Tesi di dottorato in Scienze biomediche integrate e bioetica, di Antonio Di Mauro,
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Università Campus Bio-Medico di Roma

PhD course in Scienze Biomediche Integrate e Bioetica

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A Mediterranean-pattern Meal Increases GLP-1 and Oxyntomodulin more than an Energy-matched High Fiber Plant-Based Meal in Type 2 Diabetes patients: A Crossover, Randomized, Controlled Inpatient Physiology Study.

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ABSTRACT

Background: Type-2 diabetes (T2D) is a multifactorial metabolic burden whose metabolic features include alterations in GLP-1 secretion and ultimately hunger/satiety circuit derangement. Manipulating the composition of the diet in order to promote GLP-1 secretion may represent a promising lifestyle strategy for obesity and T2D management.

Aims: The objective of this study was to assess the post-prandial profile of appetite-regulating hormones and assessing the post-prandial appetite ratings using a Visual Analogue Scale (VAS), as well as measuring the fasting and postprandial glucose/insulin responses in overweight and/or obese, well controlled patients with T2D.

Materials and Methods: Twelve T2D patients (M:F = 7:5) aged 63.1±8.5 years were enrolled in a randomized, controlled, crossover trial. Subjects consumed on two different days, at one-week interval, an experimental High Fiber Vegetarian meal (HFV) rich in dietary carbohydrate and fiber in comparison with a standard, Mediterranean-like meal (MED). The two meals were isocaloric. Appetite ratings, glucose/insulin and gastrointestinal hormone responses were assessed either at fasting and every 30' until 210' for GLP-1 and Oxyntomodulin and 240' for glucose and insulin after the ingestion of the meal. Subjects consuming the MED meal exhibited significant and higher levels of GLP-1 and oxyntomodulin across the 210' compared to the HFV group ($p<.05$ one-sided for both the hormones). The 210'-GLP-1 and Oxyntomodulin AUC were significantly increased in the MED group ($P < .022$ and $P < .023$, respectively). Both the MED and HFV meals consumption induced a biphasic-shaped secretion pattern over time but the MED consumption produced a significant

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delayed second GLP-1 peak at 150' compared to the HFV meal (56 ± 21 pg/mL Vs 44 ± 18 pg/mL, respectively ***P* <.05**), delaying the second peak one hour and half after the HFV-M group. The MED group maintained significant and consistent decreased levels of plasma over time compared to the HFV group (***P*< .039**) and the 240-minute glycemic AUC was significantly higher in the HFV compared to the MED meal, even after the adjustment for age and gender, BMI and HbA1c (***P*<.006**). In addition, the 240-minute glycemic iAUC was significantly higher in the MED meal (***P*<.002).** No major significant changes in VAS and postprandial insulin profile between the two groups were assessed.

Conclusions: A Mediterranean type of meal is more effective in increasing postprandial secretion of GLP-1 and oxyntomodulin and reducing postprandial plasma glucose levels in overweight/obese T2D patients. These changes did not influence the appetite ratings evaluated through the visual analogue scale. These findings suggest that, in acute, diet-related endocrine release may not be related to changes in self-rated hunger/satiety, possibly because of T2D metabolic features. Either on short and long term, diet influences gut hormone levels in T2D.

Keywords – type-2 diabetes; GLP-1; oxyntomodulin; Visual Analogue Scale; hunger; satiety; nutrition; diet.

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INTRODUCTION

Epidemiology and features of diabetes

Diabetes is a multifactorial burden worldwide whose implications in world populations can be introduced by a few considerations. The International Diabetes Federation (IDF) has established that in 2017 almost 425 million people were affected by this metabolic condition, such number expected to rise to almost 629 million people throughout the world within 2045 [1]. Disregarding of metabolic features, diabetes diagnosis has been related to the death of half a million people in Europe alone in 2017, such number reaching 1.3 million people in western pacific area (Australia, China, Indonesia, Japan, etc.). As diabetes treatment is much more costly than prevention, diabetes-related healthcare expenditure in 2017 was estimated around 727 USD billion worldwide for adult population only (20 to 79 years) – 377 USD million in the United States and 166 in Europe [1]. The prevalence of diabetes for women aged 20 to 79 years is estimated to be 8.4%, slightly lower than among men (9.1%). As of 2017, Italy ranked 8th in top 10 countries with the number of people with diabetes older than 65 years (2.6 million) [1].

The field of diabetes care is rapidly changing as new research, technology, and treatments that can improve the health and well-being of people with diabetes continue to emerge. As the American Diabetes Association (ADA) has stated in the last few annual guidelines, "there is no universal ideal macronutrient distribution", which is why eating plans should be individualized [2]. Over the past 10 years, the proportion of patients with diabetes who achieve recommended glycated hemoglobin (A1C), blood pressure, and low-density lipoprotein (LDL) cholesterol levels has increased [3]. The mean A1C nationally among people with diabetes has declined from 7.6% (60

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mmol/mol) in 1999–2002 to 7.2% (55 mmol/mol) in 2007–2010 based on the National Health and Nutrition Examination Survey (NHANES), with younger adults less likely to meet treatment targets than older adults [3]. This has been accompanied by improvements in cardiovascular outcomes and has led to substantial reductions in end-stage microvascular complications. However, 33–49% of patients still do not meet targets for glycemic, blood pressure, or cholesterol control, and only 14% meet targets for all three measures while also avoiding smoking [3].

Numerous interventions to improve adherence to the recommended standards have been implemented but still several factors are hindering an acceptable target, including nutritional aspects. Food insecurity (FI) is the unreliable availability of nutritious food and the inability to consistently obtain food without resorting to socially unacceptable practices. Over 14% (1 out of 7 people) of the United States population is food insecure. The rate is higher in some racial/ethnic minority groups, including African American and Latino populations, in low-income households, and in homes headed by a single mother [1], with the risk for T2D being increased twofold in those with FI [4]. Finally, in the last years World Health Organization (WHO) has issued a commission to address health equity and the determinants of health [5].

Diabetes classification

Diabetes can be classified into the following general categories:

1. Type-1 diabetes, due to autoimmune b-cell destruction, usually leading to absolute insulin deficiency, which represents about 10% of total cases [1].
2. Type-2 diabetes, due to a progressive loss of b-cell insulin secretion frequently on the background of insulin resistance

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3. Gestational diabetes mellitus, diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation
4. Specific types of diabetes due to other causes, e.g. monogenic diabetes syndromes such as neonatal diabetes and maturity-onset diabetes of the young (MODY), latent autoimmune diabetes of the adult (LADA), diseases of the exocrine pancreas such as cystic fibrosis and pancreatitis, and drug- or chemical-induced diabetes (such as with glucocorticoid use, in the treatment of HIV/AIDS, or after organ transplantation)

Type-1 diabetes (T1D) and type-2 diabetes (T2D) are heterogeneous diseases in which clinical presentation and disease progression may vary considerably. Classification is important for determining therapy, but some individuals cannot be clearly classified as having T1D or T2D at the time of diagnosis. The traditional paradigms of T2D occurring only in adults and T1D only in children are no longer accurate, as both diseases occur in both age-groups. Children with T1D typically present with the hallmark symptoms of polyuria/polydipsia, and approximately one-third present with diabetic ketoacidosis (DKA) [2]. The onset of T1D may be more variable in adults, and they may not present with the classic symptoms seen in children. Occasionally, patients with T2D may present with DKA, particularly ethnic minorities. Although difficulties in distinguishing diabetes type may occur in all age-groups at onset, the true diagnosis becomes more obvious over time. In both T1D and T2D, various genetic and environmental factors can result in the progressive loss of b-cell mass and/or function that manifests clinically as hyperglycemia. Once hyperglycemia occurs, patients with all forms of diabetes are at risk for developing the

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same chronic complications, although rates of progression may differ. The identification of individualized therapies for diabetes in the future will require better characterization of the many paths to b-cell demise or dysfunction [6].

Characterization of the underlying pathophysiology is more developed in T1D than in T2D as it is now clear from studies of first-degree T1D relatives that the persistent presence of 2 or more autoantibodies is an almost certain predictor of clinical hyperglycemia and diabetes. The rate of progression is dependent on the age at first detection of antibody, number of antibodies, antibody specificity, and antibody titer. Glucose and glycated hemoglobin (A1C) levels rise well before the clinical onset of diabetes, making diagnosis feasible well before the onset of DKA.

The paths to b-cell demise and dysfunction are less well defined in T2D, but deficient b-cell insulin secretion, appears to be the common denominator, particularly in the setting of insulin resistance. Characterization of subtypes of this heterogeneous disorder have been developed and validated in Scandinavian and Northern European populations but have not been confirmed in other ethnic and racial groups. T2D is primarily associated with insulin secretory defects related to inflammation and metabolic stress among other contributors, including genetic factors. Future classification schemes for diabetes will likely focus on the pathophysiology of the underlying b-cell dysfunction and the stage of disease as indicated by glucose status (normal, impaired, or diabetes) [6].

Diabetes diagnosis may be based on plasma glucose criteria, either the fasting plasma glucose (FPG) or the 2-h plasma glucose (2-h PG) value during a 75-g oral glucose tolerance test (OGTT), or A1C criteria [7]. Generally, FPG, 2-h PG during 75-g OGTT, and A1C are equally appropriate for diagnostic testing. It should be noted

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that the tests do not necessarily detect diabetes in the same individuals. The efficacy of interventions for primary prevention of type 2 diabetes [8,9] has primarily been demonstrated among individuals who have impaired glucose tolerance (IGT) with or without elevated fasting glucose, not for individuals with isolated impaired fasting glucose (IFG) or for those with prediabetes defined by A1C criteria. The same tests may be used to screen for and diagnose diabetes and to detect individuals with prediabetes. Diabetes may be identified anywhere along the spectrum of clinical scenarios: in seemingly low-risk individuals who happen to have glucose testing, in individuals tested based on diabetes risk assessment, and in symptomatic patients.

The FPG and 2-h PG may be used to diagnose diabetes as well. The concordance between the FPG and 2-h PG tests is imperfect, as is the concordance between A1C and either glucose-based test. Numerous studies have confirmed that compared with FPG and A1C cutoffs, the 2-h PG value diagnoses more people with diabetes. The A1C has several advantages compared with the FPG and OGTT, including greater convenience (fasting not required), greater preanalytical stability, and less day-to-day perturbations during stress and illness. However, these advantages may be offset by the lower sensitivity of A1C at the designated cut point, greater cost, limited availability of A1C testing in certain regions of the developing world, and the imperfect correlation between A1C and average glucose in certain individuals. National Health and Nutrition Examination Survey (NHANES) data indicate that an A1C cutoff >6.5% (48 mmol/mol) identifies a prevalence of undiagnosed diabetes that is one-third of that using glucose criteria [10]. When using A1C to diagnose diabetes, it is important to recognize that A1C is an indirect measure of average blood glucose levels and to take other factors into consideration that may impact hemoglobin

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glycation independently of glycemia including age, ethnicity, and anemia/hemoglobinopathies. The epidemiological studies that formed the basis for recommending A1C to diagnose diabetes included only adult populations so far, leaving uncertainties on whether A1C and the same A1C cutoff point should be used to diagnose diabetes in children and adolescents [10, 11]. In addition, African Americans heterozygous for the common hemoglobin variant HbS may have, for any given level of mean glycemia, lower A1C by about 0.3% than those without the trait [12]. Another genetic variant, X-linked glucose-6-phosphate dehydrogenase (G6PDH) G202A, carried by 11% of African Americans, was associated with a decrease in A1C of about 0.8% in hemizygous men and 0.7% in homozygous women compared with those without the variant [13]. Even in the absence of hemoglobin variants, A1C levels may vary with ethnicity independently of glycemia [14-16]. For example, African Americans may have higher A1C levels than non-Hispanic whites with similar fasting and post-glucose load glucose levels [17], and A1C levels may be higher for a given mean glucose concentration when measured with continuous glucose monitoring devices [18].

Regarding diabetes diagnosis, unless there is a clear clinical context (e.g., patient in a hyperglycemic crisis or with classic symptoms of hyperglycemia and a random plasma glucose >200 mg/dL [11.1 mmol/L]), a second test is required for confirmation. It is recommended that the same test be repeated, or a different test be performed without delay, using a new blood sample for confirmation. For example, if the A1C is 7% (53 mmol/mol) and a repeat result is 6.8% (51 mmol/mol), the diagnosis of diabetes is confirmed. If two different tests (such as A1C and FPG) are both above the diagnostic threshold, this also confirms the diagnosis. On the other hand, if a

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patient has discordant results from two different tests, then the test result that is above the diagnostic cut point should be repeated, with consideration of the possibility of A1C assay interference. The diagnosis is made on the basis of the confirmed test. For example, if a patient meets the diabetes criterion of the A1C (two results >6.5% [48 mmol/mol]) but not FPG (<126 mg/dL [7.0 mmol/L]), that person should nevertheless be considered to have diabetes.

Since all the tests have preanalytical and analytic variability, it is possible that an abnormal result (i.e., above the diagnostic threshold), when repeated, will produce a value below the diagnostic cut point. This scenario is likely for FPG and 2-h PG if the glucose samples remain at room temperature and are not centrifuged promptly. Because of the potential for preanalytical variability, it is critical that samples for plasma glucose be spun and separated immediately after they are drawn. If patients have test results near the margins of the diagnostic threshold, the health care professional should follow the patient closely and repeat the test in 3–6 months.

Prediabetes

Prediabetes occurs for individuals whose glucose levels do not meet the criteria for diabetes but are too high to be considered normal [19, 20]. Patients with prediabetes are defined by the presence of IFG and/or IGT and/or A1C 5.7–6.4% (39–47 mmol/mol). Prediabetes should not be viewed as a clinical entity in its own right but rather as an increased risk for diabetes and cardiovascular disease (CVD). Prediabetes is associated with obesity (especially abdominal or visceral obesity), dyslipidemia with high triglycerides and/or low HDL cholesterol, and hypertension.

