Salivary α-amylase gene 1 (AMY1) copy number variation and association with inter-individual differences in body composition and response to carbohydrates intake

By

Seham Almasoudi

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Supervisors:

Dr. Colin Moran

Dr. Naomi Brooks

Physiology, Exercise and Nutrition Research Group

Faculty of Health Sciences and Sport

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“The greatest glory in living lies not in never falling, but in rising every time we fall”

Nelson Mandela

1918-2013
ABSTRACT

Copy number variation has been linked to the development of several syndromes. In a 2014 publication, evidence reported the association of the copy number of salivary amylase 1 (AMY1) with obesity. Several studies confirmed these findings; however, well-powered replication studies in various populations failed to replicate this link. This controversy may be explained by the different study designs and participant populations. A significant association may still be found under specific conditions, such as gender, age, and level of physical activity. Further, if the copy number of AMY1 does influence obesity risk, its mechanisms are yet to be uncovered. We hypothesised that an increased copy number of AMY1 would be associated with body composition more strongly than body mass index (BMI) in athletes and healthy male and female adults. That led us to hypothesise that an increased copy number of AMY1 may be associated with strength athletes and muscle performance due to their lower fat mass and increased lean mass. To understand the association between AMY1 copy number and obesity from a clinical perspective, we hypothesised that healthy adults with a high copy number of AMY1 would have a healthy, controlled glycaemic response after complex CHO ingestion and a higher rate of complex CHO oxidation during rest and exercise compared to a low copy number AMY1 group. This thesis reports on three studies.

The first study (Chapter 3) investigated the association of AMY1 copy number with underlying anthropometric aspects of body composition, precisely strength and measures of muscle performance. The study included 388 young adult Lithuanian males divided into non-exercising controls (CON; n= 187, aged 23.91 ± 4) and athletes (n=...
201). The latter included the subgroups of strength athletes (STP; n= 50, aged 21.06 ± 3), team sports athletes (TEA; n= 67, aged 22.31 ± 3), and endurance athletes (END; n= 84, aged 21.68 ± 4) were controls recruited between 2006 to 2009. All athletes trained a minimum of twice a week, whereas participants in the control group did not participate in any organised physical activity more than twice a week and did not compete in competitive sports. The copy number of target genes was determined using a quantitative polymerase chain reaction (qPCR). Percentage body fat (% BF) was calculated by Faulkner’s (1968) equation considering the modified Yuhasz method. We observed that the copy number of AMY1 did not differ between the athletes and the control group. Nor was it associated with athletes’ fat mass or FFM; however, a strong association was found with their height.

The second study (Chapter 4) assessed the association of increasing AMY1 copy number and body composition, including fat mass and lean mass and assessed the influence of gender differences on fat distribution among males and females. This study included 228 healthy volunteers aged 22.2 ± 3, of which 108 were male and 220 were female recruited between 2016 to 2019. Dual-energy X-ray absorptiometry (DXA) were used for BF % and FFM measurements. Participants completed a 3-day self-reported food questionnaire to assess macronutrient and energy intake and one week of physical activity level questionnaire (SPAQ). The AMY1 copy number distribution did not differ between males and females. No association was noticed between increasing copy number of AMY1 and fat mass or lean mass across the whole study sample nor in males and females. WC (cm) was negatively associated with high AMY1 in female only. The reginal body fat distribution and lean mass distribution did not correlate with
AMY1 CN in either gender. Increasing Total EI was associated with decreasing AMY1 CN in males. However, physical activity level (PAL) did not associate with AMY1 CN in either gender.

The third study (Chapter 5) examined the effects of high copy number on glycaemic response after starch and glucose ingestion. This pilot study was conducted on 15 healthy young adult participants, divided into two groups, a high copy number group ≥ 9 copies (n= 10) and a low copy number group ≤ 5 copies (n= 5). We observed that the high AMY1 copy number group had higher iAUC plasma glucose concentration post starch ingestion than low CN group post glucose ingestion. However, iAUC plasma insulin concentration was post glucose ingestion higher post glucose ingestion in low CN group than high CN group. plasma lipid concentration did not differ between examined groups after glucose or starch ingestion. 60 % (n = 3) of the low CN group are insulin resistance compared to 10% (n= 1) in the high AMY1 CN group. The total sample size of 62 adults is recommended to detect 80 % differences between the AMY1 CNV groups in the tested variables in future research.
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AUTHOR’S DECLARATION

I wish to submit the thesis detailed above according with the University of Stirling research degree regulations. I declare that the thesis embodies the results of my own research and was composed by me. Where appropriate I have acknowledged the nature and extent of work carried out in collaboration with others included in the thesis.

Signature:  
Date: 20/07/2022
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BIA</td>
<td>Impedance Analysis method</td>
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>CHO</td>
<td>Carbohydrate</td>
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<td>SF</td>
<td>Skinfold</td>
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<td>EI</td>
<td>Energy intake</td>
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<tr>
<td>EE</td>
<td>Energy expenditure</td>
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<tr>
<td>TDEE</td>
<td>Total daily energy expenditure</td>
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<tr>
<td>NEAT</td>
<td>Non-exercise activity thermogenesis</td>
</tr>
<tr>
<td>TEF</td>
<td>Thermic effect of food</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>MFO</td>
<td>Maximal fat oxidation</td>
</tr>
<tr>
<td>LCD</td>
<td>Low-carbohydrate diet</td>
</tr>
<tr>
<td>HCD</td>
<td>High carbohydrate diets</td>
</tr>
<tr>
<td>CN</td>
<td>Copy number</td>
</tr>
<tr>
<td>MPA</td>
<td>Moderate intensity physical activity</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy number variation</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual energy X-ray absorptiometry</td>
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<tr>
<td>DZ</td>
<td>Dizygotic</td>
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<tr>
<td>$H^2$</td>
<td>Heritability</td>
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<tr>
<td>kb</td>
<td>Kilo base</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>mrem</td>
<td>Millirem</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MZ</td>
<td>Monozygotic</td>
</tr>
<tr>
<td>NCD</td>
<td>Non-communicable disease</td>
</tr>
<tr>
<td>QCT</td>
<td>Quantitative computed tomography</td>
</tr>
<tr>
<td>sAA</td>
<td>Saliva α-amylase</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SNV</td>
<td>Single nucleotide variant</td>
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<tr>
<td>SV</td>
<td>Structural variation</td>
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<td>V_E</td>
<td>Environmental variance</td>
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<td>V_G</td>
<td>Genetic variance</td>
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<td>V_P</td>
<td>Phenotypic variance</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>BC</td>
<td>Body composition</td>
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<tr>
<td>ICW</td>
<td>Intracellular water</td>
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<tr>
<td>ECW</td>
<td>Extracellular water</td>
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<tr>
<td>CM</td>
<td>Cell mass</td>
</tr>
<tr>
<td>ECF</td>
<td>Extracellular fluid</td>
</tr>
<tr>
<td>ECS</td>
<td>Extracellular solids</td>
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<tr>
<td>IVNNA</td>
<td><em>In vivo</em> neutron activation analysis</td>
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<tr>
<td>FM</td>
<td>Fat mass</td>
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<tr>
<td>FFM</td>
<td>Fat-free mass</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
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</table>
3D-PS  The three-dimensional photonic scanning
FDA   Food and Drug Administration
qPCR  Quantitative real-time polymerase chain reaction
CON   Controls
STP   Strength athletes
TEA   Team sports athletes
END   Endurance athletes
WC    Waist circumference
HC    Hip circumference
ddPCR droplet digital polymerase chain reaction
MM    Muscle mass
LM    Lean mass
BMR   Basal metabolic rate
RMR   Resting metabolic rate
ESRD  End-stage renal disease
RNA   Ribonucleic acid
VAT   Visceral adipose tissue
SAT   Subcutaneous adipose tissue
FBG   Fasting blood glucose
OGTT  Oral glucose tolerance test
OPTT  Oral polymers tolerance test
AUC   The areas under curve
NEFA  Non-esterified fatty acids
HDL   High-density lipoproteins
LDL   Low-density lipoproteins
Chapter 1 Introduction

1.1 Human Body Composition

Human body composition (BC) research is a branch of human biology that studies various body components and the characteristics of the tissue’s quantitative measurement (Wang et al., 1992). The approach of human BC is rooted in antiquity. Hippocrates (circa 460 BC), the father of medicine, proposed that the human body comprises four elements: blood, phlegm, black bile, and yellow bile. Chinese scholars suggested that there were five elements in the human body: metal, wood, water, fire, and earth and that an imbalance of these elements resulted in disease (Wang et al., 1999). Modern evaluation of human BC has developed considerably; some of these methods will be covered in the following thesis sections.

1.1.1 Human Body Composition Levels

The human body comprises more than thirty measurable components (Wang et al. 1992). The measurement of these components is a valuable and promising approach to understanding nutritional assessment, growth, muscle development, and water homeostasis. BC is known to be associated with several diseases, such as obesity, cardiovascular disease, diabetes, cancer, osteoporosis, and osteoarthritis, which interest to nutritionists, health professionals, and sports scientists. The Academy of Nutrition and Dietetics/American Society for Parenteral and Enteral Nutrition’s (ASPEN.) Consensus Statement underlines the importance of body composition when characterising nutrition status (White et al., 2012). Understanding BC components such as body fat levels and bone mineral density (BMD) may help in clinical diagnosis with
implications for formulating appropriate treatment, interventions, prevention, and nutritional guidelines.

Human BC is studied at five levels: the atomic, molecular, cellular, tissue, and whole-body levels (Wang et al., 1992) (**Figure 1-1**).

![Figure 1-1 The Five Levels of Human BCECF. ECF. Extracellular Fluid; ECS. Extracellular Solids. Figure adapted from (Wang et al., 1992).](image)

At the atomic level of BC, the human body contains ≈ 50 types of atoms distributed in tissues and organs. Six chemical elements oxygen, carbon, nitrogen, hydrogen, calcium, and phosphorus together account for > 98 % of body weight, and more than
60% of the chemical elements in human body weight is oxygen (Heymsfield et al. 1991). The equation for bodyweight as defined at the atomic level of BC is as follows:

\[
\text{Body weight} = O + C + H + N + Ca + P + S + K + Na + Cl + Mg + \text{residual mass}
\]

(Heymsfield and Wiki, 1991)

At the molecular level, the chemical elements are bound in molecules, mainly water, lipids, proteins, minerals, and carbohydrates. Water in the human body increases depending on the amount of adipose tissue and can be as high as 60–70 % of total body weight. Total body water is divided into intracellular water (ICW) and extracellular water (ECW), and the ratio of the two is an essential health parameter in many health conditions. For example, the ECW/ICW ratio is higher in obese people as a result of obesity-related oedema and hormonal responses related to adipose tissue, which may lead to irreversible changes in hemodynamics in morbid obesity (Van Marken, Lichtenbelt, and Fogelholm, 1999; Stookey et al., 2006). Furthermore, the body water content varies with age and gender. Children have more body water than adults, and males have more body water than females (Wake et al., 2002). The second component of the molecular level is lipids. These are divided into essential and non-essential lipids. Essential lipids are split in two forms of essential structural lipids: the sphingomyelin in the nervous system and phospholipids in cell membranes. Non-essential lipids are the energy store formed mainly by triacylglycerol. Moreover, the body fat of healthy adults’ accounts for between 10 %-25 % in males and 15 %-35 % in women. Protein is a functionally important component at the molecular level and accounts for 10.6 kg or 15.1 % of body mass in the reference man. The quantity of minerals in the human
Body varies between 3% - 5% of total body weight, depending on body fat. Carbohydrates can be stored in the body as glycogen in the liver (~100 g) and in muscle (~400 g) for short-term energy storage. The equation for body weight, as defined by the molecular level of BC, is the following:

\[ \text{Body weight} = \text{Lipid} + \text{Water} + \text{Protein} + \text{Minerals} + \text{Glycogen} + \text{Residual Mass} \]

(Heymsfield and Wiki, 1991)

BC at the cellular level is described in three compartments: cell mass (CM), extracellular fluid (ECF) and extracellular solids (ECS). The body’s cells possess the characteristics of life, including metabolism, growth, and reproduction. Cells have various shapes, sizes and functions and can be broadly divided into four categories: connective (adipocytes, bone cells, and blood cells), epithelial tissue, nervous, and muscle. Examples of extracellular solids are the mainly non-metabolism protein such as collagen and elastin (Wang et al., 1992). Extracellular fluid is a fluid contains 95% water and surrounds the body cells. Body weight at the cellular level is described in the following equation:

\[ \text{Body weight} = \text{CM} + \text{ECF} + \text{ECS} \]

(Wang et al., 2007)
At the tissue level, body weight equals adipose tissue, skeletal muscle, bone and organ. Tissue level is represented in the classic body composition model and is the level that is most used in clinical and epidemiological studies.

\[ \text{Body weight} = \text{Adipose tissue} + \text{Skeletal muscle} + \text{Bone} + \text{Organs} + \text{Blood} + \text{Residual mass} \]

(Wang et al., 2007)

### 1.1.2 Body Composition Evaluation Methods

Three techniques can assess BC: direct, indirect, and doubly indirect, depending on the type of method and the particular measurements made. For direct techniques, the body component of interest is determined directly with or without minor assumption. The classic example of this technique is chemical analysis by *vivo* neutron activation analysis (IVNNA) for the atomic level. *In vivo* chemical data of body composition techniques can be used as references for indirect methods. Indirect techniques are the most frequently applied models employed to assess BC in epidemiology and clinical practice. These are usually imaging techniques and provide estimates of BC based on statistical relationships between body parameter results from direct or criterion methods and components of BC. Typically, indirect techniques divide the body into fat mass (FM), which indicates the water-free body component and fat-free mass (FFM) that includes the remaining components (skeletal muscle, organs, and interstitial fat tissue). Several indirect methods have been developed for BC assessment such as DXA, the three-dimensional photonic scanning (3D-PS), magnetic resonance imaging (MRI), and computed tomography (CT). These methods are powerful tools for visualising and
quantifying regional tissues, organs, or constituents, such as muscle mass and abdominal adipose tissue deposits, using imaging techniques of the three-compartment model (Heymsfield, 2005; Kuriyan, 2018; Marra et al., 2019).

Doubly indirect methods of BC use a variety of different equations to estimate BC based on results from direct or indirect methods such as Bio Impedance Analysis (BIA), skinfold (SF) thickness measurements, anthropometry measurement and BMI, which is the most widely used index today as a predictor for body fat and obesity (Heymsfield et al., 2005). Skinfold thickness measurement for BC is based on the hypothesis that there is a strong relationship between the amount of subcutaneous adipose tissue at several specific locations on the body and total BF. Body composition for the whole body can be estimated using statistical formulae, established in earlier studies, that combine parameters such as skinfold thickness and height, weight, age, and gender (Heymsfield et al., 2005). Doubly indirect methods are also used to validate estimated of BC data from another indirect measure (Toomey et al., 2015).

The available BC measurement methods vary in precision and accuracy and range from simple to complex with all methods having limitations and associated measurement error (Kuriyan, 2018). The appropriate method is the most accurate tool to assess target components (Table 1-1). For instance, 3D-PS system offers a novel approach for large-scale epidemiologic research into associations between body shape, health risks, and outcome (Lee and Gallagher, 2008). The 3D-PS scans the body surface using four eye-safe lasers, produces a 3D image within 12 seconds, and does not require the participants to be in a fasting state or to restrict physical activity (Adler et al., 2017).
Further, imaging techniques such as CT scanning and MRI are powerful tools in visualising and quantifying regional tissues, organs, or constituents such as muscle mass and abdominal adipose tissue deposits (Kuriyan, 2018). However, DXA continues to be considered the gold standard technique for screening and the follow-up method to evaluate therapy for osteopenia and osteoporosis since its approval for clinical use in 1988 by the Food and Drug Administration (FDA). DXA not only provides a measurement of bone mineral density, but also accurate estimates of fat and lean soft tissue (three-compartment model) (Heymsfield et al., 2005; Marra et al., 2019). Though, DXA does require the participants to be in a fasting state or to restrict physical activity before the scan to minimize the biological changes in related to exercise and its related practices of fluid and food intake, which are associated with changes in the mean estimate of the total and regional body composition that range from trivial to small but substantial (Nana et al., 2013).

1.1.2.1 Assessment of Body Composition by Dual-Energy X-ray Absorptiometry (DXA) Compared with Other Methods

DXA is used widely on patients of all age groups with chronic diseases in clinical and research settings (Erlandsson et al., 2016; Messina et al., 2020). Among different methods for measuring body composition, DXA provides whole body and regional estimates of bone-free mass which has three main components: FFM, FM, and BMD explained by Blake (1997). Originally, DXA was designed to estimate bone density using two X-ray beams with different energy levels, aimed at the patient’s bones. When soft-tissue absorption is subtracted out, the BMD, FFM, and FM can be determined from the absorption of each beam, giving the total body composition (Messina et al., 2020).
Clinically, DXA is widely used to diagnose osteoporosis which estimates fracture risk and monitor therapy. Furthermore, the ability of DXA to measure lean mass has led to its use in diagnosing sarcopenia and providing a comprehensive picture of musculoskeletal health (Erlandson et al., 2016). DXA continues to be considered the gold standard technique for screening and the follow-up method to evaluate therapy for osteopenia and osteoporosis since its approval for clinical use in 1988 by the Food and Drug Administration (FDA). Further, in a systematic review conducted on behalf of ASPEN, evaluating the best available evidence regarding the validity of applicable body composition methods, DXA was recommended for the assessment of FM in patients with a variety of disease states; however, the validity of DXA for FFM assessment in any clinical population remains unknown. Even though DXA can only poorly distinguish between bones, lean tissue, and some other parts of the body (Norgan, 2005), it is correlated with MRI and CT in lean mass measurement (calculated as the difference between total body weight and body fat weight) (Maden-Wilkinson et al., 2013). In addition, its excellent precision and reproducibility means it can accommodate the evaluation of the composition of regions of the body and the status of nutrition in the presence of disease and growth disorders. DXA and BIA have made great leaps in terms of time needed for the data acquisition; it can take as little as 5–10 minutes to estimate data for a whole-body composition (Pateyjohns et al., 2006). In that time DXA scans the body by low-emission X-rays to measure the attenuation of incident X-ray beams, while BIA measures the electrical properties of body tissue and estimates BC parameters as total body water (TBW) and FFM BC parameters (Marra et al., 2019). No recommendations can be made to support the use of ultrasound or BIA in the clinical setting (Sheean et al., 2019).
Quantitative computed tomography (QCT) has the potential to measure the correct volume of bone mass density and has the advantage of differentiating between cortical and trabecular components but with more significantly exposure to radiation compared with DXA. DXA incorporates a small amount of ionising radiation (2-5.4 µSV) /≤ 5 millirem (mrem), which is considered low compared with CT, which ranges from 1–10% of a chest radiograph. The scanning bed has an upper weight limit of 150 kg and an entire body field of perspective (Norgan, 2005). In addition, DXA and BIA have a high correlation in assessing body fat in normal adiposity (Mialich et al., 2014). Although highly correlated, in an experimental study conducted by Sun et al. (2006), it was found that the correlation coefficient became wider in terms of stratifying the measurement by gender (Sun et al., 2006). These results confirmed among Singapore Chinese adults. That may make BIA an unsuitable method for measuring BF percentage for clinical purposes in adults (Gupta et al., 2011). BIA underestimated BF percentage and overestimated FFM compared with DXA in older individuals (Meier, Bai, Wang and Lee, 2020).

From a practical point of view, each method of BC assessment has limitations and strengths (Table 1-1). Knowing the differences between the various ways of estimating, the approach, and the suitability of the method for participants’ characteristics and the aim of the assessments, are essential for accurate BC evaluation. For instance, to monitor a weight loss programme, it is more important to know the accuracy of technique's observation in weight change. If there are differences among BC changes, the interpretation of the results should include the selected technique (Benito et al., 2019). DXA uses in weight and fat percentage intervention studies, due to its ability to detect the individual and independent differences in peripheral fat (arm and leg) and
central fat (trunk). DXA is an overly sensitive and consistently reliable technique for
detecting changes in fat distribution over a relatively short period time (e.g., months)
(Marra et al., 2019). A study that compared body fat changes measured by DXA, BIA,
and SF during a weight loss program found an underestimation of fat percentage by
BIA compared with the DXA—2.4 % in females, whilst among males BIA estimated
FM significantly greater, 13.3 % (Benito et al., 2019). Finally, DXA became the
standard method used in BC assessment because it is non-invasive, quick, gives high-
quality images, and has minimal radiation exposure. Therefore, it is the method chosen
for the study reported in Chapter 4.
**Table 1-1** Comparisons of Methods for Body Composition Assessment

<table>
<thead>
<tr>
<th>Methods</th>
<th>Level</th>
<th>Approach</th>
<th>Primary measurements</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dual-energy X-ray absorptiometry</strong></td>
<td>Image</td>
<td>Indirect: Measure and transform</td>
<td>Total body fat, lean mass, bone’s mineral density, and its contents</td>
<td>Little exposure to radiograph radiation, easy to use, suitable for assessing regions of fat. Can be used for all ages</td>
<td>Expensive equipment that requires an expert radiology technician to operate. Biased against sex, body size, and fatness</td>
</tr>
<tr>
<td><strong>Bio Impedance Analysis</strong></td>
<td>Estimate</td>
<td>Double indirect</td>
<td>Intracellular and extracellular fluid space, total body water</td>
<td>Can be inexpensive, simple, portable quick, and safe</td>
<td>Low accuracy in individuals and groups. Cannot be applied for some population (e.g., obese and pregnancy)</td>
</tr>
<tr>
<td><strong>Quantitative Computed Tomography (QCT)</strong></td>
<td>Image</td>
<td>Indirect</td>
<td>Regional bone density</td>
<td>High accuracy and reproducibility</td>
<td>A high radiation exposure and expensive</td>
</tr>
<tr>
<td><strong>Dilution techniques</strong></td>
<td>Estimate</td>
<td>Indirect</td>
<td>Total body water and extracellular fluid</td>
<td>Easy to use, can be used for all age groups,</td>
<td>Inaccurate if any sort of illness present, expensive, requires highly technical staff, a large number of analyses</td>
</tr>
<tr>
<td><strong>Three-dimensional photonic scanning</strong></td>
<td>Image</td>
<td>Indirect</td>
<td>Total body volume, regional</td>
<td>It can be used to assess extremely obese subjects, convenient to use, and suitable for both clinical and research applications.</td>
<td>Very few scanners are available.</td>
</tr>
<tr>
<td>Methods</td>
<td>Level</td>
<td>Approach</td>
<td>Primary measurements</td>
<td>Advantages</td>
<td>Disadvantages</td>
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<tr>
<td>Quantitative magnetic resonance</td>
<td>Image</td>
<td>Indirect</td>
<td>Total body fat, TBW, and body weight in general</td>
<td>Safe, easy, and fast</td>
<td>Costly, few systems are available.</td>
</tr>
<tr>
<td>MRI/MRS</td>
<td>Image</td>
<td>Indirect</td>
<td>Total and regional adipose tissue (subcutaneous, visceral, and intermuscular), organs (liver, kidney, heart, spleen and pancreas), lipid content in muscle and liver, skeletal muscle</td>
<td>Accurate and reproducible for the entire body and regional adipose tissue and skeletal muscle</td>
<td>Costly equipment</td>
</tr>
<tr>
<td>Skinfold</td>
<td>Estimate</td>
<td>Double indirect</td>
<td>Total body fat muscle mass</td>
<td>Low-cost equipment. Not very painful and easy to perform</td>
<td>Practice is required, subject to error. Less reliable in older adults due to their weak skin and muscles. Less reliable in patients with chronic muscle diseases, dehydration, and oedema</td>
</tr>
</tbody>
</table>
1.1.3 Variation in Body Composition

In healthy adults, body composition is maintained over the short-term within narrow limits. Changes in BC are an essential in evaluating the risk of disease in both lean and obese individuals. For example, high body FM % was linked with several health conditions. Accumulation of visceral adipose tissue (VAT), referred to as abdominal or central obesity, has been implicated as an independent risk factor for various medical conditions, such as insulin resistance, diabetes, hepatic steatosis, dyslipidaemia, and hypertension and more recently in the development of subclinical atherosclerosis and adverse cardiovascular outcomes (Sharma, 2019). VAT has also been associated with colorectal, pancreatic, and gastro-oesophageal cancer (Silveira et al., 2020). Some people with abdominal obesity or VAT can be classified as normal by BMI i.e., 18.5–24.9 kg·m\(^{-2}\), if the fat is distributed primarily as central or visceral fat (Bosomworth et al., 2019). Studying the variation of body composition is essential for preventing and assessing disease.

Body composition components are anatomically distributed in different proportions throughout the human body, and the pattern of distribution is dependent upon many factors, including age, gender, ethnicity, genotype, meal patterns and eating habits, physical activity, hormonal status, and medication (National Academies Press, 2004). Percentage body FM increases across age groups in both genders. Males have less body FM and more FFM than females (Reid et al., 2009; Kirchengast, 2010). A study of 5,225 healthy males and females in different age groups showed that males have greater FFM than females in all age groups (Kyle, Genton, Slosman and Pichard, 2001). The mean FFM was 8.9 kg (14.8 %) lower in males older than 85
years than in males 35 to 44 years old and 6.2 kg (14.3 %) lower in females older than 85 years than in females 45 to 54 years old. FM increases based on age group in females from 5.6 to 9.4 kg and from 3.7 to 7.4 kg in males (Kyle, Genton, Slosman and Pichard, 2001). BC varies among ethnic groups mainly due to differences in body build, which influence TBW and BF % (Deurenberg et al., 2002; Deurenberg et al., 2003). Sarcopenia, or gradually wasting muscles, is age-related, and the rate of wasting can be influenced by sex and ethnicity (Gonzalez et al., 2019). However, results from the National Health and Nutrition Examination Survey have shown that ethnic variances in body composition and shape are less apparent in older (≥ 70 years) Mexican - American and non-Hispanic white and black populations (Heymsfield et al., 2016). A recent study compared differences in fat and muscle mass among three ethnicities and found that Mexicans have higher FM and VAT than Germans with the same BMI. Further, normal weight Japanese are similar to Mexicans, whereas overweight Japanese are more similar to Germans. The skeletal muscle index (SMI) is highest in Germans, and they have a higher FFM per FM compared with Mexicans and Japanese. The Japanese group had the highest extracellular/TBW ratio (Jensen et al., 2019).

Health status may have a significant effect on BC. The classic example is malnutrition, which refers to deficiencies, excesses, or imbalances in macronutrients (protein, carbohydrates, and fat) leading to protein–energy malnutrition, micronutrients (electrolytes, minerals and vitamins, leading to specific micronutrient deficiencies), or both (Millward and Jackson, 2004). Undernutrition, where the lack of weight leads to organ dysfunction, reduced body cell mass, abnormal blood chemistry, and worsened clinical outcomes. Increased nutritional intake (positive
imbalance energy intake) in the absence of increased physical activity leads to increasing FM mass, obesity, and obesity-related diseases (WHO, 2022). Recent evidence from longitudinal studies has shown differences in the pattern of body composition changes in adults by diabetic status explained by an overall greater gain of FM and a more rapid loss of LM in adults with diabetes versus those without (Al-Sofiani et al., 2019). Park et al. examined the changes in body composition by diabetes status in 2,675 adults, who had an average age of 73 years. Participants with no diabetes, diagnosed diabetes, and previously undiagnosed diabetes had an annual decline rate of total FM of 0.025 kg, 0.066 kg, and 0.094 kg, respectively, and an annual decline of total LM of 0.198 kg, 0.22 kg, and 0.34 kg, respectively. Most of the decline in total LM in individuals with diabetes was due to loss of appendicular LM (Park et al., 2007). Muscle wasting is also prominent among patients with chronic kidney disease. The prevalence of sarcopenia or muscle wasting among patients with end-stage renal disease (ESRD) ranges from 20 to 44 % (Johansen and Lee, 2015).

1.1.3.1 Body Composition in Obese Individuals

Obesity is a condition involving excessive and abnormal accumulation of adipose tissue, and the health consequences represent a universal epidemic (Ulijaszek, 2003). BMI is the most accepted and widely used albeit crude index to classify obesity. Individuals with a BMI of ≥ 25 (kg/m²) are categorised as overweight, and those with ≥ 30 (kg/m²) are categorised as obese (Bimali and He, 2015). BMI determines the health risks of excess weight such as the proportion of body weight which consists of fat and its distribution (as described in body composition methods above). Body fat percentage in healthy adults varies between 10–25 % in males and 15–35 % in
females. In severe obesity body fat can be increased to 60–79 % of total body weight (WHO, 1995). Studies suggested that assessing body composition in obese individuals should be taken in two components: FM and FFM. The ratio of TBW in overweight decreased with increasing BMI and was lower in obese females than in males (Ritz et al., 2008). The distribution of TBW is unhealthy in obese individuals they have more ECW compared to ICW (Heyward and Wagner, 2004).

1.1.3.2 Body Composition in Athletes

Unlike obese individuals, athletes tend to have low FM and high muscle mass that are desirable to enhance and optimise physical performance in strength and power activities. Further, female athletes have higher body fatness than male athletes, and the average FM depends on the type of sport and for team sports, athlete position (Wilmore, 1983). For example, the essential FM of male bodybuilders is minimal to 5 % of total body weight; however, the essential FM of female bodybuilders ranges from 12 % to 16 % of total body weight (Heyward and Wagner, 2004). WHO recommends that the healthy BF % in adult males is 14 -17 % and healthy BF % in adult females is 21-24 %. The body fat percentage for male athletes ranges from 6-13 % and that of female athletes ranges from 14–20 % (Jonee and Bray, 1997).

1.2 Obesity

1.2.1 Prevalence of Obesity

In 2018, the WHO report that obesity has increased dramatically over the last four decades. The prevalence of obesity in adults has tripled since 1975. Currently, the overall prevalence rate for male is 11 % and for women is 15 % (WHO, 2018). A recent
WHO obesity report in 2020 stated that 38 million children under the age of five were overweight or obese in 2019 (WHO, 2020). In the United States, the adult obesity rate passed 42.4 % nationally for the first time in 2017–2018, compared to 26 % in 2008 according to the National Health and Nutrition Examination Survey (NHANES, 2020). In a recent comparative long-term project on age and sex-specific obesity prevalence up to the year 2100 for 18 European countries and the United States, obesity is expected to be prevalent between 2026 and 2054, with the United States and the United Kingdom predicted to reach the highest maximum levels followed by other European countries (Vidra, Trias-Llimós, and Janssen, 2019). Further, the Organisation for Economic Cooperation and Development (OECD) performed obesity projections for selected countries up to 2030, assuming that “BMI will continue to rise as a linear function of time.” According to OECD data, the adult obesity rate has increased steadily since 1990, by an average of 19.5 %, led by the US and Mexico at 38.2 % and 32.4 %, respectively. The lower rates in Japan and Korea 3.7 % and 5.3 %, respectively. In 2020 more than two in three adults (73 %) in the United States are overweight, and 42.4 % of them are obese. The number of obese is expected to rise to more than 46 % by 2030 (Figures 1-2 and 1-3) (OECD Health Statistics 2020;2017–2018). In the UK, 63 % of adults in England in 2018 are overweight; and 28 % of them are obese (NHS Digital report, 2020). According to OECD data, the percentage of obese people in England may increase to 35 % by 2030 (Figure 1-3) (adapted from OECD Health Statistics 2017–2018; Lee, 2019).
Figure 1-2 Standardised Rates of Overweight (including obesity) defined as BMI > 25 Kg/m² in adults aged 15–74 years. Height and weight were measured in England, Hungary, Korea, Mexico, and the United States but self-reported in other OCEC selected countries (Data adapted from OECD Health Statistics 2020)

Figure 1-3 OECD Projections if BMI Continues to Rise as a Linear Function of Time. Obesity was defined as BMI > 30Kg/m² in adults aged 15–74 years. Age and gender-adjusted rates using the 2005 OECD standard population. Height and weight were measured in England, Hungary, Korea, Mexico, and the United States but self-reported in other OCEC selected countries. (Adapted from OECD Health Statistics 2017–2018)
1.2.2 Obesity Impact on Health

If trends continue, this will have significant economic and social implications. A study in 26 European countries and the United States suggests this will result in a decline in life expectancy of, on average, 1.22 years for men and 0.98 years for women (Vidra, Trias-Llimós, and Janssen, 2019). Keaver et al. projected disease burden and direct healthcare costs for obesity-related conditions in Ireland in 2030. The study found that by 2030 the levels of overweight and obesity have the potential to reach 89% and 85% in males and females, respectively. This will lead to an increase in the obesity-related prevalence of coronary heart disease (CHD) by 97%, cancers by 61%, and type 2 diabetes by 21%. Thereupon, the direct healthcare costs related with these increases will amount to €5.4 billion by 2030. A 5% reduction in population BMI levels by 2030 is estimated to have the potential to result in a € 495 million decrease in obesity-related expenditure (Keaver et al., 2013).

Obesity is associated with several metabolic abnormalities, such as hyperglycaemia, insulin resistance, and dyslipidaemia (Whitlock, Lewington, and Mhurchu, 2002). Consequently, obese people are at risk of many adverse outcomes, including non-communicable diseases (NCDs), like cardiovascular disease (CVD), diabetes, and several types of cancer (Bimali and He, 2014). World Cancer Research (2016) reported that obesity is associated with 11 types of cancer. For example, the development and progression of breast cancer can be the result of chronic inflammation in adipose tissue, which is increased in obese people (Khan, Chan, Revelo and Winer, 2020).
The increase in obesity has played a part in the increased prevalence of CVD risk factors, including dyslipidaemia, glucose intolerance, hypertension (HTN), and obstructive sleep apnoea/hyperventilation (Bastien et al., 2014). In addition, an increase in BMI of 1 kg/m² or greater has been linked with increases in CHD risk of around 5–7 %, while a BMI increase of 5 kg/m² or higher is associated with a rise of approximately 30 % in the overall mortality rate (40 % from CVD; 60–120 % from diabetes and renal and hepatic diseases, and 30 % from respiratory disease and all other causes of mortality) (Whitlock et al., 2002). Being obese or overweight has been epitomised as a major cause of serious health problems in the category of NCDs (WHO, 2006). In a study of 1.46 million white adults, being overweight and obese was linked to increases in all causes of mortality. All such causes were the lowest among healthy-weight individuals with a BMI of 20.0 to 24.9 (kg/m²) (Berrington de Gonzalez et al., 2010).

Overweight and obesity have been clinically implicated with musculoskeletal disorders involving different body parts, including the back, hip, knee, ankle and foot, tissue injuries of the upper body and wrist, and strain on soft-tissue structures, such as tendons and fascia. Overweight unduly increases stress within connective tissue structures, such as bones, joints, and soft tissues of the locomotor system (Wearing et al., 2006).

### 1.3 Cause of Obesity

#### 1.3.1 Unbalancing Energy and Obesity

The world has seen a dramatic change in dietary patterns, physical activity and inactivity patterns, body composition, and the prevalence of obesity and diabetes. This,
combined with urbanisation, industrialisation, and globalisation of the marketplace in the past few decades, has led to easy food supply. This phenomenon, known as “nutrition transition, has an essential consequence on the global population’s health and nutrition” (Ulijaszek, 2003; Drewnowski and Popkin, 2009). Combined with increased food marketing, this has led to people feeling more motivated to eat out and purchase more highly processed food (Cohen, 2008). This means that people are replacing a plant-based diet with highly energetic choices which contain more animal fats and added sweeteners. (Ulijaszek, 2003; Drewnowski and Popkin, 2009).

In recent decades, the rate of obesity has risen with increasing interest in the role of specific dietary energy sources in obesity. Obesity results from a positive energy balance, i.e., excessive overfeeding (high energy intake) relative to energy needs (low energy expenditure). The principal dietary sources for energy intake (EI) are carbohydrates (45%–55%), proteins (35%), and fats (20%). Energy expenditure (EE) is an assessment of the oxidation of these nutrients (of whole-host cellular metabolic activity in the body) and can be expressed as either kilocalories or kilojoules per day (Cordoza, Chan, Bridges and Thompson, 2020). Total daily energy expenditure (TDEE) in humans consists of three components: resting metabolic rate (RMR), non-exercise activity thermogenesis (NEAT), and activity-induced energy expenditure (AEE) (FAO, 2001; Melanson, 2017).

1.3.1.1 Resting Metabolic Rate (RMR)

RMR is the rate of body energy expenditure during rest by prediction equations, accounts for 60–70% of TDEE. This energy is used in metabolic and cellular processes,
along with mechanical work, such as breathing and heartbeats. The minimum rate of body energy expenditure necessary for life is called Basal Metabolic Rate (BMR). RMR/ BMR varies between individuals and is affected by sex, age, body size and composition, and thyroid hormones (FAO, 2001). In terms of gender, males have a higher RMR than females by approximately 210 kJ/day. In both cases, RMR decreases at a rate of 1–2 % per decade after 20 years of age. This reduction in RMR is linked with lower FFM (Heymsfield et al., 2002). Further, FFM is the primary determinant of energy expended at rest (Bogardus et al., 1986). FFM is made up of many organs and tissues with different metabolic rates (Javed et al., 2010). Despite comprising < 6 % of total body weight, organs are the major drivers of RMR with brain, heart, kidneys, and liver collectively accounting for 60 – 80 %. Even though FFM is mainly made of skeletal muscle, it has lower metabolic activity during resting conditions (Muller et al., 2002; Javed et al., 2010).

Krems and colleagues found that RMR remains significantly lower in the older adults than young and middle-aged adults, even after correcting for differences in body composition (Krems et al., 2005). Age-lowering in RMR may be explained by changing organ masses, homogeneity of specific metabolic rates (Manini, 2010), and lower EI and EE (Van Belt et al., 2001). Active individuals have a higher RMR compared to non-active individuals. However, RMR might paradoxically decrease in intensive training due to decreased energy availability due to the training load and increased risk of physiological dysfunction (Woods et al., 2018). Lean mass is the primary determinant for declining RMR with advancing age in sedentary populations (Van belt et al., 2001) and increased RMR in active populations. Collectively, these factors
account for 80 – 90% of the variance in RMR. Although, specific sources have not yet been identified, genetic factors may explain the remainder.

1.3.1.2 Non-Exercise Activity Thermogenesis (NEAT)

In addition to RMR, there is an increase in EE in response to food intake. Digestion, absorption, and assimilation, all falling under NEAT, account for approximately 10% of total EE for an average diet. Thus, increased food intake increases TDEE through an increase in NEAT. Similarly, exchanging fat by protein in an isoenergetic diet increases EE by an equivalent to the difference in processing rates between fat and protein (Manini, 2010). It is worth noting that differences in this expenditure, termed thermic effect of food (TEF) between lean and obese subjects, where found, are small, and there is little evidence that defects in TEF play a major role in the development of obesity or the degree of obesity-related insulin resistance, which may be influenced by a low level of sympathetic activity (De Jonee and Bray, 1997).

1.3.1.3 Activity-Induced Energy Expenditure (AEE)

The third source of energy intake is AEE which is the result of physical activity (PA). PA is defined as any bodily movement produced by skeletal muscles that result in EE (Paola et al., 2013). AEE captures energy expended on all physical activity, including moderate and vigorous physical activity (for example, jogging, walking and biking) and low-intensity physical activity such as housework, daily chores, and postural allocation. People with higher levels of AEE have reduced rates of mortality and incident mobility impairment (Manini et al., 2006; Shahar et al., 2009; Middleton et al., 2011). AEE constitutes between 20% and 70% of TDEE, depending on the type
of activity (Gibney et al., 2005). AEE is influenced by age, body size, EI, and disease. Young children have a low AEE (Westerterp, 2013). It increases from 20% at age one to ~35% at age 18 (Butte et al., 2012). Even though young children may be highly active, the EE is lower because less energy is needed to move around with lower body weight (Westerterp, 2013). However, that is not the case in adults; overweight and obesity are not associated with a lower AEE under free-living conditions. AEE is like or even higher in with greater body weight (Prentice and Jebb, 2004). However, several studies reported similar findings. The investigators also compared PA between obese and non-obese individuals. AEE was lower in the obese group after adjusting for body weight (Johannsen et al., 2012; Carneiro et al., 2016; Butte et al., 2012; Elbelt et al., 2010; DeLany et al., 2012).

1.3.2 Energy Expenditure and Obesity

Maintaining a healthy body weight is achieved by balancing EI and EE. Unbalanced energy metabolism may play a role in long-term body weight dysregulation and the pathogenesis of human obesity and obesity-related comorbidities (Ravussin et al., 1988; Malik, Willett and Hu, 2013). Several studies on the Pima Indian population in the United States have sought to determine predictors or risk factors that may predispose humans to obesity. Within this population, obesity is common among young adults, and at least four metabolic parameters were found to be predictive of weight gain. These were low metabolic rate, low spontaneous PA, low sympathetic nervous system (SNS), and low-fat oxidation (Galgani and Ravussin, 2008).
Low rates of EE and fat oxidation predict body weight gain. It has been found that under experimental conditions of overfeeding, the associated increases in EE and fat oxidation were greater than expected, given the change in body composition (Weyer et al., 2000). Their prospective study in a subgroup of 66 siblings from 28 Pima Indians families found that those with a high 24-h respiratory quotient (RQ; ≥ 90th percentile) independent of 24-h EE, were at a 2.5 times higher risk of gaining > 5 kg body weight compared to those with low 24-h RQ (≤ 10th percentile), a low EE and a low ratio of fat to carbohydrate oxidation (Zurlo et al., 1990). Another follow-up study by Weyer et al. found that long-term weight changes are accompanied by small metabolic adaptations in both EE and fat oxidation. Furthermore, the metabolic responses to weight changes are highly variable between individuals (Weyer et al., 2000).

Galgani and Ravussin (2008) offer a different perspective when considering the physiological conditions that predispose humans to becoming obese. They suggest that using a fat balance equation instead of the commonly used energy balance equation might lead to more understanding, given that fat is the only nutrient that can cause a chronic imbalance between intake and oxidation because it can be stored long-term as adipose tissue. However, the other nutrients contribute indirectly through the overall energy balance. Therefore, if the composition of nutrient intake is a significant factor in weight gain, the composition of nutrient oxidation is also a factor to be considered in the aetiology of obesity. The non-protein RQ is an index of the ratio of carbohydrate to fat oxidation ratio, and fasting values of 0.70 after a 12-hour fast indicate a major reliance on fat oxidation. However, reliance on carbohydrate as the major energy substrate would be indicated by values approaching 1.00 after ingestion of a meal of carbohydrates (Galgani and Ravussin, 2008). Exercise – induced changes in RQ at rest
affected fat loss along with the energy expended during an exercise training programme.

Identifying the factors that influence fat oxidation will allow for appropriate measures to be recommended for the exercise intensity that best supports fat oxidation to allow individuals to lose weight and athletes concerned with peak performance strategies (Barwell et al., 2009). Purdom et al. (2018) investigated the factors that affect maximal fat oxidation (MFO), noting that MFO has been reported to occur between 47% and 75% of VO$_2$ max and varies between trained and untrained men and women. However, it is known to range from 0.17 – 1.27 g/min (Randell et al., 2017), where ketogenic adapted individuals can exceed >1.5 g/min (Volek et al., 2015). Lipid oxidation is affected by many factors, including level of fitness, the intensity of energy expended and duration, sex, and dietary intake and as such facilitates or restricts physiological changes that influence fatty acid oxidation (Purdom et al., 2018). The variability in fatty acid oxidation attributed to sex because of the inherent hormonal differences between men and women (Varlamov et al., 2015). It has been shown in several studies that the ability of premenopausal females to oxidise fat during exercise is far greater than that of males because those greater oestrogen levels have on increasing the expression of cellular proteins responsible for increased fatty acid transport and oxidation (Dasilva et al., 2011).

