quantified very-early serum insulin levels after starch consumption, since the first postprandial sample was drawn at 15 minutes. Second, we have not taken into account the effect of differences in the time/intensity of mastication among subjects, although all of them finished chewing and swallowing the starch within 10 minutes. As mentioned before, the small sample size clearly limited the possibility of finding significant associations. On the other hand, the strengths of this study are based on the use of an adequate design (cross-over study) and the use of a type of starchy food with a highly controlled composition/structure [13].

In summary, we found a strong association between copy-number of *AMY1* gene with salivary amylase activity and concentration. However, we did not find evidences for a major role of salivary amylase activity on glycemic response after starch consumption.

Acknowledgements

Supported by grants FONDECYT 11121602 and VRAID-PUC 11 (Pontificia Universidad Católica de Chile).

Conflict of Interest

The authors declare no conflicts of interest in this article.

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