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Regarding prediabetes diagnosis, this occurs with FPG levels between 100 and 125 mg/dL (5.6 and 6.9 mmol/L) [20, 21] and IGT as 2-h PG during 75-g OGTT levels between 140 and 199 mg/dL (7.8 and 11 mmol/L) [19]. It is worth mentioning that WHO defines the IFG cutoff at 110 mg/dL (6.1 mmol/L).

As with the glucose measures, several prospective studies that used A1C to predict the progression to diabetes as defined by A1C criteria demonstrated a strong, continuous association between A1C and subsequent diabetes. In a systematic review of 44,203 individuals from 16 cohort studies with a follow-up interval averaging 5.6 years (range 2.8–12 years), those with A1C between 5.5 and 6% (37 and 42 mmol/mol) had a substantially increased risk of diabetes (5-year incidence from 9 to 25%). Those with an A1C range of 6–6.5% (42-48 mmol/mol) had a 5-year risk of developing diabetes between 25 and 50% and a relative risk 20 times higher compared with A1C of 5% (31 mmol/mol) [22]. In a community-based study of African American and non-Hispanic white adults without diabetes, baseline A1C was a stronger predictor of subsequent diabetes and cardiovascular events than fasting glucose [23]. Other analyses suggest that A1C of 5.7% (39 mmol/mol) or higher is associated with a diabetes risk similar to that of the high-risk participants in the Diabetes Prevention Program (DPP) [24], and A1C at baseline was a strong predictor of the development of glucose-defined diabetes during the DPP and the study follow-up [25].

Hence, it is reasonable to consider an A1C range of 5.7–6.4% (39–47 mmol/mol) as identifying individuals with prediabetes. Similar to those with IFG and/or IGT, individuals with A1C of 5.7–6.4% (39–47 mmol/mol) should be informed of their increased risk for diabetes and CVD and counseled about effective strategies

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to lower their risks. Similar to glucose measurements, the continuum of risk is curvilinear, so as A1C rises, the diabetes risk rises disproportionately [22].

Type-1 diabetes

In a patient with classic symptoms, measurement of plasma glucose is sufficient to diagnose diabetes (symptoms of hyperglycemia or hyperglycemic crisis plus a random plasma glucose above 200 mg/ dL [11.1 mmol/L]). In these cases, knowing the plasma glucose level is critical because, in addition to confirming that symptoms are due to diabetes, it will inform management decisions. Some providers may also want to know the A1C to determine how long a patient has had hyperglycemia.

T1D is clinically defined as immune-mediated diabetes, previously known as "insulin- dependent diabetes" or "juvenile-onset diabetes," accounts for 5–10% of diabetes and is due to cellular-mediated autoimmune destruction of the pancreatic b-cells. Autoimmune markers include islet cell autoantibodies and autoantibodies to GAD (GAD65), insulin, the tyrosine phosphatases IA-2 and IA-2b, and ZnT8. T1D is defined by the presence of one or more of these autoimmune markers. The disease has strong HLA associations, with linkage to the DQA and DQB genes. These HLA-DR/DQ alleles can be either predisposing or protective. The rate of b-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Children and adolescents may present with DKA as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and/or DKA with infection or other stress. Adults may retain sufficient b-cell function to prevent DKA for many years; such individuals eventually become dependent on insulin for survival and are at risk for

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DKA. At this latter stage of the disease, there is little or no insulin secretion, as manifested by low or undetectable levels of plasma C-peptide. Immune-mediated diabetes commonly occurs in childhood and adolescence, but it can occur at any age, even in the 8th and 9th decades of life. Autoimmune destruction of b-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined. Although patients are not typically obese when they present with T1D, obesity should not preclude the diagnosis. Patients with T1D are also prone to other autoimmune disorders such as Hashimoto thyroiditis, Graves' disease, Addison disease, celiac disease, vitiligo, autoimmune hepatitis, myasthenia gravis, and pernicious anemia. In addition, some forms of T1D have no known etiologies. These patients have permanent insulinopenia and are prone to DKA but have no evidence of b-cell autoimmunity. Although only a minority of patients with T1D fall into this category, of those who do, most are of African or Asian ancestry [1]. Individuals with this form of diabetes suffer from episodic DKA and exhibit varying degrees of insulin deficiency between episodes. This form of diabetes is strongly inherited and is not HLA associated. An absolute requirement for insulin replacement therapy in affected patients may be intermittent. According to estimates provided by the International Diabetes Federation (IDF) in 2017 Diabetes Atlas, the incidence and prevalence of T1D is increasing, with more than 130,000 newly diagnosed cases each year for 0-19-year-old subjects only [1]. Patients with T1D often present with acute symptoms of diabetes and markedly elevated blood glucose levels, and approximately one-third are diagnosed with life-threatening DKA [26]. Several studies indicate that measuring islet autoantibodies in relatives of those with T1D may identify individuals who are at risk for developing the disease [27]. Such testing, coupled with education about

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diabetes symptoms and close follow-up, may enable earlier identification of T1D onset. A study reported the risk of progression to T1D from the time of seroconversion to autoantibody positivity in three pediatric cohorts from Finland, Germany, and the U.S. Of the 585 children who developed more than two autoantibodies, nearly 70% developed T1D within 10 years and 84% within 15 years [28]. These findings are significant because while the German group was recruited from offspring of parents with T1D, the Finnish and American groups were recruited from the general population. Remarkably, the findings in all three groups were the same, suggesting that the same sequence of events led to clinical disease in both sporadic and familial cases of T1D. Indeed, the risk increases as the number of relevant autoantibodies detected increases [29]. Although there is currently a lack of accepted screening programs, one should consider referring relatives of those with T1D for antibody testing for risk assessment in the setting of a clinical research study. Widespread clinical testing of asymptomatic low-risk individuals is not currently recommended due to lack of approved therapeutic interventions. Individuals who test positive should be counseled about the risk of developing diabetes, diabetes symptoms, and DKA prevention.

Type-2 diabetes

Type 2 diabetes (T2D), previously referred to as "noninsulin-dependent diabetes" or "adult-onset diabetes," accounts for 90-95% of all diabetes [1]. This form encompasses individuals who have relative rather than absolute insulin deficiency and have peripheral insulin resistance. At least initially, and often throughout their lifetime, these individuals may not need insulin treatment to survive.

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There are various causes of T2D. Although the specific etiologies are not known, autoimmune destruction of b-cells does not occur, and patients do not have any of the other known causes of diabetes. Most but not all patients with T2D are overweight or obese. Excess weight itself causes some degree of insulin resistance. Patients who are not obese or overweight by traditional weight criteria may have an increased percentage of body fat distributed predominantly in the abdominal region. DKA seldom occurs spontaneously in this condition; when seen, it usually arises in association with the stress of another illness such as infection or with the use of certain drugs (e.g., corticosteroids, atypical antipsychotics, and sodium-glucose cotransporter 2 inhibitors known as SGLT2) [30, 31]. T2D frequently goes undiagnosed for many years because hyperglycemia develops gradually and, at earlier stages, is often not severe enough for the patient to notice the classic diabetes symptoms. Noteworthy, half of all people aged 20 to 79 years in the world is not aware of such condition. That is, more than 212 million people as of 2017 were undiagnosed, 69.2% in Africa and almost 40% in Europe and in North America – Caribbean region [1]. These alarmingly data include economic repercussions upon healthcare services in comparison with people without diabetes. As diseased subjects may benefit from screening, early diagnosis, and appropriate care since the early stages of the disease, this would result in lower total expenditure worldwide. According to World Bank income classification, the latest IDF Atlas shows that 76.5% of all cases in low income countries is undiagnosed (9 million cases), such number being considerably reduced in high income countries (37.3%, equal to 32.9 million cases) [1]. Whereas patients with T2D may have insulin levels that appear normal or elevated, the higher blood glucose levels in these patients would be expected to result in even higher insulin values had their b-

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cell function been normal. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. Insulin resistance may improve with weight reduction and/or pharmacologic treatment of hyperglycemia but is seldom restored to normal.

The risk of developing T2D increases with age, obesity, and lack of physical activity. It occurs more frequently in women with prior GDM, in those with hypertension or dyslipidemia, and in certain racial/ethnic subgroups (African American, American Indian, Hispanic/Latino, and Asian American). It is often associated with a strong genetic predisposition or family history in first- degree relatives, more so than T1D. However, the genetics of T2D is poorly understood. In adults without traditional risk factors for T2D and/or younger age, consider antibody testing to exclude the diagnosis of T1D (i.e., GAD).

Screening for prediabetes and T2D through an informal assessment of risk factors or with an assessment tool, such as the ADA risk test (diabetes.org/socrisktest) or the Diabetes Risk Score test [32] is recommended to guide providers on whether performing a diagnostic test is appropriate. Prediabetes and T2D meet criteria for conditions in which early detection is appropriate. Both conditions are common and impose significant clinical and public health burdens. There is often a long pre-symptomatic phase before the diagnosis of T2D. The duration of glycemic burden is a strong predictor of adverse outcomes. There are effective interventions that prevent progression from prediabetes to diabetes and reduce the risk of diabetes complications such as those micro- and macrovascular, dietary ones being among the cornerstones.

A large European randomized controlled trial compared the impact of screening for diabetes and intensive multifactorial intervention with that of screening

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and routine care [33]. General practice patients between the ages of 40 and 69 years were screened for diabetes and randomly assigned by practice to intensive treatment of multiple risk factors or routine diabetes care. After 5.3 years of follow-up, CVD risk factors were modestly but significantly improved with intensive treatment compared with routine care, but the incidence of first CVD events or mortality was not significantly different between the groups [33]. The excellent care provided to patients in the routine care group and the lack of an unscreened control arm limited the authors' ability to determine whether screening and early treatment improved outcomes compared with no screening and later treatment after clinical diagnoses. Computer simulation modeling studies suggest that major benefits are likely to accrue from the early diagnosis and treatment of hyperglycemia and cardiovascular risk factors in T2D [34]; moreover, screening, beginning at age 30 or 45 years and independent of risk factors, may be cost-effective and result in more than 11,000 USD per quality-adjusted life-year gained [35].

Additional considerations regarding testing for T2D and prediabetes in asymptomatic patients include age, body mass index (BMI), ethnicity, and possibly dietary habits. Age is a major risk factor for diabetes; testing should take place for all patients aged 45. Screening should be considered in overweight or obese adults of any age with one or more risk factors for diabetes. In general, BMI >25 kg/m^2 is a risk factor for diabetes, although data suggest that the BMI cut point should be affected by ethnicity – lower for the Asian American population [36, 37]. The BMI cutoff points fall consistently between 23 and 24 kg/m^2 (sensitivity of 80%) for nearly all Asian American subgroups, with levels slightly lower for Japanese Americans. This makes a rounded cutoff point of 23 kg/m^2 practical. An argument can be made to push the

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BMI cutoff point <23 kg/m^2 in favor of increased sensitivity; however, this would lead to low specificity (13.1%). Data from the WHO also suggest that a BMI >23 kg/m^2 should be used to define increased risk in Asian Americans [38]. The finding that half of diabetes in Asian Americans is undiagnosed suggests that testing is not occurring at lower BMI thresholds [39, 40].

Evidence also suggests that other populations may benefit from lower BMI cutoff points. For example, in a large multiethnic cohort study, for an equivalent incidence rate of diabetes, a BMI of 30 kg/m^2 in non-Hispanic whites was equivalent to a BMI of 26 kg/m^2 in African Americans [41].

In the last decade, the incidence and prevalence of T2D in adolescents has increased dramatically, especially in racial and ethnic minority populations [26]. Some studies question the validity of A1C in the pediatric population, especially among certain ethnicities, and suggest OGTT or FPG as more suitable diagnostic tests. However, many of these studies do not recognize that diabetes diagnostic criteria are based on long-term health outcomes, and validations are not currently available in the pediatric population [42].

Gestational diabetes mellitus

For many years, gestational diabetes mellitus (GDM) was defined as any degree of glucose intolerance that was first recognized during pregnancy [43], regardless of whether the condition may have predated the pregnancy or persisted after the pregnancy. The ongoing epidemic of obesity and diabetes has led to more T2D in women of childbearing age, with an increase in the number of pregnant women with undiagnosed T2D [1, 44]. Because of the number of pregnant women with

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undiagnosed T2D, it is reasonable to test women with risk factors for such condition at their initial prenatal visit, through standard diagnostic criteria such as those shown on Standard Medical Care for diabetes [2]. Women diagnosed with diabetes by standard diagnostic criteria in the first trimester should be classified as having preexisting pregestational diabetes (T2D or, very rarely, T1D or monogenic diabetes). GDM is diabetes that is first diagnosed in the second or third trimester of pregnancy that is not clearly either preexisting T1D or T2D. The International Association of the Diabetes and Pregnancy Study Groups (IADPSG) GDM diagnostic criteria for the 75-g OGTT as well as the GDM screening and diagnostic criteria used in the two-step approach were not derived from data in the first half of pregnancy, so the diagnosis of GDM in early pregnancy by either FPG or OGTT values is not evidence based [45].

Because GDM confers increased risk for the development of T2D after delivery [1] and because effective prevention interventions are available [46, 47], women diagnosed with GDM should receive lifelong screening for prediabetes and T2D. GDM carries risks for the mother and neonate, with not all adverse outcomes being of equal clinical importance. The Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study [48], a large-scale multinational cohort study completed by more than 23,000 pregnant women, demonstrated that risk of adverse maternal, fetal, and neonatal outcomes continuously increased as a function of maternal glycemia at 24–28 weeks of gestation, even within ranges previously considered normal for pregnancy. For most complications, there was no threshold for risk. These results have led to careful reconsideration of the diagnostic criteria for GDM.