The macronutrient content of EI also affects the cellular protein expression and subsequently the endogenous or systematic substrate oxidation varies. Diets high in fat content (> 68 % total daily EI) have been shown to have positive effects on lowering
respiratory exchange ratio (RER) values (Webster et al., 2016) during moderate intensity exercise (~ 64-70 % VO₂ max) and for increased exercise time (~ 3 hours) (Vorlek et al., 2016). A study designed to investigate the link between meal-induced thermogenesis and obesity by assessing the risk factor of a high-fat meal and fat gain in children. The study concluded that the lowered postprandial thermogenesis following the high-fat meals in the long-term and conjunction with other significant factors, such as the high energy density, palatability of fatty foods, and genetic predisposition, may contribute to the progressive development of weight (fat) gain and the maintenance of obesity in children (Maffeis et al., 2001). However, the lack of sufficient means to measure EI from fat, carbohydrate, and protein in non-controlled environments and the insufficiently precise methods able to detect the small chronic imbalances in energy metabolism that lead to obesity mean that it is still not clear how the metabolic parameters they found to be important alter the balance between intake and expenditure of energy (Galgani and Ravussin, 2008).

1.3.3 Carbohydrates and Weight Loss

Given the prevalence of obesity and its impact on health as outlined and discussed in the previous section, there has been a resulting focus on weight loss diets, producing a multimillion-dollar industry, with competing advocates promoting conflicting advice as to the most effective means of losing weight. A recommended balanced diet for energy to maintain health and weight in terms of macronutrient, micronutrient, and dietary quality has been established by nutrition specialists with macronutrient rangers having been developed in the United States, Canada, Australia, New Zealand, and Europe and are similar across these countries (Naude et al., 2014).
Carbohydrates (CHO) are a major source of energy in the diet of humans (Jebb, 2014). The recommended range for CHO varies between 45% and 55% of total energy, for protein intake it is between 10% and 35%, and between 20% and 35% for fat (Jebb, 2014). Balanced weight loss diets can then be defined as reducing the overall EI across each category of macronutrient in accordance with the accepted and established guidelines of what constitutes a healthy and balanced diet. Many currently popular weight loss diets, for example the ketogenic diet and the Atkins diet, widely promoted through the media, are based on reducing carbohydrate intake alone and increasing the fat and protein ratios. Advocates of these diets promote them as more effective for weight loss; however, the evidence does not support the claims. Nordmann et al. (2006) conducted a meta-analysis of randomised controlled trials that investigated the effects of low-carbohydrate vs low-fat diets on weight loss and cardiovascular risk factors and concluded that low-carbohydrate, energy restricted diets are at least as effective as low-fat, energy restricted diets in inducing weight loss for up to a year. Naude et al (2014), in their systematic review and meta-analysis of low-carbohydrate vs isoenergetic balanced diets for reducing weight and cardiovascular risk. The trials showed weight loss in the short-term irrespective of whether a low CHO or balanced diet is followed (Naude et al., 2014).

The evidence to date suggests that the vital factor in weight loss over time is not the restriction of carbohydrates more than protein or fat, but that longer-term weight loss is related to the overall reduction of EI. A systematic review that investigated the efficacy of low-carbohydrate diets for overweight and obesity reported that from 12 systematic reviews, 10 with meta-analysis, only 2 had high review quality, 3 were moderately high, and 7 critically low. Among the meta-analyses, 4/5 with critically low
quality showed low-carbohydrate diet (LCD) advantage for weight loss (0.7–4.0 kg), whereas high-quality meta-analysis showed little or no difference between diets (Churuangsuk et al., 2018). These findings suggest well conducted systematic reviews of improved quality reduced carbohydrate trials are need before LCDs can be recommended as a superior approach to energy reduction for weight loss.

This is essential as the long-term effects of LCDs have still to be established. The issues of concern relate to ketosis when ketone bodies replace glucose as a primary source of energy. Primarily, these are long-term cardiovascular safety, lipid and renal effects. However, ketogenic diets were used to treat epilepsy in the 1920s before medications were available. Additionally, recent studies have shown how ketogenic diets have improved outcomes for acne, non-alcoholic fatty liver disease, polycystic ovary syndrome, and Alzheimer disease. Currently, testing is being done on the benefits of a keto diet for the provision of sustained and steady fuel in endurance sports and the optimisation of body composition in high-intensity training (Paoli et al., 2013; Burch and Ciapponi, 2020).

The issue of whether LCDs compared to high carbohydrate diets (HCDs) are better for weight control and metabolic health was investigated in a recent study conducted by Minderis and his colleagues (2020). They investigated the effects of EI restriction using both diets on obese mice and concluded that body composition and metabolic health were not affected when EI was restricted with fixed energy and protein intake (Minderis et al., 2020).
The popularity and intense interest in low-carbohydrate diets may be partly due to the perceived influence of CHO on current obesity levels (Minderis et al., 2020). Several studies in the 1980s found excessive CHO intake to be an important contributor to obesity (Andersen et al., 2005). Most of contemporary weight loss diets advocate increased CHO consumption and decreased protein and fat intakes. The possibility of promoting, rather than suppressing CHO cravings has been proposed as a novel strategy for achieving weight loss (Drewnowski et al., 1992). In addition, assessing the association between total CHO and body weight is a complex matter due to the need to differentiate between various types of CHO. It is estimated that roughly 60–70% of current human EI comes from starch, but substantial variation exists within and between populations (Robyt, Yoon and Mukerjea, 2008; Fermandez and Wily, 2017). The specific type and amounts consumed are key considerations for weight control (Jebb, 2014).

1.3.4 Physical Activity and Obesity

Weight loss and weight control are also significantly influenced by PA. The World Health Organisation Global Action Plan on PA 2018–2030 states that regular PA is proven to help prevent and treat NCDs as well as helping to prevent hypertension, overweight, and obesity and can contribute to better mental health, quality of life and overall general well-being (WHO, 2018). They reported that globally 25 % of adults and 75 % of adolescents (aged 11–17) do not currently meet the worldwide recommendations for PA set by WHO (150 minutes per week of moderate intensity physical activity [MPA] or 75 minutes of vigorous intensity physical activity [VPA]), and as countries develop economically, levels of inactivity increase (WHO, 2018).
The UK Chief Medical Officers’ Physical Activity Guidelines (2019) report that since 2011 the evidence to support the benefits of regular PA across all age groups has become even more compelling and highlights the risks of inactivity and prolonged periods of sitting in adults and children. The report suggested that long periods of sedentary behaviour can be harmful, even in people who achieve the recommended levels of moderate PA for some aspects of health. The new guidelines promote a whole life approach to improved health through continuous PA, sport, exercise, and active travel.

Although the benefits of PA are accepted as means of maintaining a healthy weight and overall good health, PA on its own has not been shown to be effective in reducing weight. A study conducted by Kerksick et al. (2009) on the effects of a popular exercise and weight loss programme on weight loss, body composition, EE and health in obese women found that exercise alone had minimal impact on the measured outcome, whereas positive results were achieved for the study participants when exercise was done in conjunction with a hypo energetic diet (Kerksick et al., 2009).

Insufficient PA alone, however, has been shown to be a leading risk factor for NCDs and has a negative effect on mental health and quality of life (Guthold et al., 2021). A pooled analysis of 358 population-based surveys with 1.9 million participants from 2001 to 2016, suggests that if their findings on the current global trends on the lack of PA continue, it will not meet the 2025 global activity target set by WHO to reduce inactivity by 10%. This has implications for the continued and increased prevalence of obesity as a growing global concern across all age ranges (Guthold et al., 2018).
A prospective study conducted by Williams and Wood (2005) consisting of 8080 male and 4871 female runners to investigate whether physical activity can prevent age-related weight gain and whether changing levels of activity affect body weight. The study found that age-related weight gain occurs even among the most active individuals when exercise is constant. And suggested that vigorous exercise may need to increase 4.4 km/week annually in men and 6.2 km/week annually in women to compensate for the expected gain in weight associated with aging (Williams and Wood, 2005). This has led them to theorise that vigorous exercise must increase significantly with age to compensate for the expected gain in weight associated with ageing. So, exercise as a strategy to prevent age induced body weight and body fat gain may again on its own not be sufficient. However, a recent study by Arner et al. (2019) found that lipid turnover in the fat tissue decreases during ageing and makes it easier to gain weight, even if participants did not eat more or exercise less than before.

1.4 Carbohydrate Functions

Dietary carbohydrates are a diverse group of substances with a range of chemical, physical, and physiological properties. Carbohydrates are principally substrates for energy metabolism. Carbohydrate intake also affects satiety, blood glucose and insulin, and lipid metabolism and through fermentation, exert major control on colonic function, including bowel habit, transit, the metabolism and balance of the commensal flora and large bowel epithelial cell health (Born, 2007; Bolla et al., 2019; Mills et al., 2019, Part I & Part II). In addition, intake of dietary CHO modifies the function of the immune system and influence calcium absorption (Statthos, Shulman, Schanler and Abrams, 1996; Cummings & Stephen, 2007). These properties impact on our overall health and are particularly relevant to the control of body weight, diabetes and ageing,
cardiovascular disease, BMD, large bowel cancer, constipation, and resistance to gut infection (Cummings and Stephen, 2007).

1.4.1 Carbohydrate Classification

The primary classification of dietary carbohydrates, as proposed at the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Consultation on Carbohydrates in human nutrition convened in Rome in 1997 (FAO, 1998), is by molecular size, as determined by the degree of polymerisation (DP), the type of linkage (a or non- a) and character of individual monomers. Using a chemical approach, this classification divides carbohydrates into three main groups: sugars (DP1-2), oligosaccharides (short-chain carbohydrates) (DP3-9), and polysaccharides (DP> 10). Sugars comprise (i) monosaccharides, (ii) disaccharides, and (iii) polyols (sugar alcohols). Oligosaccharides are either (a) malto-oligosaccharides (a-glucans), principally occurring from the hydrolysis of starch, and (b) non-a-glucan such as raffinose and stachyose (galactosides), fructo- and galactooligo saccharides and other oligosaccharides. Polysaccharides can be divided into starch (a-1:4 and a-1:6 glucans) and non-starch polysaccharides (NSPs). The main components are the polysaccharides of the plant cell wall, such as cellulose, hemicellulose, and pectin but also includes plant gums, mucilages, and hydrocolloids. Some carbohydrates, like inulin, deviate from this pattern because they are found in nature in multiple molecular forms. Inulin, polydisperse 1-kestose-based (GFₙ), from plants may have from 2 to 200 fructose units and so falls between oligosaccharides and polysaccharides (Roberfroid, 2005; Cummings and Stephen, 2007).
Although a classification system based on chemical structure is necessary for assessing properties and estimation of food intakes, it does not translate well into nutritional effects as each class of carbohydrates has overlapping physiological properties and effects on health. Depending on their DP, chemical composition, and linkages, dietary fibre sources have varying physicochemical characteristics, such as solubility and viscosity and physiological properties, including fermentability, bulking effects, blood glucose-lowering, and blood lipid-modifying effects which account for their beneficial impacts on health (Stephen et al., 2017). There is considerable variation in bioavailability among the CHO classes and in different foods. CHOs must be broken down into their constituent monosaccharide units by hydrolytic enzymes in the mouth and pancreas.

1.4.1.1 Digestible Carbohydrates

Ninety-five percent of the CHO in human diet is digested into glucose and absorbed via the small intestine; these are called glycaemic carbohydrates. Glucose, galactose, and fructose are the end products of CHO digestion, which are absorbed in the small intestine via the portal vein to the liver. The glucose concentration in the portal vein after a meal can rise to almost 10 mM/L when monosaccharides are transferring during the first pass through the liver via specific receptors on hepatocytes (Suzuki, 2003, Heymsfield et al., 2005; Seo et al., 2020).

1.4.1.2 Digestion of Starch Metabolism

Starch is catalysed by hydrolysis via amylase enzymes. Starting by salivary α- amylase enzymes (sAA) which is secreted in the mouth by food chewing stimulation processes.
sAA are primarily involved in the digestion of starch in the mouth to yield a mixture of di-saccharides, tri-saccharides, and some glucose Figure 1-4 (Peyrot des Gachons and Breslin, 2016; Qi et al., 2018). A function of sAA on starch digestion in the small intestine was also proposed, given the significant passage of sAA through the stomach due to incomplete inactivation by low pH. In vitro, amylase was inactivated in gastric juice as pH range from 3.8 to 3.3 (Fried, Abramson and Meyer, 1987). If trapped within a large bolus of food inside the stomach, salivary α-amylase can continue to digest starch until the bolus is broken up and exposed to gastric acid. Consequently, up to 30 – 40 % of the digestion of complex carbohydrates can take place before the food entered into the small intestine (Binder and Reuben, 2009; Barrett et al., 2018).

In the small intestine, pancreatic juice enters the lumen through the hepatopancreatic sphincter, and its high bicarbonate concentration begins to neutralize gastric acid. Thus, pancreatic α-amylase is secreted by the pancreas into lumen and actively continues to digest complex carbohydrates. Pancreatic α-amylase acts mostly in the duodenum shortly after its entry through the hepatopancreatic sphincter and generates maltose, maltotriose, trisaccharide, larger oligosaccharides, and α-limit dextrin (Marks and Lieberman, 2009; Goodman, 2010). Since di-, tri-, and oligosaccharides result from the hydrolysis of starch by α-amylase (Binder and Reuben, 2009; Goodman, 2010). These starch hydrolysis products must be additionally digest by the disaccharidases found as membrane-spanning enzymes in the plasma membranes of the brush borders of intestinal epithelial cells and enter the bloodstream either by passive diffusion (fructose) or through a sodium and energy-dependent active transport mechanism (glucose and galactose) Figure 1-4 (Lunn and Buttriss, 2007).
By the end of this process, rapidly hydrolysed starch (with a glycaemic index near 1) is completely hydrolysed. However, a substantial proportion of slowly hydrolysed starch (with a glycaemic index less than 1) remains in the gut lumen and moves to the colon to be fermented. Another type of starch, resistant to hydrolysis (with a glycaemic index near zero), remaining in the gut lumen and consequently being fermented (Lunn and Buttriss, 2007).

The structure of starch is complex and varies widely. Notwithstanding, the most significant structural aspect that has most influences on its ability to be digested is the degree and crystallinity within the granule. So, starch with long linear chains is more liable to form crystalline structures than starch with short highly branched chains (Birt et al., 2013). Therefore, as the amylose component of starch is less branched than amylopectin, high-amylose starch is more likely to be resistant to digestion than low-amylose starch.
1.4.1.3 Non-Digestible Carbohydrates

About 5% dietary carbohydrates are complex CHO, such as resistant starch, polysaccharides, and oligosaccharides (except maltodextrins) are not absorbed in the small intestine and enter the large intestine and fermented by gut bacteria. They are called non-glycaemic carbohydrates as they are not converted into monosaccharide such as glucose (Bond and Levitt, 1978; Heymsfield et al., 2005). The major portion of dietary fibres are commonly non-glycaemic (Ahsan, 2021).

Stephen et al. assessed current knowledge on the health effects of dietary fibres and how different kinds of fibre affect health, summarising the most current findings on the relationship between fibres and the major diseases that are associated with it. Specifically, individuals who followed diets rich in dietary fibres have a lower risk of non-communicable diseases such cardiovascular diseases, and coronary heart diseases, and cerebrovascular diseases outcomes. However, large gaps remain in our understanding of the relationship between the chemical and physical characteristics of dietary fibres and their physiological properties. And, in turn, how this effects disease risk. Most countries throughout Europe have developed recommendations for total fibre intake based on an amount per MJ converted to grams per day: 3-4 g/MJ per day, equating to 25-32 g/d for adult women and 30-35 g/d for adult men and less for children and the elderly based on age (Bond and Levitt, 1978; Heymsfield et al., 2005; Stephen et al., 2017).

Carbohydrates (dietary fibre, prebiotics) are necessary to ensure metabolic activity towards carbohydrate fermentation, which contributes to the physiological levels of the
short-chain fatty acids that are vital for health and well-being (Makki et al., 2018). The fermentation processes are mainly controlled by the volume and different kinds of substrate, in particular the complex carbohydrates that are available to gut microbiota (Sawicki et al., 2017). Gut microbiota are positive correlated with AMY1 copy number and Prevotella abundance in Mexican population (León-Mimila et al., 2018). Another recent study findings by Atkinson et al., investigating the physiologic and phenotypic significance of variation in human AMY1 CN, suggest that microbial fermentation in the large bowel and postprandial glycemia are influenced by AMY 1 CN and sAA activity. This suggests that individuals with high AMY 1 CN seem to digest starch faster, so there is a higher glycaemic response after consuming starchy foods but not sugary foods (Atkinson et al., 2018).

1.4.1.4 Absorption of Carbohydrates

More than 95% of carbohydrates in human diet are absorbed from small intestine into the bloodstream as monosaccharides such as glucose, galactose, and fructose (Tan et al., 2021). Nonetheless, absorption of different types of monosaccharides are mediated through different mechanisms. Generally, glucose and galactose transports are an energy requiring process and therefore a Na⁺-coupled secondary active transporter known as Na⁺-glucose transporter 1 (SGLT1) assist in absorption of glucose from intestinal lumen into enterocytes. From enterocytes, glucose entrance into bloodstream is mediate via a specific glucose transporter (GLUT) known as GLUT2 (Martínez-Delgado et al., 2021; Kurzyna-Szklarek et al., 2022).
Contrary to glucose, fructose is taken up on the brush-border side of intestinal lumen and is absorbed through a facilitated passive transport mediated by GLUT5 to enterocytes and then into the bloodstream (Havel, 2005; Sun and Empie, 2012; Merino, Fernández-Díaz, Cózar-Castellano, and Perdomo, 2019; Hernandez-Hernandez et al., 2019). GLUT5s exhibit the weakest homology to other members of the GLUT family of all GLUTs and serve primarily as fructose transporters with a Km of 6 mM, with minimal amounts entering the systemic circulation. Following absorption, uptake by the liver is mediated by GLUT2 (SLC2A2), a high-capacity, glucose-dependent fructose co-transporter primarily localized on the basolateral membrane of enterocytes and hepatocytes. Fructose is transported to the liver via the portal vein, with minimal amounts entering the systemic circulation (Figure 1-5). Only GLUT2 and GLUT5 can transport fructose, and GLUT5 has a very limited capacity for transporting glucose (Manolescu et al., 2007). Finally, GLUT2s are found in intestinal and kidney basolateral membranes (predominantly), in the liver, and in pancreatic β-cells and mediate the uptake and efflux of glucose, galactose, or fructose (Manolescu et al., 2007; Goodman, 2010).
Chapter 1: General Introduction

1.4.1.5 Metabolism of Glucose and Fructose

Metabolism of monosaccharides, particularly, glucose has been studied for a long time to clarify the metabolic alterations that occur in obesity and diabetes mellitus (Kolderup, 2015). Glucose enters circulation through the hepatic portal vein; hence, it reaches the liver in high concentrations: approximately 10 mmol/L have been measured in the portal vein while the systemic concentration is stable at 5–4 mmol/L.

Absorption of glucose is a key stimulus for the secretion of insulin from pancreatic β-cells. The pancreatic β-cells are highly vascularized cells, clustered in islets of Langerhans which can sense the absorption of glucose from intestine into bloodstream(Ding et al., 2018). The pancreatic β-cells sense the presence of glucose
through GLUT2 (Zhang et al., 2022). GLUT2 assist in the entry of glucose into the hepatocytes.

Glucose is phosphorylated in the hepatocytes, and glucose-6-phosphate is produced. The phosphorylation of glucose is assisted by glucokinase. The glucose is then either stored as glycogen in the liver or released to blood to be transported to the tissues for energy production (Chiu et al., 2018). Similar, pathway is followed for fructose metabolism, nonetheless, fructose phosphorylation is mediated by fructokinase (Figure 1-6) (Muriel et al., 2021, Jang et al., 2020).

The liver has a high capacity for soaking up glucose, more still passes into systemic circulation, resulting in increased glucose concentration and stimulation of insulin secretion. The increasing insulin/glucagon ratio switches off gluconeogenesis and glycolysis and stimulates glycogen synthesis in the liver (Lanham-New, Macdonald, and Roche, 2011).

So, most of the reactions that occur in the liver during fructose metabolism are similar to glycolysis (Embden-Meyerhof-Parnas’s pathway), except that fructose enters the glycolytic pathway at a later stage. As shown in Figure 1-6, fructose bypasses one of the main regulatory steps of glycolysis, the phosphofructokinase reaction, which is tightly regulated by available energy or ATP. The first step in fructose metabolism is the phosphorylation of fructose by fructokinase to fructose 1-phosphate (Kolderup, 2015). Unlike the phosphofructokinase in glycolysis, the fructokinase enzyme is not inhibited by ATP and will continue to metabolise fructose to fructose 1-phosphate. A
higher amount of fructose 1-phosphate increases the amount of glycerol 3-phosphate, which serves as a precursor in triglyceride and phospholipid formation (Goodman, 2010). Since these differences in metabolism of fructose and glucose by the liver yield different end products, the study of their role in the diet may be important to health (Lunn and Buttriss, 2007).

![Metabolic Pathway of Dietary Glucose and Fructose in Liver. Modified from Elliott et al., 2002; Sun and Empie, 2012](image)

**Figure 1-6** Metabolic Pathway of Dietary Glucose and Fructose in Liver. Modified from Elliott et al., 2002; Sun and Empie, 2012

### 1.4.1.6 Insulin Signalling

Most carbohydrate in the diet enters the bloodstream as final products of carbohydrates (glucose and galactose) through a SGLT1 or fructose via the GLUT5 (Lunn and Buttriss, 2007). Pancreas has highly vascularised and trained to sense glucose whenever
glucose is in its proximity. They do so with the help of the glucose transporter (GLUT) isoform - GLUT2, which is also expressed in these β-cells. (Petersen and Shulman, 2018). Glucose sensing has two roles. 1) the entry of glucose into the cell is mediated via GLUTs. 2) glucose metabolism through phosphorylation by glucokinase (Navale and Paranjape, 2016).

At the basolateral membrane, glucose can enter or leave the cell via GLUT2, depending on the concentration gradient. For luminal glucose concentrations ≥ 5 mM and inlet blood glucose of 4 mM, flux across the basolateral GLUT2 (bottom row) is always negative (glucose leaves the cell). Net glucose absorption (magnitude of the flux) increases with SGLT1 density and luminal glucose concentration. It increases with apical GLUT2 only at high luminal glucose concentrations. At 5- and 10-mM luminal glucose, an increase in apical GLUT2 leads to decreased glucose uptake since the cell-blood concentration gradient is reduced. Moreover, in the absence of luminal glucose, the concentration gradient changes sign, and glucose enters the cell from the blood (positive flux) and then is secreted into the lumen via increasing apical GLUT2 (Afshar et al., 2021).

It was suggested that rising apical SGLT1 is effective at increasing glucose uptake, even at high luminal glucose concentrations (> 15% increase at 50 mM luminal glucose as SGLT1 density trebled). It increases apical GLUT2 uptake by about 10% for the same conditions. At low luminal glucose (5 and 10 mM), an increase in apical GLUT2 leads to a decrease in glucose uptake due to glucose secretion from the cell to the lumen (Lunn and Buttriss, 2007; Afshar et al., 2021).
When glucose enters the cell via GLUT2, the insulin is secreted by the pancreatic β-cells, which are highly vascularised and trained to sense glucose whenever it is in proximity. They do so with the help of the glucose transporter GLUT2, which is also expressed in these β-cells. (Petersen and Shulman, 2018). Further, insulin also suppresses metabolic pathways in the liver, which synthesise glucose from amino acids, lactic acid, or glycerol. Thus, provided the tissues are sensitive to the actions of insulin, blood glucose concentrations will decrease. Concurrently, insulin concentrations decrease, preventing glucose uptake into the muscle, liver, and adipose tissue, and leaving a readily available source of fuel for the brain (Lunn and Buttriss, 2007).

With an influx of glucose in the body, the tricarboxylic acid cycle (TCA cycle) springs into action, leading to an increased ratio of ATP: ADP and, ultimately, membrane depolarisation - the closure of potassium channels and an influx of calcium ions, which causes insulin to be finally released into the circulation for carrying out its respective function. The insulin signalling cascade is an amplification process that it diligently performs in the three major sites: skeletal muscles, adipose tissues and hepatic tissues. Abnormalities of insulin signalling are responsible for insulin resistance. Insulin evokes its action on the target organs through the phosphorylation of a transmembrane-spanning tyrosine kinase receptor, the insulin receptor (IR). In all cell types, the binding of insulin to the α-subunit of its receptor activates the tyrosine kinase of the β-subunit of the receptor, leading to the own autophosphorylation, which, in turn, induces the tyrosine phosphorylation of insulin receptor substrates (IRS) family of proteins, as IRS-1 through IRS-6, which act as scaffolds to organise and mediate signalling complexes
(Sun et al., 1991). In an insulin sensitive state (Figure 1-7), the phosphorylation/activation of these substrates evokes the activation of the enzyme phosphoinositide 3-kinase (PI3K), which, in turn, stimulates the activation of the main downstream effector Akt or as called protein kinase B, and atypical protein kinases C. Akt is serine/threonine kinase, which stimulates the glucose uptake via the translocation of the major glucose transporter GLUT-4 to the plasma membrane. Further, Akt inactivates, by phosphorylation, the glycogen synthase kinase 3 (GSK-3), and this enzyme inhibits glycogen synthase, which is a significant regulator of the glycogenic process, therefore, facilitating the glycogenesis (Figure 1-8) (glycogen breakdown) (Boucher, Kleinridders and Kahn, 2014; Iaccarino et al., 2020).

Several polymorphisms in human IRS-1 are observed in type 2 diabetes. These polymorphisms are associated with insulin resistance, hyperinsulinemia, dyslipidemia, adiposity, and risk of coronary disease. Also, it reduced IRS-1 protein levels and decreased IRS-1–associated PI3K activity.

Insulin resistance was proposed as early as 1936 and identified as an impaired biologic response to insulin stimulation of tissues, mainly the liver, muscle, and adipose tissue. Insulin resistance impairs glucose disposal, resulting in a compensatory increase in insulin production from β-cells leading to hyperinsulinemia (Sinaiko and Caprio, 2012; Freeman and Pennings, 2021).

Insulin resistance may be either due to defects at some point before insulin binding to its receptor or at the insulin receptor level or defects in downstream signalling
components. Defects of the insulin receptor that may contribute to insulin resistance include abnormalities in receptor structure and number, binding affinity, and signalling capacity. Many factors, including oxidative stress, neurohormonal stimulation, and hyperinsulinemia, have been described as responsible for the inhibition of insulin-stimulated tyrosine phosphorylation of insulin receptors and their substrates (Iaccarino et al., 2020).

1.4.1.7 Skeletal Muscle

Skeletal muscle performs key role in maintaining glucose homeostasis in blood. Skeletal muscles are considered as prime site for glucose regulation, which accounts for up to 70% of the whole-body glucose disposal in healthy individuals (Abdul-Ghani and DeFronzo, 2010; Sharabi et al., 2015; Hargreaves and Spriet, 2020). Skeletal muscle also is an energy-consuming tissue. The glucose transported to the myocytes stores in myocytes which is mostly used for energy production, with the exception of 3-carbon units (lactate, alanine) generated by glycolysis (Shimizu et al., 2018), and mostly cycled to the liver via a cascade of biochemical reactions known as Krebs cycle or citric acid cycle that are involved in the metabolism of carbohydrates in skeletal muscles (Impey et al., 2018).

Insulin plays an important role in the transportation of glucose into muscles (Yaribeygi et al., 2019). Binding of insulin to skeletal muscle membrane assist in the autophosphorylation of the insulin receptors on the skeletal muscle membrane (Rahman et al., 2021). As described previously, the phosphorylation of PKB also works as a significant factor in suppressing glycogen synthesis and augmenting glucose uptake.
from the blood, which leads to decrease in the intracellular glucose concentration and increase uptake of the glucose by skeletal muscles (Ferrari et al., 2019). If muscle need energy for performing specific functions, glucose is used to produce energy and if muscle is at rest and does not have immediate requirement of energy, the glucose is stored as glycogen (Sylow et al., 2021).

All these effects lead to decreased intracellular glucose concentrations and increased skeletal muscle glycogen synthesis. With impaired muscle uptake, excess glucose returns to the liver, increasing de novo lipogenesis (DNL) and circulating free fatty acids, further contributing to ectopic fat deposition and insulin resistance (Freeman and Pennings, 2021). With excess calorie loads, glucose uptake by muscle exceeds capacity, and excess glucose returns to the liver, where it triggers DNL. Increased DNL increases triglyceride and FFA production, causing ectopic fat deposition into the liver, muscle, and adipose tissue. As a result, insulin resistance increases, as well as the production of inflammatory markers. Additional factors influencing insulin resistance in muscle tissue include physical inactivity and genetic risk.
1.4.1.8 Insulin Resistance

Insulin resistance is the abnormal condition when muscles cannot respond to insulin and thus cannot use glucose from blood for energy and storage (Petersen and Shulman, 2018). Insulin resistance is also called impaired insulin sensitivity. In conditions when there is insulin resistance, glucose is not stored in the skeletal muscles and therefore excess glucose are return to the liver (James et al., 2021). The resultant increase in lipogenesis and circulating free fatty acids contribute to fat deposition which further worsen the effects of insulin resistance. The vicious cycle remains continue and may produce inflammatory markers Figure 1-7 & Figure 1-8 (Shimobayashi et al., 2018).
1.4.1.9 Adipose Tissue

Although adipose tissues are mainly involved in the metabolism and storage of triglycerides (TGs), nevertheless, when insulin is released, its functionality related to lipids is reduced. The action of insulin on the adipose tissue is sufficient to promote glucose uptake from the bloodstream directly into the adipose tissues (Song et al., 2018). Lipolysis is most sensitive to insulin. Failure of insulin to suppress lipolysis in insulin-resistant adipose tissue, particularly visceral adipose tissue, increases circulating free fatty acids. Higher levels of circulating FFAs affect both liver and muscle metabolism, further exacerbating insulin resistance (Freeman and Pennings, 2021). Besides, lipid metabolism also plays a significant role in insulin resistance. Excess lipid intake by the body results in an accumulation of the lipid derivatives that play a major role in downregulating the insulin protein signalling activation.
The potency of insulin to control plasma nonesterified fatty acid (NEFA) levels is critical to the maintenance of euglycemia; suppression of lipolysis is an important physiological function of insulin in adipose tissues. Moreover, elevated TG (triglyceride) levels are also involved in the pathogenesis of insulin resistance. It is seen that there is a linear increase in the synthesis of triglycerides and diacylglycerol, both of which occur in cases of insulin resistance only. Intramuscular ceramide levels are also seen to be a part of insulin resistance. They act as lipid derivatives and work to reduce the uptake of glucose, thus playing their part in giving rise to the development of insulin resistance.

Adipose tissue could play a crucial part in buffering the flux of fatty acids in the circulation in the postprandial period, analogous to the roles of the liver and skeletal muscle in buffering postprandial glucose fluxes. Adipose tissue provides its buffering action by suppressing the release of non-esterified fatty acids into the circulation and by increasing triacylglycerol clearance. In particular, the pathway of 'fatty acid trapping' (adipocyte uptake of fatty acids liberated from plasma triacylglycerol by lipoprotein lipase) (Frayn, 2002).

1.4.1.10 Hepatic Tissue

Several mechanisms promote glucose regulation in the liver cells, with GLUT2 and GLUT4 translocations being the most prominent ones. Insulin reduces or suppresses the glucose output from the liver and increases the glucose uptake into the skeletal muscle, which leads to normalising glucose levels. However, in insulin resistance
conditions, the liver, adipose tissues, and skeletal muscles fail to respond to insulin action (Metcalfe et al., 2019).

Insulin resistance in muscle leads to increased delivery of glucose substrate to the liver, which triggers DNL, associated inflammation, and ectopic lipid deposition. Insulin resistance in adipose tissue results in increased lipolysis in adipocytes, increasing circulating FFA and further exacerbating steatosis and insulin resistance in muscle tissue. In the postprandial state, insulin reduces hepatic glucose production via inhibition of glycogenolysis, limiting the postprandial rise in glucose. This feedback mechanism is impaired with insulin resistance, and hepatic glucose production continues to rise, even as postprandial glucose rises. Glucotoxicity, associated with elevated glucose levels, further contributes to insulin resistance. Further, glycogen synthesis is mainly responsible for the pathogenesis of insulin resistance. An impaired insulin signal transduction ultimately leads to a blunted response to the phosphorylation of kinases.

1.5 Genetic Variation of Obesity

Hereditary influence on obesity has been investigated for more than a century, beginning in 1907 with Von Noorden’s delineation of exogenous and endogenous obesity. Exogenous obesity refers to obesity resulting from energy intake more than energy expenditure, while endogenous obesity is caused by hypometabolism or other thyroid disorders (Chin, 2014; Thaker, 2017).
In 1986, Stunkard and colleagues offered landmark evidence for genetic influence on obesity, using a Danish adoption registry of 540 adults. The study compared the BMIs of both biological and adoptive parents with those of the adoptees. It was found that the adoptees’ BMIs approximated those of their biological parents rather than the parents who had raised them. Genetic factors have a strong influence in determining adult body fatness, while the family environment alone has no apparent effect on study samples (Stunkard et al., 1986; Chin, 2014). A systematic review of twin studies reported that the variable heritability of weight across lifetime with an overall effect estimated between 45–90% (Silventoinen et al., 2010).

Heritability ($H^2$), as defined by Lush (1943), is the proportion of variance within individuals in a population due to genetic factors, the extent to which phenotypic traits are affected by genetic variations (Bell, 1977). It is estimated simply from the functions of the regression of offspring on parental phenotypes, the correlation of full or half siblings, and the difference in correlation of monozygotic (MZ) and dizygotic (DZ) twin pairs (Frankham, 1996).

Because of the variation in additive and non-additive genetic factors and population-specific environmental variances, heritability depends on the population (Visscher et al., 2008). Variance is a measurement of the degree to which a trait is dispersed away from the mean. An estimation of high variance means that there are more individual phenotypic differences for the trait than for an estimation of low variance (Oftedal, 2005). Phenotypic variance ($V_P$), genetic variance ($V_G$) and environmental variance
(\(V_E\)) can be linked in mathematical terms by the following formula (Oftedal, 2005; Visscher et al., 2008):

\[ V_P = V_G + V_E \]

Similarly, phenotypic variance or broad heritability (\(H^2\)) is assumed by the ratio of total genetic variance to phenotypic variance:

\[ H^2 = \frac{V_G}{V_P} \]

Since variables such as traits, allele frequencies, size of the variants, and mode of gene actions can vary across populations, like environmental variances, heritability is considered similarly variable (Visscher et al., 2008).

A 1990 study of twins reaffirmed the influence of genetics regardless of environmental variation. The study involved 247 pairs of identical twins, 154 pairs raised together, and 93 pairs adopted by different parents. The twins in each pair turned out to have similar weight, regardless of whether they had grown up together or separately. In the same year, Claude Bouchard and colleagues conducted an experimental study overfeeding identical twins with the same number of calories over a period of 100 days. The study showed a correlation of weight gain within twin pairs, much higher than between pairs (Bouchard et al., 1990). Correlations persisted in follow-up studies of these twin pairs for five years (Bouchard et al., 1996).
Over the past 50 years, meta-analysis studies of twins (involving more than 14 million pairs) have provided compelling evidence that all human traits are heritable. Not one trait investigated had a weighted heritability estimate of zero. In addition, approximately 2/3 of heritable traits exhibit a pattern of monozygotic (MZ) and dizygotic (DZ) transfer (Polderman et al., 2015). This pattern, known as twin correlation, is consistent with a simple model, as explained by Polderman et al. (2015). Family and twin genetic studies have clarified that there is a 67% BMI variance in males and females, of which 50% is due to dominance and, the remainder of the variance is accounted for by unique environmental factors, of which only 7% is correlated across twins (Ronnemaa, 1997).

Heritability can vary by sex and age within the same population. Many studies have shown that BMI is highly heritable (Schousboe et al., 2003; Hjelmborg et al., 2008). In a systematic review of healthy MZ and DZ twins conducted by Nan et al. (2012), it was found that the heritability of BMI is high in young to later adulthood, whereas the influence of environmental factors on BMI is stronger from adolescence to young adulthood. There is then less impact on BMI from an individual difference in late adulthood, whereas unique environmental influences rise steadily from pre-adolescence to late adulthood.

Genetic variations comprise single nucleotide variants (SNVs), copy number variations (CNVs), and large structural variation (SVs) (Zhao et al., 2013). Single-nucleotide polymorphism (SNP) is the predominant form of genomic variation associated with normal variations (Freeman et al., 2006) and is also the most studied form (Zhao et al.,...
From databases of public and private individuals, about 4 million SNPs had been validated by 2005 and more than 5 million collected (Sobrion et al., 2005). According to the latest information, there are around 32 million reference SNPs, around one every 200 bps. This rapid increase in data was thanks to two massive NHGRI-driven efforts: the International HapMap Project (2003) and its sequel, the 1,000 Genomes Project (2008).

SNPs occur when only a single nucleotide is exchanged for another in the DNA sequence, presenting at least 1% of the population. For example, some chromosomes within a population can have a C (C-allele) at a given site, while others have a T (T-allele) at the same site (The International HapMap Consortium, 2003). SNPs can occur more frequently in non-coding intronic regions of genes than in protein-coding exons and intragenic regions (Barreiro et al., 2008). Generally, SNPs do not affect the function of a gene; however, it may be impacted if intronic SNPs impinge on splicing and gene expression. The difference in the effect of SNPs is apparent in two types of SNPs in gene-coding regions: synonymous (silent mutations) SNPs, which do not alter the amino acid composition of the encoded proteins, and non-synonymous SNPs, which change the amino acid of the protein sequence. Recently, there has been a growing awareness that other types of genetic variations may impact the risk of disease and treatment outcomes (Barreiro et al., 2008). Synonymous SNPs influence fitness, but over the past decade, it has been shown that they can result in abnormal mRNA splicing, which may lead to human diseases (Sauna and Kimchi-Sarfaty, 2011).
Structural variations (SVs) greater than 1 kilobase (kb) in size are defined as insertions, inversions, and deletions. These range from single-base pairs to large, chromosomal-level alterations. Two models have been proposed for the association between structural variation and disease. The first model is large variants, which entail gains and losses of several hundred kb in a DNA length. Although large variants are rare in a population (< 1 %), they cause a significant fraction of disease (Alkan et al., 2011). The second model is a type of intermediate-scale SV with a copy number change involving a DNA fragment, which is typically greater than one kb and less than five megabases (mb) (Alkan et al., 2011).

Copy number variation (CNV) includes a genetic polymorphism with regard to DNA segments larger than 1 kb; it is found in variable numbers and is compared with a reference gene. This type of structural variation may account for a substantial proportion of differences in the assembled human genome (currently estimated at > 12 %), altering gene dosage and potentially modulating the expression of genes (Santos et al., 2012).

1.5.1 Copy Number Variation Determination Methods

CNVs can be detected and analysed by several methods at the genome-wide and locus-specific levels. The genome-wide level CNVs can mainly be detected by two DNA chip-based methods: Comparative genomic hybridization (CGH)-based CNV detection and SNP array-based CNV detection. The difference between these two methods is that CGH-based method uses two different fluorescent dyes for labelling test and reference DNA samples, and the samples can be hybridised to the same microarray spot.
However, the SNP array-based method uses one fluorescent dye for each sample, and comparison between the samples can offer the location of the CNV. Both methods use log2 ratios for identification of the locations of CNVs. Locus-specific analysis largely uses the following tools: pulse field gel electrophoresis (RFGP) followed by Southern blot analysis, pyrosequencing, ligation detection reaction (LDR) and the invader assay, and quantitative real-time PCR (Leea and Jeonb 2008). RFLP-Southern blot is the most conventional tool for separating CNVs in the large DNA (range of 5–500 kb). RFLP–Southern blot isolation of high molecular weight DNA, digestion with unique restriction enzymes, and resolving digested DNA fragments by pulsed field gel or low percentage (< 1 %) agarose gel electrophoresis (Pulimamidi, Ravula and Battu, 2016). The pyrosequencing method was developed by Ronaghi et al. (1998) using the principle of pyrosequencing. With this technique the DNA fragments are incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase, and the substrates adenosine 5’ phosphosulfate (APS) and luciferin. The ATP takes part in the luciferase-mediated conversion of luciferin to oxyluciferin, creating visible light proportionately to the amount of ATP. Then, there is the quantification for CNV analysis at the same time with sequence analysis for SNP detection. In ligation detection reaction (LDR), oligonucleotide ligation assay (OLA) is performed directly to genomic DNA. During the hybridized two oligonucleotide probes, DNA ligate can covalently link these two oligonucleotides. If a mismatch at either the 3-end of the first probe or the 5-end of the second probe is introduced, the biallelic state of the SNP locus is converted into a biomarker state of ligated detector oligonucleotides (Leea and Jeonb 2008). The quantitative real-time PCR has become one of the most popular and effective methods for analysing CNVs. It is basically performed on an apparatus uniting a thermal cycler and an optical instrument to capture spectral fluorescence consisting of the hydrolysis
probes, hybridizing probes, and DNA-binding agents (Leea and Jeonb, 2008). The TaqMan assay is a typical system using the hydrolysis probe for fluorescence resonance energy transfer (further information is provided in Chapter 2).

Today, molecular genetics is central to obesity research. For BMI, genome-wide association studies (GWAS) have identified up to 941 near-independent genome-wide significant SNPs at 536 polygenic loci (Yengo et al., 2018). In 2007, McCarthy and colleagues identified a common variant in FTO, a gene associated with FM and obesity, and cutting-edge sequencing technology continue to identify gene variants or mutations (Chin, 2014). FTO has been clearly identified as an obesity-associated gene via GWAS. Each additional copy of the rs9939609 risk allele is associated with increased BMI of \(~0.4\, \text{kg/m}^2\). FTO appears to have a greater effect on obesity than all other obesity loci, and this has been confirmed through replication studies throughout lifetimes and across ethnicities. The FTO rs9939609 SNP is the most commonly reported population obesity gene in association studies. It has been estimated that the population-attributable risk of FTO for obesity is as high as 20\% (Loos and Boucard, 2008). The risk allele (A allele) of rs9939609 is associated with greater total energy (food intake) and increased protein and fat intake in children and adults. FTO SNPs, mainly located in intron 1, have been reported to be associated with individual variation in appetite rating scales, loss of control overeating and eating in the absence of hunger. The FTO genotype was associated with changes in body composition as a result of regular exercise (Rankinen et al., 2010). Furthermore, in a recent study to determine the role of the FTO SNPs rs1421085, rs17817449, and rs9939609 in the effects of a 4-week hypocaloric diet on body composition in 47 exercise-trained men and women, the results showed that FTO genotype had no effect on short-term weight and fat loss (Antonio et al., 2019).
CNV is noted for its frequency in healthy individuals. It has been estimated that about 12% of the genomes in human populations are subject to CNV. Meanwhile, about 50% of reported CNVs overlap with protein-coding regions (Freeman et al., 2006). These losses and gains in gene copies may directly influence gene dosage within the CNV regions, a differential level of gene expression (Zhao et al., 2013) that is associated with (Alkan et al., 2011) or directly involved in diseases such as cancer and neuropsychiatric disorders (Zhao et al., 2013). However, the influence of CNVs does not necessarily have a negative impact on human health (Zhao et al., 2013). For example, high copy numbers of AMY1 have been shown to be associated with a reduced risk of obesity (Falchi et al., 2014). The AMY1 locus is located in a CNV region on human chromosome 1p21.1 (Falchi et al., 2014). The AMY1 copy number is correlated significantly with surrounding SNPs, with an average difference of 0.6 to 2.0 AMY1 copies per SNP minor allele in individuals with European ancestry (Usher et al., 2015).