The conflicting recommendations from expert groups underscore the fact that there are data to support each strategy. A cost-benefit estimation comparing the two

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strategies concluded that the one- step approach is cost-effective only if patients with GDM receive postdelivery counseling and care to prevent T2D [49]. The decision of which strategy to implement must therefore be made based on the relative values placed on factors that have yet to be measured (e.g., willingness to change practice based on correlation studies rather than intervention trial results, available infrastructure, and importance of cost considerations).

Neonatal diabetes

Diabetes occurring under 6 months of age is termed "neonatal" or "congenital" diabetes, and about 80–85% of cases can be found to have an underlying monogenic cause [50]. Neonatal diabetes occurs much less often after 6 months of age, whereas autoimmune T1D rarely occurs before 6 months of age. Neonatal diabetes can either be transient or permanent. Transient diabetes is most often due to overexpression of genes on chromosome 6q24, is recurrent in about half of cases, and may be treatable with medications other than insulin. Permanent neonatal diabetes is most commonly due to autosomal dominant mutations in the genes encoding the Kir6.2 subunit (KCNJ11) and SUR1 subunit (ABCC8) of the b-cell KATP channel. Correct diagnosis has critical implications because most patients with KATP-related neonatal diabetes will exhibit improved glycemic control when treated with high-dose oral sulfonylureas instead of insulin. Insulin gene (INS) mutations are the second most common cause of permanent neonatal diabetes, and, while treatment presently is intensive insulin management, there are important genetic considerations, as most of the mutations that cause diabetes are dominantly inherited.

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Diabetes comorbidities

Besides assessing diabetes-related complications, clinicians and their patients need to be aware of common comorbidities that affect people with diabetes and may complicate management [51-54]. Diabetes comorbidities are conditions that affect people with diabetes more often than age-matched people without diabetes. Many of the common comorbidities observed in patients with diabetes are autoimmune diseases, neoplastic diseases, cognitive impairment, fatty liver disease, and pancreatitis.

People with T1D are at increased risk for other autoimmune diseases including thyroid disease, primary adrenal insufficiency, celiac disease, autoimmune gastritis, autoimmune hepatitis, dermatomyositis, and myasthenia gravis [55-57]. T1D may also occur with other autoimmune diseases in the context of specific genetic disorders or polyglandular autoimmune syndromes [58]. In autoimmune diseases, the immune system fails to maintain self-tolerance to specific peptides within target organs. It is likely that many factors trigger autoimmune disease; however, common triggering factors are known for only some autoimmune conditions (i.e., gliadin peptides in celiac disease).

In those with T2D, the degree and duration of hyperglycemia are related to dementia. More rapid cognitive decline is associated with both increased A1C and longer duration of diabetes [59]. The Action to Control Cardiovascular Risk in Diabetes (ACCORD) study found that each 1% higher A1C level was associated with lower cognitive function in individuals with T2D [60]. However, the ACCORD study found no difference in cognitive outcomes in participants randomly assigned to intensive and standard glycemic control, supporting the recommendation that intensive

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glucose control should not be advised for the improvement of cognitive function in individuals with T2D [61].

In T2D, severe hypoglycemia is associated with reduced cognitive function, and those with poor cognitive function have more severe hypoglycemia. In a long-term study of older patients with T2D, individuals with one or more recorded episode of severe hypoglycemia had a stepwise increase in risk of dementia [62]. Likewise, the ACCORD trial found that as cognitive function decreased, the risk of severe hypoglycemia increased [63].

Diabetes is associated with the development of nonalcoholic chronic liver disease and with hepatocellular carcinoma [64]. Elevations of hepatic transaminase concentrations are associated with higher BMI, waist circumference, and triglyceride levels and lower HDL cholesterol levels. Interventions that improve metabolic abnormalities in patients with diabetes (weight loss, glycemic control, and treatment with specific drugs for hyperglycemia or dyslipidemia) are also beneficial for fatty liver disease [65, 66].

Diabetes is also linked to diseases of the exocrine pancreas such as pancreatitis, which may disrupt the global architecture or physiology of the pancreas, often resulting in both exocrine and endocrine dysfunction. Up to half of patients with diabetes may have impaired exocrine pancreas function [67]. People with diabetes are at an approximately twofold higher risk of developing acute pancreatitis [68]. Conversely, prediabetes and/or diabetes has been found to develop in approximately one-third of patients after an episode of acute pancreatitis [69], thus the relationship is likely bidirectional. Post-pancreatitis diabetes may include either new onset disease or previously unrecognized diabetes [70]. Studies of patients treated with incretin-based

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therapies for diabetes have also reported that pancreatitis may occur more frequently with these medications, but results have been mixed [71, 72].

Islet autotransplantation should be considered for patients requiring total pancreatectomy for medically refractory chronic pancreatitis to prevent postsurgical diabetes. Approximately one-third of patients undergoing total pancreatectomy with islet autotransplantation are insulin free one year postoperatively, and observational studies from different centers have demonstrated islet graft function up to a decade after the surgery in some patients [73-77]. Both patient and disease factors should be carefully considered when deciding the indications and timing of this surgery. Surgeries should be performed in skilled facilities that have demonstrated expertise in islet autotransplantation.

Age-specific hip fracture risk is significantly increased in people with both T1D (RR 6.3) and T2D (RR 1.7) diabetes in both genders [78]. T1D is associated with osteoporosis, but in T2D, an increased risk of hip fracture is seen despite higher bone mineral density (BMD) [79]. The pathophysiological mechanisms underlying bone fragility in diabetes are complex, including an alteration in glycemic homeostasis, oxidative stress, the accumulation of advanced glycation end products (AGEs) [80], with the involvement of mesenchymal stem cells playing a role in osteoblast fate and therefore in tissues alteration(s) – enrichment/deprivation of skeletal muscle/adipose/bone tissue. In three large observational studies of older adults, femoral neck BMD T score and the WHO Fracture Risk Assessment Tool (FRAX) score were associated with hip and non-spine fractures. Fracture risk was higher in participants with diabetes compared with those without diabetes for a given T score and age or for a given FRAX score [81, 82]. Providers should assess fracture history

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and risk factors in older patients with diabetes and recommend measurement of BMD if appropriate for the patient's age and sex. Fracture prevention strategies for people with diabetes are the same as for the general population and include vitamin D supplementation. For patients with T2D with fracture risk factors, thiazolidinediones [83] and sodium-glucose cotransporter 2 inhibitors [84] should be used with caution.

Current pharmacologic interventions: an overview

Pharmacologic agents including metformin, a-glucosidase inhibitors, orlistat, glucagon-like peptide 1 (GLP-1) receptor agonists, and thiazolidinediones have each been shown to decrease incident diabetes to various degrees in those with prediabetes in research studies [85-90], though none are approved by the U.S. Food and Drug Administration specifically for diabetes prevention. Metformin has the strongest evidence base and demonstrated long-term safety as pharmacologic therapy for diabetes prevention [90]. For other drugs, cost, side effects, and durable efficacy require consideration. Metformin was overall less effective than lifestyle modification in the DPP and DPPOS, though group differences declined over time [91] and metformin may be cost-saving over a 10-year period [92]. It was as effective as lifestyle modification in participants with BMI >35 kg/m^2 but not significantly better than placebo in those over 60 years of age [85]. In the DPP, for women with history of GDM, metformin and intensive lifestyle modification led to an equivalent 50% reduction in diabetes risk [90], and both interventions remained highly effective during a 10-year follow-up period [94]. Metformin should be recommended as an option for high-risk individuals (e.g., those with a history of GDM or those with BMI >35 kg/m^2).

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Consider monitoring B12 levels in those taking metformin chronically to check for possible deficiency.

Psychosocial/emotional disorders in diabetes mellitus patients

Prevalence of clinically significant psychopathology diagnoses are considerably more common in people with diabetes than in those without the disease [94]. Symptoms, both clinical and subclinical, that interfere with the person's ability to carry out daily diabetes self-management tasks should be addressed: providers should consider an assessment of symptoms of depression, anxiety, disordered eating, and of cognitive capacities using patient-appropriate standardized/ validated tools at the initial visit, at periodic intervals, and when there is a change in disease, treatment, or life circumstance. Caregivers and family members inclusion in this assessment is warmly recommended. In 2016, the American diabetes association issued a position statement regarding psychological care for diabetic subjects in which complex emotional interactions in these patients have been described, specifically in daily care of diabetes at both personal and social dimension [95]. Diabetes distress and psychological comorbidities are considered as well, with evidence-based guidelines useful for both healthcare provider and patient.

Anxiety symptoms and diagnosable disorders (e.g., generalized anxiety disorder, body dysmorphic disorder, obsessive-compulsive disorder, specific phobias, and posttraumatic stress disorder) are common in people with diabetes [96]. The Behavioral Risk Factor Surveillance System (BRFSS) estimated the lifetime prevalence of generalized anxiety disorder to be 19.5% in people with either T1D or T2D [97]. Common diabetes-specific concerns include fears related to hypoglycemia

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[98, 99], not meeting blood glucose targets [96], and insulin injections or infusion [100]. Onset of complications presents another critical point when anxiety can occur [101]. People with diabetes who exhibit excessive diabetes self-management behaviors well beyond what is prescribed or needed to achieve glycemic targets may be experiencing symptoms of obsessive-compulsive disorder, as described by the American Psychiatric Association (APA) [102].

General anxiety is a predictor of injection-related anxiety and associated with fear of hypoglycemia [103]. Fear of hypoglycemia and hypoglycemia unawareness often co-occur, and interventions aimed at treating one often benefit both [104]. Fear of hypoglycemia may explain avoidance of behaviors associated with lowering glucose such as increasing insulin doses or frequency of monitoring. If fear of hypoglycemia is identified and a person does not have symptoms of hypoglycemia, a structured program, blood glucose awareness training, delivered in routine clinical practice, can improve A1C, reduce the rate of severe hypoglycemia, and restore hypoglycemia awareness [105, 106].

History of depression, current depression, and antidepressant medication use are risk factors for the development of T2D, especially if the individual has other risk factors such as obesity and family history of diabetes [107-109]. A meta-analysis of Anderson et al. showed elevated depressive symptoms and depressive disorders to affect one in four patients with T1D or T2D [110]. Thus, routine screening for depressive symptoms is indicated in this high-risk population including people with diabetes, including gestational diabetes mellitus, and postpartum diabetes. Regardless of diabetes type, women have significantly higher rates of depression than men [111]. Routine monitoring with patient-appropriate validated measures can help to identify if

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referral is warranted. Adult patients with a history of depressive symptoms or disorder need ongoing monitoring of depression recurrence within the context of routine care [106]. Integrating mental and physical health care can improve outcomes. When a patient is in psychological therapy (talk therapy), the mental health provider should be incorporated into the diabetes treatment team [112].

Estimated prevalence of disordered eating behaviors and diagnosable eating disorders in people with diabetes varies [113-115]. For people with T1D, insulin omission causing glycosuria in order to lose weight is the most commonly reported disordered eating behavior [115, 116]; in people with T2D, bingeing (excessive food intake with an accompanying sense of loss of control) is most commonly reported. For people with T2D treated with insulin, intentional omission is also frequently reported [117]. People with diabetes and diagnosable eating disorders have high rates of comorbid psychiatric disorders [118]. People with T1D and eating disorders have high rates of diabetes distress and fear of hypoglycemia [119]. When evaluating symptoms of disordered or disrupted eating in people with diabetes, etiology and motivation for the behavior should be considered [114, 120]. Adjunctive medication such as glucagon-like peptide 1 receptor agonists [121] may help individuals not only to meet glycemic targets but also to regulate hunger and food intake, thus having the potential to reduce uncontrollable hunger and bulimic symptoms.

Nutritional implications for prediabetes and type 2 diabetes risk

Screening for prediabetes and T2D risk through an informal assessment of risk factors or with an assessment tool, such as the American Diabetes Association risk test, is recommended to guide providers on whether performing a diagnostic test for

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prediabetes and previously undiagnosed T2D is appropriate. Those determined to be at high risk for T2D, including people with A1C 5.7–6.4% (39–47 mmol/mol), impaired glucose tolerance, or impaired fasting glucose, are ideal candidates for diabetes prevention efforts. Using A1C to screen for prediabetes may be problematic in the presence of certain hemoglobinopathies or conditions that affect red blood cell turnover. At least annual monitoring for the development of diabetes in those with prediabetes is suggested [2].

The strongest evidence for diabetes prevention comes from the Diabetes Prevention Program (DPP) [9]. The DPP demonstrated that an intensive lifestyle intervention could reduce the incidence of T2D by 58% over 3 years. Follow-up of three large studies of lifestyle intervention for diabetes prevention has shown sustained reduction in the rate of conversion to T2D: 43% reduction at 20 years in the Da Qing study [122], 43% reduction at 7 years in the Finnish Diabetes Prevention Study (DPS) [123], and 34% reduction at 10 years [9] and 27% reduction at 15 years [91] in the U.S. Diabetes Prevention Program Outcomes Study (DPPOS). The two major goals of the DPP intensive, behavioral, lifestyle intervention were to achieve and maintain a minimum of 7% weight loss and 150 min of physical activity per week similar in intensity to brisk walking. The DPP lifestyle intervention was a goal-based intervention: all participants were given the same weight loss and physical activity goals, but individualization was permitted in the specific methods used to achieve the goals [124].

The 7% weight loss goal was selected because it was feasible to achieve and maintain and likely to lessen the risk of developing diabetes. Participants were encouraged to achieve the 7% weight loss during the first 6 months of the intervention.

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The recommended pace of weight loss was 0.5 to 1 kg/week. Calorie goals were calculated by estimating the daily calories needed to maintain the participant's initial weight and subtracting 500–1,000 calories/day (depending on initial body weight). The initial focus was on reducing total dietary fat. After several weeks, the concept of calorie balance and the need to restrict calories as well as fat was introduced [124].

The goal for physical activity was selected to approximate at least 700 kcal/week expenditure from physical activity. For ease of translation, this goal was described as at least 150 min of moderate-intensity physical activity per week similar in intensity to brisk walking. Participants were encouraged to distribute their activity throughout the week with a minimum frequency of three times per week with at least 10 min per session. A maximum of 75 min of strength training could be applied toward the total 150 min/week physical activity goal [124].

To implement the weight loss and physical activity goals, the DPP used an individual model of treatment rather than a group-based approach. This choice was based on a desire to intervene before participants had the possibility of developing diabetes or losing interest in the program. The individual approach also allowed for tailoring of interventions to reflect the diversity of the population [124]. The DPP intervention was administered as a structured core curriculum followed by a more flexible maintenance program of individual sessions, group classes, motivational campaigns, and restart opportunities. The 16-session core curriculum was completed within the first 24 weeks of the program and included sections on lowering calories, increasing physical activity, self-monitoring, maintaining healthy lifestyle behaviors, and psychological, social, and motivational challenges. Villareal et al. led a trial assessing weight loss, physical activity or both and physical function in almost 100

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obese older adults in which dietary advices (1g protein per kg/body weight) and/or exercise were introduced to assess body composition and performance, including bone mineral density and overall quality of life, suggesting a combination of the two factors is better than an intervention alone in physical function [125].

Reducing caloric intake is of paramount importance for those at high risk for developing T2D, though recent evidence suggests that the quality of fats consumed in the diet is more important than the total quantity of dietary fat [126-128]. For example, the Mediterranean diet, which is relatively high in monounsaturated fats, may help to prevent T2D [129-131].

Whereas overall healthy low-calorie eating patterns should be encouraged, there is also some evidence that particular dietary components impact diabetes risk. Higher intakes of nuts [132], berries [133], yogurt [134], coffee, and tea [135] are associated with reduced T2D risk. Conversely, red meats and sugar-sweetened beverages are associated with an increased risk [128]. As is the case for those with diabetes, individualized medical nutrition therapy (MNT) is effective in lowering A1C in individuals diagnosed with prediabetes [136].

For many individuals with diabetes, the most challenging part of the treatment plan is determining what to eat and following a meal plan. There is not a one-size-fits-all eating pattern for individuals with diabetes, and meal planning should be individualized. Nutrition therapy has an integral role in overall diabetes management, and each person with diabetes should be actively engaged in education, self-management, and treatment planning with his or her health care team, including the collaborative development of an individualized eating plan [137, 138]. All individuals with diabetes should be offered a referral for individualized MNT, preferably provided

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by a registered dietitian who is knowledgeable and skilled in providing diabetes-specific MNT. MNT delivered by a registered dietitian is associated with A1C decreases of 1% to1.9% for people with T1D [139-142] and 0.3–2% for people with T2D [142-146].

The American diabetes association has released the following MNT goals for diabetic adults [2]:

1. To promote and support healthful eating patterns, emphasizing a variety of nutrient-dense foods in appropriate portion sizes, to improve overall health and a) achieve and maintain body weight goals, b) attain individualized glycemic, blood pressure, and lipid goals, c) delay or prevent diabetes complications.
2. To address individual nutrition needs based on personal and cultural preferences, health literacy and numeracy, access to healthful foods, willingness and ability to make behavioral changes, and barriers to change.
3. To maintain the pleasure of eating by providing nonjudgmental messages about food choices.
4. To provide an individual with diabetes the practical tools for developing healthy eating patterns rather than focusing on individual macronutrients, micronutrients, or single foods.

Evidence suggests that there is not an ideal percentage of calories from carbohydrate, protein, and fat for all people with diabetes, which is why macronutrient distribution should be based on an individualized assessment of current eating patterns, preferences, and metabolic goals. Consider personal preferences (e.g., tradition, culture, religion, health beliefs and goals, eco- nomics) as well as metabolic goals

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when working with individuals to determine the best eating pattern for them [138, 147]. It is important that each member of the health care team be knowledgeable about nutrition therapy principles for people with all types of diabetes and be supportive of their implementation. Emphasis should be on healthful eating patterns containing nutrient-dense foods with less focus on specific nutrients [148]. A variety of eating patterns are acceptable for the management of diabetes [147, 148]. The Mediterranean [149, 150], Dietary Approaches to Stop Hypertension (DASH) [128, 151, 152], and plant-based diets [153, 154] are all examples of healthful eating patterns that have shown positive results in research, but individualized meal planning should focus on personal preferences, needs, and goals. The diabetes plate method is commonly used for providing basic meal planning guidance [155] as it provides a visual guide showing how to control calories (by featuring a smaller plate) and carbohydrates (by limiting them to what fits in one-quarter of the plate) and puts an emphasis on low-carbohydrate (or non-starchy) vegetables.

Management and reduction of weight is important for overweight and obese people, either with and without T1D-T2D. Lifestyle intervention programs should be intensive and have frequent follow-up to achieve significant reductions in excess body weight and improve clinical indicators. There is strong and consistent evidence that modest persistent weight loss can delay the progression from prediabetes to T2D [147, 156, 157] and is beneficial to the management of T2D. Studies of reduced calorie interventions show reductions in A1C of 0.3% to 2.0% in T2D adults, as well as improvements in medication doses and quality of life [147]. Sustaining weight loss can be challenging [64] but has long-term benefits; maintaining weight loss for 5 years is associated with sustained improvements in A1C and lipid levels [158]. Weight loss

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can be attained with lifestyle programs that achieve a 500–750 kcal/day energy deficit or provide 1,200–1,500 kcal/day for women and 1,500–1,800 kcal/day for men, adjusted for the individual's baseline body weight. For many obese T2D individuals, weight loss 0.5% is needed to produce beneficial outcomes in glycemic control, lipids, and blood pressure, and sustained weight loss of >7% is optimal [64]. The meal plans often used in intensive lifestyle management for weight loss may differ in the types of foods they restrict (e.g., high-fat vs. high-carbohydrate foods), but their emphasis should be on nutrient-dense foods, such as vegetables, fruits, legumes, low-fat dairy, lean meats, nuts, seeds, and whole grains, as well as on achieving the desired energy deficit [159-162]. The approach to meal planning should be based on the patients' health status and preferences.

Studies examining the ideal amount of carbohydrate intake for people with diabetes are inconclusive, although monitoring carbohydrate intake and considering the blood glucose response to dietary carbohydrate are key for improving postprandial glucose control [163, 164]. The literature concerning glycemic index and glycemic load in individuals with diabetes is complex often yielding mixed results, though in some studies lowering the glycemic load of consumed carbohydrates has demonstrated A1C reductions of –0.2% to –0.5% [165, 166]. Studies longer than 12 weeks report no significant influence of glycemic index or glycemic load independent of weight loss on A1C; however, mixed results have been reported for fasting glucose levels and endogenous insulin levels. The role of low-carbohydrate diets in patients with diabetes remains unclear [165]. Part of the confusion is due to the wide range of definitions for a low-carbohydrate diet [166, 167]. While benefits of low-carbohydrate diets have been described, improvements tend to be in the short term and, over time, these effects

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are not maintained [168-170]. While some studies have shown modest benefits of very low–carbohydrate or ketogenic diets (less than 50-g carbohydrate per day) [171, 172], this approach may only be appropriate for short-term implementation (up to 3–4 months) if desired by the patient, as there is little long-term research citing benefits or harm. Most individuals with diabetes report a moderate intake of carbohydrate (44–46% of total calories) [147]. Novel, surprising findings by Soare et al. have reported significant improvements in glycemic control, over a 21-day period, in T2D obese subjects following an experimental dietary pattern rich in complex carbohydrate and fiber, with carbohydrate content >70% of macronutrient daily distribution [173]. Interestingly, these findings were assessed in comparison with a standard, Mediterranean-like diet and in a crossover design, with similar results over a 6-month follow-up [174]. However, efforts to modify habitual eating patterns are often unsuccessful in the long term; people generally go back to their usual macronutrient distribution [147]. Thus, the recommended approach is to individualize meal plans to meet caloric goals with a macronutrient distribution that is more consistent with the individual's usual intake to increase the likelihood for long-term maintenance. Either children and adults with diabetes are encouraged to reduce intake of refined carbohydrates and added sugars and instead focus on carbohydrates from vegetables, legumes, fruits, dairy (milk and yogurt), and whole grains. The consumption of sugar-sweetened beverages and processed "low-fat" or "non-fat" food products with high amounts of refined grains and added sugars is strongly discouraged [175-177]. Individuals with T1D or T2D taking insulin at mealtime should be offered intensive and ongoing education on the need to couple insulin administration with carbohydrate intake. For people whose meal schedules or carbohydrate consumption is variable,

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regular counseling to help them understand the complex relationship between carbohydrate intake and insulin needs is important. In addition, education on using the insulin-to-carbohydrate ratio for meal planning can assist them with effectively modifying insulin dosing from meal to meal and improving glycemic control [140, 147, 163, 178-180]. Individuals who consume meals containing more protein and fat than usual may also need to make mealtime insulin dose adjustments to compensate for delayed postprandial glycemic excursions [181-183]. For individuals on a fixed daily insulin schedule, meal planning should emphasize a relatively fixed carbohydrate consumption pattern with respect to both time and amount [138]. By contrast, a simpler diabetes meal planning approach emphasizing portion control and healthful food choices may be better suited for some older individuals, those with cognitive dysfunction, and those for whom there are concerns over health literacy and numeracy [138-140, 163, 179]. The modified plate method, which uses measuring cups to assist with portion measurement, may be an effective alternative to carbohydrate counting for some patients to improve glycemia [155].

There is no evidence that adjusting the daily level of protein intake (typically 1– 1.5 g/kg body weight/day or 15–20% total calories) will improve health in individuals without diabetic kidney disease, and research is inconclusive regarding the ideal amount of dietary protein to optimize either glycemic control or cardiovascular disease (CVD) risk [165]. Therefore, protein intake goals should be individualized based on current eating patterns. Some research has found successful management of T2D with meal plans including slightly higher levels of protein (20–30%), which may contribute to increased satiety [128]. For those with diabetic kidney disease (with albuminuria and/or reduced estimated glomerular filtration rate), dietary protein

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should be maintained at the recommended daily allowance of 0.8 g/kg body weight/day. Reducing the amount of dietary protein below the recommended daily allowance is not recommended because it does not alter glycemic measures, cardiovascular risk measures, or the rate at which glomerular filtration rate declines [184, 185]. In individuals with T2D, protein intake may enhance or increase the insulin response to dietary carbohydrates [186]. Therefore, carbohydrate sources high in protein should not be used to treat or prevent hypoglycemia due to the potential concurrent rise in endogenous insulin.

The ideal amount of dietary fat for individuals with diabetes is controversial. The National Academy of Medicine has defined an acceptable macronutrient distribution for total fat for all adults to be 20–35% of total calorie intake [187]. The type of fats consumed is more important than total amount of fat when looking at metabolic goals and CVD risk, and it is recommended that the percentage of total calories from saturated fats should be limited [188-192]. Multiple randomized controlled trials including patients with T2D have reported that a Mediterranean-style eating pattern [190-197], rich in polyunsaturated and monounsaturated fats, can improve both glycemic control and blood lipids. However, supplements do not seem to have the same effects as their whole food counterparts. A systematic review concluded that dietary supplements with n-3 fatty acids did not improve glycemic control in individuals with T2D [165]. Randomized controlled trials also do not support recommending n-3 supplements for primary or secondary prevention of CVD [198-202]. People with diabetes should be advised to follow the guidelines for the general population for the recommended intakes of saturated fat, dietary cholesterol, and trans fats [40]. In general, trans fats should be avoided. In addition, as saturated

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fats are progressively decreased in the diet, they should be replaced with unsaturated fats and not with refined carbohydrates [197].

As for the general population, people with diabetes are advised to limit their sodium consumption to <2,300 mg/day [138]. Lowering sodium intake would in turn improve blood pressure in certain circumstances [203, 204]. However, other studies [205, 206] suggest caution for universal sodium restriction to 1,500 mg in people with diabetes. Sodium intake recommendations should take into account palatability, availability, affordability, and the difficulty of achieving low-sodium recommendations in a nutritionally adequate diet [207].

Benefit from herbal or non-herbal (i.e., vitamin or mineral) supplementation for people with diabetes without underlying deficiencies are controversial as well [138]. Metformin is associated with vitamin B12 deficiency, with a recent report from the Diabetes Prevention Program Outcomes Study (DPPOS) suggesting that periodic testing of vitamin B12 levels should be considered in patients taking metformin, particularly in those with anemia or peripheral neuropathy [208]. Routine supplementation with antioxidants, such as vitamins E and C and carotene, is not advised due to lack of evidence of efficacy and concern related to long-term safety. In addition, there is insufficient evidence to support the routine use of herbals and micronutrients, such as cinnamon and vitamin D, to improve glycemic control in people with diabetes [135, 138, 209, 210].

For some people with diabetes who are accustomed to sugar-sweetened products, nonnutritive sweeteners, containing few or no calories, may be an acceptable substitute for nutritive sweeteners (those containing calories such as sugar, honey, agave syrup) when consumed in moderation. While use of nonnutritive sweeteners

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does not appear to have a significant effect on glycemic control, they can reduce overall calorie and carbohydrate intake [138, 211]. Most systematic reviews and meta-analyses show benefits for nonnutritive sweetener use in weight loss [212, 213]; however, some research suggests an association with weight gain [214]. Regulatory agencies set acceptable daily intake levels for each nonnutritive sweetener, defined as the amount that can be safely consumed over a person's lifetime [138, 204].