1.6 Salivary α-amylase

In 1831, Ethard Leuchs reported that starch is broken down chemically when mixed with ptyalin (the agent’s name of saliva) (Butterworth et al., 2011). Amylase has several isoforms, which can be secreted by the pancreas (P form) and the salivary glands (S form) but can also be found in smaller quantities in some other tissue types. Saliva is predominantly (55–65%) secreted from three major salivary glands: the parotid, submandibular and sublingual glands. In healthy humans 40–45% of the daily saliva amount produced from pancreas accounted between 500 to 1500 ml (Santos et al., 2012; Pieper-Bigelow, Strocchi and Levitt, 1990). Saliva α-amylase (sAA) is the most common protein in human saliva, accounting for between 40–50% of total saliva
protein. sAA has two protein forms: glycosylated sAA and non-glycosylated sAA (Yang et al., 2015). Saliva plays several roles. It maintains the health of the oral cavity and gastrointestinal tract in general by inhibiting harmful microbes, and it promotes healing in oral tissues. It also helps with lubrication of food, and it contains a high concentration of the enzyme α-amylase, which is essential in the digestion of complex carbohydrates (Pedersen et al., 2002). sAA is an endo-enzyme that catalyses the hydrolysis of α-1, 4 glycosidic links in starch, resulting in maltose and larger oligosaccharides; The gene for sAA is AMY1 (Figure 3) (Yang et al., 2015).

The human AMY1 gene was reported as copying three genes in the original assembly of the locus sequence, corresponding to the position of paralogues AMY1A, AMY1B and AMY1C. These three copies are 99% identical in the DNA sequence, and a similar sequence identity extends for a region of about 26.5 kb for each copy, which corresponds to the underlying copy variable unit of AMY1 (Carpenter et al., 2015). Individuals are estimated to have even numbers of AMY1 copies four times more often than odd, mostly sharing parity with AMY2A (AMY1 and AMY2A are always either both odd or both even) (Usher et al., 2015). Further, AMY1 is very similar to AMY2 (the gene code for pancreatic amylases) in its coding region; but AMY2 is shorter in the 5′ untranslated region than the equivalent region in AMY1. The salivary amylase gene contains 511 amino acids, which differ by 3% from AMY2 protein (Meiser and Ting, 1993).
1.7 AMY1 Copy Number and Population Diet

High salivary amylase activity is associated with a predominantly CHO diet. Squires (1952) compared four healthy, young and middle-aged adult groups in relatively close geographical propinquity with different amounts of carbohydrates in their diets. Squires discovered that 48–50% of the first group, which was primarily consuming carbohydrates, had an average salivary activity rate of between 150 and 300 units/ml. As for the second group, which was made up of participants with a mixed-diet, and the third group, which consisted of adults with a low-CHO-diet, had activity rates of 101 and 22 units/ml, respectively. Then, he tested the fourth groups who lived on a predominantly carbohydrate diet over a three-month period, in which the salivary amylase activity of the group did not vary significantly over the stated timeframe (Squires, 1953). Population with high CHO consumption tend to have higher salivary activity rate and high copy number of AMY1.

Recently, it was confirmed that the activity, expression, and enzyme concentration of amylase are partially correlated with their gene copy numbers (CNs) and vice versa (Mandel & Breslin, 2012, Perry et al., 2007). Human salivary amylase genes display an extensive copy number variation (Yang et al., 2015). An individual’s genomes can have anywhere from 1 to 20 copies of AMY1 (Iafrate et al., 2004; Parry et al., 2007; Santos et al., 2012). It has been demonstrated that the average copy number of AMY1 genes are higher in populations that consume high-starch diets. Therefore, an increased number of AMY1 copies seems to represent an advantage in populations that have evolved to have starch-eating habits, such as in parts of China (where the daily intake is as high as 371g/day), while it is probably neutral in cultures of low starch intakes (Santos et al., 2012). Perry et al. (2007) asserts that the copy number of the salivary
amylase gene (AMY1) is positively correlated with salivary amylase protein levels. The study also showed that more copies of AMY1 and higher protein levels are likely to improve the digestion of starchy food and may reduce intestinal disease. This result agrees with Mathew et al. (2008), who determined that populations depending on high-starch diets have higher diploid copy numbers of AMY1 than those with low-starch diets.

1.8 Recent Studies on the Influence of High AMY1 Copy Numbers on Obesity

Obesity is a complex disorder that is influenced by the interaction of both genetic and non-genetic factors. The heritability of BMI is estimated at 40–70 % (El-Sayed Moustafa & Froguel, 2013). It has been suggested that genomic copy number variants (CNVs) may be a contributor, which in turn explains the heritability of complex diseases and common traits.

There appears to be a strong genetic link between carbohydrate metabolism and BMI (Falchi et al., 2014). This study examined the susceptibility to obesity in British twins and a French adult population by measuring AMY1 copy numbers and the findings highlighted that, subjects with a lower estimated AMY1 copy number demonstrated a significantly higher risk of obesity when the BMI is increased to 0.15 kg/m² per one low copy. The distribution of AMY1 copy numbers ranged from 1 to 18 copies and approximately 10 % of subjects were carrying more than 9 AMY1 copy numbers, with fewer having less than 4 copies (Falchi et al., 2014). A clinical study identified the putative benefits of high AMY1 copy numbers on obesity and energy metabolism among children in (Mejia-Benitez et al., 2015; Marcovecchio et al., 2016), while
another clinical study showed that low CNVs of AMY1 were associated with early-onset female obesity (Viljakainen et al., 2015), and adult males and females (Pinho et al., 2018; Leon-Mimila et al., 2018; Venkatapoorna et al., 2019; Heianza et al., 2020; Rossi et al., 2021). In contrast, other well-powered replication studies failed to confirm an association between AMY1 copy number and measurements of obesity (Usher et al., 2015; Yong et al., 2016; Rukh et al., 2017; Shwan et al., 2019; Valsesia et al., 2019; Shown & Armour, 2019; Al-Akl et al., 2020; Vázquez-Moreno et al., 2020). These inconsistent results may be due to the heterogeneity in patient samples in terms of number, ethnicity and genetic background, age, gender, and the use of a different study design and methods such as the type of CNV determined and the data collection on the diet and physical activity.

A study conducted on seven healthy participants, by Mandel and Breslin (2012), demonstrated that a healthy-weight population with high salivary amylase activity and concentration had notably better glycaemic control following an ingestion of liquid corn starch compared to a low amylase activity group (Mandel & Breslin, 2012). This finding was confirmed by a crossover study of ten healthy participants, which substantiated those participants with high amylase activity may have a greater early increase in plasma insulin concentration and a low glycaemic response after an ingestion of gelatinised starch when compared with a low-amylase activity group. However, this result did not achieve statistical significance (Alberti et al., 2015). In addition, a cohort study that applied a homeostasis model assessment of insulin resistance in 1,257 asymptomatic Korean men, who were not being treated for diabetes, established that low CNV of AMY1 had a correlation with insulin resistance in the subjects, even after adjusting for BMI (Choi et al., 2015). None of these studies
examined the major role of AMY1 after the ingestion of complex carbohydrates or evaluated the influence of AMY1 on the glycaemic response after starch ingestion, according to the high / low CVN of AMY (as shown in Table 3).
## Table 1-2 Summary of the Recent Studies Examined the Influence of AMY1 Copy Numbers on BMI and Obesity

<table>
<thead>
<tr>
<th>Study</th>
<th>Aim</th>
<th>Population</th>
<th>CNV determination</th>
<th>Result</th>
<th>AMY1 CN mean (range)</th>
<th>Conclusion</th>
</tr>
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<tbody>
<tr>
<td>Mejia-Benitez et al. (2015)</td>
<td>Assessing the association between a highly polymorphic CN of AMY1 and obesity.</td>
<td>597 Mexican children (293 of them are obese and 304 are normal weight controls)</td>
<td>dPCR and a logistic regression model used to adjust for age and sex</td>
<td>Strong associations between AMY1 copy number and obesity risk (OR per copy = 0.84). All children with AMY1 copies &gt;10 was normal weight.</td>
<td>~7.0 (1-16)</td>
<td>Marked effect of high number of AMY1 on energy metabolism among Mexican children</td>
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<td>Falchi et al. (2014)</td>
<td>Examined the influence of gene dosage effects on adiposity through a CN association.</td>
<td>Total 6,200 subjects. Family – based 149 Swedish families (98 M and 244 F), 972 Twins from UK (1,479 F), 2,137(942 M and 1,195 F) from French.</td>
<td>qPCR</td>
<td>Both UK and DERIR had a similar copy number distribution (Wilcoxon test P &gt; 0.05) and median copy number of 6, ranging from 1 to 18. Rise AMY1 copy number was positively associated with Low copy number of AMY1 is associated with increased BMI (change in BMI per estimated copy = −0.15 (0.02) kg/m²)</td>
<td>6.2 (2-17)</td>
<td>Low copy number of AMY1 is related with increased BMI</td>
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<td>Usher et al. (2015)</td>
<td>Understand these issues at the amylase locus in association with BMI</td>
<td>1,000 obese or lean Estonians plus two other Europeans - cohorts totalling ~3,500 individuals.</td>
<td>ddPCR and whole genome sequence analysis, and genome mapping</td>
<td>No association between obesity and the CN of any amylase gene (P= 0.70 for AMY1)</td>
<td>N/A</td>
<td>No association between AMY1 CN and obesity or BMI</td>
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<td>Yong et al. (2016)</td>
<td>A novel calibration method combining qPCR and array-based dPCR. This combined method was used to validate the previously reported association of AMY1 CN with obesity in two East Asian populations.</td>
<td>1,077 males ranging from ages 18 to 21 (519 Chinese controls, 413 Chinese obese, 30 Malay controls, and 115 Malay obese)</td>
<td>qPCR and dPCR.</td>
<td>AMY1 distribution was from 1 to 24 copies. CN deduced high correlations between qPCR and dPCR. No significant association in all four groups including Logistic regression.</td>
<td>~8.2</td>
<td>No association between AMY1 with obesity</td>
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<td>Study</td>
<td>Methodology</td>
<td>Results</td>
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<td>Marcovecchio et al. (2016)</td>
<td>Assessed the potential association between AMY1CN and a wide range of BMI in a population of Italian schoolchildren.</td>
<td>744 children (354 boys, 390 girls, mean age (±SD): 8.4±1.4 years) qPCR. A significant increase of BMI z-score by decreasing AMY1 copy number was observed in boys (β: -0.117, p = 0.033), but not in girls.</td>
<td>In boys, lower AMY1 CN associated with higher BMI</td>
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<td>Viljakainen et al. (2015)</td>
<td>Investigated the relationship between AMY1 CN, BMI and serum amylase in childhood-onset obesity.</td>
<td>61 males and females with a history of childhood-onset obesity (mean age 19.1 years) and 71 matched controls (19.8 years) ddPCR Mean AMY1 CN did not differ between obese and control subjects. Obese men showed the highest and obese women the lowest number of AMY1 copies (p=0.045). Only in affected females, AMY1 CN correlated significantly with whole body fat percent (r=-0.512, p=0.013) and BMI (r=-0.416, p=0.025).</td>
<td>In obese women, AMY1 CN inversely correlated with whole body fat % and BMI</td>
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<td>Rukh et al. (2017)</td>
<td>Investigated the association between AMY1 copy number and obesity traits The effect of the interaction between AMY1 copy number and starch intake on obesity traits</td>
<td>4800 obese individuals ddPCR AMY1 copy number was not associated with BMI (P= 0.80) or body fat percentage (P= 0.38). A significant effect of the interaction between AMY1 copy number and starch intake on BMI (P-interaction = 0.007) and body fat percentage (P-interaction = 0.03).</td>
<td>No association between AMY1 with obesity</td>
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<td>Pinho et al. (2018)</td>
<td>Investigating the association between AMY1 CNV and obesity in young adults of Portuguese origin.</td>
<td>262 adults’ individuals (155 females and 107 males) ddPCR A significant association was found between lower AMY1 copy number and risk of obesity (OR = 0.532; p = 0.034), even when adjusted for age and sex (OR = 0.527; p = 0.039). However, AMY1 did not show a significant association between AMY1 CN and risk of overweight/obesity in the whole population (p = 0.489)</td>
<td>The high CN of AMY1 protects against obesity in Portuguese young adults</td>
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<td>Study</td>
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<tr>
<td>León-Mimila et al. (2018)</td>
<td>Evaluated the association of AMY1 with obesity and gut microbiota in Mexican children and adults</td>
<td>AMY1 CN was significantly associated with obesity in both age groups. Gut microbiota analyses revealed a positive correlation between AMY1 copy number and Prevotella abundance.</td>
<td>The high CN of AMY1 is significantly associated with obesity in children and adults. AMY1 is correlated with gut microbiota in humans</td>
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<tr>
<td>Shown &amp; Armour (2019)</td>
<td>Tested the BMI-AMY1 association at different age points in the same individuals using longitudinal BMI information from participants in the UK 1958 Birth Cohort study.</td>
<td>No significant association was observed (P &gt; 0.05). Examined the trajectory of BMI with age by testing the interaction between AMY1 copy number, BMI, and age, detecting no significant interaction term (P = 0.27) an association between AMY1 copy number and obesity.</td>
<td>No evidence, even at nominal significance between AMY1 copy and BMI at any age point</td>
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</tr>
<tr>
<td>Valsesia et al. (2019)</td>
<td>Investigating the association of AMY1 CN with anthropometrics and glycaemic outcomes in obese individuals. Investigating the interaction between nutrient intakes and AMY1 CNs.</td>
<td>At baseline, a modest association between AMY1 CN and BMI (P = 0.04) was observed. AMY1 CN was not associated with baseline glycaemic variables. Additionally, AMY1 CN was not associated with anthropometric or glycaemic outcomes following either LCD or WMD.</td>
<td>The associations between AMY1 CN and BMI changed after LCD. The AMY1 CN cannot be considered as an important biomarker for response to a clinical weight loss and weight maintenance programs in overweight/obese subjects.</td>
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<tr>
<td>Al-Akl et al. (2020)</td>
<td>Assessing this relationship in a cohort from Qatar, where obesity affects 43% of adults.</td>
<td>The sAA was significantly lower in obese individuals. Significant inverse correlations were found between adiposity markers and plasma sAA in both sexes but were marginally stronger in men. A significant effect of high sAA, but not AMY1 CN</td>
<td>CNV 7 plasma sAA activity 4.73 and 65.82 U/L. No evidence, even at nominal significance between AMY1 copy and BMI but this is association between. Weight and SAAs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Methodology</td>
<td>Sample Size</td>
<td>Results</td>
<td></td>
<td></td>
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<tr>
<td>-----------------------</td>
<td>-----------------------------------------------------------------------------</td>
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<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>Heianza et al. (2020)</td>
<td>Investigating associations of AMY1 genetic variations with general and central adiposity changes considering dietary carbohydrate intake</td>
<td>2,054 adults (17,171 women and 14,883 men)</td>
<td>Changes in general and central adiposity over 5.5–10 years&lt;br&gt;AMY1-GRS using nine SNPs associated with copy number variation&lt;br&gt;The carbohydrate food intake significantly altered associations of AMY1-GRS with changes in weight, BMI and WC.&lt;br&gt;Significant in female cohorts rather than in male cohorts.&lt;br&gt;Among women, higher AMY1-GRS was associated with more increases in adiposity if dietary carbohydrate food intake was high, while higher AMY1-GRS was associated with less gains in adiposity when the dietary intake was low.</td>
<td>AMY1-GRS ranged 8.5–8.6 points&lt;br&gt;In women, higher AMY1-GRS is associated with more increases in adiposity if dietary carbohydrate food intake was high.&lt;br&gt;While higher AMY1-GRS is associated with less gains in adiposity when the dietary intake was low.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vázquez-Moreno et al. (2020)</td>
<td>Investigating the association of AMY1A/AMY2A CN and AMY1/AMY2 serum enzymatic activity with childhood obesity</td>
<td>427 and 337 obese Mexican cases and controls&lt;br&gt;Anthropometric and dietary starch intake data were collected.</td>
<td>ddPCR&lt;br&gt;The serum enzyme activity of AMY1 and AMY2 was negatively associated with childhood obesity risk, and the association was restricted to kids eating medium/high amount of starch.&lt;br&gt;No association between AMY1A and AMY2A CN and childhood obesity was observed in the study sample.&lt;br&gt;The study confirmed a significant association between AMY1A CN and obesity in a meta-analysis of 3100 Mexican children.</td>
<td>NA&lt;br&gt;No evidence, even at nominal significance between AMY1 and AMY2 CN. A significant association between AMY1A CN and obesity in a meta-analysis of 3100 Mexican children.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rossi et al. (2021)</td>
<td>Assessing the impact of AMY1 CNs on adiposity report conflicting findings in different global populations, likely reflecting the impact of ancestral and ethnic-specific environment</td>
<td>2935 Qatari individuals who underwent whole-genome sequencing (WGS) as part of the Qatar Genome Programme.</td>
<td>NA&lt;br&gt;A negative association between AMY1 CNs and trunk fat percentage in the Qatari population ($P = 7.50 \times 10^{-3}$) and show that Qatars of Arab descent have significantly lower CN at AMY1</td>
<td>NA&lt;br&gt;Lower AMY1 CN was associated with increased total and trunk fat</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 1-3 The Table Summary of the Studies Examined the Role on AMY1 Copy Number in Glycaemic Response After Starch Intake.

<table>
<thead>
<tr>
<th>Study</th>
<th>Aim</th>
<th>Population</th>
<th>Method</th>
<th>Result</th>
<th>SAA (mean ± SD CN)</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mandel and Breslin (2012)</td>
<td>A cohort study assessing influence of high CN of AMY1 and salivary amylase enzymatic activity with starch digestion</td>
<td>N=7 high amylase (HA) level N=7 low amylase (LA) level Adult, healthy, BMI &lt;25</td>
<td>Plasma glucose, plasma insulin, Measuring salivary amylase enzymatic activity, AMY1 CN. OGTT in 50g (10 % solution) of a glucose and OGTT of 50 g of a corn Starch. Blood sampling at -5.0, each 3 min first 15 min then each 15 min up to 2 h 5 ml blood used for AMY1 assessing FFQ</td>
<td>HA group was greater in 1- Salivary flow rate was 1.58±0.25 mL/min (P&lt;0.05).  2- Amylase concentration 120±24kU/L (P&lt;0.05)  3- Amylase activity level was 202±50 U/min (P&lt;0.01)  4- AH had more AMY1 (P&lt;0.05)</td>
<td>120 6 24 k U/L</td>
<td>No significant difference in plasma insulin concentration between HA and sAA at any time.</td>
</tr>
<tr>
<td>Alberti et al (2015)</td>
<td>Aims to assess the relationship between salivary amylase (AMY1) activity and glycaemia response</td>
<td>N- 10 adult male Age:20.6± 0.97 with normal weight BMI:22.74± 1.36</td>
<td>Assessed CN of AMY1, saliva flow and α-amylase activity. Measured blood serum insulin levels and serum glucose levels.</td>
<td>A significant direct correlation between copy number of AMY1 and amylase concentration and activity. The change in amylase activity is not statistically associated with increase postprandial plasma insulin level.</td>
<td>NA</td>
<td>A strong association between CN AMY1 with salivary amylase activity and concentration.</td>
</tr>
</tbody>
</table>
### Chapter 1: General Introduction

<table>
<thead>
<tr>
<th>Study (Year)</th>
<th>Methods</th>
<th>Participants</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Choi et al. (2015)</strong></td>
<td>A cohort study assessing influence of high CN of AMY1 and lowering the risk of insulin resistance.</td>
<td>N: 1257 Korean men (Age: 20–65 years) smokers and regular alcohol drinkers</td>
<td>CN of AMY1 show negative correlate with HOMA-IR even after adjusting for covariates. Excluded people with fasting plasma glucose levels &gt; 10.0 mmol/l, HbA1c ≥ 64 mmol/l or with insulin or oral hypoglycaemic agents. Low AMY1 CN correlated with high insulin resistance in men. This relationship is differ depending on the status of smoking and alcohol consumption.</td>
</tr>
<tr>
<td><strong>Aldossari et al. (2019)</strong></td>
<td>Aims to assess the relationship between salivary amylase (AMY1) activity and body mass index (BMI) in Saudi male and female adults in Riyadh.</td>
<td>200 (100 individuals who were overweight and obese and 100 who had normal body weight)</td>
<td>Food intake assessed using a 24-hour dietary recall. The activity of the AMY was measured using a microplate fluorescence reader. AMY1 activity significantly lower than the reference values in overweight and obese group. Lower AMY1 CN (6.6 (2-19)) associated with increased risk of obesity.</td>
</tr>
<tr>
<td><strong>Higuchi et al. (2020)</strong></td>
<td>Aimed to clarify the conflicting results of previous studies by examining AMY1 expression and metabolic indices in a homogenous group of healthy participants.</td>
<td>60 healthy non-obese (&lt; 25.0 kg/m²) young Japanese women aged 20 - 39 years</td>
<td>Participants examined for AMY1 CN, salivary amylase, BMI and serum parameters including HbA1c, ketones, and total, salivary and pancreatic amylase. Respiratory quotient at rest and changes in blood glucose after starch loading also examined. Total serum amylase was positively correlated with blood glucose at 30 and 45 min after starch loading (P = 0.04) and were correlated inversely with HbA1c (r = -0.36, P = 0.003 and r = -0.30, P = 0.02, respectively). The level of serum total amylase, but not AMY1 CN, was correlated inversely with BMI (r = -0.29, P = 0.02). Lower AMY1 CN (range, 4 - 14) 66.0±10 associated with increased risk of obesity.</td>
</tr>
</tbody>
</table>
1.9 Aims and Objectives

The existing research has substantial gaps, with no clear evidence of a major role for AMY1 after the ingestion of complex carbohydrates in relation to the number of AMY1 copies. Furthermore, CHO oxidation has not been addressed to understand whether individuals with high CN of AMY1 are better-able to use CHO as fuel, and although inconsistent across populations, no previous studies have examined AMY1 in athletes with controlling body weight. Moreover, most of these studies have assessed the link between AMY1 and obesity by using BMI and total fat percentage, whereas body fat distribution may better explain part of the inconsistency in the research outcomes.

Understanding environmental and genetic factors may help reduce the risk of obesity, promote well-being as well as influence athletic performance. Such studies can result in a better understanding of the mechanisms involved in the complex CHO metabolism responses of weight loss. An example of this is enhancing the exogenous complex CHO metabolism, which may increase athletes’ endurance capacity and performance. This may also increase the exercise metabolic rate, resulting in a reduced fat mass gain. To achieve an understanding of this, four key objectives have been set in four studies for this thesis:

- To determine the association between AMY1 CNV and BMI, anthropometric aspects of body composition and strength in male endurance athletes.

- To determine the influence of the AMY1 copy number on body fat distribution in adult males and females.
• To examine a new protocol designed to determine the association of a high copy number variation on the glycaemic response and insulin resistance after CHO ingestion.
Chapter 2 Overview of Methodology

2.1 Studies Participants

The athlete cohort (Genetics of Lithuanian Athletes; GELA) described in Chapter 3 were recruited from the Lithuanian Sports University (Kaunas, Lithuania). The data collected from this cohort included anthropometrics measurements, strength measurements and biological samples of Lithuanians (n= 388), male athletes or controls (17–37 years old). All molecular and statistical analyses were conducted at the University of Stirling. The GELA study was ethically approved by the Lithuanian State Bioethics Committee and the University of Stirling, School of Sport Research Ethics Committee. Participants for study 2, 3, and 4 described in Chapters 4 and 5 were recruited through adverts posted around the University of Stirling’s Campus and on the University of Stirling internal website. All testing, molecular and statistical analyses were conducted at the laboratories at the University of Stirling.

The Body Composition DEXA (BCD) cohort reported in Chapter 4 recruited 228 volunteers from the University of Stirling's student population, comprising males and females aged 18–40 years. The data was collected in the period between February 2016 and April 2019. The study protocol was approved by the NHS research committee at West of Scotland Research Ethics Service, Glasgow; REC reference: 12/WS/0240 and IRAS project ID: 114700.

The studies reported in Chapter 5 were a subset of participants from those recruited at the University of Stirling, aged 18–40 years. The participants of the study only included those subjects who held copy numbers ≥ 9 of AMY1 or copy number ≤ 5. Participants
with $\geq 9$ copy of AMY1 were considered as a high copy number group, whereas those holding $\leq 5$ copy of AMY1 were a low copy number group. The study in Chapter 5 recruited fifteen participants. The study protocols were approved by the Health Science and Sport School Ethics Committee at the University of Stirling.

In all the three studies in this thesis, the participants were asked to attend the laboratory in the morning, having fasted for 8–12 hours and rested overnight. Additionally, they were asked to refrain from any PA and alcohol ingestion for the 24-hours prior to each experimental trial, except the athlete’s cohort participants who attended at least 12-hours post-exercise.

### 2.2 AMY1 Copy Number Determination

### 2.3 DNA Extraction

A blood sample is the main source of DNA isolation beside saliva sample and buccal epithelial cells (Ooi et al., 2017; Philibert et al., 2008). In a study comparing all three ways of DNA isolation for AMY1 CN determination, the saliva derived DNA was found to produce the highest quality compared with other methods. This variance may be accredited to the amount of the biological samples used for DNA extraction in the study. The Biological sample of 500 $\mu$L of saliva used to DNA extraction compared to only 200 $\mu$l of blood and a single buccal swab. Moreover, Ooi et al found that blood, saliva samples, and buccal cell yielded good quality DNA for downstream qPCR (Ooi et al., 2017). In contrast, another study showed that DNA prepared from whole blood performed significantly better than that prepared from saliva in genotyping studies (Philibert et al., 2008). As the saliva-derived genomic DNA is easy to collect and
handle, it has been the choice of large genetic studies (Sun and Reichenberger, 2014). Similar to the first two studies in this thesis, we used this method in the second study presented in Chapter 4. However, the DNA of athlete’s study was extracted from blood provided from GELA cohort.

All analysis was performed at the University of Stirling. In the Athletes Cohort reported in Chapter 3, genomic DNA was extracted from 200 µl of whole blood using the QIAamp Spin DNA extraction protocol using silica columns (Macherey-Nagel GmbH and Co. KG, Germany) and extraction buffers (Qiagen Ltd, UK). In the Body Composition Cohort reported in Chapter 4, genomic DNA was extracted from a 500 µl saliva sample using the prepIT•L2P purification protocol (Figure 2-1). All DNA samples were stored at -20 °C prior to analysis.

![Figure 2-1 DNA Extraction Using the prepIT•L2P Purification Protocol From Saliva Sample](image-url)
RNA can co-purify with DNA also absorbs at 260nm absorbance. as this was used in the study there is the potential for an overestimation of the amount of DNA, therefore RNA was removed from the original DNA using double-RNase digestion and following DNA Genotek instruction. This protocol uses two ribonucleases to treat RNA. The first one is ribonuclease A (Sigma-Aldrich, Cat. No. R4875) used to cleave only at U- and C- nucleotides, resulting in large fragment apply to precipitate with alcohol. By subsequently adding ribonuclease T1 (Sigma-Aldrich, Cat. No. R1003) which cleaves at G-nucleotides, the RNA can be digested into very small fragments and therefore is no longer precipitated by alcohol (Figure 2-2).

![Figure 2-2 RNA Removal by double-RNase Digestion Using DNA Genotek Inc Protocol](image)

The DNA yield and purity of samples was measured using the absorbance method on a DS-11 Spectrophotometer (DeNovix, Wilmington, DE, USA). The absorbance of ultraviolet light (optical density; OD) at wavelengths of 260 nm and 280 nm was measured. These values were used to compare the ratio of acid concentration in the
sample (OD 260 nm) to that of protein and organic contaminants (OD 280 nm). An OD 260/280 ratio of ~1.8 and 2.0 was considered ideal for pure DNA. RNA and protein contamination are indicated by A260/A280 ratios above and below 1.8 and 2.0 (Wilfinger, Mackey, and Chomczynski, 2006). A ratio lower than this indicates contamination with phenol, and organic matter that absorbs strongly at or near 280 nm (Shabihkhani et al., 2014). Each sample had been measured twice, and the average of these two measurements used for calculations. Samples with a yield of less than 10 ng/μL were re-extracted. All DNA samples were diluted to a working concentration of 5 ng/mL with nuclease-free water.

2.4 Copy Number Assays and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

qPCR was used to analyses the copy number of AMY1 gene. Pre-validated TaqMan copy number assays combined with a copy number reference assay were run together in a duplex reaction system. The copy number assay detects the target gene of interest, and the reference assay detects a sequence that is known to be present in two copies in the diploid genome. The primer and probe of TaqMan qPCR assay, Hs07226361_cn (Thermo Fisher Scientific, UK) was used in order to target the AMY1 gene. This targets a region within exon 1 of the human AMY1 gene that is absent in the AMYP1 pseudogene, therefore ensuring specificity of the qPCR assay for AMY1 gene. For adjustment of DNA dilution quantity variation, we used the copy number reference assay, which targets the telomerase reverse transcriptase (TERT) (Thermo Fisher Scientific, UK) location in chr.5:1253373 on NCBI build 37. This assay has an 88 bp amplicon that maps within exon 16 of the TERT gene. This is located on chromosome 5, cytoband 5p15.33.
The TaqMan copy number assay Hs07226361_cn and copy number reference assay TERT were chosen after being cross validated with another TaqMan essay for AMY1 copy number determination (Hs07226362_cn) and TaqMan Copy Number Reference Assay (RNaseP) (Thermo Fisher Scientific, UK). Briefly, all the samples of the GELA cohort, the BCD cohort, and the ‘serial dilution’ data series were amplified in 4titude Frame Star® 384-well plates (Brooks Life Sciences; USA) in a Roche LightCycler480. Reactions were 10 µl total volume comprised of 5 µl TaqMan Genotyping Master Mix (Applied Biosystems; Foster City, CA, USA), 0.5 µl of TaqMan copy number assay Hs07226361_cn, 0.5 µl of TERT reference assay, 2 µl of DNA (5 ng/µl) and 2 µl of nuclease-free water. All DNA samples were analysed in triplicate.

2.5 Copy Number of AMY1 Calculation

The median absolute deviation method was used to identify any outlier crossing point (Cp) within each triplicate for each assay including the serial dilutions. The mean of the remaining Cps in each triplicate was calculated to give the average Cp for each sample. The median absolute deviation was also used to identify any samples from the cohort that needed to be repeated because of outlying corrected values. The difference between the Cp (ΔCp) of AMY1 gene of and the TERT reference assay was calculated to determine the copy number for each participant.

qPCR shows large variability in individuals in copy number of genes like AMY1 with extensive CN. Slight changes in reaction efficiency can alter copy number estimates (Lin et al., 2008). There are a variety of statistical methods which can be used to determine qPCR efficiency. These can produce different result because of the different algorithms used. To account for this, both standard curves from serial dilutions (Ruijter
et al., 2013) and LinRegPCR (Ramakers et al., 2003) were used to calculate the efficiencies of the AMY1 qPCR reactions. No differences were found between the methods in AMY1 copy number calculation. So serial dilutions were carried out to decrease a level of uncertainty in CNV. Artificial target sequences were manufactured for the AMY1 assay target sequence and TERT reference assay target sequence using gBlocks (Integrated DNA Technologies, USA). Serial dilutions of gBlocks were used to ensure all samples fell within the linear range of the reaction (Figure 2-3) and to produce standard curves for AMY1 and TERT (Figure 2-4). Copy number was determined by converting the AMY1 and TERT Cps for each sample into arbitrary amounts and then dividing one by the other. The standard curve in Figure 2-4 shows a 1.8 cycle difference between AMY1 and TERT. Furthermore, the serial dilution was repeated in each batch of samples.

![Amplification Curves](image)

**Figure 2-3** Standard curve of qPCR used for copy number analyses of both AMY1 and TERT. The standard curves were constructed by plotting a known amount of the artificial AMY1 and TERT in serial 2-fold dilutions against the corresponding threshold cycles (Ct values) of the amplification plots.
Figure 2-4 Standard curve of qPCR used for copy number analyses of both AMY1 and TERT. The standard curves were constructed by plotting a known amount of the artificial AMY1 and (1:64) TERT in serial 2-fold dilutions against the corresponding threshold cycles (Ct values) of the amplification plots.

2.6 Body Composition Assessment

In the study described in Chapter 3 body composition data was calculated from skinfold (SF), following standard anthropometric measurements (ISAK), (Hume and Marfell-Jones, 2008; Norton and Eston, 2018) by the author of this thesis. SF measurements allow the assessment of body composition due to the strong relationship between the amount of subcutaneous fat and total BF (González-Ruíz et al., 2018). Faulkner’s equation (1968) was used to assess BF % of athletes and controls in Chapter 3 study. Faulkner’s equation using four skinfolds, today considered a modified Yuhasz method \( \%BF = 5.783 + (0.153 (Tr + Sb + Si + Ab)) \) (Faulkner, 1968). According to very recent study Faulkner’s equation may be the best candidate that can be used as replacement of BIA method for general male athlete population (Dimitrijevic, Lalovic and Milovanov, 2021). A very recent study comparing the accuracy of different skinfold-based equations in estimating FM % in a cohort of soccer referees, the Faulkner’s equation showed the highest correlation with FM % estimated by DXA.
(Petri et al., 2020). Muscle mass (MM) was estimated using standardised height and skinfold corrected girth measurements of the forearm, calf, and thigh as per the equation of Martin et al. (1990). Data from six un-embalmed cadavers were used to derive a regression equation to predict total MM (Martin et al., 1990).

The study reported in Chapter 4 that compared the body composition between males and females according to quantity of AMY1, BF %, and MM % calculated body composition by DXA. All scans were performed and analysed by the standard technique. DXA was calibrated with phantom as per manufacturer guidelines before measurement. All scans were performed using the standard thickness mode as automatically selected by the software. Participants were instructed to wear minimal clothing and remove any metallic objects and jewellery. The presence of metal rods inflated body composition variables measured by DXA. Metal had the largest impact on whole-body bone mineral content \( (p < 0.034) \), causing errors of \( 1.5\% - 3\% \). Soft-tissue mass was increased when the scan included the 100-g rod \( (p < 0.003) \) (Giangregorio and Webber, 2003). DXA measures body composition in three fundamental components of bone-free mass, which are LM, FM, and BMD; this principle was explained by Blake (1997). At the end of the scan a total body and regional analysis is automatically made by the software and presented in percentage of FM and lean mass, in total and body regions, including arms, legs, trunk, and android. Body fat % was calculated as the sum of six site skinfolds using the six-site formula for men \[ \text{BF} \% = (\text{sum of SKF} \times 0.1051 + 2.588) \] and women \[ (\text{sum of SKF} \times 0.1548 + 3.58) \], as published by International Standards for Anthropometric Assessment (2012) (Stewart et al., 2011; Marfell-Jones et al., 2012; Bi et al., 2018). The sum of six site skinfolds was used to calculate BF % in order to obtain accurate estimation. This study was
conducted on a general adult population whose body fat could be distributed in their upper and lower body.

2.7 Oral Glucose Tolerance Test (OGTT) and Oral Polymers Tolerance Test (OPTT)

A 1.1 x 32mm Cannula (BD Nexiva, USA) was inserted into the participant’s antecubital vein for blood sampling. The participants then drank either a 100 % dextrose solution as a control for OGTT (My Protein, UK) or a waxy maize starch solution for OPTT (My Protein, UK). 40 g of starch was mixed with 500 ml water to get a final concentration of 8 %. However, to get 8 % concentration of dextrose and equal energy 35.4 g of dextrose was mixed with 442.5 ml water (Table 2-1). Then, the participant was instructed to finish the drink within two minutes. Both solutions were prepared 30 minutes before the trial. A sample (5 ml) of blood was drawn at -5, 0, 15, 30, 45, 60, 90 and 120 minutes. Blood samples were collected in lithium heparin tubes and kept in ice until the end of the session (Becton, Dickinson & Company, NJ, USA).

Table 2-1 Nutritional Information of Carbohydrate Solutions

<table>
<thead>
<tr>
<th>DRINK</th>
<th>CONTROL (SINGLE CARBOHYDRATE)</th>
<th>TRIAL (COMPLEX CARBOHYDRATE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOURCE</td>
<td>Glucose Drink (single unit) 100% Dextrose Glucose</td>
<td>Polymer (long-chain) 100% Waxy Maize Starch</td>
</tr>
<tr>
<td>SERVING</td>
<td>35.4</td>
<td>40</td>
</tr>
<tr>
<td>CONCENTRATION (%)</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>ENERGY (kcal)</td>
<td>141.6</td>
<td>141.6</td>
</tr>
<tr>
<td>CARBOHYDRATE (g)</td>
<td>35.4</td>
<td>35.2</td>
</tr>
<tr>
<td>OF WHICH SATURATES (g)</td>
<td>35.4</td>
<td>0</td>
</tr>
<tr>
<td>FAT (g)</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>PROTEIN (g)</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>WATER (ml)</td>
<td>442.5</td>
<td>500</td>
</tr>
</tbody>
</table>
2.8 Plasma Analysis

Blood samples were used to assess plasma glucose (mmol/L), plasma insulin (μIU/mL) and fasting plasma total cholesterol (mmol/L), fasting plasma HDL-C (mmol/L), fasting plasma LDL-C (mmol/L) and triacylglycerol (mmol/L), and non-esterified fatty acids (NEFA) (mmol/L).

Each participant drank one glucose solution (OGTT) and one starch solution (OPTT) in two different morning sessions a minimum two-day part. At the end of each session, the plasma fraction was immediately separated by centrifuging at 3,500 rpm for 15 minutes at 4ºC. The supernatant plasma was removed and aliquoted into fresh tubes to be stored at -80ºC for analysis.

An automated Aries ILab benchtop analyser (Instrumentation Laboratories, MA, US) was used to assess the plasma glucose, a lipid panel comprising total cholesterol, HDL-C, LDL-C and triacylglycerol, and NEFA at each time points throughout OGTT and OPTT. TG and NEFA were measured to assess the impact of the source of metabolic fuel, for example, plasma NEFA and TG concentrations increasing in the fasting state, while decreasing in the post-prandial state due to an insulin-induced inhibition of adipose tissue lipolysis.

To ensure the reliability of the obtained study data, Ilab Aries were calibrated by a multicomponent calibrator ReferrIL G (00018257000) for use in the calibration of substrate assays (listed in Table 2-2). Reaction volume of 200 μL of each plasma sample run in duplicated. This resulted in the concentration being automatically
calculated by the instrument against the calibrator and ensuring quality control was obtained. Controls SeraChem® Control Level 1 (Cat. No. 0018162412) and SeraChem® Control Level 2 (Cat. No. 0018162512) were used with samples run to establish its own mean and standard deviation and adopt a quality control programme. The quality control ranges references according to participant’s age, gender, and geographical area. Data out of the reference range were excluded and re-analysed.

### Table 2-2 Assays and Reagents Used in an Automated Aries ILab Benchtop Analyser

<table>
<thead>
<tr>
<th>Catalogue Name</th>
<th>Catalogue No.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cholesterol</strong></td>
<td>000018250540</td>
<td>Total Cholesterol reagent</td>
</tr>
<tr>
<td><strong>LDL- Cholesterol</strong></td>
<td>00018256040</td>
<td>LDL- Cholesterol reagent</td>
</tr>
<tr>
<td><strong>HDL- Cholesterol</strong></td>
<td>00018258940</td>
<td>HDL- Cholesterol reagent</td>
</tr>
<tr>
<td><strong>Triglyceride</strong></td>
<td>00018258740</td>
<td>Triglyceride reagent</td>
</tr>
<tr>
<td><strong>NEFA HR II R1</strong></td>
<td>434-91795</td>
<td>NEFA reagent 1</td>
</tr>
<tr>
<td><strong>NEFA HR II R2</strong></td>
<td>436-91995</td>
<td>NEFA reagent 2</td>
</tr>
<tr>
<td><strong>Control Serum I</strong></td>
<td>410-00104</td>
<td>NEFA Control 1</td>
</tr>
<tr>
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<td>NEFA Control II</td>
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<tr>
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</tr>
<tr>
<td><strong>ReferrIL G</strong></td>
<td>00018257000</td>
<td>Calibrator</td>
</tr>
</tbody>
</table>

Plasma insulin concentrations were measured at the same time points as the plasma glucose and lipid analysis using a solid phase enzyme-linked immunosorbent assay kit (ELISA) based on the sandwich principle according to the manufacturer’s instructions (Demeditic, DE2935, Germany). The insulin results are reported in SI units: pmol/L as recommended by (Knopp et al., 2018). Gen5 software (BioTek, Winooski, VT, USA) was used for ELISA data collection. The average absorbance values were calculated for each set standards for controls and participants’ samples. The mean absorbance
value for each sample determined the corresponding concentration from the standard
curve of controls run with each calibration curve. The values and ranges stated on the
quality control manufacturer’s manual refer to each test kit lot and were used for direct
comparison of the results.

2.9 Insulin Sensitivity

Insulin sensitivity was estimated using plasma glucose and insulin concentrations in
the basal and / or during the OGTT / OPTT, using homeostasis model assessments of
insulin resistance (HOMA-IR) and whole-body Matsuda insulin sensitivity index (ISI)
(Matsuda and DeFronzo, 1999; DeFronzo and Matsuda, 2010; Hayashi et al., 2012).
Elevated HOMA-IR levels indicate low insulin sensitivity. In healthy adults mean
HOMA-IR levels are below 2.7. The HOMA-IR cut off our study consider by 75
presential of HOMA-IR, and 25 presential for Matsuda ISI. Following formulas were
used to calculate HOMA-IR and Matsuda insulin sensitivity index:

**HOMA-IR** (Matthews et al., 1985)

\[ \text{fasting glucose (mg/dL)} \times \text{fasting insulin (μU/mL)} / 405 \]

**Matsuda insulin sensitivity index.** (Matsuda and DeFronzo, 1999)

\( (10,000/\text{square root of } [\text{fasting glucose (mg/dL)} \times \text{fasting insulin (μU/mL)}] \times [\text{mean glucose (mg/dL)} \times \text{mean insulin (μU/mL)}] \) using the data derived from both OGTT.
2.10 Statistical analysis

All statistical analyses were carried out using SPSS. Statistical analyses were performed using SPSS (version 25, version 27, version 28, IBM, Armonk, NY, USA). In Athlete’s study (Chapter 3) Before merging the data, preliminary analyses were conducted to ensure it met all requirements (assumptions). All variables were converted into standardized z-scores to identify the univariate outliers. It was found that three cases had z-standard scores greater than 3.29 and were subsequently removed from the data. In all other cases, standardized z-scores were less than 3.29. Hence the sample size was reduced from 407 to 404. Moreover, out of 404, those 16 cases which exceeded the cut-off scores (using Chi-Square = 54.05) were considered as outliers and were removed from the data set. Hence, the sample size reduced from 404 to 388. All phenotype data were tested for normality using skewness and kurtosis values within the range (± 2) (West et al., 1995).