Novel gut peptides in the treatment of type-2 diabetes: oxyntomodulin

Oxyntomodulin has been described for the first time in 1948 by Sutherland and De Duve [215], who individuated glucagon-like substances in extracts of intestinal mucosa. In 1982 Bataille et al. isolated the hormone from porcine jejuno-ileum and defined as "glucagon-37" or "bioactive enteroglucagon" [216] because of its 29-amino acid domain deriving from glucagon plus an elongation at its C-terminal end by the octapeptide Lys-Arg-Asn-Lys-Asn-Asn-Ile-Ala, thus 37 amino acids. Further research has established that such gut hormone is a product of the proglucagon gene released post-prandially from the L-cells of the small intestine, with calorie intake playing a role in oxyntomodulin secretion [217]. The processing of proglucagon is tissue specific, producing from a single protein different hormone depending on the tissue considered. In pancreatic α cells, prohormone convertase 2 (PC2) generates predominantly glucagon, whereas in intestinal L cells present in the jejunum, ileum, and colon, PC 1/3 predominantly produces glicentin, oxyntomodulin, GLP1, and GLP2. Similar processing is also thought to occur in the same neurons in the nucleus of the solitary tract (NTS) in the hindbrain [218].

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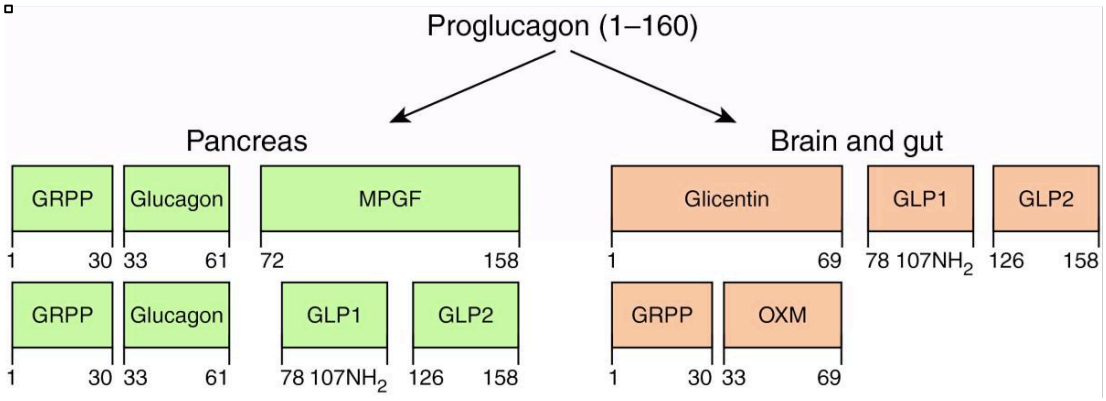

Figure 1. Tissue-specific cleavage of proglucagon gene. Adapted by Pocai A, 2012 [218]

Oxyntomodulin displays anorexigenic properties, as established by several studies based on this hormone administration to rodents and humans [219-222]. In a study led by Wynne et al. with overweight and obese subjects, oxyntomodulin was shown to ameliorate leptin and adiponectin profiles over a 4-week follow-up period in which subject self-administered oxyntomodulin subcutaneously 3 times per day, 30 min before each main meal, leading also to a significant body weight reduction in comparison with a placebo group [222]. Similar findings were obtained by a trial involving 13 healthy subjects (BMI = 22 kg/m^2), in which intravenous infusion of oxyntomodulin (3 pmol/kg/min) resulted in reduced appetite and food intake at an *ad libitum* buffet meal (19.3% ± 5.6%, P<0.01), as measured by scores for hunger [220].

Besides evidence on oxyntomodulin administration to produce weight loss in obese patients and rodents, with anorexigenic effects described in Glp1r$^{-/-}$ mice by Baggio et al. observed a lack of oxyntomodulin efficacy in these mice, demonstrating that the initial anorectic effect of oxyntomodulin is mediated solely by activation of the GLP1R [223]. Nevertheless, several findings on glucose metabolism are available as well. As Pocai reported in an elegant review on oxyntomodulin defining this hormone as "GLP-1's enigmatic brother" [218], chronic treatment with

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oxyntomodulin results in superior weight-lowering and comparable antihyperglycemic effect to a GLP1R-selective agonist [224]. This is likely achieved through body weight reduction due to the causal link between obesity and T2D [225], as well as direct enhancement of glucose-dependent insulin secretion [226, 227]. Activation of GCGR is associated with an elevation in glucose levels but the simultaneous agonism at the GLP1R would be expected to counteract this effect. Acute treatment with oxyntomodulin improves glucose tolerance during a glucose challenge in mice [226]. Moreover, oxyntomodulin administration improved glucose intolerance by enhancing glucose disposal during a hyperinsulinemic clamp study performed in diet-induced insulin-resistant mice [228]. It has been proposed that following a single injection, oxyntomodulin acts solely via GLP1R to modulate glucose homeostasis [226]. However, oxyntomodulin was reported to increase hepatic glucose production during a euglycemic–hyperinsulinemic clamp performed in diet-induced obese mice, suggesting activation of the hepatic GCGR *in vivo* [228]. Recently, it was demonstrated that while acute treatment with oxyntomodulin improves glucose metabolism during a glucose tolerance test and during a hyperglycemic clamp in mice, a matched pair peptide without GCGR activity (oxyntomodulin Q3E) exerted better glucose-lowering properties compared with oxyntomodulin administration [227]. The same authors showed decreased glucose tolerance in oxyntomodulin -infused compared with vehicle-infused *Glp1r*$^{-/-}$ mice. The lack of effect observed following a single intraperitoneal injection of oxyntomodulin during a glucose tolerance test in *Glp1r*$^{-/-}$ mice [226] may be explained by the fact that *Glp1r*$^{-/-}$ mice are glucose intolerant and resistant to diet-induced obesity; hence, the acute glucoregulatory effect of a single injection of oxyntomodulin could be confounded by compensatory

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mechanisms associated with chronic deletion of the GLP1R. To further strengthen these data, hyperglycemic clamps performed in *Gcgr*$^{-/-}$ mice showed a similar effect of oxyntomodulin and oxyntomodulin Q3E infusion on glucose metabolism in the absence of a functional GCGR. This study demonstrated that simultaneous activation of the GLP1R counteracts the hyperglycemic effect of glucagon *in vivo*. The glucose-lowering effect of oxyntomodulin is mostly mediated by GLP1R activation and activation of the GCGR appears to limit the acute antihyperglycemic efficacy of oxyntomodulin while contributing to the insulinotropic properties of oxyntomodulin [227]. Mighiu et al. recently demonstrated that intrahypothalamic glucagon suppresses hepatic glucose production and counteracts the direct hepatic stimulatory effect of circulating glucagon on liver glucose production in rodents during a pancreatic clamp [229]. Therefore, activation of the GCGR in discrete CNS areas may contribute to the improvement of whole-body glucose metabolism in animals treated with oxyntomodulin. More recent literature appears to be in contrast with the aforementioned findings, addressing oxyntomodulin limited biological significance and a minimal role in metabolic or surgical conditions such as T2D or post-gastric bypass surgery, although co-agonism with GLP-1 has been recognized to be of interest to counteract diabetogenicity [230].

Dietary implications in hunger/satiety circuit

Hunger and satiety are both related to caloric sensing, respectively to a drop and to an abundance of nutrients. When appetite suppression is blunted, metabolic conditions such as obesity ensue [231]. Considering todays' ease of use of foods such as sweets, junk food and refined carbohydrates, dietary lifestyle is today crucial in daily

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hunger/satiety management. A specific brain area of primates has been identified in food taste and temperature perception, even before this is eaten and disregarding of current hunger/satiety state. The several hunger or satiety inputs related to the multiple orexigenic/anorexigenic hormones involved are integrated in the hypothalamus, particularly in the orbitofrontal cortex – such area being implicated in food-related reward value. GLP-1 secretion has been correlated with fiber [232], carbohydrate, and fat content [233].

While dietary carbohydrate restriction has long been advocated as the main intervention to halt T2D, with current scientific evidence often referring to very-low carbohydrate ketogenic diets (VLCKD), our research group has in the past few years assessed significant improvements in T2D management with a high-carb, high-fiber diet compared with a standard control diet, either in a short [173] and medium term [174].

Visual analogue scale

The Visual Analogue Scale (VAS) is a measurement instrument that aims to quantify a characteristic or attitude that is believed to range across a continuum of values and cannot easily be directly measured [234]. Although originally known for the use in pain quantification, the VAS has been frequently deployed in the last decades and is now often used in epidemiologic and clinical research to measure the intensity or frequency of various symptoms [235], including hunger/satiety perception [136]. This is a validated 100-mm scale which allows for thorough evaluation of the outcome of interest, being easy to administer at the same time for both youngsters and elderlies [136]. VAS can be presented in a variety of ways, including scales with a middle point,

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graduation or numbers (numerical rating scales), meter-shaped scales (curvilinear analogue scales), box-shaped scales with concentric circles equidistant each other (one of which has to be selected by the responder), and scales with descriptive terms at intervals along a line (graphic rating or Likert scales) [236]. Furthermore, an electronic version has recently been made available for scientific purposes, known as electronic appetite rating system (EARS) [237, 238].

The ends are usually defined as the extreme limits of the parameter to be assessed (e.g., symptom, pain, health, mood, wellness, hunger/satiety), orientated from the left (less, worst) to the right (most, best) [239]. Through a tick, the responder marks on the line the point he/she believes to be the most representative to the perceived he/she feels at that time (current state). The VAS score is then determined by measuring in millimeters from the left end of the line to the point that the person has marked, in order to get a 0-100 score.

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RATIONALE OF THE STUDY

Considering scientific literature currently available, scarce is the evidence relating incretins and oxyntomodulin release to nutrients, i.e. to a meal, which has to be considered as a matrix of macro- and micronutrients plus fiber and water. No study has thoroughly assessed the modulation of nutrients absorption and/or how oxyntomodulin release is affected by nutrients ingestion.

In the past few years, our research group has produced novel findings regarding the administration of a vegetarian dietary pattern rich in fiber and complex carbohydrate, with daily caloric content from the latter being above 70%, in patients affected by T2D [173]. Importantly, our findings were assessed in comparison with a standard Mediterranean diet currently recommended by the main medical societies for dietary treatment of T2D and obesity, either over a short period of 21 days and over a follow-up 6-month period [174], questioning if food quality has more influence on metabolic health than quantity in those patients. We have previously shown significant improvements involved fasting blood glucose values, A1C, as well as inflammatory markers such as c-reactive protein [240] using this high fiber-high carbohydrate dietary pattern. These improvements were assessed also on gut microbiota health in these subjects, confirming glycemic control is tightly related to gut flora eubiosis – the vegetarian experimental diet resulted effective in counteracting the increase of possible proinflammatory phyla such as Collinsella and Streptococcus in the gut ecosystem, supporting the recovery of health-promoting SCFA producers like Faecalibacterium, Roseburia, Lachnospira, Bacteroides, and Akkermansia [241]. Finally, the administration of this vegetarian dietary pattern significantly improved the quality of life in non-diabetic patients affected by reactive hypoglycemia over a 7-day

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period in which we assessed glycemic control through a continuous glucose monitoring device and a crossover design [242].

As this diet proved to be effective in these patients, we aimed to explore the effect of a meal based on this diet compared to a Mediterranean type of meal on the hunger/satiety circuitry alterations putative of metabolic diseases such as obesity and T2D in overweight and obese type 2 diabetic subjects.

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RESEARCH DESIGN AND METHODS

The crossover design was chosen for this trial for two main purposes, a) avoiding any possible selection bias, and b) reducing total sample size needed to pursue the study.

Aim of the study:

We aimed to explore hunger/satiety circuitry alterations putative of metabolic diseases such as obesity and T2D through the analysis of GLP-1 and oxyntomodulin profile after the meal ingestion and including the Visual Analogue Scale assessment in overweight and obese type 2 diabetic subjects.

Endpoints:

Primary endopoint:

Changes in serum GLP-1 levels after the meal ingestion were chosen as primary endpoint of the study.

Secondary endpoints:

Plasma oxyntomodulin and blood glucose and insulin levels, as well as VAS scores, were chosen as secondary outcomes.

Sample size calculation:

Considering that about 70% of total daily caloric intake in the HFV meal derives from complex carbohydrate, such number being equal to 50% in the Mediterranean diet; with a daily calorie intake estimated around 2000 kcal for men and 1800 kcal for women (adults); that in T2D subjects Toft-Nielsen et al. found GLP-

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1 levels 60' following a meal equal to 13-14 pmol/L [243]; choosing a 20% superiority criterion of the HFV meal in comparison with the MED meal, with a statistical power equal to 80% and an a error equal to 0.05, the sample size needed to conduct the analysis was 10 subject – i.e., 5 per group. As a 20% dropout rate should be taken into account before running a clinical protocol, we enrolled 6 subjects per group. Hence, our study involved 12 diabetic patients of both genders belonging to outpatients' clinics at Campus Bio-Medico University of Rome, Italy, with diagnosis of T2D obtained between the past 2 to 5 years. All cases were consecutively recruited from individuals willing to participate to the study from January to February 2018. Subjects were randomly assigned to receive either HFV or MED meal upon a crossover design, in order to control for possible selection bias. One patient withdrew between visit 1 and 2 for personal inability to follow protocol's procedures (**figure 2**).

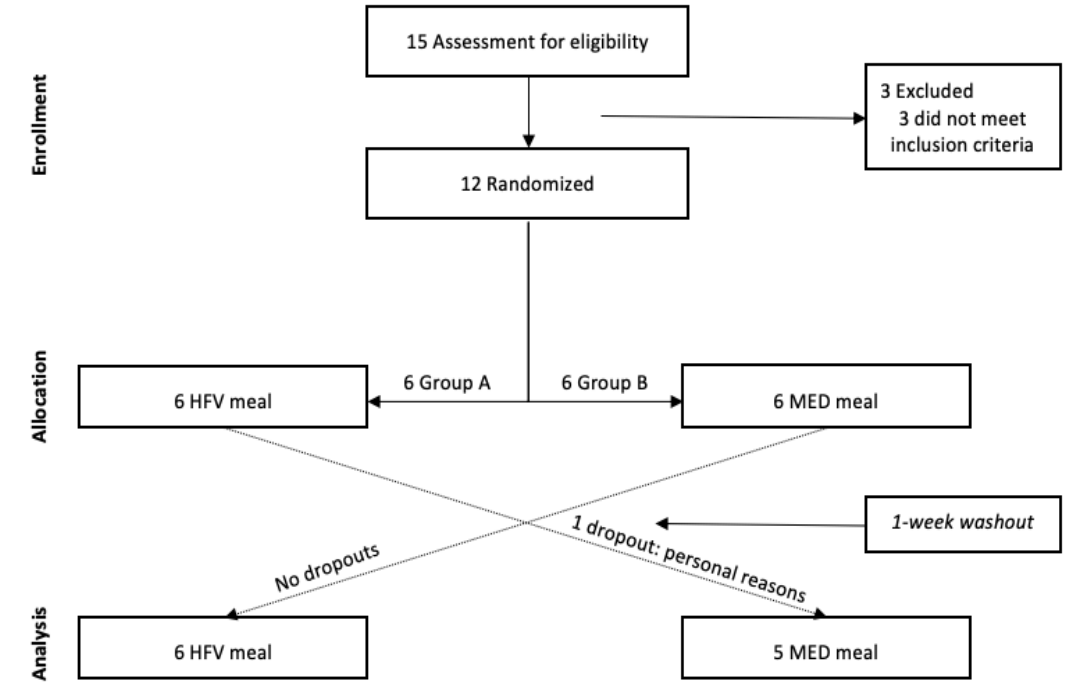

Figure 2. VAS trial flowchart

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The inclusion criteria were age above 18 years, body mass index (BMI) between 25 and 35 kg/m^2, and drug naïve and/or with metformin treatment only. Subjects with clinically significant neurological, endocrinological, or other systemic diseases, as well as those with acute illness and chronic inflammatory or infective disease, were excluded from the study. The diagnostic criteria for T2D were those provided by the American Diabetes Association (ADA) [244] – fasting blood glucose ≥126 mg/dL (7 mmol/L); 2-h post-glucose load ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test (OGTT) of 75g glucose; glycated hemoglobin (HbA1c) ≥ 6.5% (48 mmol/L); random blood draw with glycemia ≥ 200 mg/dL (11.1 mmol/L) in individuals with typical symptoms of hyperglycemia or hyperglycemic crisis. Basic characteristics, including age, gender, smoking status, alcohol consumption, physical activity level (PAL), history of any diabetes-related complication, family history of diabetes, and dietary habits were obtained by phone calls and confirmed during the screening visits. Anthropometric parameters such as weight (kg) and height (cm) were collected by trained project staff. BMI was calculated as weight (kg)/square of height (m^2). All of the participants underwent a physical examination at the screening. After an overnight fasting, a venous blood samples drawn from the antecubital vein was collected before and after the ingestion of the meal as shown below (**figure 3**).

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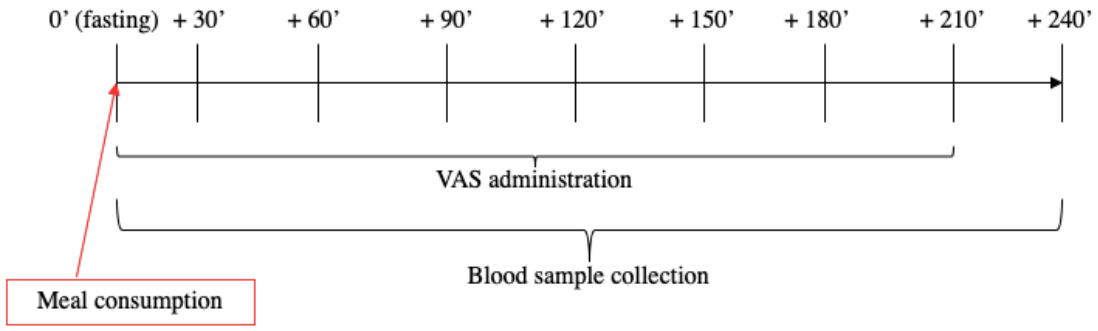
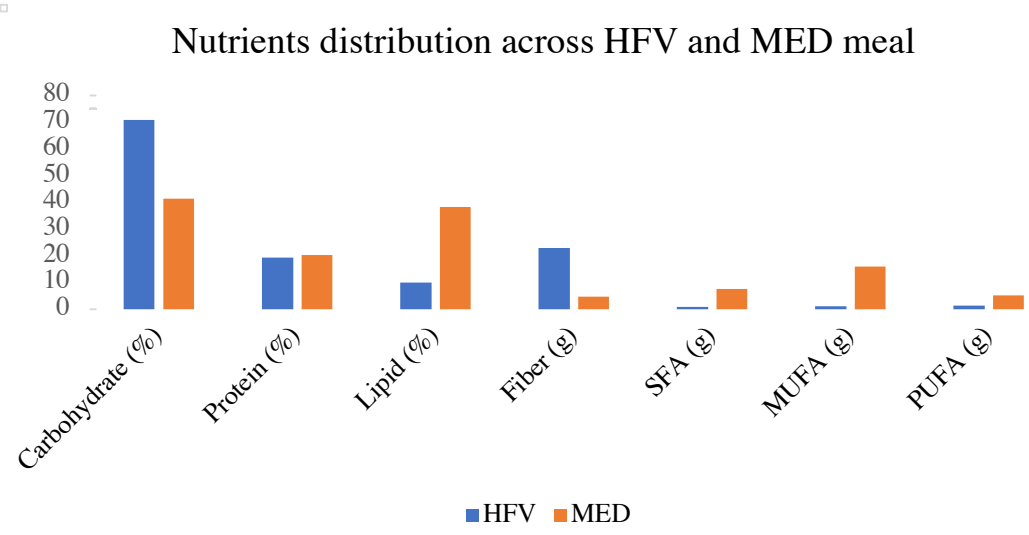


Figure 3. VAS trial timeline, with timing expressed as minute following HFV or MED meal consumption

Dietary interventions

Either the HFV and the MED meal were designed by registered nutritionists and physicians from our research group. The two meals were isocaloric (660±10 kcal) but, as shown in the graph below, they differed in nutrient composition and in fiber content. Bromatological composition was assessed through Winfood® nutritional software (Medimatica SRL, Martinsicuro, Italy).



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All of the subjects completed the two meals with no leftovers, as assessed by a registered dietitian of our research group. Both meals preparation was conducted by chefs according to the protocol indications.

HFV meal ingredients				
Ingredient	**Quantity (g)**	**GI (glucose as std)**	**GI (bread as std)**	**GL**
Whole rice	50	55	79	27.5
Millet	55	71	101	39.05
Barley	40	25	36	10
Chickpea	30	28	39	8.4
Chicory	140	15	15	21
Carrot	110	16	23	17.6
Onion	20	15	15	3
Savoy cabbage	140	15	25	21
Parsley	1	5	5	0.05
Wakame seaweed	1			0
Kombu seaweed	1			0
Nori seaweed	1			0
Miso	1			0
Tamari	1	20	20	0.2
GI: glycemic index; GL: glycemic load				

Table 1. high-fiber vegetarian (HFV) meal composition.

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MED meal ingredients				
Ingredient	**Quantity (g)**	**GI (glucose as std)**	**GI (bread as std)**	**GL**
Pasta	58	58	83	33.64
Mackerel	150	0	0	0
Lettuce	100	15	15	15
Rocket	30	15	15	4.5
Tomato sauce	100	35	35	35
Parmesan	10	0	0	0
EV olive oil	12	0	0	0
Vinegar	1	0	0	0
GI: glycemic index; GL: glycemic load				

Table 2. Mediterranean (MED) meal composition.

Outcome	**HFV meal**	**MED meal**
Calorie (Kcal) [KJ]	676.3 [2828]	651.7
Protein (g) [%]	27.6 [19.28]	39.06 [20.22]
Lipid (g) [%]	6.33 [9.95]	32.95 [38.37]
Carbohydrate (g) [%]	135.3 [70.77]	53 [41.41]
Starch (g)	72.04	39.5
Sugar (g)	15.7	9.56
Fiber (g)	23	4.8
Soluble fiber (g)	4.7	0.8
Insoluble fiber (g)	16.66	2.23
Cholesterol (mg)	0	151.6
Saturated fatty acids (g)	0.8	7.67
Monounsaturated fatty acids (g)	1.02	15.93
Polyunsaturated fatty acids (g)	1.41	5.18
Glycemic index	265[a] [358][b]	123[a] [148][b]
Glycemic load	147.8	88.14

[a] glycemic index with glucose set as standard

[b] glycemic index with bread set as standard

Table 3. Dietary features of high-fiber vegetarian (HFV) and Mediterranean (MED) meal

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All of the bromatological parameters were calculated by Winfood® nutritional software (Medimatica SRL, Martinsicuro, Italy); glycemic index and glycemic load values were checked with the 2008 international tables for glycemic index and glycemic load [245].

Visual Analogue Scale

VAS was prepared by trained staff of our research group, who also supervised individual administration to the trial participants, the collection and finally the measurements. Patients were instructed to put a vertical line in order to encounter the horizontal 100-mm scale in only one point, in order to get a reliable score, according to personal belief of hunger/satiety perceived at that moment. The VAS was administered upon a time schedule defined with the research group and withdrawn immediately upon completion. Below is shown the VAS deployed in our study (**figure 4**).

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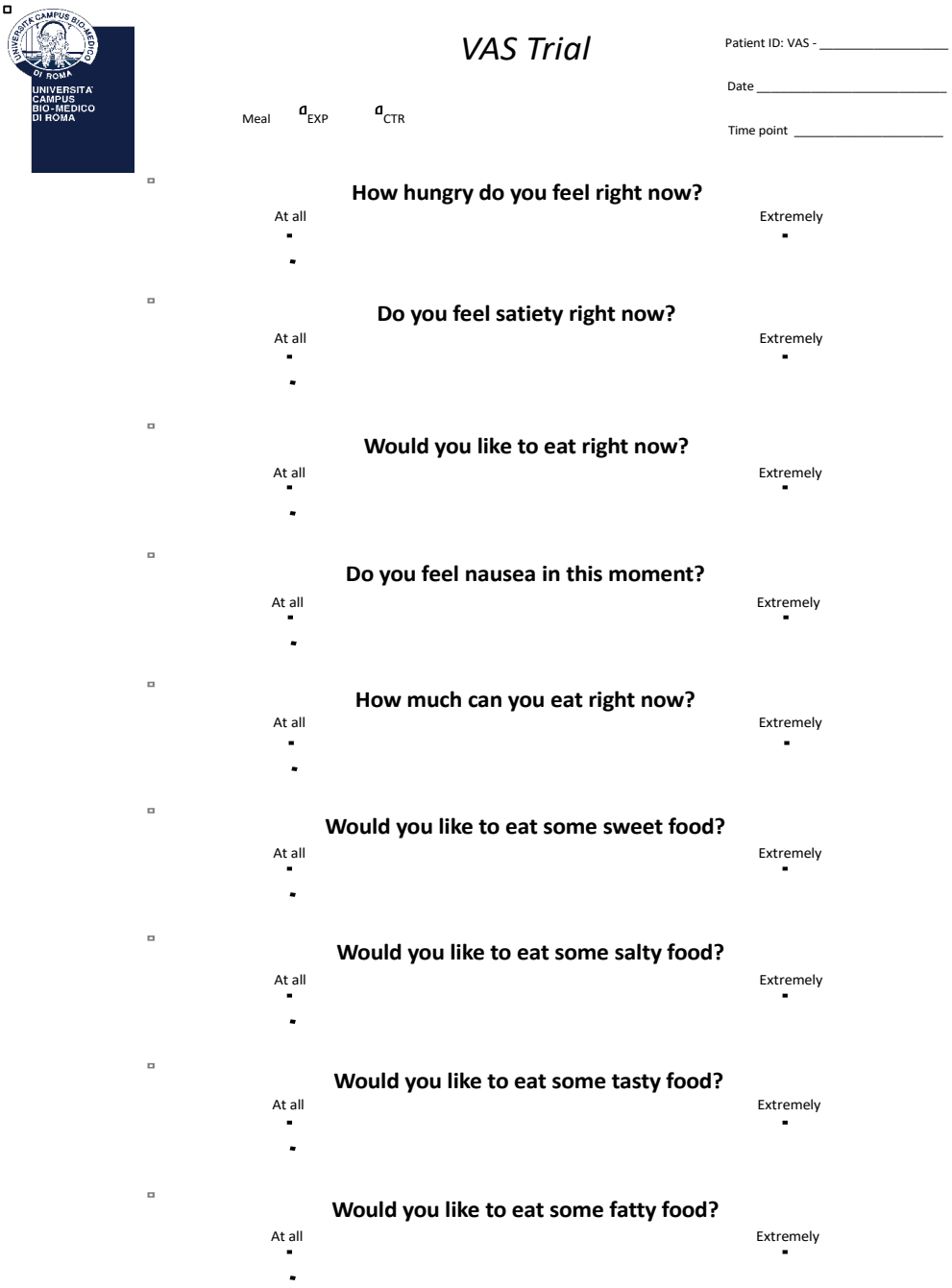

Figure 4. The 100-mm Visual Analogue Scale used in our study to evaluate hunger/satiety perception

The study received ethical approval by the ethics committee of Campus Bio-Medico University of Rome (#08/2017) before the beginning of the enrolment and any

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other procedure; all of the participants provided written informed consent before study initiation. The study has been carried out in accordance with Helsinki declaration and is currently being registered on ISRCTN registry portal.