One-way ANOVA was used for exploring the differences between group means for AMY1 CN, age, body mass, BMI, \( \dot{V}O_2 \) max, height, body fat, and FFM. Associations were assessed between AMY1 CNV, BMI, height, weight, body fat, and FMM among Lithuanian athletes. The Games-Howell post hoc test was conducted to discover the significant differences between group means. Further, Pearson correlation was used to assess the association of AMY1 CNV with major study variables among different groups.

A hierarchical linear regression analysis was used to predict the AMY1 CNV with height and from different anthropometric measurements including Pubic bone (cm),
Sitting height (cm), Chin height (cm), Upper sternum (cm), Right femur (cm), Left femur (cm), and Arm span (cm) among Lithuanian athletes and non-exercising controls. Similarly, a hierarchical linear regression analysis was also useful to predict the AMY1 CNV from different performance measures, including extension and flexion of isokinetic right and left leg, peak knee.

Because of the significant differences between tested groups, the statistical analysis was run in three models (as shows in Table 3-3 and all following presented results. Model 1 analysis was without adjustment. Model 2 analysis was adjusted for the four subgroups: SSP, TEA, END and CON. Model 3 was adjusted for height and subgroups due to the association between height and anthropometrics and body composition measurements.

Body composition study (Chapter 4) statistical analyses were performed on data from the 288 participants who completed the study. Data was assessed for normality of distribution using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Any data which were not normal were transformed using the Box-Cox transformation to give better approximations of the normal distribution (using version 20.2, Minitab, State College, PA.), and normality was confirmed. Data are expressed as mean ± standard deviation (SD). An independent sample t-test was used to assess the difference between the mean characteristics of both groups. A Non-parametric median test, the Mann Whitney U test, was used to analyse the difference between the median values of the two groups. Correlation and Linear regression analysis was performed using phenotype data with and without group corrections to identify associations between AMY1 CN and the
examined variables. As before performing the regression analysis, a correlation structure is essential to observe the association (relationship) between the underlying variables. When considering an interaction, a $p$-value of $<0.05$ was considered significant. Standardized $\beta$-coefficient was used to quantify the association and $R^2$ to describe the explanatory rate given for subgroups.

Pilot study (Chapter 5) statistical analyses were performed on data from the 15 participants who completed the study. Data was assessed for normality of distribution using the Shapiro-Wilk test (Shapiro and Wilk, 1965). All values are presented as mean $\pm$ standard deviation (SD). Independent sample t-tests were run at baseline to compare possible mean differences in measured variables between the high CN and low CN groups. A two-way repeated measures ANOVA was used to determine whether there were significant differences between the two groups (high CN and low CN) during OGTT/OPTT periods (trials $\times$ time).

The error variance for each study variable was estimated from the corresponding intrasubject coefficient of variations (CVs) based on two replicates total 15 non obese subjects. The CV is ratio of the slandered deviation of the mean. It is calculated based on the average values of the respective level (0-120 min). The incremental glucose response vs time was evaluated by area under the curve (iAUC). To measure the glucose response vs time during an OGTT / OPTT the trapezoidal method was applied (Purves, 1992). The tAUC depends on basal glucose values while the iAUC and pAUC are not related to basal glucose value. Since the tAUC is independent of the ever-
changing baseline glucose or insulin levels, it might be the preferred method for evaluation of the response during OGTT (Liu et al., 2018; Khan and Thorsten, 2017).

In clinics, for the diagnostic purpose of impaired glucose tolerance, the area under the curve (AUC) defines the glycaemic index following OGTT. It is helpful in estimating blood glucose total rise during OGTT. Later on, incremental area under curve (iAUC) was established due to presence of different levels of fasting glucose among different subjects. But it created a problematic challenge when it yielded negative values while subtracting baseline value of fasting plasma glucose. Hence, after that positive incremental AUC (pAUC) established utilizing the values above the base line. Above all it is recommended recently that the total is under curve (tAUC) best expresses the correlation with two hours OGTT and tAUC is better to be used in preference to IAUC and pAUC. (Cheng et al., 2018). Because of the big variance in baseline plasma glucose between individuals in the same group. iAUC of plasma glucose and insulin curves during OGTT/ OPTT were calculated using GraphPad Prism version 8 (Graphpad Software Inc., AC, USA).

G* Power software used to generate the power calculations for future studies based on current study data from sample size offered 80 % power ($\alpha = 0.05$) need to change of plasma concentration of glucose, insulin, TG, and NEFA in healthy adults with high / low copy number of AMY1.
Chapter 3  Copy Number Variation in Salivary $\alpha$-amylase 1 gene associated with stature but not body fatness in Lithuanian male athletes and non-exercising controls

3.1 Abstract

BACKGROUND: The high copy number variation (CNV) in the salivary amylase gene (AMY1) has been associated with obesity in different populations. However, several studies have reported inconsistent results. No study has so far assessed the association of AMY1 CNV with body composition measures in athletes.

AIM: To investigate association of AMY1 CNV with BMI, underlying anthropometric, other body composition measures, and strength in Lithuanian athletes and non-exercising controls.

METHODS: This study evaluated AMY1 gene copy number in 388 young Lithuanian adult males (17-37 years old). The study sample comprised non-exercising Controls (CON; n=187), Endurance athletes (END; n=84), Strength-Sprint Athletes (SSP; n=67) and Team Sport Athletes (TEA; n=50). Height, body mass, body fat, and fat-free mass (FFM) were measured. Faulkner’s equation (1968) using 4 skinfolds was used to calculate fat mass percentage (FM), and Martin et al. (1990) equation thickness assessed to calculate FFM. Muscle strength was measured using isokinetic dynamometry for extension and flexion of the right and left legs and arms (Biodex Pro3, USA) at 30, 90 and 180 °/s, handgrip strength using a Hydraulic Hand Dynamometer (Model J00105, Lafayette Instrument Company, Lafayette, IN) and performance of whole-body movements, i.e., 30 m sprint.
Copy number of AMY1 was estimated by TaqMan quantitative polymerase chain reaction (qPCR). All statistical analyses were carried out using SPSS (version 28). One-way ANOVA and The Games-Howell post hoc were used for exploring the differences between group means in variable and AMY. Partial correlation was used to determine the association of AMY1 CNV with major study variables. Multiple regression analysis was used to predict the anthropometric and body composition from AMY1 CNV.

**RESULTS:** The copy number distributions did not differ between study groups. In two-way ANOVA height and anthropometric measurements, and sprint were associated with increasing AMY1 CN, after adjustment for athletes’ subgroups AMY1 $p$-value decreased. However, after adding height to the interaction of subgroups and AMY1 CN no association was detected. The findings indicated a significant difference among groups in age, height, body mass, BMI, $\dot{V}O_2$ max, body fatness, and FFM and ($p < 0.05$) and all anthropometric measurements ($p < 0.001$). Body fat ($\beta = 0.34$, $p < 0.05$), and FFM ($\beta = 0.32$, $p < 0.05$) significantly predicted from the AMY1. However, anthropometric measurements, including pubic bone, sitting height, chin height, upper sternum, right femur, left femur, and arm span were not predicted from the AMY1 CNV $F (7, 380) = 1.45$, $p > 0.05$.

**CONCLUSION:** Individuals with high copy number of AMY1 are more likely to be taller. High AMY1 copy number was not associated with % body fatness, FFM and BMI in our cohort, which included athletes and non-exercising controls. Furthermore, no relationships were found between AMY1 CN and athletes’ strength. The genetic
testing of AMY1 for an association with height as a means of aiding at sport talent selection may be premature.

3.2 Introduction

Obesity is defined as excess body fat measured with a Body Mass Index (BMI) ≥ 30 kg/m². It is a complex metabolic disorder influenced by the interaction of genetic and non-genetic factors (Herrera, Keildson, and Lindgren, 2011). BMI has a high heritability factor, estimated at 40-80 % in twin and family-based studies (Stryjecki et al., 2017; Maes; El-Sayed Moustafa & Froguel, 2013; Zaitlen et al., 2012; Hjelmborg et al., 2008; Neale and Eaves, 1997). Apart from genetic predisposition, lifestyle factors also play a significant role in obesity, especially dietary factors and physical activity. These may suppress the genetic risk of adiposity through a healthy lifestyle. However, variation in physical activity also has a high heritability, estimated to be 27 %–84 % in twin and family-based studies. Additionally, genetics is still reported to contribute to obesity in these individuals (Schnurr et al., 2020).

As with most phenotypes, the identification of individual variants remains largely elusive. The cumulative impact of the known variants does not account for the estimates of heritability. This is known as missing heritability. It has been suggested that genomic copy number variation (CNV) may contribute and explain part of the missing heritability of various phenotypes (Nagao, 2015). The influence of CNV on obesity and obesity-related phenotypes requires further investigation.
Apart from the genetic predisposition, it is widely accepted that BMI and anthropometric traits are heavily influenced by physical activity levels (Corder et al., 2020). Further, physical performance strongly correlates with anthropometric characteristics in athletes, and it is considered a success factor in sports (Rienzi et al., 2000). In distance running events, winners are usually low in body mass and height (Berg, 2013). Other studies have found an association between poor muscle power and high BMI and body fat levels in basketball (Nikolaidis et al., 2015), handball (Nikolaidis and Ingebrigtsen, 2013) and soccer players (Nikolaïdis, 2012). This association has also been assessed in futsal players, finding a correlation between countermovement jump performance and body height (Nikolaidis et al., 2019; Galy et al., 2015) and bilateral asymmetries in fat-mass percentage in dominant versus non-dominant limbs of sub-elite players in the 3rd league as compared to elite players in the 1st league (López-Fernández et al., 2020; Sekulic et al., 2021). Moreover, body mass affects physical dominance, speed, and stamina, while body composition modulates agility and strength measures in handball and football. As noted, athletes benefit from different anthropometric characteristics depending on their sports (Popovic et al., 2013; Arifi et al., 2019).

The balance between fat mass and fat-free mass is helpful to assess energy expenditure levels and nutritional requirements (Burke et al., 2006). Similarly, body composition can guide dietary interventions in sports nutrition, and it is used by athletic trainers and strength coaches to create, evaluate, and optimize their training programs (Moon, 2013). In athletes, body composition varies and is modulated according to the type of physical activity (Wilmore et al., 1972), with decreased performance levels as body fat increases (Welch et al., 1958). Not surprisingly, body composition in football players
ranges between 4 and 29 % body fat depending on their position, suggesting that body composition is also variable within the same sport (Costa et al., 2002; Moon et al., 2009; Bouchard et al., 1999).

A previous study in twins estimated the role of genetics and environmental factors to determine anthropometric measurements, including standing height, knee height, sitting height, chest circumference, body weight, biiliac diameter, and arm span, finding high heritability of all the measurements, estimated in 40 % - 91 % (Chatterjee et al., 1999). In sports genomics studies involving athletes of many disciplines, the heritability of athlete status was estimated at 66 %. A closer look at individual traits would show that muscle strength heritability ranges from 30 to 80 % in various phenotypes such as isometric knee strength, handgrip strength, and elbow flexion (Maciejewska-Skrendo et al., 2019).

Further, worldwide studies confirm the influence of genetic factors over athletic performance, and more specifically, over strength, flexibility, endurance, psychological traits, neuromuscular coordination, among other essential features in sports (Maciejewska-Skrendo et al., 2019). Muscle strength is a variable trait determined by behavioural factors such as pain tolerance or motivation to train (Sallis et al., 1992; Boutelle et al., 2004). Individuals with an advantageous genetic predisposition have a higher chance of becoming an athlete when environmental factors and genetics interact in favour of strength and other physical traits (Kostek et al., 2010). The influence of genetic factors is estimated at 44 % - 68 % for endurance-related phenotypes (Miyamoto-Mikami et al., 2018). Also, as a consequence of the thrifty genotype
hypothesis (Chakravarthy et al., 2004), genetic variants that predispose modern sedentary individuals to obesity may also predispose physically active individuals to have high endurance capacity.

One of the genes linked to BMI is the human salivary amylase gene, which displays extensive CNV variations between individuals. The set of alpha-amylase 1 (AMY1) genes has been shown to range from 1 to 20 copies (Parry et al., 2007; Santos et al., 2012). A higher AMY1 copy number has been linked with lower BMI and obesity risk in a group of 6,200 adults of European and Asian origin (Falchi et al., 2014). Still, subsequent studies yielded conflicting results when replicating these findings. For instance, association with AMY1 CNVs was not replicated in a study that included 4,000 individuals of European ancestry, including individuals at the extremes of the BMI distribution and despite its 99% power to detect such an association (Usher et al., 2015). Similar results were reported in a case-control study of 932 Chinese and 145 Malay samples (Yong et al., 2016) and 1,400 participants from the UK 1958 Birth Cohort (Shwan et al., 2018). Conversely, studies specifically analysing obesity in children and young adults supported the association of BMI with amylase gene copy number in Mexican children (Mejia-Benitez et al., 2015), Italian school’s boys (Marcovecchio et al., 2016), and French adults (Bonnefond et al., 2017). These studies highlight the complexity of assessing an endpoint that comprises genetic and environmental components, suggesting that differences in ethnicity, environment, and food preferences may further influence the manifestation of this complex phenotype as genetic susceptibility. However, to this date, AMY1 CNVs have not been tested in physically active populations, nor has their influence been tested on physical performance. Rossi et al. observe a negative association between AMY1 CNVs and
anthropometric characteristics in the Qatari people, particularly in the trunk fat percentage \( (p = 7.50 \times 10^{-3}) \) (Rossi et al., 2021). Investigating the association between AMY1 CNV, BMI, and strength in different populations may provide additional insight into the role of this variation in modulating physical performance.

BMI is a useful measure in extensive epidemiological obesity studies, but it is not an accurate measure of adiposity. BMI predicts body fat percentage differently in males and females: for the same BMI, the percentage of body fat of females was \( \sim 10\% \) higher than that of males (Jackson et al., 2002). BMI has also been shown to systematically under- or over-estimate body fat percentage in females from different ethnic groups (Jackson et al., 2002; Heo et al., 2012). Alternatively, AMY1 CNVs may relate to aspects of BMI such as height or weight to discriminate between fat mass or muscle mass. Furthermore, research in adults suggests that BMI is negatively correlated with height, especially in women and older adults (Sperrin et al., 2015). Muscle mass and height are known to have significant heritable components and be affected by diet (You et al., 2018). Consequently, it is essential to assess the association of AMY1 copy number with more detailed anthropometric and body composition measurements and BMI.

This study aims to investigate AMY1 CNVs in association with BMI and body composition, including body fat percentage, FFM (kg), and anthropometric measures of body composition. It is also the aim of this study to determine the association between AMY1 CNVs and strength in physically active Lithuanian athletes and non-
exercising controls. To achieve the objective of this study, we seek to answer these questions:

1. Is the athletes’ group displaying a higher number of AMY1 copies than controls?
2. Is a high copy number of AMY1 associated with low BMI, weight, FM, and FFM, as well as increasing height among athletes or control?
3. Is an increasing copy number of AMY1 associated with athletes’ performance and strength compared to controls?

### 3.3 Methodology

Ethical approval was obtained for the Genetics of Lithuanian Athletes (GELA) study from the Lithuanian State Bioethics Committee and the University of Stirling School of Sports Research Ethics Committee. Written informed consent was obtained from all participants, and the study was conducted in accordance with the Declaration of Helsinki (World Medical Association, 2008). All physiological data were collected in 2009 at the Lithuanian University of Sport. All CNV assessments and statistical analyses were conducted by the author of this thesis at the University of Stirling.

#### 3.3.1 Study Participants

The participants’ demographics are summarised in Table 3-1. For this study, native Lithuanian (n= 388 following outlier removal) males (17-37 years old) were recruited at the Lithuanian Sports University (Kaunas, Lithuania). The study excluded any participants unable to perform the physiological tests due to injury or disease to confirm their capacity to perform close to their peak at the time of phenotype determination.
The study also excluded any athletes who failed to be included in the category of Team Sports Athletes (TEA), Strength-Sprint (SSP) or Endurance (END) users, as well as those who no longer performed at their top level. A total of 338 participants met the inclusion criteria for further analysis and remained in the study.

The study sample was divided into non-exercising controls (CON; n= 187) and athletes (n= 201). The latter included the subgroups of Strength – Sprint athletes (SSP; n= 67), Team Sports Athletes (TEA; n=50), and endurance athletes (END; n= 84). The SSP group included weightlifters, bodybuilders, strongmen, combat athletes, field athletes, gymnasts, short-distance swimmers, and runners. The TEA group included handball, volleyball, basketball, and football players. The END group included long-distance runners, cyclists, triathletes, kayakers, modern pentathletes, orienteers, skiers, and walkers. According to the athletes’ highest levels of competition, they were also divided into recreational athletes (n= 30), regional athletes (n= 38), national athletes (n= 120), and international athletes (n= 20). Regional athletes were members of their local area squad. National athletes ranked in the top 3 for their sport or had been included in a Lithuanian national sport squad. International athletes included in the study represented Lithuania at the Olympic Games or World Championships. All athletes trained a minimum of twice a week, whereas control individuals did not participate in organised exercise more than twice a week and did not participate in competitive sports.

### 3.3.2 Experimental Procedures

All participants visited the testing laboratory on three separate occasions, having rested for at least 12-hours and fasted for at least 2-hours. In their first visit, 10 ml venous
blood was drawn into an EDTA container and frozen at -80 °C prior to DNA extraction. DNA was extracted using NucleoSpin® Blood kits (Machery-Nagel, Germany). During the laboratory visits, the participants were required to fill out questionnaires and complete a range of standardised physiological tests carried out by trained examiners. The laboratory visits allowed detailed phenotypes to be collected, including anthropometric measures, body composition, endurance performance, strength performance, and cardiac size and function. For the current study analysis, a subset of the tests which require strength and power phenotypes were selected, including isokinetic dynamometry for extension and flexion of the right and left legs and right and left arms (Biodex Pro3, USA). During the muscle strength test, participants performed five repetitions of maximal isokinetic knee extensions and flexions with a range of motion at velocities of 30° and 180 °/s. A knee joint angle of 30° corresponding to full extension at 90 and 180 °/s, and whole-body movement performance, i.e., the completion of a total sprint.

Percentage body fat (BF %) was calculated by Faulkner’s (1968) equation considering the modified Yuhasz method using the sum of 4 skinfolds site measurements: Triceps (Tr) Subscapularis (Sb), Suprailiac (Si), and Abdominal (Ab). Faulkner’s equation (1968) using 4 skinfolds, today known as a modified Yuhasz method was calculated using the following formula:

\[ \%BF = 5,783 + (0,153 \times (Tr + Sb + Si + Ab)) \]

(Pires-Neto et al., 2017; Faulkner, 1968).
3.3.3 AMY1 Copy Number Determination by Quantitative Polymerase Chain Reaction (qPCR) Reactions

Genomic DNA was extracted from 200 µl of whole blood using the QIAamp Spin DNA extraction protocol. AMY1 copy numbers were determined using a pre-validated TaqMan qPCR assay, Hs07226361_cn (Thermo Fisher Scientific, UK). This targets a region within Exon 1 of the human AMY1 gene absent in the AMYP1 pseudogene, therefore ensuring the specificity of the qPCR assay for AMY1. Briefly, all samples were amplified in 384-well plates in a Roche LightCycler480. Reactions were 10 µl in volume comprised of 5 µl TaqMan Genotyping Master Mix (Applied Biosystems; Foster City, CA, USA), 0.5 µl of TaqMan Copy Number Assay Hs07226361_cn, 0.5 µl of TERT reference assay, 2 µl of DNA (5 ng/µl) and 2 µl of nuclease-free water.

All DNA samples were analysed in triplicate. Artificial target sequences were manufactured for the AMY1 assay target sequence and TERT reference assay target sequence using gBlocks (Integrated DNA Technologies, USA). Serial dilutions of gBlocks were used to ensure all samples fell within the linear range of the reaction and to produce standard curves for AMY1 and TERT. Copy number was determined by converting the AMY1 and TERT Cps for each sample into arbitrary amounts and then dividing one by the other. Samples were assessed in six batches, with batch included as a covariate in general linear model analysis.

3.3.4 Statistical Analysis

All statistical analyses were carried out using SPSS (version 28, IBM, Armonk, NY, USA). Before merging the data, preliminary analyses were conducted to ensure it met
all requirements (assumptions). All variables were converted into standardized z-scores to identify the univariate outliers. It was found that three cases had z-standard scores greater than 3.29 and were subsequently removed from the data. In all other cases, standardized z-scores were less than 3.29. Hence the sample size was reduced from 407 to 404. Moreover, out of 404, those 16 cases which exceeded the cut-off scores (using Chi-Square = 54.05) were considered as outliers and were removed from the data set. Hence, the sample size reduced from 404 to 388. All phenotype data were tested for normality using skewness and kurtosis values within the range (± 2) (West et al., 1995).

One-way ANOVA was used for exploring the differences between group means for AMY1 CN, age, body mass, BMI, $\dot{VO}_2\text{max}$, height, body fat, and FFM. Associations were assessed between AMY1 CNV, BMI, height, weight, body fat, and FMM among Lithuanian athletes. The Games-Howell post hoc test was conducted to discover the significant differences between group means. Further, Pearson correlation was used to assess the association of AMY1 CNV with major study variables among different groups.

A hierarchical linear regression analysis was used to predict the AMY1 CNV with height and from different anthropometric measurements including Pubic bone (cm), Sitting height (cm), Chin height (cm), Upper sternum (cm), Right femur (cm), Left femur (cm), and Arm span (cm) among Lithuanian athletes and non-exercising controls. Similarly, A hierarchical linear regression analysis was also useful to predict the AMY1 CNV from different performance measures, including extension and flexion of Isokinetic right and left leg, peak knee.
Because of the significant differences between tested groups, the statistical analysis was run in three models (as shows in Table 3-3 and all following presented results. Model 1 analysis was without adjustment. Model 2 analysis was adjusted for the four subgroups: SSP, TEA, END and CON. Model 3 was adjusted for height and subgroups due to the association between height and anthropometrics and body composition measurements.

3.4 Result

3.4.1 Study Participants

An ANOVA test was carried out to explore the difference between groups in terms of AMY1, age, height, body mass, BMI, \( \dot{V}O_2 \) max, body fatness, and FFM (kg). The findings indicated a significant difference between groups in age, height, body mass, BMI, \( \dot{V}O_2 \) max, body fatness, and FFM (kg) \((p < 0.05)\). However, no difference in AMY1 CN was observed between the groups. Further, findings of the post-hoc analysis based on Games-Howell indicated that the mean age in the control group was significantly higher than all three athlete groups. The mean height in the control group was significantly lower than that of the athlete groups.

The results also indicated that the mean body mass in the control group was significantly lower than the TEA and SSP subgroups. However, it was higher than the END subgroup of athletes. On the other hand, the mean BMI of the control group was found to be significantly higher than the END group while significantly lower than of the SSP group. And no differences have been found in the mean BMI between the control group and TEA group. The \( \dot{V}O_2 \) max of control group was significantly lower
than other athlete’s groups. However, the mean body fatness in the control group was significantly higher than the mean of all three athlete’s groups. Lastly, the results showed that the mean FFM of the control group was significantly lower than the TEA and SSP groups. However, it was significantly higher than the END subgroup of athletes (Table 3-1)

Table 3-1 Descriptive Characteristics of the Groups of Participants.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>Control (N = 187)</th>
<th>END (N = 84)</th>
<th>TEA (N = 50)</th>
<th>SSP (N = 67)</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMYI</td>
<td>Means ± SD</td>
<td>6.58 ± 2.12</td>
<td>6.88 ± 2.54</td>
<td>6.46 ± 2.28</td>
<td>6.82 ± 2.15</td>
<td>0.60</td>
</tr>
<tr>
<td>Age (year)</td>
<td>23.91 ± 4.28*</td>
<td>21.68 ± 3.61*</td>
<td>21.06 ± 3.02*</td>
<td>22.31 ± 3.35*</td>
<td>11.41*</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>180.17 ± 5.81*</td>
<td>179.27 ± 5.42</td>
<td>186.98 ± 7.49*</td>
<td>180.56 ± 5.42</td>
<td>20.55*</td>
<td></td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>76.37 ± 10.06*</td>
<td>70.56 ± 6.67*</td>
<td>80.56 ± 7.86*</td>
<td>80.30 ± 10.99*</td>
<td>18.14*</td>
<td></td>
</tr>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>23.50 ± 2.66*</td>
<td>21.95 ± 1.70*</td>
<td>23.03 ± 1.48</td>
<td>24.57 ± 2.69*</td>
<td>16.24*</td>
<td></td>
</tr>
<tr>
<td>VO₂ max (l/min)</td>
<td>3.84 ± 0.50*</td>
<td>4.52 ± 0.47*</td>
<td>4.40 ± 0.47*</td>
<td>4.12 ± 0.45*</td>
<td>46.03*</td>
<td></td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>13.73 ± 3.67*</td>
<td>10.50 ± 1.39*</td>
<td>11.93 ± 2.04*</td>
<td>12.40 ± 2.52*</td>
<td>24.61*</td>
<td></td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>64.04 ± 6.62*</td>
<td>62.57 ± 5.52</td>
<td>69.45 ± 6.78*</td>
<td>68.51 ± 8.31*</td>
<td>18.07*</td>
<td></td>
</tr>
</tbody>
</table>

Display mean differences among groups including: SSP; strength - sprint athletes, END; endurance athlete, TAE; team sports Player Athletes, and Control Values are the total number with the number of the group in brackets. Data are expressed as means ± SD, and p < 0.05 was considered statistically significant (*).

3.4.2 Anthropometric Measurements Difference between Athlete Groups and Controls

An ANOVA test revealed differences between groups in terms of anthropometric measurements, including pubic bone, sitting height, chin height, upper sternum, right femur, left femur, and arm span (all p < 0.001). Further, results of the post hoc analysis based on Games-Howell showed that the mean pubic bone, sitting height, chin height, upper sternum height and arm span of the control group were all significantly lower
than those of the TEA group. However, the mean right and left femur lengths were significantly lower in the control group rather than the TEA and SPSS groups (Table 3-2).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control (N = 187)</th>
<th>END (N = 84)</th>
<th>TEA (N = 50)</th>
<th>SSP (N = 67)</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pubic bone</td>
<td>93.65 ± 4.26*</td>
<td>93.06 ± 3.57</td>
<td>97.27 ± 5.85*</td>
<td>93.80 ± 4.13</td>
<td>11.22*</td>
</tr>
<tr>
<td>Sitting height</td>
<td>95.22 ± 2.89*</td>
<td>94.38 ± 3.25</td>
<td>98.42 ± 3.19*</td>
<td>95.14 ± 3.01</td>
<td>19.94*</td>
</tr>
<tr>
<td>Chin height</td>
<td>156.98 ± 5.74*</td>
<td>156.30 ± 5.09</td>
<td>164.03 ± 7.14*</td>
<td>157.60 ± 5.23</td>
<td>22.95*</td>
</tr>
<tr>
<td>Upper sternum</td>
<td>147.10 ± 5.16*</td>
<td>146.59 ± 4.61</td>
<td>153.09 ± 6.93*</td>
<td>147.44 ± 5.03</td>
<td>19.34*</td>
</tr>
<tr>
<td>Right femur</td>
<td>45.56 ± 2.80*</td>
<td>46.03 ± 2.44</td>
<td>48.64 ± 3.36*</td>
<td>46.98 ± 2.34</td>
<td>14.56*</td>
</tr>
<tr>
<td>Left femur</td>
<td>45.91 ± 2.76*</td>
<td>46.39 ± 2.38</td>
<td>48.64 ± 3.42*</td>
<td>47.27 ± 2.32*</td>
<td>15.07*</td>
</tr>
<tr>
<td>Arm span</td>
<td>186.79 ± 7.09*</td>
<td>184.95 ± 6.41</td>
<td>192.31 ± 8.65*</td>
<td>187.51 ± 7.19</td>
<td>11.40*</td>
</tr>
</tbody>
</table>

Athletes’ groups including: SSP; strength - sprint athletes, END; endurance athlete, TAE; team sports player athletes, and Control. N is the total number in each group. Data are expressed as means ± SD. P < 0.05 was considered statistically t and labelled by *.

### 3.4.3 AMY1 Copy Number Distribution

The copy number distributions were similar by group. Overall, the copy number of AMY1 in this study ranged between 1 and 14 copies (Figure 3-1 A.). The number of copies did not differ between athletes and the control group, with a mean of 6.80 copies for both (Figure 3-1 B.). Nor were there significant differences between athlete subgroups CON, END, TEA, and SSP (p = 0.957).
Figure 3-1 Distribution of AMY1 Copy Number in the Study Population. Figure A represents total participants and figure B shows participants by subgroups. Athletes’ groups including: SSP(n = 50); strength - sprint athletes; END(n = 84); endurance athlete, TAE (TSP) (n = 67); team sports player athletes, and Control(n = 187). Estimates of copy numbers have been rounded to the nearest integer and presented in percentage. Data are expressed as means ± SD. p< 0.05 was considered statistically.
3.4.4 The Association Between AMY1 CNV and Anthropometrics and Body Composition Measurements

The association between AMY1 and both BMI and FFM strengthened after adjustment for subgroups (Model 2) compared to the unadjusted model (Model 1). However, the association weakened after additionally adding height to the model (Model 3) confirming the lack of association between AMY1 CN and BMI and FFM. Further, the AMY1 $p$-value weight increased after adjustment for subgroups than before adjustment for subgroups. However, the AMY1 CN $p$-value decreased after adding height to the interaction of subgroups and AMY1 CN conforming the lack of association between AMY1 CN and weight. Moreover, the results indicated that the AMY1 $p$-value for body fat decreased in a linear pattern after adjustment for subgroups than before adjustment for subgroups and after adding height conforming the lack of association between AMY1 CN and body fat %. In contrast the AMY1 $p$-value for height was decreased after adjustment for subgroups conforming significant association between AMY1 CN and height (Table 3-3).

The findings revealed that the AMY1 $p$-value for Anthropometrics Measurements including Pubic bone (cm), Sitting height (cm), Chin height (cm), Right femur (cm), and Left femur (cm) variation pattern was constant as the AMY1 CN $p$-value were decreased after adjustment for subgroups than before adjustment for subgroups that conforming the significant association between high AMY1 CN and the BC measurements (Model 1 and Model 2). However, the AMY1 $p$-value for all the measurements increased after adding height to the interaction of subgroups and AMY1 CN. So, the detected substantial associations in Model 1 and Model 2 were disappeared after adjusted for height (Table 3-3).
3.4.4.1 The Associations Between AMY1 CNV and Measures of Strength-Isokinetic Right leg, peak knee Extension & Flexion

The AMY1 CN was not associated with the Measures of Strength-Isokinetic Right leg, peak knee Extension including 90d/sec. The AMY1 CN p-value increased after adding height to the interaction of subgroups and AMY1 CN in Model3 compared to the first two models. Further, the findings indicated that the AMY1 p-value for Isokinetic Right leg, peak knee Extension including 180d/sec and 30d/sec, increased after adjustment for subgroups than before adjustment for subgroups and after adding height to the interaction of subgroups and AMY1 CN. But all of thesis associations were not significant.

Further, the AMY1 CN p-value for Measures of Strength-Isokinetic Right leg, peak knee Flexion including 30d/sec, decreased after adjustment for subgroups in model2 than before adjustment for subgroups in model1. However, the AMY1 CN p-value increased after adding height to the interaction of subgroups and AMY1 in model3. Moreover, the AMY1 CN p-value for Measures of Strength-Isokinetic Right leg, peak knee Flexion including 90d/sec, decreased after adjustment for subgroups in model2 than before adjustment for subgroups and after adding height to the interaction of subgroups and AMY1 CN in model1 and model3. 90d/sec. In contrast, the AMY1 p-value for Measures of Strength-Isokinetic Right leg, peak knee Flexion including 180d/sec, increased after adjustment for subgroups than before adjustment for subgroups and after adding height to the interaction of subgroups and AMY1 CN in model1 and model3. But all of thesis associations were not significant (Table 3-3).
The AMY1 CN p-value for Measures of Strength-Isokinetic Left leg, peak knee Extension including 30d / sec, 90d / sec and 180d / sec, consistently increased after adjustment for subgroups in model2 than before adjustment for subgroups and after adding height to the interaction of subgroups and AMY1 CN model1 and model 3. But all of thesis associations were not significant (Table 3-3).

The AMY1 CN p-value for Measures of Strength-Isokinetic Left leg, peak knee Flexion including 30d / sec, 90d / sec and 180d / sec, uniformly decreased after adjustment for subgroups than before adjustment for subgroups. However, the AMY1 CN p-value for Measures of Strength-Isokinetic Left leg, peak knee Flexion including 30d / sec, 90d / sec and 180d / sec, uniformly increased after adding height to the interaction of subgroups and AMY1 CN. But all of thesis associations were not significant (Table 3-3).

The AMY1 CN p-value for sprint total uniformly increased after adjustment for subgroups than before adjustment for subgroups and after adding height to the interaction of subgroups and AMY1. The detected substantial associations in Model 1 and were disappeared after adjusted for subgroups and after height in model 2 and model 3(Table 3-3).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg.m⁻²)</td>
<td>0.429</td>
<td>0.334</td>
<td>0.939</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.034*</td>
<td>0.013*</td>
<td>--</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>0.668</td>
<td>1.000</td>
<td>0.911</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>0.887</td>
<td>0.460</td>
<td>0.396</td>
</tr>
</tbody>
</table>

Table 3-3 The p-values from GLM Analysis of the Associations Between AMY1 CN and Measures of Anthropometrics, Strength and Speed.
Chapter 3: Copy Number of AMY1 and Athletes

### Table 3.4.4.2

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFM (kg)</td>
<td>0.395</td>
<td>0.206</td>
<td>0.869</td>
</tr>
<tr>
<td>Pubic bone (cm)</td>
<td>0.021*</td>
<td>0.007*</td>
<td>0.105</td>
</tr>
<tr>
<td>Sitting height (cm)</td>
<td>0.047*</td>
<td>0.023*</td>
<td>0.712</td>
</tr>
<tr>
<td>Chin height (cm)</td>
<td>0.033*</td>
<td>0.014*</td>
<td>0.046</td>
</tr>
<tr>
<td>Upper sternum (cm)</td>
<td>0.082</td>
<td>0.036</td>
<td>0.138</td>
</tr>
<tr>
<td>Right femur (cm)</td>
<td>0.038*</td>
<td>0.006*</td>
<td>0.056</td>
</tr>
<tr>
<td>Left femur (cm)</td>
<td>0.025*</td>
<td>0.005*</td>
<td>0.051</td>
</tr>
<tr>
<td>Arm span (cm)</td>
<td>0.099</td>
<td>0.051</td>
<td>0.743</td>
</tr>
<tr>
<td>Isokinetic Right leg, peak knee Extension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30d/sec</td>
<td>0.622</td>
<td>0.656</td>
<td>0.869</td>
</tr>
<tr>
<td>90d/sec</td>
<td>0.409</td>
<td>0.372</td>
<td>0.982</td>
</tr>
<tr>
<td>1800d/sec</td>
<td>0.431</td>
<td>0.462</td>
<td>0.582</td>
</tr>
<tr>
<td>Isokinetic Right leg, peak knee Flexion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30d/sec</td>
<td>0.516</td>
<td>0.358</td>
<td>0.596</td>
</tr>
<tr>
<td>90d/sec</td>
<td>0.983</td>
<td>0.839</td>
<td>0.780</td>
</tr>
<tr>
<td>1800d/sec</td>
<td>0.786</td>
<td>0.805</td>
<td>0.857</td>
</tr>
<tr>
<td>Isokinetic Left leg, peak knee Extension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30d/sec</td>
<td>0.245</td>
<td>0.460</td>
<td>0.796</td>
</tr>
<tr>
<td>90d/sec</td>
<td>0.224</td>
<td>0.395</td>
<td>0.848</td>
</tr>
<tr>
<td>1800d/sec</td>
<td>0.143</td>
<td>0.220</td>
<td>0.982</td>
</tr>
<tr>
<td>Isokinetic Left leg, peak knee Flexion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30d/sec</td>
<td>0.169</td>
<td>0.113</td>
<td>0.949</td>
</tr>
<tr>
<td>90d/sec</td>
<td>0.250</td>
<td>0.186</td>
<td>0.549</td>
</tr>
<tr>
<td>1800d/sec</td>
<td>0.192</td>
<td>0.100</td>
<td>0.916</td>
</tr>
<tr>
<td>Sprint Total</td>
<td>0.041*</td>
<td>0.078</td>
<td>0.092</td>
</tr>
</tbody>
</table>

(*) $p < 0.05$ was considered statistically significant. Model 1: unadjusted, Model 2: adjusted for athletes’ groups according to type of sport SSP: strength - sprint athletes, END: endurance athlete, and TEA: team sports Player athletes, and controls and Model 3: adjusted for height.

### 3.4.4.2 Prediction of Anthropometric and Body Composition from AMY1 CNV

A hierarchical linear regression analysis was carried out to evaluate the utility of AMY1 in the prediction of anthropometric and body composition including BMI, height, weight, body fat and FFM. For the first model (Model1) the predictor variables were analysed without adjusting for subgroups and heights. The results indicated that the model was not statistically significant $F(5, 382) = 1.93, p >0.05$. Additionally, the $R^2$
value of (0.025) associated with the regression model suggests that the predictor variables account for (2.5 %) variation in the AMY1, which mean that (97.5 %) of the variation in BMI, height, weight, body fat and FFM cannot be explained by the predictor AMY1 CNV alone. Further, the findings indicated that despite the model was not statistically significant the body fat ($\beta = 0.34, p < 0.05$), and FFM ($\beta = 0.32, p < 0.05$) significantly predicted by the AMY1.

Similarly, for the second model (Model 2) the predictor variables were analysed with adjustment for subgroups. The results indicated that the model was not statistically significant $F (6, 381) = 1.70, p > 0.05$. Additionally, the $R^2$ value of (0.026) associated with the regression model suggests that the predictor variables account for (2.6 %) variation in the AMY1, which means that (97.4 %) of the variation in BMI, height, weight, body fat and FFM cannot be explained by the predictor variable alone. Further, the findings indicated that despite the model was not statistically significant and after adjustment for subgroups the body fat ($\beta = 0.36, p < 0.05$), and FFM ($\beta = 0.33, p < 0.05$) significantly predicted by the AMY1.

Moreover, for the third model (Model 3) the predictor variables were analysed with adjusting for height. The results indicated that the model was not statistically significant $F (5, 382) = 1.93, p > 0.05$. Additionally, the $R^2$ value of (0.025) associated with the regression model suggests that the predictor variables account for (2.5 %) variation in the AMY1, which means that (97.5 %) of the variation in the AMY1 cannot be explained by the predictor variables alone. Further, the findings indicated that despite the model was not statistically significant and after adjusting for height two of the
predictors including body fat ($\beta = .34$, $p < 0.05$), and FFM ($\beta = .32$, $p < 0.05$) significantly predicted the AMY1 (Table 3-5).
Table 3.4: A Hierarchical Linear Regression Analysis Results of Associations between AMY1 copy number and Anthropometric and Body Composition.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β Coefficient</td>
<td>β Coefficient</td>
<td>β Coefficient</td>
</tr>
<tr>
<td></td>
<td>(95% CI)</td>
<td>(95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.43 (-0.92, 1.77)</td>
<td>0.45 (-0.90, 1.80)</td>
<td>0.43 (-0.92, 1.77)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>0.14 (-0.20, 0.49)</td>
<td>0.15 (-0.20, 0.50)</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>-0.41 (-0.85, 0.04)</td>
<td>-0.43 (-0.88, 0.02)</td>
<td>-0.41 (-0.85, 0.04)</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>0.34 (0.03, 0.64) *</td>
<td>0.36 (0.05, 0.67) *</td>
<td>0.34 (0.03, 0.64) *</td>
</tr>
<tr>
<td>FFM (kg)</td>
<td>0.32 (0.04, 0.60) *</td>
<td>0.33 (0.04, 0.61) *</td>
<td>0.32 (0.04, 0.60) *</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.025</td>
<td>0.026</td>
<td>0.025</td>
</tr>
</tbody>
</table>

β coefficient and 95% Confidence interval, and $p < 0.05$ was considered statistically significant (*). Model 1: unadjusted, Model 2: adjusted for athletes’ groups according to type of sport SSP; strength - sprint athletes, END; endurance athlete, and TAE; team sports Player athletes, and controls and Model 3: adjusted for height and subgroups.

3.4.4.3 Prediction of Anthropometric Measurements from AMY1 CNV

A hierarchical linear regression analysis was carried out to evaluate the prediction of AMY1 from anthropometric measurements, including pubic bone, sitting height, chin height, upper sternum, right femur, left femur, and arm span. For the first model (Model 1) the predictor variables were analysed without adjusting for subgroups and heights. The results indicated that the model was not statistically significant $F (7, 380) = 1.45$, $p > 0.05$. Additionally, the $R^2$ value of (0.026) associated with the regression model suggests that the predictor variables account for (2.6 %) variation in the AMY1, which mean that (97.4 %) of the variation in the AMY1 cannot be explained by the predictor variables alone.

Similarly, for the second model (Model 2) the predictor variables were analysed with adjusting for subgroups. The results indicated that the model was not statistically
significant $F(8, 379) = 1.27, p > 0.05$. Additionally, the $R^2$ value of (0.026) associated with the regression model suggests that the predictor variables account for (2.6%) variation in the AMY1, which mean that (97.4%) of the variation in the AMY1 cannot be explained by the predictor variables alone.

Moreover, for the third model (Model 3) the predictor variables were analysed with adjusting for height. The results indicated that the model was not statistically significant $F(8, 379) = 1.28, p > 0.05$. Additionally, the $R^2$ value of (0.026) associated with the regression model suggests that the predictor variables account for (2.6%) variation in the AMY1, which mean that (97.4%) of the variation in the AMY1 cannot be explained by the predictor variables alone (Table 3-5).

### Table 3-5 A Hierarchical Linear Regression Analysis Results of Associations Between AMY1 Copy Number and Anthropometric Measurements.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$ Coefficient</td>
<td>$\beta$ Coefficient</td>
<td>$\beta$ Coefficient</td>
</tr>
<tr>
<td></td>
<td>(95% CI)</td>
<td>(95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>Pubic bone (cm)</td>
<td>0.09 (-0.02, 0.20)</td>
<td>0.09 (-0.02, 0.20)</td>
<td>0.09 (-0.02, 0.20)</td>
</tr>
<tr>
<td>Sitting height (cm)</td>
<td>0.07 (-0.06, 0.19)</td>
<td>0.07 (-0.05, 0.20)</td>
<td>0.06 (-0.09, 0.20)</td>
</tr>
<tr>
<td>Chin height (cm)</td>
<td>0.08 (-0.09, 0.26)</td>
<td>0.09 (-0.09, 0.26)</td>
<td>0.07 (-0.14, 0.28)</td>
</tr>
<tr>
<td>Upper sternum (cm)</td>
<td>-0.18 (-0.37, 0.01)</td>
<td>-0.18 (-0.38, 0.01)</td>
<td>-0.19 (-0.40, 0.02)</td>
</tr>
<tr>
<td>Right femur (cm)</td>
<td>-0.07 (-0.41, 0.27)</td>
<td>-0.07 (-0.41, 0.27)</td>
<td>-0.07 (-0.42, 0.27)</td>
</tr>
<tr>
<td>Left femur (cm)</td>
<td>0.16 (-0.19, 0.52)</td>
<td>0.16 (-0.19, 0.52)</td>
<td>0.16 (-0.19, 0.52)</td>
</tr>
<tr>
<td>Arm span (cm)</td>
<td>-0.01 (-0.06, 0.05)</td>
<td>-0.01 (-0.06, 0.05)</td>
<td>-0.01 (-0.06, 0.05)</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
</tr>
</tbody>
</table>

$\beta$ coefficient and 95% Confidence interval, and $p < 0.05$ was considered statistically significant (*). Model 1: unadjusted, Model 2: adjusted for athletes’ groups according to type of sport SSP; strength - sprint athletes, END; endurance athlete, and TEA; team sports Player athletes, and controls and Model 3: adjusted for height and subgroups.
### 3.4.4.4 Prediction Measures of Strength from AMY1 CNV

A hierarchical linear regression analysis was carried out to evaluate the prediction of AMY1 from measures of strength, including Isokinetic Right leg, peak knee Extension (30d/sec, 90d/sec, 180d/sec), Flexion (30d/sec, 90d/sec, 180d/sec) and Isokinetic Left leg, peak knee Extension (30d/sec, 90d/sec, 180d/sec), Flexion (30d/sec, 90d/sec, 180d/sec), and total sprint. For the first model (Model 1) the predictor variables were analysed without adjusting for subgroups and heights. The results indicated that the model was not statistically significant $F(13, 374) = 1.26, p > 0.05$. Additionally, the $R^2$ value of (0.042) associated with the regression model suggests that the predictor variables account for (4.2 %) variation in the variables, which mean that (95.8 %) of the variation in the variables cannot be explained by the AMY1 CN alone. Further, the findings indicated that despite the model was not statistically significant one of the predictors including sprint total ($\beta = 1.11, p < 0.05$) significantly predicted by the AMY1 CN.