Laboratory assays

Several outcomes were assessed either at fasting and every 30' until 210' after the meal (both HFV and MED), according to what shown before in **figure 3**. Serum glucose and insulin levels were assessed by our central laboratory. All of the other blood samples were immediately added with aprotinin (Sigma-Aldrich, St. Louis, Missouri) at a concentration of 500 KIU/mL blood and 10 uL DPP-IV inhibitor (Merck, Darmstadt, Germany) according to manufacturer's protocol, then separated for serum or plasma within one hour, and finally stored at -80°C until analysis. Serum levels of GLP-1 and plasma levels of oxyntomodulin were determined at multiple time points, as shown in **figure 3**. VAS administration occurred at the same time points. Serum GLP-1 and plasma oxyntomodulin were analyzed through ELISA (Merck, Darmstadt, Germany and AnshLabs, Webster, Texas, respectively).

Statistical analyses

Statistical Package for the Social Science (SPSS), version 20 (IBM) was used for analysis. Data are presented as means ± SD. Normality was assessed with graphical methods (pp plots and histograms). Non-normally distributed data were log transformed. Comparisons of the response curves of glucose, insulin, GLP-1 and oxyntomodulin in the Mediterranean meal (MED) and the High Fiber Vegetarian meal (HFV) subjects were performed using the repeated-measures analysis of covariance

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(ANCOVA), testing for a group × time effect. Post hoc comparisons were performed using Šidák correction tests. We also checked for a treatment × time × baseline values interaction. Total areas under the curve (AUC) and incremental areas under the curve (iAUC) were calculated as well. For the calculation of total AUC and the iAUC, the trapezoid rule was used.

A general linear mixed model was used to assess the treatment effect on AUCs and iAUCs variables with the variables of treatment, sequence, and visit included as fixed effects, patient-within-sequence included as a random effect. Differences were considered statistically significant when $p < .05$.

Anthropometry and body composition

Anthropometric measures were determined without shoes and in light clothing. Height was measured using a stadiometer (SECA Group, Hamburg, Germany), weight using an electronic scale (SECA Group, Hamburg, Germany), and umbilical waist and hip circumference using a stretch-resistant tape (Figure Finder, Novel Products, Rockton, IL, USA). All measures were taken in duplicates. Body composition was estimated using bioelectrical impedance analysis (BIA) (BIA 101 Akern, Florence, Italy). Fat-free mass (FFM), fat mass (FM), hydration profile (i.e., total body water, TBW, and extracellular water, ECW), and basal metabolic rate (BMR) were calculated according to manufacturer's protocol.

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RESULTS

Our study population features are described in **table 4** below. Our population included 7 men and 5 women, with a mean age of 63 years and a mean BMI range belonging to grade 1 obesity (30 to 34.99 kg/m^2). Mean A1C was 6.45% and mean fat mass was above 30%. 3 out of 12 subjects (25%) were taking metformin, whilst none of them were actually smoking or drinking alcohol. Lipid profile was in normal ranges for all of the parameters assessed (triglycerides, total cholesterol, and HDL-cholesterol).

Age (years)	63.1±8.5
Male, n (%)	7 (64%)
BMI (kg/m^2)	34.4±2.6
Waist circumference (cm)	112.2±7.4
Hip circumference (cm)	114.5±5.2
Neck circumference (cm)	40.6±3.6
Wrist circumference (cm)	20.1±1.9
Fasting plasma glucose, mg/dL (mmol/L)	134±27.9 (7.44±1.55)
Triglyceride, mg/dL (mmol/L)	136.1±77.5 (1.54±0.87)
Total cholesterol, mg/dL (mmol/L)	160.4±40.8 (4.148±1.055)
HDL cholesterol, mg/dL (mmol/L)	51.8±14.8 (1.34±0.38)
Creatinin, mg/dL	0.89±0.22
GOT, U/L	20.4±14.9
GPT, U/L	37.4±26.2
HbA1c (%)	6.45 ± 0.8
Basal metabolic rate, kcal/d (KJ/d)	1730±175 (7238.3±732.2)
Fat mass, % on body weight (kg)	31.2±6.2 (29.27±5.63)
Metformin, n (%)	3 (25)
Current smoker, n (%)	0 (0)
Current drinker, n (%)	0 (0)
Educational level, n (%)	
None or elementary school, n (%)	1 (8.3)
Middle school, n (%)	3 (25)
High school, n (%)	6 (50)
College/university, n (%)	2 (16.7)

Table 4. Demographics and clinical characteristics, data expressed as mean ± standard deviation.

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After meal ingestion hormonal profile:

Herein we have analyzed the effect of two different meals, the High Fibers Vegetarian meal (HFV) and the Mediterranean meal (MED), on postprandial profile of oxyntomodulin, Glucagon -Like Peptide 1 (GLP-1), plasma glucose and plasma insulin.

Oxyntomodulin:

We found a similar initial increase in Oxyntomodulin levels for both the groups that peaked at 30 minutes after the meals ingestion and then showed a biphasic-like pattern over time. After the first peak, the High Fiber Vegetarian meal (HFV) group exhibited a significant and more rapid decrease in oxyntomodulin levels at 90 minutes (the first phase) as compared with the Mediterranean meal (MED) group (439±242 pg/mL Vs 296±196 pg/mL respectively, ***P* <.05, Figure 5**). Oxyntomodulin levels then slightly increased reaching another spike at 150 minutes (NS between the two groups) and then progressed to the lower level after 210 minutes in both the groups (the second phase). The MED group maintained significant and consistent higher levels of oxyntomodulin over time compared to the HFV group (***P*< .05, one-sided, Figure 7**).

The 210-minute oxyntomodulin AUC was significantly higher in the MED group compared to the HFV group even after the adjustment for age, gender, BMI and HbA1c **(*P<.023,* Figure 8).** However, the 210-minute incremental oxyntomodulin AUC (iAUC) only tended to be higher in the MED group **(*P* = .13).**

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GLP-1:

We found a similar initial increase also in GLP-1 levels for both the groups that peaked at 30 minutes after the meals ingestion and then showed a biphasic-like pattern over time too. After the first peak, the HFV group exhibited a more rapid decrease in GLP-1 levels at 60 minutes (the first phase) to slightly increase again at 90 minutes (NS between the two groups) and then reaching the lowest values at 180 minutes (second phase). The MED group maintained significant and consistent higher levels of GLP-1 over time compared to the HFV group **(*P*< .05, one sided, Figure 5)** and then peaked again at 150 minutes (second phase) (56 ± 21 pg/mL Vs 44 ± 18 pg/mL, respectively ***P* <.05**, **Figure 5)**, delaying the second peak one hour and half after the HFV group.

The 210-minute GLP-1 AUC was significantly higher in the MED compared to the HFV even after the adjustment for age and gender, BMI and HbA1c **(*P*<.022, Figure 6).** However, the 210-minute GLP-1 iAUC tended to be higher in the MED meal (*P* = .10).

Glycemia:

We found a similar initial increase also in plasma glucose levels for both the groups that peaked at 30 minutes after meals ingestion. After the first peak at 30 minutes, the HFV group exhibited a significant second peak at 90 compared to the MED group (159 ± 48 mg/dL Vs 198 ± 57 mg/dL, respectively ***P* <.04, Figure 9),** and then the MED group maintained significant and consistent decreased levels of plasma over time compared to the HFV group **(*P*< .039, Figure 9).**

The 240-minutes glycemic AUC was significantly higher in the HFV compared to the MED group, even after the adjustment for age and gender, BMI and HbA1c

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(***P*<.006, Figure 10**). In addition, the 240-minute glycemic iAUC was significantly higher in the MED group (***P*<.002, Figure 10**).

Insulin:

We found a similar initial increase also in plasma insulin levels for both the groups, but the MED group peaked at 30 minutes while the HFV group peaked at 90 minutes after the meal ingestion. There was no significant difference over the 240 minutes observation between the two groups (***P* = .56 Figure 11**).

The 240-minute insulin AUC tended to be higher in the MED compared to the HFV group adjusting for age and gender, BMI and HbA1c (***P*<.10**, **Figure 12**), with this tendency being confirmed in the 240-minute insulin iAUC (***P* = .06, Figure 12).**

VAS

As shown in **Figure 13**, data analysis from VAS scores did not lead to significant differences among the variables explored (i.e., hunger, satiety, and desire to eat) between the two dietary interventions (all P = NS). For each dimension, the following questions were made:

- HUNGER: How hungry do you feel right now?
- SATIETY: How full do you feel right now?
- DESIRE TO EAT: How pleasant would it be to eat right now?

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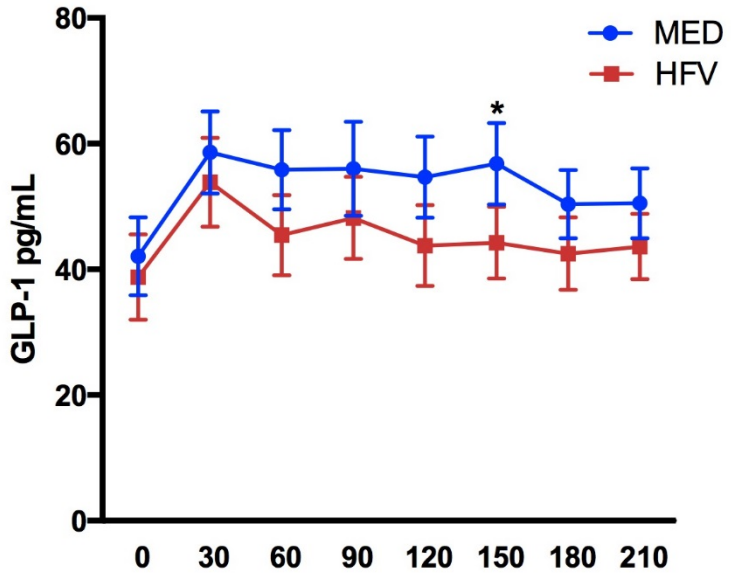

Figure 5. Serum GLP-1 levels comparison across HFV and MED meal, data represented as mean ± SEM.

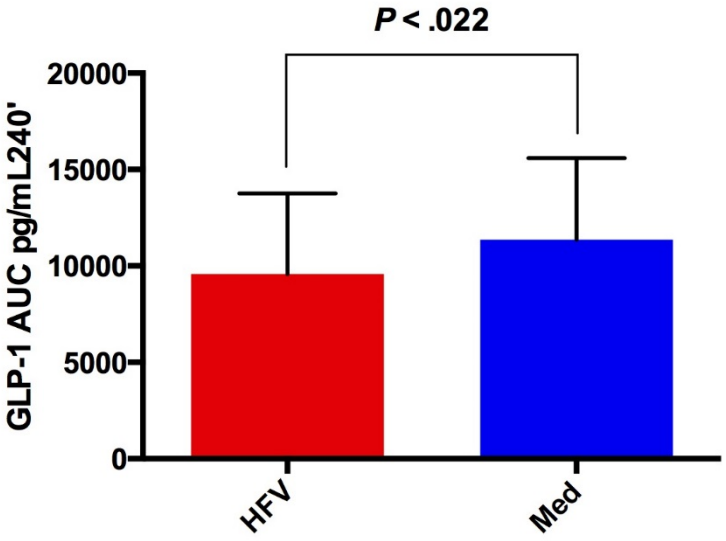

Figure 6. Serum GLP-1 area under the curve (AUC) comparison between HFV and MED meal, data represented as mean ± SEM

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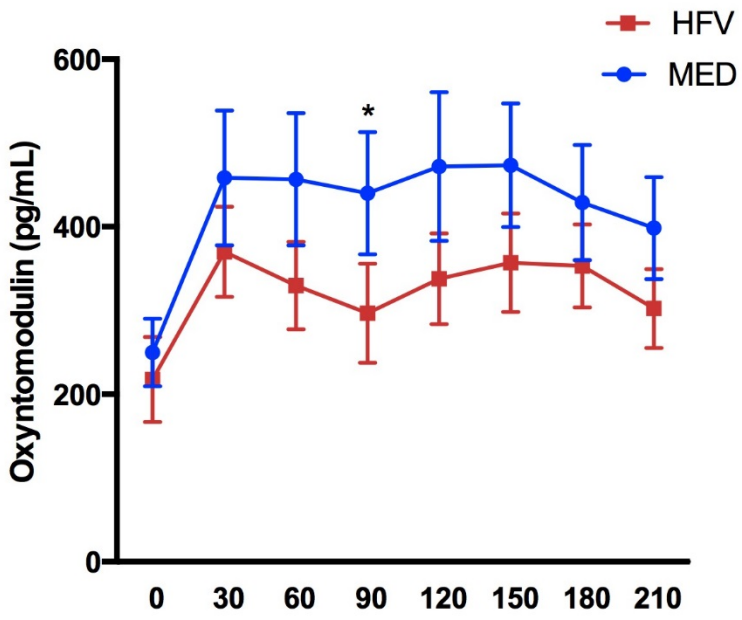

Figure 7. Plasma oxyntomodulin levels comparison across HFV and MED meal, data represented as mean ± SEM