Similarly, for the second model (Model 2) the predictor variables were analysed with adjusting for subgroups. The results indicated that the model was not statistically significant $F(14, 373) = 1.22, p > 0.05$. Additionally, the $R^2$ value of (0.044) associated with the regression model suggests that the AMY1 CN account for (4.46 %) variation in the variables, which mean that (95.6 %) of the variation in the variables cannot be explained by the predictor AMY1 CN alone. Further, the findings indicated that despite the model was not statistically significant one of the predictors after adjusting for subgroup which was sprint total ($\beta = 1.25, p < 0.05$) significantly predicted the AMY1 CN.
Moreover, for the third model (Model 3) the predictor variables were analysed with adjusting for height. The results indicated that the model was not statistically significant $F(14, 373) = 1.23, p > 0.05$. Additionally, the $R^2$ value of (0.044) associated with the regression model suggests that the predictor AMY1 CN account for (4.4 %) variation in the variations, which mean that (95.6 %) of the variations in the variables cannot be explained by the predictor AMY1 CN alone. Further, the findings indicated that after adjusting for height the total sprint which was significant (in model 1 and model 2) was become non-significant ($p > 0.05$) in this model (Table 3-6).
Chapter 3: Copy Number of AMY1 and Athletes

Table 3-6: A Hierarchical Linear Regression Analysis Results of Associations Between AMY1 Copy Number and Performance Measures.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$ Coefficient (95% CI)</td>
<td>$\beta$ Coefficient (95% CI)</td>
<td>$\beta$ Coefficient (95% CI)</td>
</tr>
<tr>
<td><strong>Isokinetic Right leg, peak knee</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 d/sec</td>
<td>-0.01 (-0.02, -0.01)</td>
<td>-0.01 (-0.03, -0.01)</td>
<td>-0.01 (-0.02, -0.01)</td>
</tr>
<tr>
<td>90 d/sec</td>
<td>0.01 (-0.02, 0.04)</td>
<td>0.01 (-0.02, 0.04)</td>
<td>0.01 (-0.02, 0.04)</td>
</tr>
<tr>
<td>180 d/sec</td>
<td>-0.01 (-0.04, 0.02)</td>
<td>-0.01 (-0.04, 0.02)</td>
<td>-0.01 (-0.04, 0.02)</td>
</tr>
<tr>
<td>Flexion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 d/sec</td>
<td>0.01 (-0.01, 0.03)</td>
<td>0.01 (-0.01, 0.03)</td>
<td>0.01 (-0.01, 0.03)</td>
</tr>
<tr>
<td>90 d/sec</td>
<td>-0.01 (-0.04, 0.02)</td>
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<td><strong>Isokinetic Left leg, peak knee</strong></td>
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<td>Total (s)</td>
<td>1.11 (0.04, 2.18) *</td>
<td>1.25 (0.13, 2.36) *</td>
<td>1.02 (-0.06, 2.11) *</td>
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<tr>
<td>$R^2$</td>
<td>0.042</td>
<td>0.044</td>
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$\beta$ coefficient and 95% Confidence interval, and $p < 0.05$ was considered statistically significant (*). Model 1: unadjusted, Model 2: adjusted for athletes’ groups according to type of sport SSP; strength - sprint athletes, END; endurance athlete, and TEA; team sports Player athletes, and controls and Model 3: adjusted for height and subgroups.
3.5 Discussion

To the best of our knowledge, this research was the first to evaluate variations in AMY1 copy numbers in the athletic population. The aim was to assess whether a higher AMY1 copy number was linked to BMI, body fat percentage, anthropometric measures of body fat, strength, and FFM in a group of athletes as compared to non-athlete controls. For the first time, our study reports that increases in AMY1 copy number is positively linked with strength and height variations in athletes.

3.5.1 Copy Number and Athlete Body Composition

AMY1 is the gene encoding salivary amylase. Recently, a high CNV AMY1 has been investigated as a newly identified genetic variant that could explain BMI's missing heritability not related to single nucleotide polymorphisms. However, the contribution of AMY1 copy numbers on BMI remains controversial. The first study conducted in a large adult population indicated a remarkably strong association between AMY1 copy numbers and increased BMI, with a significantly increased risk of obesity in subjects with low compared to those with high AMY1 copy numbers (Falchi et al., 2014). Subsequently, several studies supported this association among different populations and age groups (Viljakainen et al., 2016; Benitez et al., 2015; Yong et al., 2016; León-Mimila et al., 2018).

This study found a similar variation in the number of copies between all groups, even though the AMY1 copy number ranged between 1 and 14. No association was found with increasing AMY1 copy number and lower BMI, healthy body fatness, and FFM in the investigated population. However, significant variations in all the tested
variances were detected among athlete’s groups and controls study participants, which make the association harder to be observed. Our study reproduces the findings reported by Ushar et al. (2017), who found no association between AMY1 copy numbers and BMI in two cohorts of ~3,500 individuals, 569 individuals from phase 1 of the 1000 Genomes Project, and 114 parent-offspring trios from HapMap. Ushar et al. (2017) suggested that this result may be due to the use of ddPCR, a potentially more accurate method to detect copy numbers than that used in previous studies. In this study, similar results were found when the TaqMan assay was applied, suggesting that the difference in the reported observation likely comes from dissimilarities in the populations, as most of the participants in this study were athletes. However, Marcovecchio et al. (2016) did not detect an overall difference in AMY1 relative copy number between obese and lean school children. Still, this study showed significant association between high AMY1 copies and increased BMI in boys when gender-based analyses were performed (Marcovecchio et al., 2016).

3.5.2 Height and Copy Number of AMY1

CNVs have been recognized as a significant contributor to the genetic variation between individuals in addition to single-nucleotide polymorphisms (Kang et al., 2010). Dauber et al. (2011) suggested that CNVs may contribute to genetic variations in stature among the general population (Dauber et al., 2011). Kang et al. assessed the relationship between 405 and 431 (unfortunately not including AMY1 gene) commonly segregating CNV regions (frequency > 5 %) and height or BMI in genome-wide association analysis of anthropometric traits in ~ 2,000 individuals of African-ancestry, finding no significant associations (Kang et al., 2010). The current study’s finding was the first to link high CN of AMY1 with height.
Anthropometric measurements were tested to understand further the influence of high copy number of AMY1 in relation to height. Increased leg length, sitting height, chin height, upper sternum, femur, and arm span were significantly associated with a higher copy number. But, after adjustment for height, these associations were no longer significant. This is likely to be due to their strong correlation with height. Arm span is positively correlated with height (Steele & Chenier, 1990), and height can also be predicted reliably from arm measurement (Jarzem & Gledhill, 1993). Stature was associated with leg length and sitting height. Adult height is determined by both genetic and environmental factors, including nutrition, illness, socioeconomic status, and psychosocial stress in early life (Bogin and Varela-Silva, 2009; Batty et al., 2009; Sawada and Takasaki, 2017).

The early childhood linear growth appears to be highly predictive of adult height. A recent cohort study was conducted to estimate the associations between exposure to early life growth faltering at the population level and adult height in 425 birth cohorts across 126 regions in 21 low-and-middle countries. The study found decreasing growth in the most malnourished populations and reported average Height for Age Z-scores (HAZ) < −3, which translates in an increase of 8.8 cm in adult male height and a 5.6 cm increase in adult female height (Karra & Fink 2019).

These variations in height seem to be significant from a cross-country perspective; a study of European populations of 10 countries found a height range of 170 to 179 cm in males and 160 to 167 cm in females (Cavelaars et al., 2000). Given that the average adult in the presented study population included an average height mean ± SD in cm of
180.2 ± 5.8 in control, 179.3 ± 5.4 in END 186.58 ± 7.4 in TEA, and 180.6 ± 5.4 in SSP.

Karra & Fink suggested that these individual-level correlations may reflect, to a large extent, genetic differences that impact both childhood and adult outcomes and, therefore, should not be interpreted as an indication for a causal effect of nutrition on development (Karra & Fink, 2019). The association between height and CNV of a gene like AMY1 CN, linked with carbohydrate intake, may explain more of a causal link to catch-up growth or rapid growth in adolescence and height in future research.

A possible explanation is that AMY1 interacts with other gene (s) variants. If that is so, AMY1 copy number influence on BMI is more evident in users with a specific set of gene variants. Such gene variants may trigger changes in energy metabolism or adipose tissue development. For instance, a recent meta-analysis evaluated gene expression in obese and non-obese individuals, finding 821 gene variants predominant in the adipose tissue of obese subjects (Goutzelas et al., 2022). A combination of such genes could potentially modulate the interaction between AMY1 copy number and BMI. However, no research up to this date has evaluated the interaction between previously identified obesity-associated genes and AMY1 copy number in modulating BMI. Moreover, studies correlating AMY1 CN and other amylase genes located in the same region report that AMY1 copy number is independent of the other two amylase genes, AMY2A and AMY2B, and the evidence suggests that the genetic link can be found in carbohydrate metabolism genes, especially those involving gut microbiota (Nakajima, 2016).
The link between AMY1 copy number, energy metabolism genetics, and gut microbiota is further demonstrated by metagenomic sequencing and short-chain fatty acid quantification in the faecal material of individuals with low and high AMY1 copy number. The investigators found that participants with low AMY1 copy number displayed a higher predominance of complex carbohydrate degradation enzymes in their gut microbiota. Still, the same study transplanted the gut microbiota from low and high AMY1 copy number individuals in mice, reporting higher weight gain in mice transplanted with high AMY1 copy number gut microbiota. This only reveals the complexity of AMY1 genetics in combination with diet, microbiome, and other gene variants to modulate BMI and adiposity (Poole et al., 2019).

Another explanation of these equivocal results involves an unassessed or unrecognised environmental interaction. In the same line of thinking, Rukh et al. (2017) evaluated the link between diet and AMY1 copy number, revealing a significant interaction between starch intake and BMI in 4800 Swede nondiabetic adults (Rukh et al., 2017). The participants in another study were subject to randomized low-calorie diet interventions, resulting in significantly higher central adiposity and weight loss in rs11185098-A allele carriers, which has been recognized as a proxy of higher AMY1 copies and activity) (Usher et al., 2015) in The POUNDS Lost Trial, which included 692 European subjects (Heianza et al., 2018). Taking together the evidence, the studies bring forward environmental factors as modulators in the association between adiposity and AMY1 copy number, especially when it comes to dietary choices. Further, Rossi et al. (2021) evidenced the impact of lifestyle and environment in selecting the amylase loci in their study of the relationship between pancreatic and salivary amylase gene copy number, performed in 2935 Qatari individuals.
Understanding the cause of height is important in sports and health filed. Height is now used as an indicator in epidemiology studies to estimate the association of one's early life exposure and diseases. Emerging Risk Factors Collaboration conducted a meta-analysis of 121 cohort studies comprising over 1 million participants and stated that short stature is a well-documented risk factor for death from circulatory diseases such as coronary disease, stroke, and heart failure (Emerging Risk Factors Collaboration, 2012). In contrast, height was positively associated with the risk of death from melanoma and cancers of the pancreas, endocrine and nervous systems, ovary, breast, prostate, colorectum, blood, and lung (The Emerging Risk Factors Collaboration, 2012).

A large prospective study of 409,748 adult individuals found that overall height is positively associated with deaths from cancer but inversely associated with deaths from circulatory disease (Sawada et al., 2017). However, shorter males showed increased risk of cardiovascular mortality (HR per 5 cm increase: 0.81, 95 % CI: 0.72–0.91), and all-cause mortality (HR per 5 cm increase: 0.89, 95 % CI: 0.83–0.96). Further, shorter females showed increased risk of non-cardiovascular, non-cancer mortality (HR per 5 cm increase: 0.82, 95 % CI: 0.71–0.96), and all-cause mortality (HR per 5 cm increase: 0.88, 95 % CI: 0.81–0.96) (Zhao et al., 2019). Furthermore, a large cohort of 22,809,722 Korean men and woman adults confirmed positive associations between height and risk of all site-combined cancers, including malignancy in the oral cavity, larynx, lung, stomach, colorectum, liver, pancreas, biliary tract and gallbladder, breast, ovary, cervix and corpus uteri, prostate, testes, kidney, bladder, central nervous system, thyroid, skin, and lymphatic and hematopoietic systems. The positive association between height and cancer has been estimated, and the hazard ratios (HRs) for all-site cancers per 5 cm
increment in height is 1.09 (95% CI: 1.086–1.090), especially in thyroid, breast, lymphoma, testicular, and renal cancers (Choi et al., 2019).

Several potential underlying mechanisms explain the opposing association of adult height with cancer mortality. Positive associations may result from the fact that taller people have larger organs, and therefore a greater number of cells are at risk of proliferation. Another explanation is an increased level of insulin-like growth factor (IGF) which is the primary mediator of growth hormone activity and a hormone that has been positively associated with cancers at several anatomic sites (Choi et al., 2019). It has been reported that each 10 cm increase in height corresponded to a 4% increase in circulating IGF-1 levels (Crowe et al., 2010). Finally, several genetic factors are related to height, cancer, and cardiovascular disease. Identifying such genetic variants might shed light on potential mechanisms underlying the associations between height and mortality (Sawada et al., 2017). Further research is needed to confirm the association between increasing CNV of AMY1 and height.

This study has several limitations, including the low variance of age, fat mass, BMI within groups of athletes, and controls. Also, some of the control individuals can be considered physically active or even participating in planned training sessions as their $\dot{V}O_2$ max values were high. Further research is needed to evaluate the association between increasing AMY1 copy number and BMI and body fatness in the general adult population compared to the active population.
3.6 Conclusion

Individuals with a higher copy number of AMY1 are more likely to be taller. This may underlie the inconsistent associations with BMI observed in less active population groups. Further studies are needed to confirm this finding in large-scale studies of active and non-active populations. Further, the strong association between increasing AMY1 and height may suggest further research investigating AMY1 as a height-related gene and assessing its relationship to cancer risk and other diseases associated with height.
Chapter 4  Copy Number of AMY1, and Body Composition of Adult Male and Female University Students in Scotland

4.1 Abstract

BACKGROUND: A 2014 publication reported for the first time a possible link between higher BMI and lower copy number (CN) of the salivary amylase 1 (AMY1) gene. Since then, several replication studies have been conducted. The results are inconsistent despite being well-powered studies with a consistent methodology. Different study designs and not considering gender as a covariate may explain the current discrepancy in the literature.

AIM: To evaluate the association between AMY1 CN, anthropometrics, and body composition measures in males and females.

METHODS: A total population of 228 (males n = 108 and females n = 120) were recruited from the University of Stirling's student population. Ages ranged from 18 to 40 years. Height, weight, BMI, and other anthropometric measurements were taken. A DXA scan was performed, and the participants completed a 3-day self-reported food questionnaire to assess macronutrient and energy intake. Physical activity level was assessed by the Scottish Physical Activity Questionnaire (SPAQ). An independent sample t-test was used to assess the difference between the mean characteristics of both groups. Linear regression analysis was used to predict the anthropometric and body composition from AMY1 CNV.

RESULTS: AMY1 CN distribution did not differ between males and females ($p = 0.942$). No association was detected between AMY1 CN and adiposity markers in the
study population (Height, $p = 0.326$ Weight, $p = 0.241$; BMI, $p = 0.438$; Total Body Fat $\%$, $p = 0.644$). WC (cm) was negatively associated with high AMY1 CN in females only ($\beta = -0.473$, $p = 0.005$). The regional body fat distribution and lean mass distribution did not correlate with AMY1 CN in either gender with all ($p > 0.05$). Increased total EI was associated with decreased AMY1 CN in males ($r = -0.964$, $p = 0.001$), and females ($r = -0.902$, $p = 0.001$). However, no such association were observed between and AMY1 CN and EI from CHO, fat, or protein in males or females. Physical activity level (PAL) did not associate with AMY1 CN in either gender.

**CONCLUSION:** The gender differences is detected in the associations between AMY1 CN and WC: this association was significant in females but not in males. In addition, this study found a link between dietary intake and AMY1 CNVs in adult males and female. More research in larger populations is warranted to confirm these results.

### 4.2 Introduction

The increasing prevalence of obesity (defined in adults as a BMI $\geq 30$; kg/m$^2$) has become a major public health concern throughout the globe. According to the WHO report in 2018, the prevalence of obesity in adults has approximately tripled worldwide since 1975 (WHO, 2018). In the UK, the majority of English adults in 2018 were overweight, with a prevalence of 63%, and 28% of those overweight individuals were obese (NHS, 2020). Similarly, Scotland’s statistics in 2019 show that 66% of adults were overweight, and 29% of those were obese (NatureScot, 2021). In each report, gender differences were noticeable in both groups of overweight and obese individuals. In the overweight category, there was a significantly higher number of males compared
with females in England (67 % and 60 % respectively) and Scotland (69 % and 63 % respectively). However, obesity or morbid obesity was more common in females than males in England (29 % and 26 % respectively); although, no significant difference was found between obese males and females in Scotland (29 % and 30 % respectively). If the current trends continue, overweight and obesity will have important economic and social implications.

A study across 26 European countries including the UK and the USA suggests that increasing overweight/obesity rates will result in a decline in life expectancy, ranging from 0.66 to 1.54 years for men and 0.86 to 1.67 years for women (Vidra, Trias-Llimós and Janssen, 2019). Obesity predisposes to several chronic diseases including cardiovascular disease (CVD), type 2 diabetes mellitus (DM) (Mokdad et al., 2003; Aung et al., 2014), and some types of cancers, including thyroid and breast cancer (He et al., 2019). Furthermore, obesity may also produce psychological morbidity, especially depression among females (Stunkard et al., 2003). Gender differences appear to suggest that females suffer more than males from the impact of obesity on length and quality of life (Muennig et al., 2006).

Obesity is a multifactorial disease driven by modern Western lifestyles in which individuals find themselves living in an obesogenic environment characterised by high fat hypercaloric diets combined with low physical activity levels (PAL) and sedentary jobs (Nurwanti et al., 2019; Choi et al., 2010). Western diets cause a high level of postprandial insulin production and can produce insulin hypersecretion and hyperinsulinemia. This endocrine modification stimulates fat storage, prevents
lipolysis, and causes increased appetite, hyperphagia, and weight gain (Kopp, 2019). However, there is also a genetic component to obesity; some individuals are more susceptible to weight gain than others when exposed to the same obesogenic environment (Albuquerque et al., 2017). Several studies have identified genetic factors that influence the development of obesity, with estimates of obesity heritability ranging from 40% to 80% (Stunkard et al., 1990; Maes et al., 1997; Herrera and Lindgren, 2010) (Hjelmborg et al., 2008; Malis et al., 2005). Furthermore, gene-environment interactions explain why certain obesogenic behaviours are more problematic for some individuals than they are for others (Francis, Shodeinde, Black and Allen, 2018; Nan et al., 2012). Understanding the genetic component of obesity is vital to understanding an individual’s risk of obesity.

The genetic component of obesity is complex. It comprises hundreds of genes and their individual effects, which are usually small but overlap and become relevant when combined with environmental triggers. Genome-wide association studies (GWAS) have recently identified a large number of significant genome-wide loci with an impact on BMI, obesity, Body Composition, and body fat distribution (Peters et al., 2020). For BMI, 941 single-nucleotide polymorphisms (SNPs) at 536 polygenic loci were detected as significant near-independent genome-wide loci, explaining ~ 6.0 % of the variance of BMI in the Health and Retirement Study (Yengo et al., 2018). A very recent GWAS on whole-body lean body mass (LBM; adjusted for gender, age, and height with or without fat mass adjustments) revealed seven loci associated with LBM (Karasik et al., 2019). Another GWAS on the percentage of body fat distribution in arms, legs, and trunk on 362,499 individuals from the UK Biobank identified 98 independent loci, 29 of which had not previously been associated with anthropometric traits, including BMI.
Chapter 4: Copy Number of AMY1, and Body Composition

(Rask-Andersen, Karlsson, and Johansson, 2019). The most recent GWAS on body fat distribution as measured by waist-to-hip ratio adjusted for BMI reported 463 independent loci (Pulit et al., 2019). However, the influence of all known SNPs taken together only explains 1-3% of BMI variance (Ahmad and Imam, 2015). Hence, there is a gap between the amount of BMI variation due to observable SNPs and the estimated heritability of the BMI variance (40-80%).

Recently, attention has turned to copy number variation (CNV) as a factor that may close the gap. CNV can modify thousands of nucleotides, often including multiple genes. Therefore, it has the potential of a much larger effect than individual SNPs. One such CNV is the Salivary α-Amylase 1 gene (AMY1). Whilst humans carry two copies of most genes, AMY1 has been reported to range from 1 to 20 copies (Falchi et al., 2014; Marcovecchio et al., 2016). Increased/decreased AMY1 copy number (CN) has been associated with the low/high risk of obesity through modifications in BMI measured at 0.15 kg/m² per copy and obesity risk changes of 1.19 per copy in European and Asian adults (Falchi et al., 2014). That would be the most substantial heritable factor yet to be discovered as a modifier of the obesity epidemic.

Follow-up studies have had inconsistent results: several confirm the significant association between low AMY1 CN and lowering BMI which reported by Falchi et al. (e.g., Mejia-Benitez et al., 2015; Atkinson et al., 2018; Leon-Mimila et al., 2018; Venkatapoorna et al., 2019; Vázquez-Moreno et al., 2020; Barber et al., 2020; Mayneris-Perxachset al., 2020; Selvaraju et al., 2020); while other studies reported no association (e.g., Usher et al., 2015; Viljakainen et al., 2015; Marcovecchio et al., 2016;
Pinho et al., 2018; Shwan et al., 2019; Al-Akl et al., 2020) or a positive association between AMY1 gene CN and BMI (Ruch et al., 2017). However, these studies evaluated different population groups and age groups or used indirect measures of adiposity such as bioelectric impedance. Carefully controlled studies with direct measurements of adiposity in well-defined phenotypic groups are essential to confirm and understand the contribution of AMY1 CNV to obesity.

Dietary CHO quantity and quality have been associated with weight gain and risks of obesity complications, such as type 2 diabetes (Ludwig et al., 2018). The digestion of polysaccharide CHOs begins in the oral cavity with the generation of smaller saccharides by the action of salivary α-amylase. Individual differences in salivary amylase function are attributed to the amount and activity of AMY1 CN variations (Atkinson et al., 2018). Individuals with higher salivary amylase levels have been shown to display faster oral starch digestion (Mandel, 2010). Higher AMY1 CN is also related to improved starch digestion (Mandel, 2012; Atkinson et al., 2018). Falchi et al. in 2014 found the first link between low AMY1 CN, carbohydrate (CHO) metabolism, and BMI in European and Asian populations (Falchi et al., in 2014). Rukh et al. reported that AMY1 CNV is associated with body fat only when taking into consideration the starch intake of individuals in a Swedish cohort (Rukh et al., 2017). AMY1 copy variations are likely caused by human genetic adaptation to starch-rich diets. Individuals from populations with high-starch diets have on average, more AMY1 copies compared to those with traditionally low-starch diets (mean = 6.7; mean = 5.44 respectively) (Parry et al., 2007; Santos et al., 2012). Notably, the increase of AMY1 CN is significantly correlated with levels of salivary amylase protein and serum amylase (Parry et al., 2007;
Mandel, 2010; Carpenter et al., 2017). This suggests a genetic link between CHO metabolism and obesity (more details will be presented in the next chapter).

The genetic adaptation to improve starch digestion, reflected by changes in AMY1 CN, may also have an impact on oral and gut microbiome profiles (Poole et al., 2019; León-Mimila et al., 2018). Gut microbiota alterations have been related to energy balance, weight gain, and adiposity via interactions with dietary factors (Lazar et al., 2019). Further, Salivary α-amylase concentration is regulated by circadian rhythms and the nervous system. Physical exercise activates the sympathetic nervous system (Paterson, 1996). Antoon et al. found that high-intensity exercise leads to the anticipated increase in amylase secretion and salivary flow rate (Antoon et al., 2015). Thus, the current study will assess the intake of CHO and other macronutrients in the study sample and its association with AMY1 CN.

In 1956, Vague observed that men tend to gain fat in the upper body (android pattern), and women store fat in the lower body (gynoid pattern). These gender differences in fat distribution have become clear and are closely associated with whole-body metabolism and long-term health (Vague 1947; Vague 1956). Women have consistently been found to display higher levels of total body adiposity than men (Jensen MD, 2002). Women preferentially deposit fat subcutaneously with greater accumulation in the gluteofemoral region. This fat distribution pattern may provide a buffer for fat storage during periods of positive energy balance and improve glucose metabolism, partially protecting against the development of type 2 diabetes in premenopausal women (Mauvais-Jarvis, 2018). In contrast, men tend to accumulate fat in the abdominal region and the visceral compartment, where it contributes to an increased risk for metabolic disease (Mathieu et al., 2014). These differences in total lipid storage may have evolved
to favour the energy needs of reproduction and lactation in women and suggest fundamental differences in the handling of metabolic fuels by the two genders. Although these sex-based differences in fat distribution could respond to genetics (O'Sullivan, 2009), and there are many studies about AMY1 CNV in association with body fat, few of these studies have investigated sex differences in body fatness, and those that have done so, have generally been inconclusive. Significant correlation of AMY1 with BMI was reported in young Finnish obese adult women ($n = 29; p = 0.045$), but not in men (Viljakainen et al., 2015). One year later, another study reported a significant increase of BMI z-score by decreasing AMY1 CN in Italian boys ($n = 744; P = 0.033$), but not in girls (Marcovecchio et al., 2016). These data suggest that the association of AMY1 CNV with obesity measures might vary with gender.

Body fat distribution associated with the burden of disease attributable to obesity also varies by sex (Muennig, Lubetkin, Jia and Franks, 2006). Sex-specific designed studies of obesity-associated genes are of value to improve precision in medicine, guide sex-stratified clinical trials, and make progress towards treating and ultimately stemming the obesity epidemic worldwide. Therefore, this study aimed to evaluate whether the associations between AMY1 CN and adiposity measures are influenced by gender via assessment of the body fatness percentage and the lean mass percentage by Dual-energy X-ray Absorptiometry (DXA). This study also aimed to investigate the link between AMY1 CN and body fatness in specific body regions of males and females.
4.3 Methodology

4.3.1 Ethical Considerations

The study protocol was approved by the research committee at West of Scotland Research Ethics Service, Glasgow; REC reference: 12/WS/0240 and IRAS project ID: 114700.

4.3.2 Study Participants

The study recruited 228 participants, comprised of males n = 108 and females n = 120 aged 18 – 40 years, from the University of Stirling's student population as part of an ongoing 10-year longitudinal study of body composition changes in students. The data used in this study was collected in the period between February 2016 and April 2019. Participants were asked to arrive at the laboratory in the morning, having fasted for at least 8 hours and rested overnight. The body composition measurements were taken of girth, height, weight, BMI, and DXA scans. Each participant completed a self-reported dietary intake form using a 24-hour dietary recall (24HR). (Figure 4-1).

![Figure 4-1 Diagram of a laboratory visit](image)
4.3.3 Anthropometry Measurements

Anthropometric measurements including body weight and height, were used to calculate BMI and body composition. Weight (kg) was measured to the nearest 0.1 kg using a digital scale (Seca Quadra 808, Birmingham, UK), in light sport clothes and without footwear. Height (cm) was measured using a stadiometer to the nearest millimetre. BMI was calculated as body weight (kg) divided by height (m) squared (kg.m$^2$). Waist circumference (WC) was measured in cm. All anthropometric measurements were taken in duplicate, and if a difference of > 10% was observed between the two measurements, a third measurement was taken.

4.3.4 Assessment of Body Composition by DXA

Body composition was measured by DXA (Lunar DXA GE Healthcare) with analysis performed using GE Encore software v. 13.6 (GE Healthcare). All scans were performed and analysed by DXA, which was calibrated with phantoms as per the manufacturer’s guidelines before measurement. All scans were performed using the standard thickness mode as automatically selected by the software. Participants were instructed to drink 500 ml of water before they came for the scan (Rodriguez-Sanchez and Galloway, 2015). Just before the scan, they were asked to empty their bladder, wear minimal clothing and remove any metallic objects and jewellery. Female participants took a pregnancy test prior to scanning and took part in the DXA only if they were not pregnant. DXA measures body composition in three fundamental components of bone-free mass, which are fat-free mass (FFM), fat mass (FM), and bone mineral content, as explained by Blake (1997). At the end of the scan, a total body and regional analysis is automatically made by the software and presented as percentages of FM and FFM,
in whole-body and distinct body regions, including arms, legs, trunk, android (a portion of the abdomen included between the line joining the two superior iliac crests and extending cranially up to 20% of the distance between this line and the chin), and gynoid region (a portion of legs stretching caudally from the femoral great trochanter to a distance double that of the android region).

### 4.3.5 Diet and Physical Activity Measures

Total Energy Intake and macronutrient intake (kJ/day) were measured with a 3-day food diary: two days from normal weekdays (Monday to Friday) and one day from a weekend day (Saturday or Sunday). Participants were provided with digital food scales and full instructions on how to fill in the food diary. Nutritional analysis of three-days food records was performed using the Nutritics Nutrition Analysis Software (Academic Edition, Nutritics, Dublin, Ireland). Macronutrients from dietary supplements were included in the analysis based on the manufacturer’s specifications from brand websites. Macronutrient intakes were calculated in (kJ/24hurs). Reported EI was evaluated against presumed energy requirements (Goldberg et al, 1991; Black, 2000a). This procedure is known as the Goldberg cut-off technique, and a sensitivity analysis was undertaken to examine the impact of low energy reporting. The Goldberg cut-off for EI to the calculation of estimated Basal Metabolic Rate (BMR) (Black et al, 1991; Goldberg et al, 1991) was applied to exclude under-reporters and over-reporters of (EI_{rep}) based on physical activity level (PAL) and compared with the ratio of EI_{rep}: BMR. as recommended by the Goldberg cut-off method (Black, 2000a), is based on different categories of physical activity (low, moderate and vigorous physical activity). BMR was estimated for each individual using the Schofield equations for adults based on age, gender, height and weight (Schofield et al., 1985). Statistically if mean reported
EI<sub>rep</sub>: BMR is less than the lower 95% confidence limit or lower cut-off then it has low probability (< 2.5%) that the EI<sub>rep</sub> could represent genuinely low intakes obtained by chance. Similarly, if reported mean EI: BMR is greater than the upper 95% confidence limit or upper cut-off then it has low probability (< 2.5%) that the reported intake could represent genuinely high intakes obtained by chance. Participants with individual EI<sub>rep</sub>: BMR < 0.89 are categorised as under-reporters, and subjects with individual EI<sub>rep</sub>: BMR > 2.24 are categorised as over-reporters.

Physical activity (hours / week) was assessed using the Scottish Physical Activity Questionnaire (SPAQ), which uses a 7-day recall of leisure time and occupational physical activity as a measure of physical activity (Lowther et al., 1999).

### 4.3.6 Saliva-Sample Collection

Before saliva collection, participants were instructed to rinse their mouths using water only or water and a toothbrush to reduce remaining food, bacteria and fungi (Sun & Reichenberger, 2014; Ooi et al., 2017). The Oragene® DNA Self-collection Kit (DNA Genotek; Ottawa, ON, Canada) was used to collect saliva samples following the manufacturer's instructions (Chartier and Pinard, 2006). Participants were instructed to deposit 2 ml of saliva through a funnel into the collection tube until the amount of liquid saliva, excluding bubbles, reached the fill line. When an adequate sample had been collected, the funnel lid was firmly closed. The collection tubes are designed so that a stabilising liquid attached to the lid mixes with the saliva when the funnel lid is securely fastened, stabilizing the saliva sample for long-term storage and beginning the initial phase of DNA isolation. As recommended by the manufacturer, saliva samples were
stored at room temperature until DNA extraction (Nunes et al., 2012). (DNA Genotek, 2014)

4.3.7 DNA Extraction and Quantification by Quantitative Polymerase Chain Reaction (qPCR) for the AMY1

As explained in Chapter 2 (Section 2)

4.3.8 Statistical Analysis

Statistical analyses were performed using SPSS (version 25, IBM, Armonk, NY, USA). Data was assessed for normality of distribution using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Any data which were not normal were transformed using the Box-Cox transformation to give better approximations of the normal distribution (using version 20.2, Minitab, State College, PA.), and normality was confirmed. Data are expressed as mean ± standard deviation (SD). An independent sample t-test was used to assess the difference between the mean characteristics of both groups. A Non-parametric median test, the Mann Whitney U test, was used to analyse the difference between the median values of the two groups. Correlation and Linear regression analysis was performed using phenotype data with and without group corrections to identify associations between AMY1 CN and the examined variables. As before performing the regression analysis, a correlation structure is essential to observe the association (relationship) between the underlying variables. When considering an interaction, a p-value of < 0.05 was considered significant. Standardized β-coefficient was used to quantify the association and R-squared to describe the explanatory rate given for subgroups.
4.4 Results

4.4.1 Participant’s Characteristics

The study cohort consisted of 228 independent adults (108 males and 120 females) from the University of Stirling, with a mean ± SD age of 22.3 ± 3.7 years (range: 17 – 40 years) and a mean ± SD AMY1 CN of 7.0 ± 2.3 (range: 2–14). Table 4-1 presents the descriptive characteristics of the participants from the two groups. The study did not record any significant differences in Age, CNVs, BMI, and PA between males and females \((p = 0.694, p = 0.942, p = 0.994, \text{ and } p = 0.615 \text{ respectively})\). Expected differences were found between males and females in weight, height, waist circumference (WC), and Body Fat Percentage (BF %) as the corresponding \(p\)-values are less than 0.05.

Table 4-1 Descriptive Characteristics of the Participants in this Study Grouped According to their Gender.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Males ((n=108))</th>
<th>Females ((n=120))</th>
<th>All ((n=228))</th>
<th>(p)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>22.0 ± 3.3</td>
<td>22.4 ± 3.9</td>
<td>22.3 ± 3.7</td>
<td>0.694(^B)</td>
</tr>
<tr>
<td>CNV</td>
<td>7.0 ± 2.4</td>
<td>6.9 ± 2.2</td>
<td>7.0 ± 2.3</td>
<td>0.942(^A)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77.5 ± 12.9</td>
<td>65.1 ± 11.1</td>
<td>70.2 ± 13.4</td>
<td>&lt;0.001 (^A)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>179.6 ± 6.5</td>
<td>165.2 ± 6.1</td>
<td>172.2 ± 9.5</td>
<td>&lt;0.001 (^A)</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>82.3 ± 8.1</td>
<td>73.5 ± 8.9</td>
<td>77.5 ± 9.6</td>
<td>&lt;0.001 (^A)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.9 ± 3.4</td>
<td>23.7 ± 3.4</td>
<td>23.8 ± 3.5</td>
<td>0.994 (^A)</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>20.2 ± 7.5</td>
<td>32.3 ± 6.4</td>
<td>26.5 ± 9.2</td>
<td>&lt;0.001(^B)</td>
</tr>
<tr>
<td>PA (Hour/w)</td>
<td>17.16±13.6</td>
<td>16.26±11.9</td>
<td>16.68±12.7</td>
<td>0.615(^A)</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD and CN, copy number; WC, waist circumference; BMI, body mass index; body fat %; PA, physical activity. (A) \(p\)-values < 0.05 independent sample \(t\)-test. (B) \(p\)-values < 0.05 Mann-Whitney \(U\) test for comparison between males and females.
4.4.2 Copy Number Detection in Each Group

The distribution of AMY1 CN in male and female participants is shown in Figure 4-2A. The mean AMY1 CN did not significantly differ between males and females ($p = 0.100$). The AMY1 CN distribution ranged from 2-14 for males with a mean of 7.05; while in females, it ranged from 3-14 with a mean of 7.03. (Figure 4-2 B.).

![Figure 4-2A: Total Estimated CNV](image)

![Figure 4-2B: Estimated CNV in Groups](image)

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Males</th>
<th>Females</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.0 ± 2.3</td>
<td>7.0 ± 2.4</td>
<td>6.9 ± 2.2</td>
<td>0.942</td>
</tr>
</tbody>
</table>

Figure 4-2 Distribution of AMY1 Copy Number in the Study Population. (A) Shows AMY1 copy number percentage distribution for all participants. (B) Shows AMY1 copy number percentage distribution for males (n= 108) and females (n= 120). For this figure estimates of copy number have been rounded to the nearest integer. Mean ± SD data presented under the figures. $p$-values < 0.05 indicates a significant.
4.4.3 Body Composition and Copy Numbers of AMY1

In this study, the correlation analysis is carried out to check the inter relationships between AMY1 CN and height, weight, BMI, WC, and Fat percentage. As the correlation analysis is the best statistical tool to find the relationship between the quantitative variables. Once the underlying correlation between the variables is observed, regression analysis is performed to investigate the functional relationship between the dependent (AMY1 CN) and independent variables (height, body weight, BMI, WC, and BF %). The results of correlation and regression analysis are reported in Tables 4-2. Under the men category of Gender, no significant relationship was found between AMY1 CN and height (r = -0.073, \( p = 0.367 \)), body weight (r = 0.215, \( p = 0.324 \)), BMI (r = 0.297, \( p = 0.282 \)), WC (r = 0.303, \( p = 0.838 \)) and BF % (r = 0.236, \( p = 0.471 \)). Similarly, for females, no significant correlations were observed between AMY1 CN for height (r = 0.009, \( p = 0.599 \)), weight (\( p = 0.579 \), r = -0.015), BMI (r = -0.018, \( p = 0.497 \)) and BF (r = -0.259, \( p = 0.628 \)). A significant association was detected between AMY1 CN and WC (r = -0.259, \( p = 0.005 \)) in the females. Further, no significant association was detected between the whole population AMY1 CN with height, weight, BMI, WC or BF (\( p > 0.05 \) for all) (Table 4-2).

The below Figures 4-3 and Figures 4-4 show the scatter plots depicting the relationship between AMY1 CN and anthropometrics measurements including weight, weight, BMI and WC in males (circles and solid lines) and females (diamonds and dotted lines). The \( R^2 \) value is used to measure the percent of variation due to the respective independent variables separately. Figure 4-3 shows that only 2.9 % in males and <0.001 % in females of the variation in weight (kg) can be explained if the AMY1 CN included in the model. Similarly, the \( R^2 \) for height (cm) (Figure 4-4) alone in the regression model for the males and females are 0.7 % and <0.001 % respectively. For BMI, the \( R^2 \) for males and females are 5.9 % and <0.001 % respectively. The \( R^2 \) value reported in Figure 4-4 shows that when WC is considered alone, only 7.6 % and 0.3 % variation
is explained by AMY1 CN. All the above results regarding $R^2$ values suggests that very little portion of variation is explained when only a single independent variable is included in the model. It is also observable that under the female category of gender, the $R^2$ value is smaller as compared to the male category for all related variables.

**Table 4-2** R value From Correlation Analysis Results Between AMY1 Copy Number and Body Composition.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>Waist (cm)</th>
<th>Body Fat (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>-0.073</td>
<td>0.215</td>
<td>0.297</td>
<td>0.303</td>
<td>0.236</td>
</tr>
<tr>
<td>Females</td>
<td>0.009</td>
<td>-0.015</td>
<td>-0.018</td>
<td>-0.259</td>
<td>-0.015</td>
</tr>
<tr>
<td>All</td>
<td>-0.018</td>
<td>0.097</td>
<td>0.129</td>
<td>-0.036</td>
<td>0.085</td>
</tr>
</tbody>
</table>

Body composition measurements includes height and markers of adiposity including weight, BMI, WC, waist circumference, and body fat percentage in males and females and total participants. Data shows no significant differences $p$-value $= > 0.050$. 
Figure 4-3 Scatter Plots Depicting the Relationship Between AMY1 Copy Number and Anthropometrics Measurements (A) Weight and (B) Height by linear regression with best fit lines, males (circles and solid lines) and females (diamonds and dotted lines).
Figure 4-4 Scatter Plots Depicting the Relationship Between AMY1 Copy Number and Anthropometrics Measurements including, BMI (C) and waist circumference (WC) (D) by linear regression and best fit lines, males (circles and solid lines) and females (diamonds and dotted lines).
Table 4-3 Linear Regression Results Between AMY1 Copy Number and Height and Markers of Adiposity.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Males</th>
<th>Females</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$</td>
<td>p-value</td>
<td>$\beta$</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>1.023</td>
<td>0.367</td>
<td>0.450</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>-2.701</td>
<td>0.324</td>
<td>-1.163</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>2.619</td>
<td>0.282</td>
<td>1.298</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>-0.063</td>
<td>0.838</td>
<td>-0.473</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>0.136</td>
<td>0.471</td>
<td>0.085</td>
</tr>
</tbody>
</table>

$R^2$ 0.377 0.351 0.024

Males (n= 108), Females (n = 120), and all (n=228). Adiposity markers includes weight, BMI, waist circumference (WC), and fat percentage. (*) p-values < 0.05 indicates a significant coefficient ($\beta$), corr. Denotes the correlation coefficient between the AMY1 copy number and other variables, Adjusted R-squared ($R^2$) is also reported for each model.

In the above Table 4-3, a linear regression analysis was carried out to evaluate the utility of AMY1 in the prediction of anthropometric and body composition including Height, Weight, BMI, WC and BF % on the dependent variable AMY1 CN. It is observable that under the male’s category of the gender, a change of one copy in AMY1 gene results into increase in Height, BMI, and BF % the unite of 1.023, 2.619 and 0.136, respectively. Similarly for a unit change in AMY1 CN results in a decline in Weight and WC by 2.701 and 0.063, respectively. The $R^2$ value of (0.377), associated with the regression model suggests that the predictor variables for the subgroup men account for 37.7 % variation in the AMY1 CN, which mean that 63.3 % of the variation in BMI, height, weight, body fat and FFM cannot be explained by the predictor AMY1 CNV alone. Similarly, under the category of the females the $R^2$ value of (0.351) associated with the regression model suggests that the predictor variables account for 35.1 % of the variation in BMI, height, weight, body fat and FFM cannot be explained by the predictor AMY1 CNV alone (Table 4-3). For the total category of the participants the $R^2$ value of (0.024) associated with the regression model suggests that the predictor variables account for 2.4 % variation in the AMY1 CN, which mean that 92.6 % of the
variation in BMI, height, weight, body fat and FFM cannot be explained by the predictor AMY1 CNV alone.

4.4.4 Regional Fat Distribution and AMY1 Copy Number

The correlation analysis cared out to evaluate the association between AMY1 CN and body fat distribution (regional BF%) (Table 4-4). There was no significant association observed between all measured regional BF% and AMY1 CN in total population, males or females ($p > 0.05$ for all). In males no significant associations were found between AMY1 CN and the percentage of Arm Fat % ($r = 0.222$), Trunk Fat % ($r = 0.251$), Android Fat % ($r = 0.218$), Gynoid Fat % ($r = 0.226$) and Leg Fat % ($r = 0.251$). Similarly, no significant associations were observed between AMY1 CN and the percentage of Arm Fat % ($r = 0.007$), Trunk Fat % ($r = -0.023$), Android Fat % ($r = -0.017$), Gynoid Fat % ($r = -0.036$) and Leg Fat % ($r = 0.010$) in females.

Figures 4-5 and Figures 4-6 show the scatter plots of the regional BF: Arms Fat %, Legs Fat %, Trunk Fat %, Android Fat %, and Gynoid Fat % in male and females. The value of $R^2$ for the male and female under the linear regression models are 9 % and 0.035 % respectively showing that a very small amount of the variation in Arm Fat can be explained if we include AMY1 CN in the model. Similarly, the $R^2$ for Legs Fat alone when included AMY1 CN in the regression model for the male and female categories are 6.2 % and 0.010 % respectively. Form the above Figure 4-6, the values of the $R^2$ for Trunk Fat under both categories of gender showing that 5 % and 0.0001 % variation respectively explained by in the AMY1 CN. The $R^2$ value reported in Figure 4-6 shows that when alone Gynoid Fat % only 5.2 % and 0.055 % variation is explained AMY1 CN. All the above results regarding $R^2$ values suggests that very little portion of variation is explained when the single independent variable regarding AMY1 CN is
included in a model. It is also observable that under the female category of gender, the \( R^2 \) value is smaller as compared to the male category for all regional Fat distribution related variables.

**Table 4-4** \( R \) value From Correlation Analysis Results of Associations Between AMY1 Copy Number with Regional Fat Distributions.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Arms</th>
<th>Legs</th>
<th>Trunk</th>
<th>Android</th>
<th>Gynoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Males</td>
<td>0.222</td>
<td>0.251</td>
<td>0.222</td>
<td>0.218</td>
<td>0.226</td>
</tr>
<tr>
<td>Females</td>
<td>0.007</td>
<td>0.010</td>
<td>-0.023</td>
<td>-0.017</td>
<td>-0.036</td>
</tr>
<tr>
<td>All</td>
<td>0.068</td>
<td>0.086</td>
<td>0.085</td>
<td>0.089</td>
<td>0.068</td>
</tr>
</tbody>
</table>

Males (n = 108), Females (n = 120), and All (n = 228). Regional fat distributions in Arms, Legs, Trunk, Android Fat %, and Gynoid Fat % in male and females and total participants. Data shows no significant differences \( p \)-value = > 0.050.
Figure 4-5 Scatter Plots Depicting the Relationship Between AMY1 Copy Number and Percentage Fat Distribution including, Arm Fat (A) and Leg Fat (B) obtained from linear regression analyses and best fit lines, males (circles and solid lines) and females (diamonds and dotted lines).
Chapter 4: Copy Number of AMY1, and Body Composition

Figure 4-6 Scatter Plots Depicting the Relationship Between AMY1 Copy Number and Percentage Fat Distribution including, Trunk Fat (C) and Gynoid Fat (D) obtained from linear regression analyses and best fit lines, males (circles and solid lines) and females (diamonds and dotted lines).
**Table 4-5** Linear Regression Results of Associations Between AMY1 Copy Number with Regional Fat Distributions.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Males</th>
<th>Females</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\beta$</td>
<td>p-value</td>
<td>$\beta$</td>
</tr>
<tr>
<td>Arms Fat %</td>
<td>-0.114</td>
<td>0.774</td>
<td>0.097</td>
</tr>
<tr>
<td>Trunk Fat %</td>
<td>0.557</td>
<td>0.722</td>
<td>0.088</td>
</tr>
<tr>
<td>Android Fat %</td>
<td>-0.391</td>
<td>0.775</td>
<td>0.036</td>
</tr>
<tr>
<td>Gynoid Fat %</td>
<td>-0.537</td>
<td>0.402</td>
<td>-0.928</td>
</tr>
<tr>
<td>Legs Fat %</td>
<td>0.724</td>
<td>0.144</td>
<td>0.729</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.074</td>
<td>0.039</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Males (n = 108), Females (n= 120), and All (n = 220). Regional fat distributions: Arms (%), Trunk (%), Android (%), Gynoid (%), and Legs (%) in all participants, male and females. (*) $p$-values < 0.05 indicates a significant coefficient ($\beta$). Denotes the correlation coefficient between the AMY1 copy number and other variables, Adjusted $R$-squared ($R^2$) is also reported for each model.

The above **Table 4-5** a linear regression analysis was carried out to evaluate the utility of AMY1 CN in the prediction of regional BF %. It was observed that under subcategory of males, a copy changes in AMY1 CN resultants an increase a one unit in the Trunk Fat (%) and Leg Fat (%) results into the 0.557 and 0.724 respectively. However, a copy changes in AMY1 CN resultants a decline in the unit of the Arms Fat (%), Android Fat (%) and Gynoid Fat (%) by 0.114, 0.0391 and 0.537 respectively. The overall goodness of fit measurement $R^2$ (0.074) indicates that the predictor variables for the subgroup males account for 7.4% variation in the AMY1 CN, which mean that the rest of the variation (92.6 %) in the Arms Fat (%), Trunk Fat (%), Android Fat (%), Gynoid Fat (%), and Legs Fat (%) cannot be explained by the predictor AMY1 CNV alone. For the subgroup female the value of $R^2$ value of (0.039), which mean that 3.9 % indicating that a very low proportion of the variation in the dependent variables of Arms Fat (%), Trunk Fat (%), Android Fat (%), Gynoid Fat (%), and Legs Fat (%) is explained by the AMY1 CNV only. For the total population the value of $R^2$ for the
total participants is (0.030) indicating that 3% of the variation in the dependent variables is explained by the AMY1 CNV only.

### 4.4.5 Fat Free Mass Distribution and AMY1 Copy Number

Table 4-6 shows the results for the correlation analysis of body distributions of reginal FFM (g) and AMY1 CN according to the participant’s gender. No association were observed between total LM and AMY1 CN in males or females ($r = 0.072, p = 0.966$ for males, and $r = -0.028, p = 0.195$ for females). Reginal FFM (g) distribution in Arm, Android, Gynoid and legs were also not associated with AMY1 in males ($r = 0.085, p = 0.721; r = 0.056, p = 0.638, r = 0.079, p = 0.581; r = 0.103, p = 0.261; and r = 0.076, p = 0.890, respectively). For females, Arm, Android, Gynoid and legs were also not associated with AMY1 ($r = 0.008, p = 0.164; r = -0.013 p = 0.157; r = -0.011, p = 0.673; r = -0.023, p = 0.148; and r = -0.047, p = 0.499$) (Table 4-6 and Table 4-7).

#### Table 4-6 R value From Correlation Analysis Between AMY1 Copy Number and Regional FFM Distributions

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Arms</th>
<th>Trunk</th>
<th>Android</th>
<th>Gynoid</th>
<th>Legs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>0.085</td>
<td>0.056</td>
<td>0.079</td>
<td>0.103</td>
<td>0.076</td>
<td>0.072</td>
</tr>
<tr>
<td>Females</td>
<td>0.008</td>
<td>-0.013</td>
<td>-0.011</td>
<td>0.023</td>
<td>-0.047</td>
<td>-0.028</td>
</tr>
<tr>
<td>Total</td>
<td>0.035</td>
<td>0.017</td>
<td>0.027</td>
<td>0.047</td>
<td>0.014</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Males (n = 108), Females (n= 120), and All (n = 220). Regional FFM distributions: Arms (g), Trunk (g), Android (g), Gynoid (g), Legs (g) and Total (g) in male and females and total participants. Data shows no significant differences $p$-value $> 0.050$. 


The above Table 4-7 a linear regression analysis was carried out to evaluate the utility of AMY1 CN in the prediction of regional LM (g). It was observed that under subcategory of males, a copy changes in AMY1 CN resultants an increase a one unit in the Arms LM, Android LM, Gynoid LM, and total LM by 0.205, 0.158, 0.370, and 0.110 respectively. However, a copy changes in AMY1 CN resultants a decline in the unit of the Trunk LM, Legs LM by 0.583 and 0.151 respectively. The $R^2$ value of (0.180), associated with the regression model suggests that the predictor variables for the subgroup males account for 18 % variation in the AMY1 CN, which mean that 80.2 % of the variation in the Arms LM, Trunk LM, Android LM, Gynoid LM, legs LM and Total LM cannot be explained by the predictor AMY1 CNV alone. Similarly, under subcategory of females, a copy changes in AMY1 CN resultants an increase a one unit in the Arms LM, Trunk LM, Gynoid LM, and Legs LM by 0.488, 0.842, 0.481, and 0.425 respectively. However, a copy changes in AMY1 CN resultants a decline in the unit of the Android LM and Total LM by 0.120 and 1.874 respectively. The $R^2$ value of (0.192), associated with the regression model suggests that the predictor variables
for the subgroup females account for 19.2 % variation in the AMY1 CN, which mean that 81.8 % of the variation in the Arms LM, Trunk LM, Android LM, Gynoid LM, legs LM and Total LM cannot be explained by the predictor AMY1 CNV alone. For the total population The R² value of (0.148), associated with the regression model suggests that the predictor variables for 14.8 % variation in the AMY1 CN, which mean that 86.2 % of the variation in the Arms LM, Trunk LM, Android LM, Gynoid LM, legs LM and Total LM cannot be explained by the predictor AMY1 CNV alone.

### 4.4.6 Energy Intake and AMY1 Copy Number

Table 4-8 shows the association between AMY1 CN and the Total EI (KJ), Energy from macronutrient intakes (KJ), and PA (Hours / week) in a total of 67 participants (males n= 36 and females n= 31) met the under reported and over reported cut-off criteria. It has been observed that inverse significant association was detected between AMY1 CN and Total EI in total participants, males and females with \( P = < 0.001 \) for all, \( r = -0.921 \), \( r = -0.964 \), and \( r = -0.902 \) respectively. Further, a negative significant association was detected between AMY1 CN and EI from CHO (R = -0.330) and protein (R = -0.0385) in female only with \( P = < 0.05 \).

<table>
<thead>
<tr>
<th>Measurements</th>
<th>EI (kJ/day)</th>
<th>CHO (kJ/day)</th>
<th>Protein (kJ/day)</th>
<th>Fat (kJ/day)</th>
<th>PA (kJ/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Females</strong></td>
<td>-0.964**</td>
<td>0.107</td>
<td>0.245</td>
<td>0.287</td>
<td>0.448</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td>-0.902**</td>
<td>-0.330*</td>
<td>-0.385*</td>
<td>-0.061</td>
<td>0.161</td>
</tr>
<tr>
<td><strong>All</strong></td>
<td>-0.921**</td>
<td>-0.149</td>
<td>-0.131</td>
<td>0.025</td>
<td>0.325</td>
</tr>
</tbody>
</table>

Males (n = 36), Females (n = 31), and All (n = 67). Total EI (kJ), and energy intake from CHO (kJ), Protein(J), Fat(kJ) and PA. Significant correlation \( p \)-values \( < 0.05 \) (*) and \( p = < 0.001 \)(**).

The below Figures 4-7 to Figures 4-11 show the scatter plots of the Total EI (KJ), Energy from CHO(KJ), Protein (KJ), Fat (KJ), and PA (Hours / week) in males and
females. From Figure 4-7 it is observable that the value of $R^2$ for the male and female under the linear regression models are 11.1 % and 8.1 % respectively showing that a very small amount of the variation in Total EI can be explain if include the AMY1 CN in the model. Similarly, the $R^2$ for CHO EI reported in Figure 4-8 alone when included in the regression model for the male and female categories are 8.2 % and 0.6 % respectively showing the small percent of variation explained by AMY1 CN the underline model. The $R^2$ value reported in Figure 4-9 shows that when alone protein EI only 14.8 % and 6 % variation is explained by AMY1 CN. From Figure 4-10, the values of $R^2$ for AMY1 CN under both categories of gender show that 0.04 % and 8.2 % variation in the Fat EI respectively. Similarly, the $R^2$ reported in Figure 4-11 when PA (Hours / week) is included in the model is 2.6 % and 20.1 % respectively for males and females showing the percentage of variation explained by AMY1 CN.

![Figure 4-7](image.png)

**Figure 4-7** Scatter Plots Depicting the Relationship Between AMY1 CNV and Energy Intake, obtained from linear regression analyses and best fit lines. $R^2$ value = 0.111, $p = 0.001$ in males (circles and solid lines) and $R^2$ value = 0.081, $p = 0.001$ in females (diamonds and dotted lines).
Figure 4-8 Scatter Plots Depicting the Relationship Between AMY1 CNV and Energy Intake, obtained from linear regression analyses and best fit lines $R^2$ value = 0.092, $p = 0.322$ in males (circles and solid lines) and $R^2$ value = 0.006, $p = 0.827$ in females (diamonds and dotted lines).

Figure 4-9 Scatter Plots Depicting the Relationship Between AMY1 CNV and Protein, obtained from linear regression analyses and best fit lines. $R^2$ value = 0.148, $p = 0.941$ in males (circles and solid lines) and $R^2$ value = 0.060, $p = 0.795$ in females (diamonds and dotted lines).
Figure 4-10 Scatter Plots Depicting the Relationship Between AMY1 CNV and Fat, obtained from linear regression analyses and best fit lines. $R^2$ value = 0.004, $p = 0.477$ in males (circles and solid lines) and $R^2$ value = 0.082, $p = 0.722$ in females (diamonds and dotted lines).

Figure 4-11 Scatter Plots Depicting the Relationship Between AMY1 CNV and SPAQ, obtained from linear regression analyses and best fit lines $R^2$ value = 0.026, $p = 0.686$ in males (circles and solid lines) and $R^2$ value = 0.201, $p = 0.118$ in females (diamonds and dotted lines).
Table 4-9 Linear Regression Analysis Results for Associations of AMY1 Copy Number with Energy Intake.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Males</th>
<th>Females</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>p-value</td>
<td>β</td>
</tr>
<tr>
<td>Total Energy intake (kJ)</td>
<td>-32.0</td>
<td>0.001*</td>
<td>-32.95</td>
</tr>
<tr>
<td>Carbohydrate (kJ)</td>
<td>-0.001</td>
<td>0.322</td>
<td>-0.0021</td>
</tr>
<tr>
<td>Protein(kJ)</td>
<td>0.004</td>
<td>0.941</td>
<td>0.0060</td>
</tr>
<tr>
<td>Fat(kJ)</td>
<td>-0.0016</td>
<td>0.477</td>
<td>-0.0013</td>
</tr>
<tr>
<td>Physical Activity (h/w)</td>
<td>0.0168</td>
<td>0.686</td>
<td>0.0173</td>
</tr>
<tr>
<td>R²</td>
<td>0.907</td>
<td></td>
<td>0.968</td>
</tr>
</tbody>
</table>

Males (n = 36), Females (n = 31), and All (n = 67). Total EI (kJ), and energy intake from CHO (kJ), Protein(kJ), Fat(kJ) and PA. (*) p -values < 0.05 indicates a significant coefficient (β). Denotes the correlation coefficient between the AMY1 copy number and other variables. Adjusted R-squared (R²) is also reported for each model.

Table 4-9 above contains the results of the regression analysis, β coefficients and p-values of the Total EI (KJ), Energy from CHO(KJ), Protein (KJ), Fat (KJ), and PA (Hours / week) in total population, males, and females. It was observed that under subcategory of males, a copy changes in AMY1 CN resultants an increase a one unit in the Protein EI and PA by 0.004 and 0.0168 respectively. However, a copy changes in AMY1 CN resultants a decline in the unit of the total IE, CHO EI, Fat EI by 0.320, 0.001, and 0.0016 respectively. Further, from the overall goodness of fit for the subgroup males, the higher R² show that 90.7% of variation in the dependent variables is explained by the underlying predictor AMY1 copy number and the rest of the variation 9.3 % is not explained by the considered predictor.

It was observed that under subcategory of females, a copy changes in AMY1 CN resultants an increase a one unit in the Protein EI and PA by 0.0060 and 0.0173 respectively. However, a copy changes in AMY1 CN resultants a decline in the unit of the total IE, CHO EI, Fat EI by 32.95, 0. 0021, and 0.0013 respectively. For the
subgroup females, the overall goodness of fit measure $R^2$ is 96.8 % showing that larger amount of variation in Total EI, CHO EI, Protein EI, Fat EI, and PA is due to the AMY1 CN.

It was observed that under subcategory of females, a copy changes in AMY1 CN resultant an increase a one unit in the Protein EI and PA by 0.0045 and 0.0168 respectively. However, a copy changes in AMY1 CN resultant a decline in the unit of the total IE, CHO EI, Fat EI by 32.032, 0.0011, and 0.0016 respectively. For the total data, the $R^2$ value is 92.4 % showing that the included predictors in the regression model explains 92.4 % of variation in the AMY1 copy number and the rest of the variation 7.6 % of variation is not explained by the predictor.

4.5 Discussion

This study demonstrated an absence of a link between AMY1 CN and adiposity markers, including weight, BMI, WC, FM, and FFM in males and females. The study also evidenced the lack of association between AMY1 CN and reginal FM % distribution and reginal LM % distribution in both genders. Notably, Total EI shows a significant inverse correlation with AMY1 CN in males and females. In contrast, IE from CHO, Protein, and Fat intakes were not associated with AMY1 CN in either gender. PA was not associated with AMY1 in either males or females.

Several studies have investigated the relationship between AMY1 CN and the predisposition to metabolic disorders, including obesity, T2DM, and insulin resistance, in both children and adults. However, contradictory results have been reported. The
reported discrepancies remain unexplained. However, possible explanations may include the different techniques used to estimate AMY1 CN and the different ethnicities and ages of the studied differences between the genders will be consider in the following sections including CNV, body composition differences, energy intake, and PA.

The distribution of AMY1 CN in this study sample (2–14 copies) is comparable to what has been reported previously across modern human populations (Table 1-2). AMY1 CN did not differ between genders mean was 7 copies in males and 6.9 copies in females, which agreed with a recently reported study (Alakl et al., 2020).

This study reported an absence of the influence of AMY1 on height in males and females. However, a strong association between high AMY1 CN and male height was detected in the athlete’s study, as reported in Chapter 3. Two reasons might explain the inconsistent result in the association between AMY1 and height. Firstly, by considering height (cm) variances in the two populations. For instance, in the study conducted on Lithuanian athletes and non-exercising controls only males are included however, this study is mixture of males and females. The height (mean ± SD) consisted of 181.1 ± 6.6 cm compared to total population mean height 172.2 ± 9.5 cm in this study. However, similar mean height observed, if the mean height of Lithuanian athletes (males only) compared to only male’s height in this study (mean ± SD) 179.6 ± 6.5 cm. The second potential reason may explain the inconsistent result in the association between AMY1 and height is the limited cohort sample size, which mean that the association between AMY1 CN and height is weak to detected in 108 males
compared to 388 males in Lithuanian athlete’s study. Further, in a broader context the
hight AMY1 CN distribution might be random in the examined populations which may
make the associations stronger or weaker.

No association was observed between BMI, BF % and other measurement of adiposity
with AMY1 CN in the examined populations of the current study. Even though the
limited sample size of the current study, these results are in line with other studies with
big samples size reporting a lack of association between AMY1 and obesity such as
Ushar et al. conducted in 4,500 adults, Yong et al. in 1077 adults, Rukh et al. in 4,800
adults, Shwan & Armour in 4,235 in adults 923 Al-Akl et al. in 923( Ushar et al., 2015;
Yong et al., 2016 ; Rukh et al., 2017; Shwan & Armour, 2019; Al-Akl et al., 2020). In
contrast, several other studies did find this association between BMI, BF % and other
measurement of adiposity with AMY1 CN in a gig sample size including Falchi et al.
conducted in 6,200 in adults, León-Mimila et al. in 920 adults, Heianza et al. 2,054
adults, Rossi et al. 2935 adults and similer to our sample size Pinho et al. in 262 adults
(Falchi et al., 2014; León-Mimila et al., 2018; Pinho et al., 2018; Heianza et al., 2020;
Rossi et al., 2021). One of those did not show a significant association between AMY1
CNV and the risk of being overweight or obese in the whole population ($p = 0.489$).
However, after testing case-control data in the sub-set of samples above the third
quartile (CN ≥10), a significant association is found between lower AMY1 copy
number and risk of obesity (OR = 0.532; $p = 0.034$), even when adjusted for age and
gender (OR = 0.527; $p = 0.039$), adults’ participants with >10 AMY1 copies are normal
weight controls (n = 20) or overweight (n = 6) (Pinho et al., 2018). In children Mejia-
Benitez et al. identified a marked effect of AMY1 copy number on reduced risk of
obesity (OR per estimated copy 0.84. All children with AMY1 copies >10 was from
controls normal weight (Mejia-Benitez et al., 2015). The current study found no association between body fat percentage and CN of AMY1, even among those with a high CN (≥10) (Appendix 1).

The study aimed to assess the association between AMY1 and reginal BF and reginal LM distributions between the two genders across the general adult population. Thus, weight or BMI cut-off criteria were not applied in the selection of the sample or the analysis as the mean comparison was conducted between males and females. Sex-specific differences in the association between AMY1 CN and adiposity markers, including weight, total BF % or reginal BF distribution, BMI, and WC, were not found in this study. In a previous observation, Viljakainen et al. (2015) examined the association between AMY1 CN and 61 males and females with a history of childhood-onset obesity and 71 matched controls according to their body weight. The study reported a lack of association between AMY1 CN and body fatness in normal-weight females and males. However, an inverse correlation was found between AMY1 CN and whole-body fat% ($r = -0.512, p = 0.013$) and BMI ($r = -0.416, p = 0.025$) in obese females only. Although Viljakainen and his colleagues found that obese males carried the highest number of AMY1 copies and obese women had the lowest ($p = 0.045$), no such observation was observed in normal normal-weight males and females. Viljakainen et al. explained the increase in the number of AMY1 copies between groups as random distribution (Viljakainen et al., 2015).

Additionally, Heianza et al. investigated whether AMY1 CN is associated with two-year changes in adiposity among 692 overweight and obese considering dietary CHO
intake. The study found that those who were obese, and overweight had higher AMY1 copies and higher amylase activity. These obese with high CN may have greater weight loss and decreased central adiposity on low-energy diet interventions (Heianza et al., 2017). In contrast, the reported results were inconsistent, not only in the association between high AMY1 CN and obesity but also in explaining the influence of AMY1 on these individuals and whether AMY1 correlated with body fatness markers inversely or positively.

The full implications of the influence of AMY1 on adiposity are still not completely understood. Falchi et al. provided the first study to find a significant association between low AMY1 CN with increased BMI in a cohort of more than 6,000 adult participants of both genders with a median age of 53 years compared to the median age of 22 years in this study’s population (Falchi et al., 2014). Marcovecchio et al. reported a significant increase of BMI z-score by decreasing AMY1 CN in boys ($\beta = -0.117$, $p = 0.033$), but not in girls (Marcovecchio et al., 2016). Another recent cross-sectional study of 923 Qatari adults compared the association between salivary $\alpha$-amylase activity (psAA) and AMY1 CN with adiposity in males and females. Significant inverse correlations were found between adiposity markers and psAAa, in both gender and in males is stronger (Alakl et al., 2020).

It should, nevertheless, be noted that there is a difference in descriptive BF % between males and females in this study; 20.2±7.2 % (mean ± SD) in males compared to 32.2 ± 6.4 in females. This places males in the normal range and females just above it according to WHO criteria (WHO, 1995): normal is defined as 18 % to 24 % in females.
and 25% - 31% in males. The percentage of adipose tissue is higher in females than males, and males more commonly have central obesity (Power & Schulkin, 2008; Stevens, Katz, and Huxley, 2011). However, the low variability in fat percentage between males and females and within the same gender group is considered one of the limitations of this study. Generally, these differences stem from metabolic and hormonal variations between sexes. The differences between males and females are partly explained by the anatomic distribution of adipose tissue throughout the human body and the proportions and pattern of distribution. Body fat tissue is mainly distributed into two compartments with different metabolic characteristics: subcutaneous adipose tissue (SAT), which is more common in females, and visceral adipose tissue (VAT) present in the abdominal cavity (Power & Schulkin, 2008). In a study of 483 Caucasian adults in the US, they found that women had 1.8kg more SAT than men in any given WC, and males have greater VAT than females (Kuk et al., 2005). Females have greater adipose stores in the thighs than males. Lower body adiposity is associated with a less unhealthy metabolic profile in both genders (Goodpaster et al., 2005).

It should be noted that there is a significant difference in WC between men and women (mean ± SD WC was 82.3 ± 8.1 cm in males compared to 73.5 ± 8.9 cm in females), even though the group mean of WC for both genders was in a healthy range. WC cut-off points are 94 cm and 80 cm for European men and women, respectively, according to WHO criteria (WHO, 2008), which sets higher WC estimates in males than females (Adegbija, Hoy, and Wang, 2015). In contrast, other epidemiological studies report an association between low serum concentrations of amylase and increased WC (Nakajima et al., 2011; Lee et al., 2015; Aldossari et al., 2019; Gabry and Gawish,
Chapter 4: Copy Number of AMY1, and Body Composition

2019) as well as abdominal fat (Dias et al., 2016). In this study, WC (cm) was negatively associated with high AMY1 in female only ($\beta = -0.473, p = 0.005$). An association between low AMY1 CN with WC has also been reported in other studies (Falchi et al., 2014; Leon-Mimila et al., 2018; Venkatapoorna et al., 2019).

In this study, low AMY1 CN was significantly associated with high total EI in males and females by increasing one copy of AMY1 decreases 33 KJ of total EI. However, no such association were observed between and AMY1 CN and EI source whether it comes from CHO, fat, or protein in males or females. Also, there was an association between AMY1 CN and central adiposity measured by WC (cm) in females only. Further, there was no association between AMY1 CN and others adiposity measurements in males or females. Gender-specific differences in the influence of AMY1 CNV on adiposity related to dietary intake and CHO in food, in particular, have been reported in previous studies. For instance, a cross-sectional study of European individuals found that higher AMY1 CN was related to lower BMI among people with lower starch intake. At the same time, the trend was reversed among people with higher starch intake (Rukh et al., 2017). A very recent meta-analysis investigating interactions between AMY1 genetic risk score (GRS) and dietary intake for changes in general and abdominal adiposity found that CHO intake significantly altered associations of AMY1 GRS with changes in BMI and WC among 32,054 adults from four prospective cohort studies. The study found that, in males, higher AMY1-GRS was associated with lower gains in adiposity when the dietary CHO intake was low while, among women, higher AMY1-GRS was associated with higher increases in adiposity if dietary CHO food intake was high (Heianza et al., 2020). Both studies of Rukh et al. and Heianza et al. did observe significant associations between AMY1 and adiposity when differences in
dietary intake of starch or CHO foods were not considered. In our study we are conforming that we detected a significant association between female’s AMY1 CN and low WC along with low total EI disparate of the course of energy. We can pointe that the negative direction of associations AMY1 CN and EI from CHO protein, and fat with more than 90% the proportion of variances explained by the CN of AMY1. As noted, there remains controversy within the literature regarding the association between AMY1 CN and adiposity. Variation in dietary preferences (starch ingestion) between studied populations may explain some differences in the strength of the association between AMY1 CN and obesity markers.

In this study, only 67 participants were matched with EI: BMR cut-offs out of 112 participants who reported their intake. Further studies are needed to confirm the inverse correlation between CHO intake and CNV of AMY1 in males, especially since an inverse correlation between males and body fatness has been reported in two studies.

This study demonstrated a lack of association between AMY1 CN on fat distribution and LM among males and females, which is novel and merits further investigation in additional cohorts. The strengths of this study include a well-characterised cohort, gender-based analysis, a reasonable sample size, and considerable phenotypic detail. The gold standard method of DXA for body composition measurement was used. This study also has several limitations. The differences between males and females in weight, height, BMI, and fat % were not considered. Further, it is difficult to compare the results with other studies because of differences in the fat measurement methods.
and the characteristics of the groups. There was also a lack of detailed SAT and VAT characterisation because of limitations in the DXA software.

4.6 Conclusion

This study did not report associations between AMY1 CNVs and BMI, body fatness, and lean mass in either male or female individuals. Gender differences were found in the energy and macronutrient intake in relation to AMY1 CNVs. The data in this study suggests that high AMY1 CNVs do not predispose young male or female individuals to obesity. Future investigation is needed in larger samples to confirm these results.
Chapter 5 Impact of Copy Number of AMY1 on Postprandial Glucose Response during Rest: A Pilot Study

5.1 Abstract

AIM: The aim of this pilot study was to generate data that would inform future research to understand potential reasons behind the gaps in the conflicting results of the influence of AMY1 CN on glycaemic response after starch ingestion compared to glucose loading during rest in healthy individual adults.

METHODS: A pilot study was conducted at University of Stirling and recruited fifteen healthy adult male and female following the inclusion and exclusion criteria and divided them into two groups. (I) high AMY1 CN ≥ 9 (n=10) and (II) low AMY1 CN ≤ 5 (n=5). A blood sample was drawn at 0 min (fasted state) and at 15, 30, 45, 60, 90 and 120 min following ingestion of a starch solution (OPTT) and a dextrose solution (OGTT). An automated Aries ILab benchtop analyser was used to assess plasma glucose and lipid levels, and commercial ELISA kit was used to measure plasma insulin concentrations. G* Power software was used for sample size calculation.

RESULTS: The plasma glucose iAUC and plasma insulin iAUC were significantly different between the two groups. Plasma glucose iAUC after starch ingestion was significantly higher in the low AMY1 CN group compared to the high AMY1 CN group. Plasma insulin iAUC after starch ingestion was significantly higher in the high AMY1 CN group compared to the low AMY1 CN group. No significant differences were found in plasma TG and NEFA concentrations. The total sample size of 62 adults would be adequate number to detect differences between the groups in tested variables.
CONCLUSION: AMY1 CN showed a potential association with glycaemic levels in healthy adults upon ingestion of starch and glucose. Further research is needed to confirm this finding with a total sample size of 36 healthy adults. Sex differences and lifestyle factors should be taken into consideration in future research while assessing associations between glycaemic response and AMY1 CN.

5.2 Introduction

Various risk factors and aetiologies explain the incidence of obesity and diabetes in different ethnic groups and populations. However, the topic is and requires a broad outlook to cover a wide range of complex associations of different factors including poor diet, activity levels, body mass index and diabetes which creates a serious health burden. Among the different factors, genetic predisposition has been postulated as one of such risk factors, but our current understanding of specific genetic markers is still incomplete.

The salivary amylase gene (AMY1) has been implicated in the onset of obesity, and copy number variation (CNV) of AMY1 is associated with obesity and impaired glucose metabolism (Mandel and Bresline, 2012; Falchi et al., 2014; Mejia-Benitez et al., 2015; Choi et al., 2015; Nakajima et al., 2016; Elder et al., 2018; Pinho et al., 2018; Leon-Mimila et al., 2018; Venkatapoorna et al., 2019; Higuchi et al., 2020; Barbar et al., 2020; Rossi et al., 2021). However, the findings of these studies are inconsistent with evidence from other reports that show no association of AMY1 copy number with obesity and impaired glucose metabolism (Tan et al., 2016; yong et al., 2016; Atkinson et al., 2018; Marquina et al., 2019; Valsesia et al., 2019; Shwan et al., 2019). These
inconsistent results may be due to methodological differences between studies or the heterogeneity in participant samples in terms of number, ethnicity and genetic background (Ooi et al., 2017, Ushar et al., 2016).

Dietary carbohydrate (CHO) quantity and quality have been associated with weight gain and risks of obesity complications, such as type 2 diabetes (Ludwig et al., 2018). The digestion of polysaccharides begins in the oral cavity with its breakdown into smaller saccharides by the action of salivary α-amylase. The copy number variation and activity of AMY1 are responsible for individual differences in salivary amylase function (Atkinson et al., 2018). Individuals with higher salivary amylase levels have been shown to display faster oral starch digestion (Mandel et al., 2010). Higher AMY1 CN is also related to improved starch digestion (Almandel and Breslin, 2012; Atkinson et al., 2018). In 2014, Falchi et al. found the first link between CHO metabolism, low AMY1 CN, and BMI in European and Asian populations (Falchi et al., 2014). Rukh et al. reported that AMY1 CNV is associated with body fat only when taking into consideration the starch intake of individuals in a Swedish cohort (Rukh et al., 2017). AMY1 copy variations are likely caused by human genetic adaptation to starch-rich diets. Individuals from populations with high-starch diets have on average, more AMY1 copies compared to those with traditionally low-starch diets (mean = 6.7; mean= 5.44 respectively) (Parry et al., 2007; Santos et al., 2012). Notably, the high CN of AMY1 is significantly correlated with higher levels of salivary amylase protein and serum amylase (Parry et al., 2007; Mandel et al, 2010; Carpenter et al., 2017). This suggests a genetic link between CHO metabolism and obesity, as the next chapter will provide a pilot study to begin to address this goal.
The genetic adaptation to improve starch digestion, reflected by changes in AMY1 CN, may also have an impact on oral and gut microbiome profiles (Poole et al., 2019; León-Mimila et al., 2018). Gut microbiota alterations have been related to energy balance, weight gain, and adiposity via interactions with dietary factors (Lazar et al., 2019). Further, salivary α-amylase concentration is regulated by circadian rhythms and the nervous system. Physical exercise activates the sympathetic nervous system (Paterson, 1996), Antoon et al. (2015) found that high-intensity exercise leads to the anticipated increase in amylase secretion and salivary flow rate. Thus, the current study will assess the association of AMY CN with glycaemic control in healthy adults after intake of starch and glucose intake of CHO and other macronutrients in the study sample and its association with AMY1 CN.

AMY1 may provide a genetic link between carbohydrate metabolism and BMI (Falchi et al., 2014), which leads us to hypothesize about the association between AMY1 CNV and metabolic status. However, this link is not yet completely understood. In Almandel & Breslin’s study (n= 7), the authors suggest a potential role of salivary amylase activity in initiating glucose homeostasis pathways (Almandel and Breslin, 2012). These findings have been confirmed by another small crossover study (n= 10) which showed that individuals with high amylase activity may have a higher and earlier increase in plasma insulin concentration, and low glycaemic response after the ingestion of gelatinised starch food compared with the low amylase activity group (Alberti et al., 2015). The two studies failed to find an association between AMY1 CN and glycaemic response, but they showed that the salivary amylase activity and concentration significantly influenced the glycaemic response. It is important to note that the expression, activity, and enzyme concentration of amylase are partially correlated with
increasing AMY1 copy numbers and vice versa (Mandel and Breslin, 2012; Perry et al., 2007). A very recent study in sixty healthy non-obese young women showed that the low AMY1 CNV is associated with chronic unfavourable glucose metabolism (Higuchi et al., 2020). It has been reported that high AMY1 CN associates significantly with a favourable metabolic profile, including lower visceral fat volume, higher serum levels of adiponectin and HDL-cholesterol, and enhanced glucose absorption following an oral glucose load, although the association was not found to be significant in terms of insulin sensitivity (Barber et al., 2020).

It is documented that after ingestion of glucose and subsequent insulin signalling there is an increase in the adipocyte lipid storage via two mechanisms, one includes stimulation of triacylglycerol synthesis and the other inhibition of lipid breakdown. The reverse happens in participants who present with lower AMY CN and subsequent decreased insulin/insulin resistance thereby leading to lipid breakdown and increased levels of TAG (Ormazabal et al., 2018).

Insulin resistance exhibits as a decreased utilization of glucose at the cellular level as well as a decline in muscle glycogenesis and lipolysis. Insulin sensitivity is also decreased in different cells obtained from obese individuals, in the presence of lipids derived from lipolysis from the fat cells. This supports the theory of accumulation of excessive fat thereby clearly exhibiting the correlation of obesity, insulin resistance and altered lipid profile (Hardy, Czech and Corvera, 2012).
Reduced amylase 1 (AMY1) copy numbers are associated with obesity, insulin resistance, and inflammation. Although mechanisms linking AMY1 copy number with metabolic disorders are poorly understood. The aim of this study is to contribute to the clarification the conflicting results of previous studies by examining AMY1 copy number variation and glucose metabolism in a homogenous group of healthy participants. The study data can be used for effect size estimation and subsequent power calculation for future, larger studies. We addressed the following research questions:

1- What sample size is required to examine the interaction between AMY1 CN (high AMY1 CN ≥ 9 and low AMY1 CN ≤ 5) and postprandial glucose response after glucose or starch ingestion?

2- Is there preliminary evidence that individuals with high CN have higher glucose concentrations after starch ingestion compared to the low CN group?

3- Is there preliminary evidence that individuals with high CN have low insulin concentrations after starch ingestion compared to the low CN group?

4- What factors may affect high CN / low CN individuals’ impaired postprandial glucose response in this pilot study?
5.3 Methods

5.3.1 Ethical Approval

The study protocol was performed in accordance with the Declaration of Helsinki and was approved by the Health Science and Sports School Ethics Committee at the University of Stirling (the SSREC No #880). All the information about the participants’ identities was anonymous.

5.3.2 Study Participants

Fifteen healthy male and female adults, aged 18-40 years, were recruited from the University of Stirling to participate in this investigation. Participants were asked to sign consent forms after being provided with the full details of the study. Participants were also asked to fill out a health questionnaire, including questions about their medical history and physical activity, and were given the opportunity to ask questions. Participants in the DXA study (reported in Chapter 4) were classified into the top and bottom 10% of the AMY1 copy number distribution: high AMY1 copy number ≥ 9 and low AMY1 copy number ≤ 5. All potential participants were contacted to take part in the current study. 10% of DEXA study participants had high AMY1 CN with an eightfold greater risk of obesity based on their copy number distribution and the study results by Falchi and colleagues who published the first report of an inverse association between human AMY1 CN and BMI using a quantitative polymerase chain reaction (qPCR) approach. These study results were replicated in > 6000 participants from cohorts that included European and Singaporean Chinese ethnicities, demonstrating that each additional copy of AMY1 reduced obesity risk by 1.2-fold, and there was an eightfold greater risk of obesity in individuals with an AMY1 CN ≤ 4, compared to
those with an AMY1 CN ≥ 9 (Falchi et al., 2014). Every participant who met the study inclusion criteria was contacted. The study included only participants who were not on medications during the study period (including thyroid drugs, obesity treatment drugs and antipsychotics), as well as participants with a fasting blood glucose (FBG) ≥ 7mmol/l (126 mg/dl) during the study. Only fifteen participants agreed to take a part in this study. All DNA extraction and AMY1 CN amplification were explained in Chapter 2. The assessment of total Energy Intake (EI; kJ/day), macronutrient intake (g/day), physical activity level (Hours/week) were explained in Chapter 4.

5.3.3 Study Design

The participants were asked to attend the laboratory in the morning on five separate occasions, having fasted for 8-12-hours and rested overnight. Additionally, they were asked to abstain from alcohol consumption for 24 hours prior to their arrival. On the first visit, the participants were instructed to fill in a 24-hour dietary record for the last 24 hours before the samples were taken and were asked to repeat the same diet the day before each subsequent visit in order to minimise the influence of their diet on the results. In the first and second sessions, the participants completed either the Oral Glucose Tolerance Test (OGTT) or Oral Polymers Tolerance Test (OPTT).

5.3.4 Oral Glucose Tolerance Test (OGTT) and Oral Polymer Tolerance Test (OPTT)

A 1.1x32mm Cannula (BD Nexiva, USA) was inserted into the participant’s vein for blood sampling. The participants then drank either a dextrose solution as a control for OGTT (My Protein, UK) or a waxy maize starch solution for OPTT (My Protein, UK). 40g of starch was mixed with 500ml water to get a final concentration of 8
%. However, to get 8% concentration of dextrose and equal energy, 35.4 g of dextrose was mixed with 442.5 ml water (Table 5-1). The participant was instructed to finish the drink within two minutes. Both solutions were prepared 30 minutes before the trial. A sample (5 ml) of venous blood was drawn at correspond time points, 0, 15, 30, 45, 60, 90 and 120-mins. Blood samples were collected in Lithium Heparin tubes (Becton, Dickinson & Company, NJ, USA) and kept on ice until the end of the session.

Table 5-1 Nutritional Information of Carbohydrate Solutions

<table>
<thead>
<tr>
<th>DRINK</th>
<th>CONTROL</th>
<th>TRIAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOURCE</td>
<td>100% Dextrose Glucose</td>
<td>100% Waxy Maize Starch</td>
</tr>
<tr>
<td>SERVING</td>
<td>35.4</td>
<td>40</td>
</tr>
<tr>
<td>CONCENTRATION (%)</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>ENERGY (KCAL)</td>
<td>141.6</td>
<td>141.6</td>
</tr>
<tr>
<td>CARBOHYDRATE(G)</td>
<td>35.4</td>
<td>35.2</td>
</tr>
<tr>
<td>OF WHICH SATURATES(G)</td>
<td>35.4</td>
<td>0</td>
</tr>
<tr>
<td>FAT(G)</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>PROTEIN (G)</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>WATER (ML)</td>
<td>442.5</td>
<td>500</td>
</tr>
</tbody>
</table>

5.3.5 Plasma Analysis

After each session, the plasma fraction was immediately separated by centrifugation at 3500 rpm for 15 min at 4°C. The supernatant plasma was removed, aliquoted into Eppendorf tubes, and stored at -80°C for later analysis. An automated Aries ILab benchtop analyser with associated reagents (Table 5-1) (Instrumentation Laboratories, MA, US) was used to estimate plasma glucose concentrations (mmol/l), and a lipid panel to detect significant associations in test results. Serum lipid measurements included total cholesterol (mg/dl, converted to mmol/l), HDL-C (mmol/l), LDL-C (mmol/l), triacylglycerol (mmol/l), and non-esterified fatty acids (NEFA) (mmol/l).
These lab tests were taken at 8-time points as a part of their OGTT and OPTT tests (-5 to 120 min). To ensure the reliability of the obtained study data, the Ilab Aries was calibrated using a multicomponent calibrator ReferrIL G (00018257000) (Werven, Milano, Italy) to ensure the accuracy of substrate assays (listed in Table 2-2). A reaction volume of 200 μL of each plasma sample was run in duplicate. Analyte concentration was automatically calculated by the instrument against the calibrator. Controls SeraChem® Control Level 1 (Cat. No.0018162412) and SeraChem® Control Level 2 (Cat. No. 0018162512) (Werven, Milano, Italy) were used to establish mean and standard deviations and provide a quality control method. Data out of the reference range were excluded and samples re-analysed.

Plasma insulin concentrations were measured at the same time points as the plasma glucose and lipids using commercial ELISA kits and according to the manufacturer’s instructions (Demeditic, DE2935, Germany). The insulin results are reported in SI units: pmol/L as recommended by (Knopp et al., 2018).

5.3.6 Insulin Sensitivity Index (ISI)

Insulin sensitivity Index (ISI) was obtained after collecting plasma glucose levels and serum insulin concentration in a fasted state and as a part of the measurements provided by the OGTT and OPTT tests. After obtaining this data, whole-body insulin sensitivity was estimated through the Matsuda index using the formula outlined below (Matsuda and DeFronzo, 1999; DeFronzo and Matsuda, 2010; Hayashi et al., 2013):

**Matsuda insulin sensitivity index.** (Matsuda and DeFronzo, 1999)
Chapter 5: Pilot Study

(10,000/square root of \([\text{fasting glucose (mg/dL)} \times \text{fasting insulin (μU/mL)}] \times [\text{mean glucose (mg/dL)} \times \text{mean insulin (μU/mL)}]\) using the data derived from both OGTT and OPTT. FPG = fasting plasma glucose, FPI = fasting plasma insulin.

Based on the emerging literature on different cut-off values for the Matsuda index, we use the 25th percentile as a lower level of insulin resistance and set a Matsuda cut-off of \(\leq 4.1\) as a clinically reasonable value to differentiate between individuals with and without insulin resistance in our study.

**HOMA-IR**

Homeostasis model assessments of insulin resistance (HOMA-IR) was computed using the formula provided by Matthews et al. (1985):

\[
[f\text{asting glucose (mg/dL)}] \times [f\text{asting insulin (μU/mL)}]/405
\]

Elevated HOMA-IR levels account for low insulin sensitivity, which is indicated by a value of 1.9 or more. The cut off HOMA-IR in this study calculating according to participants conditions. The results for each insulin resistance values were ranked and divided into percentiles. As HOMA-IR correlates directly with insulin resistance, the 75th percentile was selected as the best cut-off value (HOMA-IR \(\geq 3.4\)).

5.3.7 **Statistical Analysis**

Statistical analyses were performed on data from the 15 participants who completed the study by using IBM SPSS Statistics Version 28 (SPSS Inc. Chicago, USA). Data was
assessed for normality of distribution using the Shapiro-Wilk test (Shapiro and Wilk, 1965). All values are presented as mean ± standard deviation (SD). Independent sample t-tests were run at baseline to compare possible mean differences in measured variables between the high CN and low CN groups. A two-way repeated measures ANOVA was used to determine whether there were significant differences between the two groups (high CN and low CN) during OGTT/OPTT periods (trials × time).

The error variance for each study variable was estimated from the corresponding intrasubject coefficient of variations (CVs) based on two replicates total 15 non obese subjects (Table 5-4). The CV is ratio of the standard deviation of the mean. It is calculated based on the average values of the respective level (0-120 min).

The incremental glucose response vs time was evaluated by area under the curve (iAUC). To measure the glucose response vs time during an OGTT / OPTT the trapezoidal method was applied (Purves, 1992). The tAUC depends on basal glucose values while the iAUC and pAUC are not related to basal glucose value. Since the tAUC is independent of the ever-changing baseline glucose or insulin levels, it might be the preferred method for evaluation of the response during OGTT (Khan and Thorsten, 2017; Cheng et al., 2018).

In clinics, for the diagnostic purpose of impaired glucose tolerance, the area under the curve (AUC) defines the glycaemic index following OGTT. It is helpful in estimating blood glucose total rise during OGTT. Later on, incremental area under curve (iAUC) was established due to presence of different levels of fasting glucose among different
subjects. But it created a problematic challenge when it yielded negative values while subtracting baseline value of fasting plasma glucose. Hence, after that positive incremental AUC (pAUC) established utilizing the values above the base line. Above all it is recommended recently that the total area under the curve (tAUC) best expresses the correlation with glucose concentration at two hours of OGTT and tAUC is better to be used in preference to iAUC and pAUC (Cheng et al., 2018). Because of the big variance in baseline plasma glucose between individuals in the same group, iAUC of plasma glucose and insulin curves during OGTT / OPTT were calculated using GraphPad Prism version 8 (Graphpad Software Inc., AC, USA).

G*Power3 software used to generate the power calculations for future studies based on current study data sample size offered 80% power (α = 0.05) need to change of plasma concentration of glucose, insulin, TG, and NEFA in healthy adults with high / low copy number of AMY1. G*Power is a power analysis software used for estimating sample size for research studies. Contrary to other sample size calculation softwares, G*Power offers the ability to calculate power for a wide variety of statistical tests including T-tests, F-tests, and chi-square-tests. G*Power has a built-in tool for determining effect size for calculating sample size. G*Power was first introduced in 1996 as a completely interactive, menu-driven program performing high-precision statistical power analysis, nonetheless the modern version of G*Power was developed in 2007 (Erdfelder et al., 1996; Faul et al., 2007; Faul et al., 2009).

The commonly general assumptions used to include, type 1 error value is 5% therefore we used α err prob as 0.05. Furthermore, we assumed the power of the study as 80% with two groups and seven number of measurements. We used the default values of
correlation among repeated measures (0.05) and non-sphericity correction (1). The used assumptions for calculating sample size from the pilot study, we had seven time-points of measurements after OGTT, therefore ANOVA (repeated measurement) used for calculating sample size. To estimate sample size based on ANOVA, the most appropriate test family is F-test, therefore we select F-test as test family. The calculated effect size based on partial $\eta^2$. Based on multivariate tests for different variables (Glucose, total-Cholesterol, Insulin, TG, NEFA), partial $\eta^2$ of 0.016, 0.022, 0.039, 0.059 and 0.065 were used for calculating effect sizes.

5.4 Results

5.4.1 Study Participants

Fifteen healthy adult participants were included in the study. They were divided according to their CNV of AMY1: high AMY1 CN group $\geq$ 9 n =10 (30 % females); low AMY1 CN group $\leq$ 5 n = 5 (80 % females). There were no significant differences ($p$-value > 0.05) in demographic and basic biochemical characteristics of the two groups (Table 5-2).

Mean $\pm$ SD of BMI in was 27.1 $\pm$ 3.9 kg/m2 in low CN group and 23.1 $\pm$ 2.7 in high CN group but was not statistically significant ($p = 0.060$). The Mean $\pm$ SD of PAL (hour/week) was 16.9 $\pm$ 10.09 in low CN group and 8.39 $\pm$ 5.66 in high CN group but was not statistically significant ($p = 0.053$) (Table 5-2).
Table 5-2 Descriptive Characteristics of the Participants in this Study Grouped According to Copy Number of AMY1.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>High CN</th>
<th>Low CN</th>
<th>p = value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=10 (m=7 and f=3)</td>
<td>n=5 (m=1 and f=4)</td>
<td></td>
</tr>
<tr>
<td>AGE (year)</td>
<td>26.8 ± 6.42</td>
<td>22.2 ± 1.64</td>
<td>0.145</td>
</tr>
<tr>
<td>CN of AMY1</td>
<td>10.6 ± 1.50</td>
<td>4.4 ± 0.50</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>70.79 ± 13.11</td>
<td>73.32 ± 4.57</td>
<td>0.687</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>152.59 ± 53.61</td>
<td>169.86 ± 5.07</td>
<td>0.493</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.6 ± 2.69</td>
<td>27.12 ± 3.89</td>
<td>0.060</td>
</tr>
<tr>
<td>Body Fat %</td>
<td>23.4 ± 6.75</td>
<td>30.82 ±13.21</td>
<td>0.169</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Macronutrients and Physical Activity</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EI (kJ)</td>
<td>4112 ± 3657.6</td>
<td>3401.0 ± 2744.4</td>
<td>0.801</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>242.53 ± 43.96</td>
<td>214.4 ± 22.09</td>
<td>0.379</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>76.76 ± 29.65</td>
<td>64.17 ± 19.89</td>
<td>0.574</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>85.73 ± 13.38</td>
<td>76.52 ± 22.92</td>
<td>0.580</td>
</tr>
<tr>
<td>PA (h/w)</td>
<td>8.39 ± 5.66</td>
<td>16.9 ± 10.09</td>
<td>0.053</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fasting Blood Markers</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FPG (mmol/ml)</td>
<td>5.54 ± 0.73</td>
<td>5.2 ± 0.39</td>
<td>0.395</td>
</tr>
<tr>
<td>FPI (pmol/ml)</td>
<td>62.40 ± 30.94</td>
<td>99.00 ± 89.43</td>
<td>0.253</td>
</tr>
<tr>
<td>TOTAL- COLESTROL (mmol/ml)</td>
<td>4.40 ± 0.90</td>
<td>4.81 ± 0.74</td>
<td>0.399</td>
</tr>
<tr>
<td>HDL (mmol/ml)</td>
<td>1.72 ± 0.46</td>
<td>1.53 ± 0.23</td>
<td>0.414</td>
</tr>
<tr>
<td>LDL (mmol/ml)</td>
<td>2.65 ± 0.718</td>
<td>3.08 ± 0.35</td>
<td>0.234</td>
</tr>
<tr>
<td>TG (mmol/ml)</td>
<td>1.15 ± 0.66</td>
<td>0.860 ± 0.34</td>
<td>0.371</td>
</tr>
<tr>
<td>NEFA (mmol/ml)</td>
<td>1.81 ± 1.08</td>
<td>1.01 ± 0.82</td>
<td>0.172</td>
</tr>
</tbody>
</table>

(High- CN = ≥ 9 of AMY1 and Low- CN = ≤ 5 of AMY1). BMI, body mass; EI, Energy Intake; FPG, Fasting Plasma Glucose; FPI, Fasting Plasma Insulin; HOMA-IR, Homeostasis of Model Assessment Insulin Resistance; OGTT, Oral Glucose Tolerance Test; PGTT, Oral Polymeter Tolerance Test. All values are expressed as mean ± SD. p-value <0.05 considered significant differences by one-way ANOVA test.

Though there was no significant differences (p-value > 0.05) between mean fasting plasma glucose (FPG) concentrations of high CN group (5.54 ± 0.73 mmol/ml) and low CN group (5.2 ± 0.39 mmol/ml), nevertheless, 6 (60 %) participants in the high CN group and 1 (20 %) participant in the low CN group demonstrated impaired fasting
glucose (IFG) (≥ 5.6 mmol/L - < 7.0 mmol/L) according to the WHO diagnostic criteria for diabetes and impaired glucose tolerance (Table 5-3).

Table 5-3 Percentage of Normal Participants, Prediabetes, and Diabetes Among High CN and Low CN group.

<table>
<thead>
<tr>
<th>Diagnostic Criteria</th>
<th>High CN (n=10)</th>
<th>Low CN (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO</td>
<td>%</td>
</tr>
<tr>
<td>Fasting Plasma Glucose (FPG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (&lt;5.6 mmol/L)</td>
<td>4</td>
<td>40%</td>
</tr>
<tr>
<td>Prediabetes (≥5.6 mmol/L)</td>
<td>6</td>
<td>60%</td>
</tr>
<tr>
<td>Diabetes (≥7.0 mmol/L)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (&lt;5.5 mmol/L)</td>
<td>4</td>
<td>40%</td>
</tr>
<tr>
<td>Prediabetes (≥5.5 mmol/L)</td>
<td>6</td>
<td>60%</td>
</tr>
<tr>
<td>Diabetes (≥7.0 mmol/L)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2-hour Plasma Glucose Concentration (OGTT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (&lt;7.8 mmol/L)</td>
<td>8</td>
<td>80%</td>
</tr>
<tr>
<td>Prediabetes (&gt;7.8 mmol/L)</td>
<td>2</td>
<td>20%</td>
</tr>
<tr>
<td>Diabetes (≥11.1 mmol/L)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ADA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (&lt;7.7 mmol/L)</td>
<td>8</td>
<td>80%</td>
</tr>
<tr>
<td>Prediabetes (≥7.7 mmol/L)</td>
<td>2</td>
<td>20%</td>
</tr>
<tr>
<td>Diabetes (≥11.1 mmol/L)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(High- CN = ≥ 9 of AMY1 and Low- CN = ≤ 5 of AMY1). Data display in fasting plasma glucose (FPG) and 2-h fasting plasma glucose from OGTT (2-h FPG), in high CN group and low CN group using the World Health Organisation (WHO) and American Diabetes Association (ADA) criteria.

The coefficient of variation % (CV) (SD*100/mean) is basically a relative measurement which is the ratio of the standard deviation to the mean. Higher values of CV indicate greater dispersion around the central value. It is useful to compare the values of variables having different measurement units. The lesser the CV value the greater the precision level of estimate. Generally, results indicate that the blood markers after starch ingestion are observed with lesser CV than after starch ingestion in the high CN
group compared to the low CN group (Table 5-4). It signifies that there exists higher degree of variation among the values. Total CV % of glucose concentration during the OGTT was 23.16 % in high CN and 20.19 % in low CN; in OPTT was 6.64 % in the high CN group and 5.07 % in the low CN group. The total CV % for plasma insulin concentration during OGTT was high in both groups, CV= 52.30 % in high CN and 52.31 % in low CN.

Table 5-4 Coefficient of Variations (CV) for Plasma Concentration in Different Variables.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Glucose</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>CV (%)</td>
</tr>
<tr>
<td>Glucose</td>
<td>High CN</td>
<td>6.52 ± 1.51</td>
</tr>
<tr>
<td></td>
<td>Low CN</td>
<td>6.30 ± 1.28</td>
</tr>
<tr>
<td>Insulin</td>
<td>High CN</td>
<td>160.61 ± 84</td>
</tr>
<tr>
<td></td>
<td>Low CN</td>
<td>228.04 ± 199.30</td>
</tr>
<tr>
<td>Total- C</td>
<td>High CN</td>
<td>4.49 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>Low CN</td>
<td>4.50 ± 0.15</td>
</tr>
<tr>
<td>LDL-C</td>
<td>High CN</td>
<td>2.96 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>Low CN</td>
<td>3.10 ± 0.04</td>
</tr>
<tr>
<td>HDL-C</td>
<td>High CN</td>
<td>1.77 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Low CN</td>
<td>1.40 ± 0.11</td>
</tr>
<tr>
<td>TG</td>
<td>High CN</td>
<td>1.26 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>Low CN</td>
<td>0.90 ± 0.05</td>
</tr>
<tr>
<td>NEFA</td>
<td>High CN</td>
<td>1.97 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Low CN</td>
<td>1.40 ± 0.23</td>
</tr>
</tbody>
</table>

Data display in Mean ± SD and CV (%). High CN (n = 10), Low CN (n = 5).
5.4.2 Power Calculations

The effect size was calculated based on partial $\eta^2$. Based on the current study results of ANOVA (repeated measurement) for different variables concentrations (Glucose, Total-Cholesterol, Insulin, TG, NEFA), the lowest partial $\eta^2$ of 0.016, 0.022, 0.039, 0.059 and 0.065 were used for calculating effect sizes (Appendix 2-6). Based on partial $\eta^2$ for glucose (0.016), a total of 62 participants (31 participants in each group) will be required for the study. Because partial $\eta^2$ for remaining variables (Total-Cholesterol, Insulin, TG, NEFA) was higher than glucose, therefore a smaller number of participants will be required, if we assume other variables for estimating sample size. (Table 5-5)

<table>
<thead>
<tr>
<th>Variable</th>
<th>partial $\eta^2$</th>
<th>Effect size</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.016</td>
<td>0.1275153</td>
<td>62</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.022</td>
<td>0.1499830</td>
<td>46</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.039</td>
<td>0.2014515</td>
<td>26</td>
</tr>
<tr>
<td>TG</td>
<td>0.059</td>
<td>0.2503982</td>
<td>18</td>
</tr>
<tr>
<td>NEFA</td>
<td>0.065</td>
<td>0.2636640</td>
<td>16</td>
</tr>
</tbody>
</table>

5.4.3 Plasma Glucose Responses following Carbohydrate Ingestion

The plasma glucose curves for the high AMY1 CN and low AMY1 CN groups are presented in Figure 5-1. Figure 5-1 A. shows that plasma glucose concentration following glucose ingestion was comparable between the high CN and low CN groups. Following glucose ingestion an increase was observed from the baseline to a peak at 30 minutes, followed by a decrease to 90 mins, and then stable increase from 90 min continuing to 120 min. Figure 5-1 B. shows that plasma glucose concentration after starch ingestion was comparable between low and high CN groups. The mean plasma glucose post- starch was lower in low CN group than high CN group ($p = 0.048$) only
at 60 min time point. However, there were no significant statistical differences between
groups at the remaining time points ($p = > 0.050$; two-way ANOVA).

Peak plasma glucose concentrations and the corresponding total time-points in both
tests (following the glucose solution and starch solution) were not significantly altered
between groups ($p$-value = 0.781; two-way ANOVA) (Table 5-6). Figure 5-1 C.
shows that the plasma glucose iAUC has non-significant difference post-glucose
ingestion ($p$-value = 0.831; $t$-test), and plasma glucose iAUC over 120 min has
significantly higher post-starch ingestion in high CN group then low CN groups ($p$
-value = 0.013; $t$-test).
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A.

Plasma Glucose concentration (mmol/L)

Time (min)

High CN

Low CN

B.

Plasma Glucose concentration (mmol/L)

Time (min)

High CN

Low CN

*
Figure 5.1 Comparing Plasma Glucose Concentrations in of Two Examined Groups (Mean ± SEM): the high CN (n=10) group and the low CN (n=5). (A) shows Plasma Glucose concentrations between the two groups in post-glucose ingestion during OGTT (0-120 min), (B) shows Plasma Glucose concentrations between the two groups in post-starch ingestion during OPTT (0-120 min). (C) comparand Plasma Glucose iAUC between high CN group and low CN group in post-glucose ingestion and post-starch ingestion. (*) considered significant \( p < 0.05 \) (\( t \)-test).

5.4.4 Plasma Insulin

Plasma insulin curves for the high CN and low CN groups are presented in Figures 5-2. Figure 5-2 A. shows plasma insulin after glucose ingestion; a large increase occurred in the high CN group plasma insulin from the baseline to 30 mins post-ingestion and steadily decreased up to 120 minutes. The low CN group’s plasma insulin concentration increased from the baseline to 30 minutes, followed by a decrease at 45 mins to 90 mins. The mean plasma insulin post-glucose was higher in low CN group than high CN group (\( p = 0.038 \)) only at 15 min time point. However, there were no significant statistical differences between groups at the remaining time points (\( p = > 0.050 \); two-way ANOVA).
Figure 5.2 B. shows plasma insulin after starch ingestion; were not stable in high CN group compared to low CN group. Peak plasma insulin concentrations and the corresponding total time-points in both test (following the glucose solution and starch solution) was not significantly altered between groups ($p$-value = 0.342; two-way ANOVA) (Table 5-6).

Figure 5-2 C. shows that the plasma insulin iAUC over 120 min has significant higher in low CN group then high CN group ($p$-value = 0.003; $t$-test) post glucose ingestion and non-significant difference after starch ingestion ($p$-value = 0.430; $t$-test) among high and low CN groups.
Chapter 5: Pilot Study

A.

- Plasma insulin concentration (pmol.L)

B.

- Plasma insulin concentration (pmol.L)
Figure 5-2 Comparing Plasma Insulin Concentrations in of Two Examined Groups (Mean ± SEM): the high CN (n= 10) group and the low CN (n= 5) (A) shows Plasma Insulin concentrations between the two groups in post-glucose ingestion during OGTT (0-120 min), (B) shows Plasma insulin concentrations between the two groups in post-starch ingestion during OPTT (0-120 min). (C) comparand Plasma Insulin iAUC between high CN group and low CN group in post-glucose ingestion and post-starch ingestion. (*) considered significant p <0.05 (t-test).

Table 5-6 Glucose and Insulin Concentrations Over 120 Minutes of OGTT and OPTT.

| Glycaemic response | Glucose (mmol/l) | Insulin (pmol/l) |
|--------------------|-----------------|-----------------
|                    | High CN         | Low CN          | p-value         |
|                    | Post-Glucose    | Post-Starch     | Post-Glucose    | Post-Starch     |
|                    | 5.54 ± 0.72     | 5.61 ± 0.77     | 5.23 ± 0.39     | 5.86 ±0.79      | 0.781^A 0.480^B |
| -5 min             | 5.53 ± 0.75     | 5.67 ± 0.73     | 5.62 ± 0.36     | 5.41 ± 0.51     |              |
| 0 min              | 7.76 ± 2.10     | 6.20 ± 1.31     | 7.68 ± 1.78     | 6.00 ± 0.81     |              |
| 15 min             | 8.97 ± 2.19     | 5.82 ± 0.22     | 8.35 ± 2.85     | 5.82 ± 0.22     |              |
| 30 min             | 7.85 ± 3.10     | 6.56 ± 1.49     | 7.28 ± 2.46     | 5.39 ± 0.90     |              |
| 45 min             | 6.35 ± 1.63     | 6.60 ± 1.45     | 6.31 ± 2.91     | 5.75 ± 0.39     |              |
| 60 min             | 4.59 ± 1.30     | 6.52 ± 1.40     | 5.20 ± 1.99     | 6.05 ± 1.60     |              |
| 90 min             | 5.54 ± 1.82     | 6.05 ± 1.82     | 5.03 ± 1.33     | 5.23 ± 1.08     |              |
| 120 min            | 71.30 ± 41.36   | 45.76 ± 22.37   | 99.00 ±89.43    | 77.76 ± 50.96   | 0.342^A 0.136^B |
| -5 min             | 109.29 ± 123.3  | 60.45 ± 38.96   | 95.88 ±63.30    | 78.96 ± 49.67   |              |
| 0 min              | 198.20 ± 133.5  | 86.26 ± 54.97   | 357.0 ± 323.6   | 92.76 ± 54.50   |              |
| 15 min             | 274.51 ± 163.2  | 84.10 ± 62.62   | 381.0 ±298.38   | 84.36 ± 41.99   |              |
| 30 min             | 267.32 ± 217.74 | 92.28 ± 65.29   | 384.60 ± 290.59 | 82.56 ± 50.28   |              |
| 45 min             | 153.36 ± 152.0  | 79.16 ± 36.09   | 304.60 ± 248.41 | 81.48 ± 46.16   |              |
5.4.5 Plasma Lipids

The concentrations of plasma triglycerides (TG) and NEFA were not significantly different between high CN and low CN group, and the values in each group did not differ following starch ingestion ($p$-value > 0.05) or glucose ingestion ($p$-value > 0.05). The two-way repeated measures ANOVA was used to test the significant difference among test, time and groups. The Triglycerides and NEFA have a non-significant difference among test, time and groups (Table 5-7).

Triglycerides iAUC post glucose and post starch ingestion have a non-significant difference ($p$-value > 0.05) for high and low CN groups. NEFA iAUC post glucose ingestion and post starch ingestion have a non-significant difference ($p$-value > 0.05) for high and low CN groups (Figure 5-3).

Table 5-7 Triglyceride and NEFA Concentrations Responses of Over 120 Minutes of OGTT and OPTT.

<table>
<thead>
<tr>
<th>Lipids response</th>
<th>High CN</th>
<th>Low CN</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-Glucose</td>
<td>Post-Starch</td>
<td>Post-Glucose</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5 min</td>
<td>1.15 ± 0.66</td>
<td>1.42 ± 0.72</td>
<td>0.86 ± 0.34</td>
</tr>
<tr>
<td>0 min</td>
<td>1.27 ± 0.63</td>
<td>1.32 ± 0.60</td>
<td>0.9 ± 0.34</td>
</tr>
<tr>
<td>15 min</td>
<td>1.27 ± 0.73</td>
<td>1.28 ± 0.51</td>
<td>0.99 ± 0.30</td>
</tr>
<tr>
<td>30 min</td>
<td>1.21 ± 0.6</td>
<td>1.21 ± 0.49</td>
<td>0.95 ± 0.44</td>
</tr>
<tr>
<td>45 min</td>
<td>1.32 ± 0.71</td>
<td>1.31 ± 0.58</td>
<td>0.86 ± 0.32</td>
</tr>
<tr>
<td>60 min</td>
<td>1.50 ± 0.89</td>
<td>1.22 ± 0.51</td>
<td>0.90 ± 0.38</td>
</tr>
<tr>
<td>90 min</td>
<td>1.20 ± 0.64</td>
<td>1.33 ± 0.50</td>
<td>0.82 ± 0.34</td>
</tr>
<tr>
<td>120 min</td>
<td>1.18 ± 0.58</td>
<td>1.13 ± 0.53</td>
<td>0.87 ± 0.34</td>
</tr>
<tr>
<td>NEFA (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-5 min</td>
<td>1.81 ± 1.08</td>
<td>2.12 ± 0.97</td>
<td>1.01 ± 1.05</td>
</tr>
<tr>
<td>0 min</td>
<td>1.87 ± 0.88</td>
<td>2.20 ± 0.86</td>
<td>1.33 ± 0.67</td>
</tr>
</tbody>
</table>

High CN (n = 10), Low CN (n = 5). OGTT, Oral Glucose Tolerance Test; PGTT, Oral Polymer Tolerance Test. Data presented in mean ± SD. $p$-values; two-way ANOVA repeated measurement. A (time x CN groups), B (time x test x CN groups).
Figure 5-3 Comparing iAUC Triglycerides and NEFA Concentration Over 120 Minutes of in Post-glucose Ingestion During OGTT (0-120 min) and Post-starch Ingestion During OPTT (0-120 min). OGTT and OPTT. (*) considered significant p <0.05 (t-test). (Mean ± SEM):
5.4.6 AMY1 Copy Number and Insulin Resistance

Of the total 15 participants, 4 participants had diagnosis of insulin resistance (IR). Out of 4 participants with diagnosis of IR, 3 (30%) were from high CN group and 1 (20%) participant was from low CN group (Table 5-8). Results showed that mean HOMA-IR for participants in low CN group were just below the threshold value for diagnosis of IR. Nonetheless, mean HOMA-IR for participants in high CN group were far below the threshold value for diagnosis of IR (Figure 5-4).
Figure 5-4 Comparing HOMA-IR Values Between High CN Group (n= 10) and Low CN Group (n= 5). (A) presented individuals values from both groups (B) explained the Mean ± SEM of HOMA-IR values in high CN low CN group.

5.4.7 AMY1 Copy Number and Insulin Sensitivity

Results showed that 3 (30%) participants in high CN group and 1 participants (10 %) in low CN group were below the threshold value for impaired insulin sensitivity/ inulin resistance. Remaining participants were above the threshold value for IS Figure 5-6 A (Table 5-8). The mean ISI (Matsuda Index) for participants in high CN group and low CN groups were above the threshold value for diagnosis of IR. Indicated that the study participates individuals with low insulin sensitivity and high insulin sensitivity Figure 5-6 B.
Figure 5-6 Comparing Matsuda Index Values Between High CN Group (n= 10) and Low CN Group (n= 5). (A) presented indviduals values from both groups (B) explained the Mean ± SEM of I Matsuda Index values in high CN low CN group.
Table 5-8 Displayed the Mean ± SD for HOMA-IR and ISI for the High and Low CN Groups.

<table>
<thead>
<tr>
<th>Insulin Resistance Index</th>
<th>High CN mean ± SD / n-%</th>
<th>Low CN mean ± SD / n-%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HOMA-IR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal: ≤ 3.4</td>
<td>2.25 ± 1.17</td>
<td>3.29 ± 2.95</td>
</tr>
<tr>
<td>IR: ≥ 3.4</td>
<td>7 (70 %)</td>
<td>4 (80 %)</td>
</tr>
<tr>
<td><strong>ISI (Matsuda Index)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal: ≥ 4.1</td>
<td>9.94 ± 9.27</td>
<td>6.95 ± 5.63</td>
</tr>
<tr>
<td>IR: ≤ 4.1</td>
<td>7 (70 %)</td>
<td>4 (80 %)</td>
</tr>
</tbody>
</table>

Number and percentage of patients according to insulin resistance diagnoses. Participants below HOMA-IR = ≥3.2 consider insulin resistant, Matsuda Index = ≤ 3.9 consider insulin resistance.

5.5 Discussion and Prospects for the Future

Fifteen healthy adult adults took part in this pilot study. Participants were grouped according to AMY1 CN; high CN ≥ 9 n = 10, and low CN ≤ 5 n = 5. The aim of the present pilot study was to generate data that would inform future research to understand potential reasons behind the gaps in understanding of AMY1 CN influence on the glycaemic response after starch ingestion compared to glucose loading during rest in healthy individual adults. Further, we aimed for assimilating an appropriate sample size to achieve that goal.

The baseline characteristic did not defer between participants. However, age was different between our examined groups (high CN mean ± SD age = 26.8 ± 6.42, low CN group = 22.2 ± 1.64), with non-significant (p = 0.145). Further, Low CN participants had a higher (mean ± SD BMI = 27.12 ± 3.89) than the high CN group (mean ± SD BMI = 23.6 ± 2.69), but the data did not reach statistical significance (p = 0.060). Higher PA Level (h/w) noticed among Low CN group (mean ± SD PAL = 16.9 ± 10.09) than high CN group (mean ± SD PAL = 168.39 ± 5.66).
5.5.1 Plasma Glucose and Insulin Responses Following Carbohydrate Ingestion

The influence of AMY1 CNV and/or salivary α-amylase enzyme on glycaemic response and glucose homeostasis after starch and glucose ingestion has been a topic of interest in the last ten years and a potential explanation for the link between AMY1 CNV and obesity (Fernandez and Wiley, 2017). In the current study, glycaemic responses following ingestion of a glucose solution did not differ between the AMY1 CN groups using two-way ANOVA with repeated measurements of time x CNV or time x trials x CNV. However, low CN groups had significant higher insulin concentration at 15 min compared to high CN groups ($p = 0.038$). Furthermore, the iAUC of insulin concentration after glucose ingestion increased significantly in the low CN group compared to the high CN group. Moreover, iAUC glucose concentration after starch ingestion was significantly higher in the high CN group compared to the low CN group. The Glucose concentration at 60 min post – starch was higher in the high CN group compared to low CN group. These results come in agreement with a study by Barbar et al. with subgroups: high AMY1 CN 9–12, $n = 10$; low AMY1 CN 4–6, $n = 7$, which showed a high AMY1 CN association with enhanced glucose absorption following an oral glucose load (Barbar et al., 2020).

The study showed a low variance in fasting plasma glucose concentration between individuals, as noted by the CV = 6.64 % in the high CN group versus CV = 5.07 % in the low CN group. However, there was a high variance between individuals in plasma insulin concentration, as noted by the CV = 52.30 % in the high CN group versus CV = 52.31 % in the low CN group. Thus, we examined both individual groups in the following points.
1- Two methods and two sets of criteria (FPG and 2- h FPG from OGTT) were used to diagnose diabetes according to the WHO and ADA (Table 5-3). Two individuals (20 %) of the high CN group were diagnosed with prediabetes using the WHO and ADA methods, and all individuals (100 %) of the low CN group were normal. Both WHO and ADA criteria were valid with the examined groups as the same results provided FPG and 2- h FPG from OGTT (Table 5-3). Here, we suggest that both WHO and ADA criteria are valid for the examined groups as the same results provided FPG and 2- h FPG from OGTT (Table 5-3).

2- iAUC was used in the study to examine the glycaemic response in the groups. The suggestion for future research is to continue using iAUC to highlight the differences between groups, which showed clearly in iAUC but not in the two-way ANOVA analysis of actual concentrations. Also, the differences between groups in the two-way ANOVA analysis of actual concentration might be explained by the low sample size and variance between individuals in the same testing group, which makes the differences difficult to detect from the direct result.

5.5.2 Plasma Lipids Responses Following Carbohydrate Ingestion

Fasting blood lipid levels did not vary between the high and low CN groups. The groups had a healthy fasting concentration of total- Cholesterol, LDL- C, TG, and NEFA, but low HDL-C concentration. It is important to highlight that TG concentration and NEFA concentrations were not statistically significant between the study groups in both trials.
A novel understanding of lipid changes considers these alterations both a cause and a consequence of impaired glucose metabolism, especially in terms of TG and HDL-C levels. An elevation in TG is associated with free fatty acids and induces β-cell dysfunction and insulin resistance. The exact mechanism is not entirely understood, but free fatty acid increases appear to modulate or disrupt normal β-cell function (Sobczak et al., 2019). This evidences the interaction between lipid and glucose metabolism (Parhofer, 2015). According to recent studies, AMY1 CN affects the gut microbiome, inflammatory markers, and lipid and glucose homeostasis, modulating obesity and cardiometabolic disorders. Thus, lipids can be essential in explaining why AMY1 CN influences obesity and metabolic disorders (Hariharan, Mousa and Courten, 2021).

The lipidomic signature was negatively associated with AMY1 CN in overweight and obese subjects without diabetes, suggesting a link between chronic low-grade inflammation and obesity (Mayneris-Perxachs et al., 2020). In previous reports, subjects with low AMY1 CN showed higher β-oxidation levels and reduced cell glucose uptake (Arredouani et al., 2016). The association between AMY1 CN and metabolic disorders is not yet entirely understood. Still, the previous findings listed above prompt the recommendation of including plasma lipid concentration in future research of this type.

5.5.3 Insulin Sensitivity/ Resistance

This study also evaluated insulin sensitivity / resistance in subjects from both groups. We did not find an association between AMY1 CN with either insulin sensitivity or insulin resistance. This is in accordance with previous research by Albirìti et al. (2015)
and Courten et al. (2019), who reported no association between AMY1 CN and insulin sensitivity. Additionally, our results confirm a very recent study by Barbar et al., which found an association between AMY1 CN and differences in measures of insulin sensitivity in males and females.

Low insulin sensitivity was detected in pilot study participants of both groups from the beginning of the study. The selected sample of this pilot study are adults with insulin sensitivity/resistance after OGTT, but this was not confirmed when the FPG and FPI results were collected. Further, insulin sensitivity/resistance assessment should be considered during the exclusion and inclusion criteria phase.

The estimation of insulin resistance can be done using various techniques and indices. The most trustworthy procedures for determining insulin resistance are the oral glucose tolerance test (OGTT) and the hyperinsulinemia euglycemic clamp; both are considered benchmarks. A few straightforward techniques have been verified, such as the homeostasis model assessment (HOMA-IR) developed by Mathews and the quantitative insulin sensitivity check index (QUICKI). HOMA-insulin resistance, QUIKI, and Matsuda are appropriate for research and therapeutic use (Gutch et al., 2015). At present, however, the glucose clamp technique is used to quantify beta-cell sensitivity to glucose and insulin. The hyperinsulinemic/euglycemic clamp (glucose clamp) technique offers a highly reproducible method of assessing sensitivity to glucose and tissue sensitivity to insulin, but it is complex and difficult to use. The Matsuda index is an index of whole-body insulin sensitivity derived from a frequent
assessment of insulin and glucose concentrations during an OGTT and was developed by Matsuda and Defronzo.

HOMA-IR and QUICKI are the simplest indirect techniques for measuring fasting serum insulin and glucose. Both are the most commonly used surrogate measures and provide a reliable alternative to the glucose clamp. However, 60 % of the high CN group and 20 % of the low CN group had abnormal FPG concentrations ≥ 5.5 mmol/L. So, as a fasting insulin-resistant test, the insulin sensitivity surrogate marker, Matsuda index based on OGTT, was validated against the hyperinsulinemic-euglycemic clamp (Otten, Ahrén, and Olsson, 2014).

HOMA-IR cut-off values differ according to gender, age, ethnicity, disease, and complications. Even so, they are not consistent across studies. Bonora et al. (1998) suggested a top quintile for the HOMA-IR of ≥ 2.77 to detect isolated insulin resistance without metabolic disorders. Tripaty et al. (2000) reported that insulin resistance was more severe in individuals with impaired fasting glucose than in those with normal glucose tolerance (HOMA-IR, 2.64 vs. 1.73). Yeni-Komshian et al. (2000) suggested a HOMA-IR cut-off value of 2.7 after examining steady-state plasma glucose in 490 healthy non-diabetic subjects. Ascaso et al. (2004) defined a HOMA-IR cut-off point of 2.6 to determine insulin resistance according to the 75th percentile value.

In this pilot study, results for each insulin resistance values were ranked and divided into percentiles. As HOMA-IR correlates directly with insulin resistance, the 75th percentile was selected as the best cut-off value (HOMA-IR ≥ 3.4). 3 out of 10 (30 %)
individuals of the high CN group were insulin resistant compared to 1 out of 5 (20 %) individuals of the low CN group.

Across the literature, the suggested cut-off values to define insulin resistance using the Matsuda index are not consistent. They range from < 2.5 to 6.417, including measures of < 3.530 and < 4.331. Based on the diverging literature on different cut-off values for the Matsuda index, we use the 25th percentile as a lower level of insulin resistance and set a Matsuda cut-off of ≤ 4.1 as a clinically reasonable value to differentiate between individuals with and without insulin resistance in our study. Three of 10 (30 %) individuals of the high CN group were low insulin sensitive / insulin resistance compared to 1 out of 5 (20 %) individuals of the low CN group. The outcomes from HOMA-IR values were identical to the Matsuda index values in the low CN group and in the high CN group. The study had total of 4 insulin resistant individuals in both groups, that may explain the higher insulin secretion in low CN group at 15 min post – glucose. The results showed that high CN group might have healthier glycaemic response. It has been notated that high CN group might have more ability to digested starch in first two hours compared to low CN group. We had inconsistent results in insulin concentration and high intra-assay average CV among individuals, which can indicate that the significant differences lifestyle factors that can play a key role in the collected biological sample in the study.

5.5.4 Lifestyle Factors

Here are the lifestyle factors which may cause high variance in the glycaemic response among the individuals in our study. These factors are also well-known determinants of
obesity and T2DM. More attention should be given to designing future research, including lifestyle factors such as heavy alcohol consumption, smoking status, dietary intake, and physical activity level, as well as additional parameters such as gender, weight, diastolic blood pressure, and blood cholesterol.

**Heavy alcohol consumption** plays a vital role in the process of weight gain as it provides a potential source of energy that increases body fat, and consumption amplifies the harmful effect of BMI on lipid profiles (Gao et al., 2021).

**Smoking** increases fasting glucose levels (Nakanishi et al., 2000). It also affects factors that modulate lipid and glucose metabolism, including adiponectin, lipoprotein lipase, tumor necrosis factor-alpha, and peroxisome proliferator-activated receptors. These outcomes of smoking and the associated metabolic risk can sometimes be reversed with smoking cessation, but the risk may increase initially after smoking cessation, which is likely a result of weight gain (Jeong, Joo, Kwon, and Park, 2021; Behl, Stamford and Moffatt, 2022). Still, it is evident that not all smokers develop T2DM, and the link between T2DM and smoking is heterogeneous, possibly modulated by genetic factors (Erzurumluoglu et al., 2019; Wu et al., 2020).

An interesting study by Choi et al. found that AMY1 CN correlated negatively with HOMA–IR in 1257 asymptomatic Korean men even after adjusting for covariates, e.g., BMI, systolic blood pressure, triacylglycerol, alcohol consumption, smoking, and physical activity. When the participants were divided according to current smoking and alcohol consumption habits, negative correlations between AMY1 CNVs and HOMA–
IR was more evident among non-smokers and regular drinkers and were non-significant among smokers and non-regular drinkers. Such a relationship presented differently according to the status of smoking and alcohol consumption (Choi et al., 2015).

Our participants were asked to avoid alcohol consumption 24 hours before attending the lab and avoid smoking overnight. However, smoking status was not recorded. Such data is recommended to be collected in future research to understand the interaction between smoking status and AMY1 CN to develop insulin resistance and weight gain in a healthy population.

**Gender** and biological sex impact the pathogenesis of numerous diseases, including metabolic disorders such as diabetes. There are accepted sex-specific differences that influence substrate metabolism. Whereas the current study recruited both male and female participants, it did not adjust for gender as we only have one man and four females in the low AMY1 CN group.

A previous study has shown that adult males have higher insulin resistance and a greater risk for type 2 diabetes (Mauvais-Jarvis, 2018). Women with normal glucose tolerance have lower levels of FPG and HBA1c and higher levels of 2hPG-OGTT compared with men with normal glucose tolerance. Such differences remain after challenging participants with the same glucose load, regardless of physical fitness and body size (Faerch et al., 2010). Insulin sensitivity differences between genders have been attributed to sexually dimorphic body composition and sex hormones (Tramunt et al., 2020). The lower proclivity of insulin resistance among women is conflicting,
considering that they tend to have higher circulating NEFA levels, higher lipid content in myocytes, increased fat mass, and lower skeletal muscle mass. A likely mechanism is that females are more resistant to lipotoxicity and protected from NEFA-induced insulin resistance, which has been found experimentally in skeletal muscle tissue (Frias et al., 2001).

Further, sex hormones have been implicated in this gender difference in glucose homeostasis. Indeed, menopausal estrogen therapy decreases fasting glucose while impairing glucose tolerance (van Genugten et al., 2006; Mauvais-Jarvis, 2018). Further research is needed to examine the role of gender-related factors influencing glycaemic homeostasis in AMY1 CN.

**Physical Activity Level**, it is noticeable that participants in this study were active. The low CN group (four females and one male) was more active than the high CN group (3 females and seven males). Nevertheless, both groups were very active. High CN group PAL (h/w) mean ± SD = 8.39 ± 5.66, and low CN group PAL (h/w) mean ± SD = 16.9 ± 10.09. A good assessment method (SPAQ) was used for assessing PAL, which uses a 7- day recall of leisure time and occupational physical activity as a measure of physical activity (Lowther et al., 1999). It was recorded that several participants involved in part-time jobs reported running in Stirling during data collection time. We asked these participants to record a week before to reflect on their usual activity, but according to the results, the subjective influence is clear.
Generally, APL data are essential for our study and future related research. It is known that during physical exercise, glucose uptake by the working muscles rises 7 to 20 times over the basal concentration, depending on the intensity of the work performed. Physical exercise improves the reduced peripheral tissue sensitivity to insulin in impaired glucose tolerance, abnormal lipid metabolism, and Type II diabetes (Helmrich et al., 1994; Sato, Nagasaki, Nakai, and Fushimi, 2003). In our study the low CN group were more active than the high CN groups. It has been that may partially explain why noticed that low CN group in this study may has a healthier glycaemic control comparing than high CN group.

**Dietary intake**, the majority of the CHO and other macronutrients enter the bloodstream as glucose to be used for energy, stored as glycogen in the liver and the muscles, or converted into fat. The fate of the glucose circulating in the bloodstream is determined by the relative concentrations of the hormone insulin. Insulin is released from the β-cells of the pancreas in response to glucose absorption and stimulates the liver cells and muscle tissue to receive glucose, which is used as an energy source or stored. Insulin modulates liver metabolism and suppresses the conversion of amino acids into glucose. Thus, blood glucose concentrations will reduce when the tissues respond to insulin appropriately (Lunn and Buttriss, 2007) (as discussed in Chapter 1).

Postprandial studies show that common meals may have significant adverse effects (Shapira, 2019), including the promotion of chronic inflammation, especially in people at high risk (Biobaku et al., 2019). This effect can be sustained by multiple meals and
last for many hours daily, potentially covering 18 hours or a major part of the day (Jackson, Poppitt, and Minihane, 2012; Vries et al., 2014).

The proinflammatory effects of macronutrients, such as high-fat, high-calorie (HFHC) meals, are essential in the pathogenesis of the pro-inflammatory states of insulin resistance, obesity, and type 2 diabetes. On the contrary, fiber-rich meals exert anti-inflammatory effects that can significantly suppress the inflammation caused by HFHC meals when these meals are compared (Ghanim et al., 2017). HFHC intake predisposes to visceral adiposity and intrahepatic fat accumulation, creating the milieu for inflammation, which leads to insulin resistance. Intrahepatic fat accumulation is even more strongly correlated to the metabolic derangements associated with obesity compared with visceral adiposity (Fabbrini et al., 2009).

This study showed no differences in macronutrient intake between the high and low AMY1 CN groups. This agrees with study findings by Arredouani et al. (2016) and Barbar et al. (2020). Shin and Lee found that AMY1 influences the incidence of type 2 diabetes in Korean women after considering their dietary carbohydrate intake (Shin and Lee, 2021). It has been suggested that high AMY1 CN with increased carbohydrate intake resulted in enhanced salivary digestion of starch to maltose, followed by its conversion into glucose. This enhancement optimized the efficiency of glucose absorption across the upper gastrointestinal tract. Thus, increasing serum insulin levels following oral glucose ingestion occurred in response to enhanced early glucose absorption in those with a high AMY1 CN (Barbar et al., 2020).
This pilot study performed three-day food records using the Nutritics Nutrition Analysis Software (Academic Edition, Nutritics, Dublin, Ireland). Macronutrient intakes were calculated in (kJ/24hurs). Reported EI was evaluated against presumed energy requirements in a procedure known as the Goldberg cut-off technique. (Goldberg et al, 1991; Black, 2000a). It is also important to note that the disadvantage of self-reported 24-h food records is that it relies upon memory and can be affected by subjective error. However, the dietary assessment method in this study helped assess the participants’ dietary intake.

**Salivary α- amylase activity levels** (sAA). This enzyme processes starch in the mouth and converts this substrate into di-saccharides, tri-saccharides, and some glucose (Peyrot des Gachons and Breslin, 2016). A function of sAA on starch digestion in the small intestine was also proposed, given the significant passage of sAA through the stomach due to incomplete inactivation by low pH. In vitro, amylase was inactivated in the gastric juice as pH fell between 3.8 and 3.3 (Fried, Abramson, and Meyer, 1987).

A study by Mandel and colleagues (2010) found that individuals with higher amylase activity levels exhibited faster starch digestion in the oral cavity. Thus, they expected a decrease in salivary amylase pre-digestion would also decrease starch digestion, resulting in a lower glycaemic response. Mandel and colleagues study results in 2010 and 2012 reported a strong link between amylase activity and increased AMY1 copy number (Perry et al., 2017; Alberti et al.,2015). Mandel and Breslin (2012) suggested that AMY1 CN may play a role in the development of insulin resistance. They investigated the influence of salivary amylase and AMY1 CN on starch digestion in a
total of seven healthy, non-obese adults classified into high amylase concentration and low amylase concentration groups and found that individuals with more elevated salivary amylase had significantly lower postprandial blood glucose responses to starch ingestion, and a more pronounced postprandial excursion of insulin within the first 9 minutes following starch ingestion (Mandel and Breslin, 2012).

**Other related genes**, insulin resistance and other metabolic disorders result from an interaction of environmental or lifestyle variables and genetic predisposition. For instance, insulin sensitivity can be lowered by changes to genes coding for a protein in the insulin-signalling pathway and/or factors that cause elevated levels of circulating substances that interfere with insulin-signalling system and metabolic pathways. Despite having no signs of metabolic syndrome, it is widely established that people with a family history of type 2 diabetes are more likely to be insulin resistant than those without a history (Kendall et al., 2003).

However, the superposition of modifiable factors, common in many Westernised societies, such as increased energy consumption and decreased physical exercise is believed to be responsible for the rising prevalence of insulin resistance. Humans have evolved systems that encourage fat storage during times of abundance and induce lipolysis in times of food scarcity because fat is a very effective way to store energy (Lebovitz, 2006). Insulin resistance is linked to adipose tissue accumulation, such as in obesity. A lack of fatty tissue, such as that seen in lipodystrophy, is also connected with insulin sensitivity (Frayn, 2001).
A recent genetic study conducted by Shin and Lee (2021) evaluated individual differences in AMY1 protein expression by six genetic SNPs variants of the AMY1 gene: rs10881197, rs4244372, rs6696797, rs1566154, rs1930212, and rs1999478. This was a large-scale prospective research based on the Korean Genome and Epidemiology Study. It found that T2DM incidence in the high carbohydrate-intake group influenced the A allele of rs6696797, A allele of rs4244372, and G allele of rs10881197 in females but not Korean males (Shin and Lee, 2021).

5.5.5 Experiment Methods

It should be noted that this study had a significant variation in insulin concentrations with intra- and inter-assay (17.8 % in the high CN group and 9.5 % in the low CN group). This inter-variance in insulin concentration might be due to the assessment method of plasma insulin.

Several methods are prompting a good selectivity towards insulin detected with fewer interferences and high throughputs, e.g., ELISA, chemiluminescence immunoassay (CLIA), radioimmunoassay (RIA) and on-chip immunoassay. The ELISA, in particular, sandwich ELISA is the most popular for insulin determination for clinical purposes (Shen, XU, 2019). Moreover, Chromatographic assays, including high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV), micellar electrokinetic capillary chromatography (MECC), and liquid chromatography with tandem mass spectrometry (LC-MS/MS) are highly sensitive and capable of simultaneous detection of insulin.
This pilot study used a Demeditec ELISA kit based on the sandwich principle. The sandwich ELISA has the highest sensitivity among all the ELISA types, direct ELISA, indirect ELISA, and competitive ELISA. However, sandwich ELISA has disadvantages e.g., the time and expense and the necessary use of a "matched pair" (divalent/multivalent antigen) and secondary antibodies (Engvall, 2010; Alhajj and Farhana, 2022). The high sensitivity of ELISA reflects the measurement sensitivity for target proteins. Thus, it might be a suitable choice for the practical analysis of plasma insulin in repeated collection samples such as OGTT or among diabetes participants, where the biological plasma sample can be adequate. However, it also affected by common ELISA issues, e.g., weak or no signal in ELISA because of incorrect storage of components or reagents are at room temperature.

Further, the excessively high signal in ELISA is due to insufficient washing, testing time, or wells contamination. Also, the inconsistent pipetting between samples or other regular laboratory technical issues resulted in poor standard curve linearity. The insulin-ddetected methods should be selected according to target participant characteristics.

Further, our participants did complain about the glucose drink. However, they find the starch drink is inconvenient to sallow, which is expected because of its starchy texture. We recommended using starch for eating in a regular human diet instead of row starch powder and / or sport's polymers powder solution. The next chapter (Chapter 6) will provide experimental design, including further details and recommendations for future research.
5.5.6 Power Calculations

Based on partial $\eta^2$ for glucose (0.016), a total of 62 participants (31 participants in each group) will be required for the statistical study power (>80%) to find a significant association between the copy number variation of the AMY1 gene and glucose concentration within and between CNV AMY1 groups. Because partial $\eta^2$ for remaining variables (Cholesterol, Insulin resistance, TG, NEFA) was higher than glucose; therefore, a lower number of participants will be required if we assume other variables for estimating sample size.

Mandel and Breslin's (2012) study included seven participants. It relied on data by Perry et al. (2007), according to which a sample size of $n=10$ is required to provide adequate statistical power (>80%) with a confidence of 95% to find a significant association between AMY1 CN with salivary amylase activity/concentration.

Mandel and Breslin (2012) investigated the influence of salivary amylase and AMY1 CN on starch digestion in a total sample size of 7 healthy adults who were classified as having high amylase concentrations and low amylase concentrations and found that those individuals with higher salivary amylase had significantly lower postprandial blood glucose responses to starch ingestion. Two years before, Mandel and colleagues (2010) expected a decrease in salivary amylase pre-digestion would also decrease starch digestion, resulting in a lower glycaemic response. Perry et al. 2017; Alberti et al., 2015 reported a strong link between amylase activity and increased AMY1 copy number. Thus, Mandel and Breslin's study included seven participants and based on data by Perry et al. (2007), a sample size of $n=10$ is required to provide adequate statistical
power (>80 %) with a confidence of 95 % to find a significant association between the copy number variation of AMY1 gene with salivary amylase activity/concentration. The assuming that adequate sample size for detecting differences between salivary amylase enzyme and glycaemic response can be used to detect glycaemic response differences among healthy adults according to their AMY1 CNV. However, both studies reported a significant statistical association between salivary starch digestion and high salivary amylase enzyme activity but not AMY1 copy number. It is essential to note the low sample size of the previously published experiments, behind the inconsistency in the result of the association between CNV and insulin resistance and glycaemic response after starch ingestion. We recommended that future related research include 62 participants to conforming the role of the result of the AMY1 CN starch digestion or insulin resistance.

This study has several limitations. First, the study did not include data on salivary amylase concentration, which limited the study from further exploring the link between AMY1 CNV and salivary amylase concentrations and comparing the influence of salivary amylase with AMY1 CN on substrate metabolism of starch. The activity of the salivary amylase is the factor that best reflects its current capacity and determines the degradation rate of polysaccharides like starch. Therefore, despite the positive link between salivary amylase activity and high AMY1 CNV, salivary amylase is the most suitable parameter to investigate the relationship between salivary amylase and the association of metabolic disorders. Further, the low CN group comprised four females and one male, which may influence gender-related differences in body composition of the group, such as fat % and BMI, all factors related to impaired glucose response.
Moreover, 4 of study participants had higher than expected HOMA-IR values and low Matsuda insulin sensitivity indexes.

Our findings may be applicable as recommendations to design future related studies. The number of subjects in each group was not equal, and the statistical difference might be hard to confirm. Future studies should investigate lifestyle factors and conduct a follow-up study of the influence of AMY1 CNV in insulin and glucose response groups.

5.6 Conclusion

Individuals with low AMY1 CN tend to be more sensitive to insulin, according to OGTT. AMY1 CN was associated with starch digestion in adult individuals with a high copy number. Further research is needed to confirm this finding with a sample size of more than 62 adults. Gender differences and the abnormal glucose concentration were the most challenging factors, which should be considered in future research to assess the associations between glycaemic response and AMY1 CN.
Chapter 6  General Discussion

Phenotypic variations due to a change in gene copy number were first identified in 1936 in the Bar gene of *Drosophila melanogaster* species (HARLAND, 1936). For decades, geneticists have also found copy number variations in the human genome. They were unaware of their significance for many years until much more recently. Since the public domain release of the human genome sequencing, several reports have described copy number variation (CNV) of DNA segments. It became clear that CNV research is essential for a complete understanding of genetic variability and evolution. Current CNV research will eventually be extended to various areas of health and disease to delineate complex genetic phenomena. CNV can significantly affect phenotype by altering gene transcription levels (Hegele, 2007). AMY1 is a gene of interest, and recent studies have thrown conflicting results regarding the effect of AMY1 CNV on obesity and glucose/starch metabolism. This thesis has attempted to contribute to the nutrigenetic field by examining the influence of AMY1 CNV on body composition and CHO metabolism.

This thesis aimed to address the current knowledge gap regarding the influence of AMY1 CNV on adiposity and body composition among athletes, controls, and non-obese adults. It also aimed to consider whether a higher AMY1 copy number impacts CHO metabolism and consider this from a clinical perspective. These two primary aims were achieved by the completion of the following four key objectives:

- To determine the association between AMY1 CNV and BMI, anthropometric measures of body composition, and strength in male athletes (Chapter 3).
• To determine the influence of AMY1 copy number (CN) on body fat and lean mass distribution in adult males and females (Chapter 4).

• To investigate the impact of AMY1 CNV on the glycaemic response after starch/glucose ingestion in a pilot study (Chapter 5).

The principal findings of this research have been discussed in detail throughout the four experimental chapters and were primarily fivefold:

• We observed that AMY1 CN did not differ between adult athletes and non-athletes in Chapter 3 or between male and female non-obese adults in Chapter 4.

• As a novel finding, a significant association was found between AMY1 CN and athletes’ height. However, no such associations were observed between AMY1 CN and athletes’ fat mass percentage and lean mass.

• AMY1 CN was not associated with measures of adiposity and lean mass measurements in male and female normal-weight adults.

• In a pilot study, plasma glucose concentration, insulin concentration, and plasma lipid concentration following starch ingestion did not differ significantly between the high AMY1 CN (≥ 9 copies) and low AMY1 CN (≤ 5 copies) groups. The iAUC plasma insulin concentration post-starch was significantly higher in the low CN group compared to the high CN group. The high CN group was also found to have significant higher glucose concentration post-glucose ingestion. Other than that, no significant differences were found
in the case of remeasurements performed in the study. Sample size of 62 healthy adults is recommended for repeated future research.

From these principal findings, the AMY1 CN was not associated with BMI or body composition measures among athletes, recreationally active males, and the general male and female adult population with average body weight. For the past seven years, five studies have reported an association between AMY1 CN and obesity presence among adult males, females, and children (Falchi et al., 2014; Marcovecchio et al., 2016; Pinho et al., 2018; Leon-Mimila et al., 2018; Venkatapoorna et al., 2019). In contrast, another five well-powered replication studies did not find an association between AMY1 copy number and obesity measurements (Usher et al., 2015; Yong et al., 2016; Rukh et al., 2017; Shwan et al., 2019; Valsesia et al., 2019). The present studies in this thesis are in alignment with these well-powered negative findings. These inconsistent results may be due to the heterogeneity in patient samples in terms of number, ethnicity, genetic background, age, gender, and the use of different study designs and methods, including the CNV detection method, as well as data collection of diet and physical activity. This thesis covers a number of these methods.

Sex differences can also play a role in these inconsistencies. The probable causes of these differences between males and females could be attributed to several factors. The hormonal surges in females, the level of stress hormones, and the apparent differences in physiology and metabolism all contribute to causing inconsistencies in the data, which are otherwise a normal part of either gender. The same could be applied to the differences reported in adults and children. Children have a significantly different physiology than adults, making it easy to compare differences between age groups. A
study on Italian school children found that AMY1 CNV did not associate with girls’ BMI. However, it found that BMI was negatively associated with AMY1 copy number in boys (Marcovecchio et al., 2016). In another study in Finland, Viljakainen et al. (2015) found no difference in AMY1 copy number between healthy subjects and subjects with a history of childhood-onset obesity. Still, they reported that obese men had a higher copy number than obese females. In our non-obese adult study reported in Chapter 4, we examined gender differences between healthy-weight males and females. We found that AMY1 CN was not associated with BMI and body composition measurements (the amount and distribution of BF % and FFM (g) in either males or females). However, clear and significant expected differences were present between males and females in the baseline measurements of weight, height, WC, BMI, and BF %, which is taken as a limitation of the study.

The CN distributions of AMY1 did not differ between experimental groups in this thesis. Males and females in chapter 4 or male athletes and controls in Chapter 3 were not reported to display significant differences in CN distribution. This was expected, as comparable results had been reported in previous studies. The mean AMY1 CN in athletes’ study (Chapter 3) was mean = 6.80 (range: 1-14), and the mean AMY1 copy number among the non-obese adults’ study (Chapter 4) was mean = 6.98 (range: 2–14). This thesis's average copy number distribution agrees with the mean copy number in previous AMY1 copy number studies, mean = 6.8 (Chapter 1, Table 1-2). However, the frequency of AMY1 copies in several studies was as high as 27 (Perry et al., 2007; Santos et al., 2012; Carpenter et al., 2015).
It is worth mentioning that a study of 597 obese and normal-weight Mexican children with a copy number ranging between 1 and 16 copies suggested benefits of a high number of AMY1 CN since normal-weight children had an AMY1 CN greater than ten copies (Mejia-Benitez et al., 2015). We considered this result and assessed the association between fat % and AMY1 among people with >10 copies of AMY1 in the healthy weight adults’ study (Chapter 4) (Appendix 1). However, no evidence was observed about the high copy number of AMY1 with adiposity.

It has been suggested that these conflicting results may be due to different study genotyping methods to detect the CNV of AMY1. This explanation is possible in the case of complex CNV such as that exhibited in the AMY1 loci (Ooi et al., 2017). In such cases, it may be difficult to estimate CN variation using traditional methods for CNV assessment (Usher et al., 2015).

Height has been examined as a separate component of BMI to comprehend further the influence of AMY1 copy number on this measure. The thesis has found for the first time a link between increasing AMY1 copy number and height in athletes and controls (Chapter 3). Height is highly heritable, with 90% of heritability estimates (Hirschhorn & Lettre, 2009; Visscher et al., 2006; Silventoinen et al., 2012). CNVs have been recognized as a significant contributor to genetic variation between individuals in addition to SNPs (Kang et al., 2010). Dauber et al. (2011) suggested that CNVs may contribute to genetic variation in stature among the general population (Dauber et al., 2011). For further confirmation of the association between height and AMY1 CN, we also evaluated this measure in the general population of the second study (Chapter 4).
However, we found no evidence of the influence of AMY1 on height. Two reasons can explain the inconsistent results in the association between AMY1 and height. Firstly, by considering height (cm) variances in the two populations. For instance, only males were included in the study conducted on Lithuanian athletes and non-exercising controls.

In contrast, this study joins data from males and females. The mean height ± SD in this study was 181.1 ± 6.6 cm, compared to the total population mean height of 172.2 ± 9.5 cm. However, a similar mean height was observed between Lithuanian athletes (males only) and male’s height in this study (mean ± SD) 179.6 ± 6.5 cm. The second potential reason to explain the inconsistent result in the association between AMY1 and height is the limited cohort sample size, which makes the association between AMY1 CN and height weak in 108 males compared to 388 males in the Lithuanian athlete’s study. Further, in a broader context, the distribution of height and AMY1 CN might be random in the examined populations, making the associations stronger or weaker.

Height is now used as an indicator in epidemiology studies to estimate the association between one's early life exposure and diseases. The Emerging Risk Factors Collaboration conducted a meta-analysis of 121 cohort studies comprising over 1 million participants. They reported short stature as a well-documented risk factor for death from circulatory diseases such as coronary disease, stroke, and heart failure (Emerging Risk Factors Collaboration, 2012). In contrast, height was positively associated with the risk of death from melanoma and cancers of the pancreas, endocrine, and nervous systems, ovary, breast, prostate, colorectum, blood, and lung (The
Emerging Risk Factors Collaboration, 2012). A large prospective study of 409,748 adult individuals found that overall height is positively associated with deaths from cancer but inversely associated with deaths from circulatory disease (Sawada et al., 2017). Shorter men showed increased risk of cardiovascular mortality (HR per 5 cm increase: 0.81, 95 % CI: 0.72–0.91), and all-cause mortality (HR per 5 cm increase: 0.89, 95 % CI: 0.83–0.96). For shorter women there was increased risk of non-cardiovascular, non-cancer mortality (HR per 5 cm increase: 0.82, 95 % CI: 0.71–0.96), and all-cause mortality (HR per 5 cm increase: 0.88, 95 % CI: 0.81–0.96) (Zhao et al., 2019). Furthermore, a large cohort of 22,809,722 Korean men and women positively associated height with the risk of all site-combined cancers and with malignancy in the oral cavity, larynx, lung, stomach, colorectum, liver, pancreas, biliary tract and gall bladder, breast, ovary, cervix and corpus uteri, prostate, testes, kidney, bladder, central nervous system, thyroid, skin, and lymphatic and hematopoietic systems. The positive association between height and cancer as hazard ratios (HRs) for all-site cancers per 5 cm increment in height was 1.09 (95 % CI: 1.086–1.090), and the highest association was found in thyroid, breast, lymphoma, testicular, and renal cancers (Choi et al., 2019). The influence of high CNV of AMY1 on height requires confirmation. Further studies are also warranted to build on the associations between CNV of AMY1 and height-related diseases such as cancers, coronary disease, stroke, and heart failure.

This thesis also found that the high CN group showed significant higher iAUC glucose concentrations compared to the low CN group after glucose ingestion. However, iAUC plasma insulin was significantly higher among the group with low CN than those with high CN. No differences between groups were noticed after starch digestion. Overall,
the plasma TG concentration did not differ between the high and low CN groups after ingesting either the glucose or starch solution (Chapter 4). Several studies have shown that low serum amylase concentration but not CNV of AMY1 is associated with low-speed starch metabolism and insulin resistance. It has been observed that insulin secretion occurs early after starch consumption, which is enhanced in subjects with higher salivary amylase activity. (Mandel & Breslin, 2012, Peyrot et al., 2016). (Chapter 1, Table 1-3). One thing worth mentioning about this study (Chapter 4) is the fact that it covers the significant calculations required to establish designs for future studies, along with providing substantial insight into what factors need to be considered when carrying out further research in the same domain.

6.1 Thesis Limitations

The data presented in this thesis adds important and novel findings to the literature that will facilitate advancing knowledge within the field. However, several limitations exist that warrant consideration.

- The first observational study reported in Chapter 3 included a comparison analysis between low variance age, fat mass, and BMI between recruited athletes and controls. In this stage, some control participants in this study were included in the ‘active’ category after admitting to working out and training at least twice a week maximum.

- This thesis addressed the association between male athletes’ body composition and height, but female athletes were not included in the cohort. This gap needs to be addressed and bridged accordingly in future research by doing another
large-scale study that includes both males and females in an equal ratio. Both inactive and active participants from either sex need to be part of any future study to bridge the knowledge gap and work out the associations between the females’ body composition and height, too.

- As detailed in the first part of this discussion, the general population body composition study (Chapter 4) was designed based on sex-specific differences to examine variations in fat distribution within and between the sexes according to AMY1 copy number. However, there is a lack of details on the distribution of SAT and VAT because of the limitations of the DXA technique. These particular limitations can undoubtedly give rise to a gap in the study in terms of sex-related fat distribution in both males and females and the response of this fat distribution to insulin after ingesting starch. The differences in insulin sensitivity between genders have been attributed to sexual dimorphisms in the body, such as fat distribution and adipose tissue biology (e.g., men have more abdominal and VAT, which is associated with insulin resistance). In contrast, women have more SAT, which has been shown to be protective against insulin resistance. Increasing SAT is also associated with rising serum adiponectin levels. A practical way of addressing this could be to equally include both the genders in the same manner in future studies – both the control and participating groups should be built according to the same criteria, with similar inclusions and exclusions.

- This thesis examined gender differences concerning the influence of high CN on the body composition of an adult population. However, the mean weight of
both groups was considered healthy, and the number of overweight or obese individuals in the study sample was deficient.

- This present thesis did not consider carbohydrate foods in the assessment of the association between AMY1 and gender adiposity as a result of dietary intake, among those who reported their intake in chapter 4, 68 out of 112 participants matched with EI: BMR cut-offs.

- A further limitation of this thesis is the low number of participants, especially in the low copy number group, which may play a role in the lack of statistical differences in Chapter 5.

- A thorough and elaborate analysis of salivary amylase concentration is required. Thus, the study could not further explore the linkage between AMY1 CNV and salivary amylase concentrations. Additionally, Chapter 5 was limited to low AMY1 CNV participants to pick up the statistical differences. Therefore, our findings may serve as recommendations to design future plans to help put the AMY1 to its correct usage while avoiding unwanted limitations that interfere with the results. This study will also serve as a helping guide for future research programs that will take place to elaborate on or expand research in this domain.

- The pilot studies did not consider the sex factor. This is regarded as a limitation because glucose tolerance can differ between males and females (Mauvais-Jarvis, 2018). In the experimental study in Chapter 5, the low AMY1 CN group comprised four females and one male to assess gender-related differences.
in body composition measures of the group, such as BF% and BMI, all of which are associated with an impaired glucose response. The study also included an assessment of the insulin sensitivity index in the participants, which was calculated using OGTT. It was found that females had a higher insulin sensitivity index than males, even after adjusting for age and BMI (Kautzky-Willer et al., 2012), which influences the results significantly since the low CN group has one male and four females. It is noticeable that our participants were active, and women carrying low CN were more active than the high CN group. The study by Lundsgaard and Kiens confirmed that healthy women are more sensitive to insulin than men when matched for physical fitness (41 % increase in whole-body insulin sensitivity) because of enhanced glucose uptake by the skeletal muscle in women (Lundsgaard and Kiens, 2014). Generally, gender differences in this regard have been reported to respond to muscle characteristics. Women have a higher proportion of type I fibers and capillary density, which favours enhanced insulin action (Lundsgaard and Kiens, 2014).

- This study did not assess the influence of AMY1 CN over glucose response during exercise or CHO metabolism at rest compared to exercise. Thus, we expect our study design to provide a direction for future research.

- In pilot study Chapter 5, moreover, 4 of study participants had higher than expected HOMA-IR values and low Matsuda insulin sensitivity indexes. Further, significant inter-assay CV % in insulin concentration in both groups, with may influenced the reliability of our data.
6.2 Future Directions

- Further research is needed to assess the association between increasing AMY1 copy number, BMI, and body fatness in the general adult population according to BMI classification and physical activity in males and females.

- Further studies are needed to confirm the current findings about the glycaemic responses to CHO solutions using larger-scale active and non-active samples. The relation between AMY1 CN and BMI depends on several other underlying factors, including weight, height, and metabolic responses. Therefore, it would be incorrect to hold response to dextrose or CHO solutions as the only factor responsible for this association. A more thorough approach should also be considered before any conclusions or theories are driven from the given data.

- There remains controversy in the literature about the association between AMY1 CN and BMI. Variation in dietary preferences (starch ingestion) between study populations may explain some of the differences in the strength of the association between AMY1 CN and BMI. Two studies found such an association between CNV of AMY1 and obesity only among people who consumed more starch. So, the influence of a healthy diet and the level of energy expenditure and physical activity needs to be examined as prevention tools to minimize the potential impact of low CN of AMY1 in increasing BMI. The AMY1 gene was also found to be responsible for influencing several other factors as well. A novel observation now includes the impact that AMY1 has on the development or progression of diabetes.
mellitus and HbA1c. Age, alcohol consumption, gender, systolic and diastolic blood pressure, and blood cholesterol levels all seem to play one or the other role in interacting with AMY1 and causing possible incremental or decremented changes.

- The link between salivary amylase enzyme activity and the increasing copy number of its gene has been confirmed in published studies. Salivary amylase enzyme activity has suggested predisposing to obesity. However, the study’s findings were still conflicting in the association between AMY1 CNV and obesity. Such conflicting results warrant a reassessment of the association between salivary amylase enzyme activity and CNV of AMY1. Also, a large sample size should assess the degree of association between the amylase enzyme, CNV, and obesity.

- Besides insulin, many other hormones are associated with overweight, obesity, weight loss, and weight regain. For instance, previous studies have shown that fasting ghrelin concentration correlates with weight regain in patients trying to lose weight (Thom et al., 2020). Moreover, appetite-related hormones such as leptin and ghrelin are known to modulate not only perceived hunger but also perceived stress levels and adaptive thermogenesis (Thom et al., 2021).

Here, we provide a study design as a background for further research. The study proposal comprised two phases: phase I included recommendations from our findings in the pilot study in Chapter 5, whereas phase II included suggestions to have in future
research topics to provide a more accurate comprehension of AMY1 CN and its role in carbohydrate metabolism (Figure 6-3). We suggest conducting the two phases of the proposal in one single study to compare the data of both phases in the same population.

**Phase 1 (CHO Metabolism During Rest Status)**

Repeating the pilot study design reported in Chapter 5 and taking the below list into consideration (Figure 6-3):

- According to our power calculation results, a sample size of 62 (males n= 31 and females n= 31) should be used.

- The participants should be in similar categories regarding gender, age, BMI, body fat, alcohol consumption, smoking status, and physical activity level.

- Insulin sensitivity/resistance rather than FBI and FBG should be assessed during inclusion and exclusion criteria.

- The relation between AMY CN 1 and BMI / glycaemic responses to CHO solutions depends on several other factors, most of which rely solely on the people under observation. Therefore, it would be incorrect to hold response to dextrose or CHO solutions as the only responsible approach. Factors involving individual and lifestyle factors such as dietary intake, physical activity, alcohol consumption, systolic and diastolic blood pressure, and blood cholesterol levels should also be considered.
• The participants should follow the same diet according to each individual’s recommended daily intake for 24 hours before testing to control for dietary influences on metabolism during exercise and biomarker results.

• OGTT is an excellent method to assess the glycaemic response during resting status. But the glucose/starch ingestion should be increased to 75 g, and the time should be extended to an interval of 4 hours and 15 minutes.

• A minimum of two days apart between experimental days.

**Phase II (CHO Metabolism During Exercise Status)**

Phase II is the second part of the study, including the research about AMY1 CNV on CHO metabolism during rest (Phase I). The participants are asked to attend the laboratory in the morning on three separate occasions, having fasted for 8-12 hours and rested overnight. Participants should perform each exercise session at the same time of the day, between 07:00 and 11:00 AM. There should be a minimum of two days apart from testing to minimize the influence of circadian variance. Researchers should also request to avoid alcohol consumption, strenuous exercise, caffeine, and tobacco for 24 hours before each experimental exercise session.

In the first session, a lactate threshold test should be carried out on each participant. In the second and third sessions, RMR and exercise metabolic testing should be completed with either glucose or starch ingestion.
Lactate Threshold (LT): This test aims to establish the individual’s lactate threshold and determine the level of energy they used from CHO through the anaerobic system. Consequently, it is possible to use 100 % force of the individual’s lactate threshold force in testing for their exercise metabolism. All participants will arrive at the lab at about 7:00 am - 8:00 am, rest for 15 minutes, then receive an attached heart rate monitor to allow continuous monitoring of their heart rate during exercise. A blood sample should be taken via a finger prick. Participants should then start their test on the resistance bicycle, with the starting power at 50 watts (suitable for both genders). The degree of intensity of the test continues to rise until the participants become exhausted. The power increases should be set at 30w for males and 20w for females. There should be at least six levels, each lasting three minutes with no rest in-between. Towards the end of each level, a finger-prick blood sample should be taken (Figure 6-1).

![Diagram of Lactate Threshold Test in the First Session.](image)
6.2.1 Metabolic Measurements

6.2.1.1 Resting Metabolic Rate (RMR)

Resting metabolic rate will be measured by indirect calorimetry with Oxycon pro (Carefusion, CA). Flow-volume sensor calibration was performed to ensure the system measuring Oxycon (consisting of the amplifier, Triple V, and the pressure transducer) is functioning correctly. A calibrated 3-L syringe connected to the Triple V assembly should be used for this purpose. A series of completed pumps of the syringe are repeated until the percentage difference between the current and the previous volume calibrations is less than 1%, as explained by Carter and Jeukendrup (2002).

Participants will come to the lab between 7 and 10 am; a heart rate monitor (Polar H10) then will attach to their chest before being put in a supine position. A ventilation hood is placed over the head of the participant and while connected to an oxygen supply. Measurements from the first 15 minutes of recording are not used in the analysis as this period reflects the participant coming to rest and getting acquainted with the experimental conditions. However, the following 30 minutes or more are recorded on the software.

6.2.1.2 Exercise Metabolic Testing

The participants will provide a glucose or starch solution (as explained in Table 5-2, Chapter 5). Five minutes after ingesting, a blood sample was taken. Participants then warm up on the power bicycle with a 50% power of their lactate threshold force (as determined by the LT in the first session) for 2 minutes, followed by 100% of the individual lactate threshold force. The duration and intensity of the exercise is 60 min
cycling at 100% force LT (It will occur in untrained individuals at about 50-60% of VO₂ max and about 70-80% of VO₂ max in trained individuals). This methodology is in accordance with the American College of Sports Medicine guidelines for prescribing exercise for health benefits. As such, it was thought to reflect one type of exercise session typical of those undertaken by the general population rather than a performance trial, more typical in athletic performance. Blood samples are taken every 10-minute interval from the cannula during the formal test. A meal is provided after all the procedures are finished (Figure 6-2).

Figure 6-2 Diagram of RMR Exercise Metabolic Testing in the Second and Third Sessions.

6.2.1.3 Suggestions Calculations for Carbohydrate and fat oxidation during rest and exercise by Indirect Calorimetry (IC)

IC allows measuring respiratory exchange ratio RER. It is the term utilised to represent fuel oxidation by IC made at the cellular level, similar to respiratory quotient (RQ), the ratio of CO₂ produced / O₂ consumed (Mtaweh et al., 2018). RQ is measured in the study reported in chapter 6 with other parameters that can be derived from IC, such as the substrate of fuel utilization (CHO and Fat). During CHO metabolism, there is an equal amount of CO₂ produced for every O₂ consumed (RER = 1.0). During fat
metabolism, there is less CO₂ produced for every O₂ consumed (Haugen et al., 2007; Gupta et al., 2017).

The accuracy and reliability of CI in metabolic acquired data can lead to significant differences in the results. The following point should be considered.

- The variables of age, gender, PA level, lean mass, and other metabolic variations should be considered during the studies’ repetition to minimize possible confounding effects on the analysis of CHO metabolism.

- Gas analysers should be calibrated following the manufactural instructions.

- Alcohol burns should be performed when measuring energy expenditure (EE) and calculated substrate utilisation using the respiratory exchange ratio (RER = VCO₂ / VO₂) and calculating CHO and fat oxidation from the Oxycon instrument. A known volume of alcohol is burned, and the actual yield of O₂ and CO₂ should be compared to theoretical values calculated based on the moles of alcohol used. Thus, the study has an extensive variability within-subject data obtained on RQ substrate oxidation in the same group.

- The accuracy and reliability of CI in metabolic acquired data can lead to significant differences in the results, following point should be considered.
The Resting metabolic rate (RMR) measured by indirect calorimetry is suggested to measure oxygen consumption during the resting state. Breathing generates rapid readings by measuring short intervals of gas samples (Delsoglio et al., 2019). This method can be used during exercise for the measurement of oxygen consumption ($\text{VO}_2$, ml / min), carbon dioxide production ($\text{VCO}_2$, ml / min), and respiratory exchange ratio (RER). It is a standard tool of exercise physiology used to assess aerobic capacity and energy expenditure. In this regard, one study performed in postmenopausal women showed that the respiratory quotient and substrate use accounts for up to 40.2% of the variance in energy expenditure and changes in fat mass (Barwell et al., 2009). RMR, CHO, and fat combustion can be calculated using the Weir formula and were expressed as follows (Frayn, 1983; Péronnet et al., 1991; Carter and Jeukendrup, 2002):

\[
\text{RQ} = \frac{\text{VCO}_2}{\text{VO}_2}
\]

\[
\text{RMR (Kcal/24h)} = \{(3.9 \times \text{VO}_2) + (1.1 \times \text{VCO}_2) \times 1.44\}
\]

\[
\text{CHO-oxidation (g/min)} = \{(4.55 \times \text{VCO}_2 \text{~min/L}) - (3.23 \times (\text{VO}_2 \text{~min/L})}\}
\]

\[
\text{Fat oxidation (g/min)} = \{(1.69 \times \text{VO}_2 \text{~min/L}) - (1.69 \times (\text{VCO}_2 \text{~min/L})}\}
6.3 Conclusion

To summarise, after comparing data from the present studies with all the previous research on the topic, it is clear that some changes are required to build the methodology for future investigations. Several changes need to be made, such as including an equal ratio of both males and females in the same study. The dietary intake of the glucose and starch solution also needs regulation in the context of adjusting their dosages and timings.
Additionally, the sample size criteria need to be strictly checked. Several other factors, ranging from the participants’ physiological factors to their dietary responses, should also be considered to improve the accuracy and relevance of the results of the upcoming studies.
List of References


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Appendix 1

1.1 Participant’s Characteristics

The following (Table 4-1) contains the mean ± SD for the demographic variables for overall group, as well as in two categories of the gender for the respective variables along with the $P$-values. Furthermore, the data contains the respondents having the CNV greater than 10 copies of AMY1. This study did not record any significant differences in age, CNVs, body fat and PA between males and females. Expected differences were found between males and females in weight, height, WC, BMI as the corresponding $P$-values were < 0.05.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male ($n=15$)</th>
<th>Female ($n=11$)</th>
<th>All ($n=26$)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>22.50 ± 4.2</td>
<td>23.60 ± 4.3</td>
<td>22.40 ± 3.7</td>
<td>0.551B</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>82.31 ± 12.4</td>
<td>59.59 ± 7.5</td>
<td>70.22 ± 13.4</td>
<td>&lt;0.001A</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>178.1 ± 6.2</td>
<td>163.9 ± 8.0</td>
<td>172.1 ± 9.70</td>
<td>&lt;0.001A</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>86.55 ± 10.4</td>
<td>62.00±2 2.2</td>
<td>77.40 ± 9.41</td>
<td>0.004A</td>
</tr>
<tr>
<td>BMI (kg.m$^2$)</td>
<td>25.89 ± 4.3</td>
<td>22.16 ± 2.6</td>
<td>23.94 ± 3.52</td>
<td>0.020A</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>25.57±8.4</td>
<td>31.3 ± 5.4</td>
<td>26.70 ± 9.19</td>
<td>0.059B</td>
</tr>
<tr>
<td>PA (Hour/w)</td>
<td>13.0 ± 8.9</td>
<td>19.19 ± 7.8</td>
<td>16.68 ± 12.76</td>
<td>0.081A</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD and CN = copy number, WC = waist circumference, BMI = body mass index. Fat % from DEXA scan. PA = physical activity. (A) $P$ values < 0.05 independent sample t-test. (B) $P$ values < 0.05 Maan-Whitney U test to comparison between males and females.

1.2 AMY1 Copy number Detection in Each Group

The distribution of CN in male and female participants is shown in Figure 1-1 and 1-2 respectively. The mean CNs did not significantly differ between males and females ($P = 0.614$). reported in below table 1-2 along with the number of patients having CNV>=10.
Figure 1-1 Distribution of CNV copy number>=10 in males and females.
Table 1-2 Comparison of CNV>=10 in two categories of gender

<table>
<thead>
<tr>
<th>Groups</th>
<th>No</th>
<th>Mean ± SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>15</td>
<td>11.53 ± 1.356</td>
<td>0.614</td>
</tr>
<tr>
<td>Females</td>
<td>11</td>
<td>11.82 ± 1.471</td>
<td></td>
</tr>
</tbody>
</table>

1.3 Body Composition and CNV greater than 10 (CNV>=10)

The correlation and regression analysis results are showing in the following Table 1-3 along with the goodness of fit measure for the underlined model having CNV >=10 as a dependent and Height, weight, BMI, WC, and Total BF% as dependent variables. Under the men category of Gender, no significant relationships were found between CNV>=10 and height (r = -0.362, p = 0.186), body weight (r = 0.186, p = 0.362), BMI (r = 0.227, p = 0.415), WC (r = -0.010, p = 0.977) and BF% (p = 0.661, r = -0.123).

Furthermore, there exist a positive correlation between the CNV and Weight, BMI, but the correlation between the CNV and Height, WC and Body fat are negative. Similarly, for females, no significant correlations are observed between CNV for height (r = -0.173, p = 0.661), weight (r = -0.173, p = 0.611); BMI (r = 0.136, p = 0.899), WC (r = -0.315, p = 0.409) and BF (r = -0.109, p = 0.749). Further, no significant correlation has been detected between the whole population CNV and Height, Weight, BMI, WC and BF as the corresponding p-values for the β coefficients of linear regression are greater than or equals to 0.05.
Table 4-2: Linear regression results between CNV copy number greater than or equals to 10 and height, markers of adiposity.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Males (n=15)</th>
<th>Females (n=11)</th>
<th>All (n=26)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>R</td>
<td>B</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>1.985</td>
<td>-0.362</td>
<td>14.36</td>
</tr>
<tr>
<td>Weight(kg)</td>
<td>-7.687</td>
<td>0.362</td>
<td>-30.49</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>7.376</td>
<td>0.227</td>
<td>29.75</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>0.174</td>
<td>-0.010</td>
<td>-0.309</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>-0.760</td>
<td>-0.123</td>
<td>-1.556</td>
</tr>
<tr>
<td>R²</td>
<td>0.154</td>
<td>0.845</td>
<td>0.281</td>
</tr>
</tbody>
</table>

Adiposity markers including weight, BMI, waist circumference (WC), and fat percentage in all participants, male and females. P values < 0.05 indicates a significant coefficient (β), corr. Denotes the correlation coefficient between the CNV copy number greater than or equals to 10, and other variables, R-squared (R²) is also reported for each model.

The above Table 4-2 contains the results of the regression analysis used to check the effect of the independent variables namely Height, Weight, BMI, WC and Body fat on the dependent variable AMY1 CN greater than or equals to 10 copy number. It is observable that under the male category of the gender, a one unit change in the Height, BMI, and Body fat results into the 1.985, 7.376 and 0.174 unit increase in the value of CNV copy number. Similarly for a unit change in CNV CN resultants a decline in Weight and WC by 7.687 and 0.760, respectively. The value of the R² (0.154) means that 15.4% of variation in Height, Weight, BMI, WC and Body fat explained by AMY1 CN, but 84.6% variation in Height, Weight, BMI, WC and Body fat cannot explained by AMY1 CNV only. Similarly, under the category of the female total of 84.5% variation in the variables is explained by AMY1 CN and the rest of the variation 15.5% of the variations cannot explained the underline model. For the total population samples, only 28.1% variation in the Height, Weight, BMI, WC and Body Fat is explained by the AMY1 CN, and rest of the variation 71.9% of the variance cannot be explained by AMY1 CNV only.
Appendix 2

Sample size calculation in the result of glucose in the pilot study, reported in Chapter 5. Sample size was calculated using G Power software. A total of 62 participants (31 participants in each group) will be required to conduct the study, based on following assumptions.

- Effect size $d = 0.1275$
- $\alpha$ err prob $= 0.05$
- Power (1-$\beta$ err prob) $= 0.80$
- Number of groups $= 2$
- Number of measurements $= 7$
- Corr among repeated measures $= 0.5$

Effect size was calculated based on lowest Partial Eta$^2$ for Glucose $= 0.016$
Appendix 3

Sample size calculation in the result of insulin in the pilot study, reported in Chapter 5. Sample size was calculated using G Power software. A total of 26 participants (13 participants in each group) will be required to conduct the study, based on following assumptions.

- Effect size $d = 0.2014515$
- $\alpha$ err prob = 0.05
- Power (1-$\beta$ err prob) = 0.80
- Number of groups = 2
- Number of measurements = 7
- Corr among repeated measures = 0.5

Effect size was calculated based on lowest Partial Eta$^2$ for Insulin = 0.039

(Screenshot for G*Power software)
Sample size calculation in the result of total-cholesterol in the pilot study, reported in Chapter 5

Sample size was calculated using G Power software. A total of 46 participants (23 participants each group) will be required to conduct the study, based on following assumptions.

\[
\text{Effect size } d = 0.1499830
\]
\[
\alpha \text{ err prob} = 0.05
\]
\[
\text{Power (1-}\beta \text{ err prob)} = 0.80
\]
\[
\text{Number of groups} = 2
\]
\[
\text{Number of measurements} = 7
\]
\[
\text{Corr among repeated measures} = 0.5
\]

Effect size was calculated based on Partial Eta\(^2\) for Cholesterol= 0.022

(Screenshot for G*Power software)
Appendix 5

Sample size calculation in the result of TG in the pilot study, reported in Chapter 5

Sample size was calculated using G Power software. A total of 18 participants (9 participants in each group) will be required to conduct the study, based on following assumptions:

\[
\begin{align*}
\text{Effect size } d &= 0.2503982 \\
\alpha \text{ err prob} &= 0.05 \\
\text{Power (1-}\beta \text{ err prob)} &= 0.80 \\
\text{Number of groups} &= 2 \\
\text{Number of measurements} &= 7 \\
\text{Corr among repeated measures} &= 0.5
\end{align*}
\]

Effect size was calculated based on Partial Eta\(^2\) for TG= 0.059

![Screenshot for G*Power software](image-url)

(Screenshot for G*Power software)
Appendix 6

Sample size calculation in the result of NEFA in the pilot study, reported in Chapter 5. Sample size was calculated using G Power software. A total of 16 participants (8 participants in each group) will be required to conduct the study, based on following assumptions:

- Effect size $d = 0.2636640$
- $\alpha$ err prob = 0.05
- Power (1-$\beta$ err prob) = 0.80
- Number of groups = 2
- Number of measurements = 7
- Corr among repeated measures = 0.5

Effect size was calculated based on Partial Eta$^2$ for NEFA= 0.065