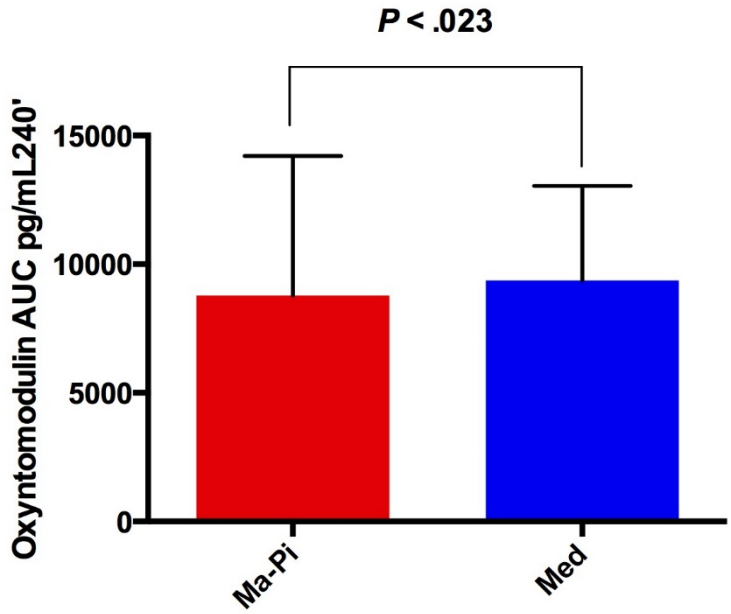

Figure 8. Plasma oxyntomodulin area under the curve (AUC) comparison between HFV and MED meal, data represented as mean ± SEM

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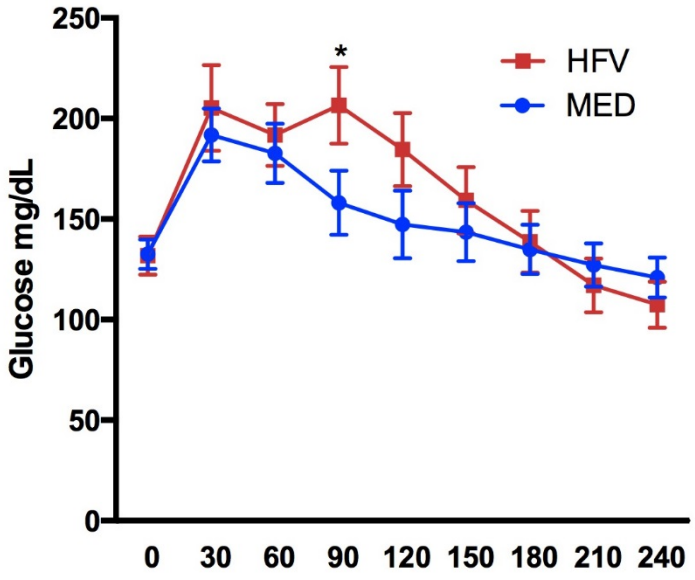

Figure 9. Serum glucose levels comparison across HFV and MED meal, data represented as mean ± SEM

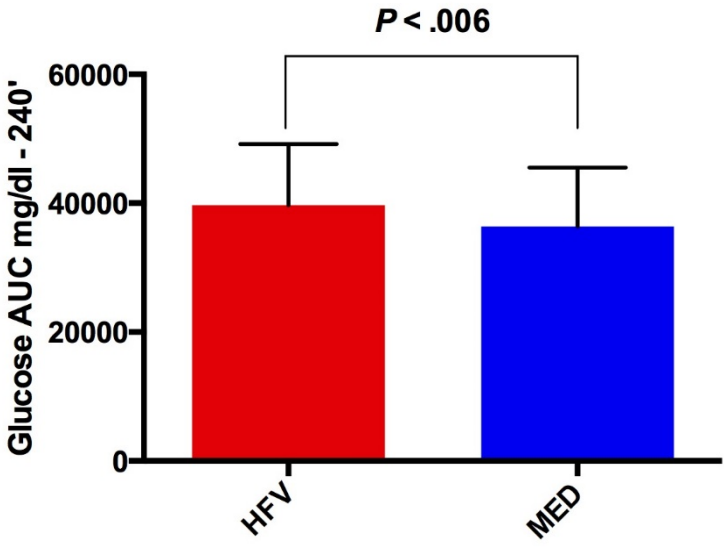

Figure 10. Serum glucose area under the curve (AUC) comparison between HFV and MED meal, data represented as mean ± SEM

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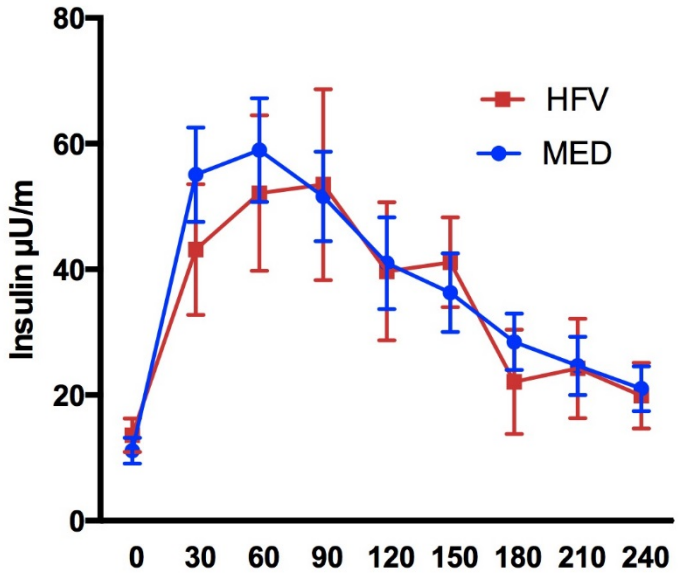

Figure 11. Serum insulin levels comparison across HFV and MED meal, data represented as mean ± SEM

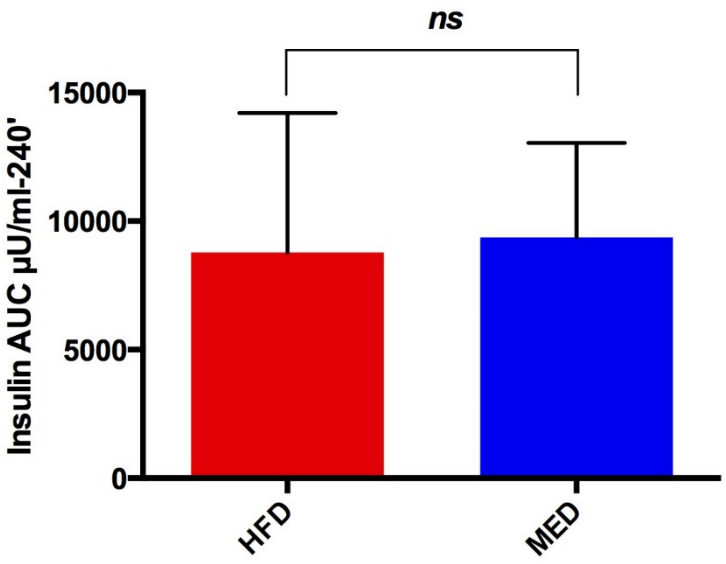

Figure 12. Serum insulin area under the curve (AUC) comparison between HFV and MED meal, data represented as mean ± SEM

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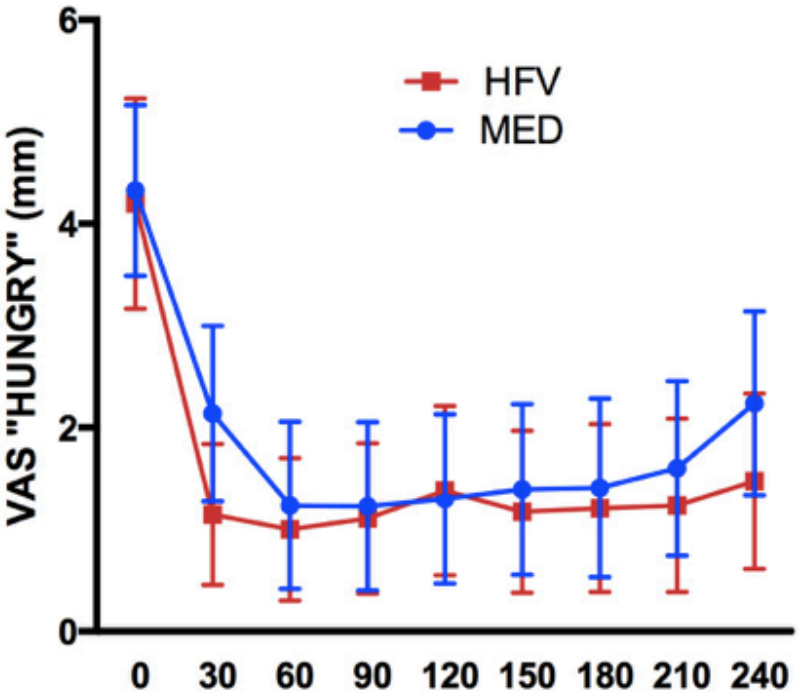

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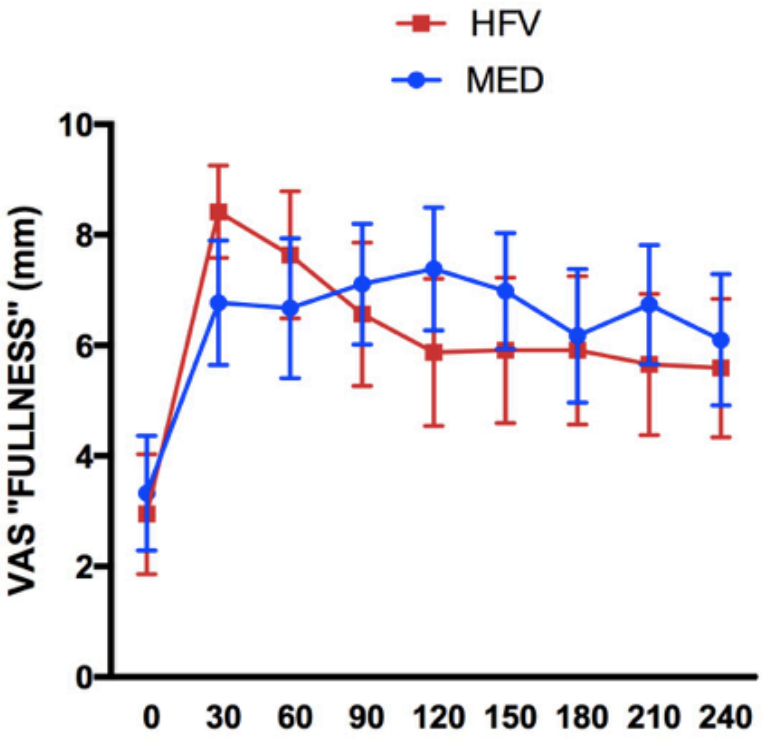

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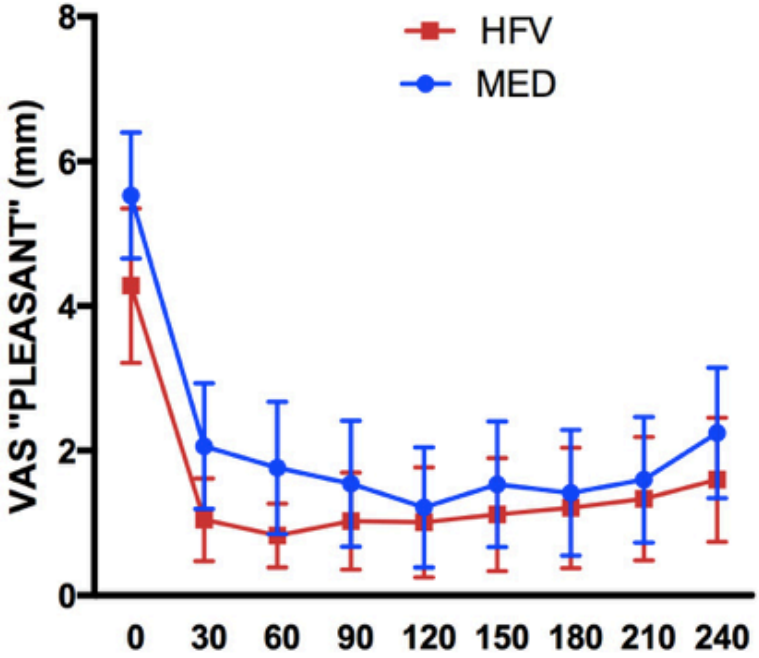

Figure 13. Comparison of self-reported VAS scores on hunger (A), satiety (B), and desire to eat (C) across HFV and MED meal, data represented as mean ± SEM

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DISCUSSION

The implications of macronutrients in glycemic control has long been debated, either alone (i.e., isolated protein or carbohydrate or fat) or combined, and still uncertainties in this field exist. The composition of ingested foods and meals has long been advocated to modulate, either to lessen or to increase, energy homeostasis of an individual. This includes metabolic responses such as glycemic control (both glucose and insulin level), and ultimately appetite behavior. Furthermore, this occurs in a same person over the course of the day as a result of circadian hormonal rhythms [246], in addition to the differences existing between multiple dietary patterns. One of the metabolic pathways which is receiving considerable attention is GLP-1 regulation, either in terms of release and in terms of cleavage inhibition. These are represented by the drugs belonging to GLP-1 receptors agonists (GLP-1RA) and dipeptidyl peptidase IV-inhibitors (DPP-IVi), respectively. Besides these recent scientific advancements, nutritional modulation of GLP-1 levels by carbohydrate, protein, and lipid by-products represent another field of interest and novelty. Indeed, it has been described how L-cells' GPCRs bind to several products of food and macronutrients' breakdown such as monosaccharides, amino acids and peptides, as well as SCFAs, medium and long-chain fatty acids, all of which have been correlated to GLP-1 secretion.

In our research, we assessed incretin release and self-reported hunger/satiety in subjects affected by T2D, a multifactorial disease whose body of evidence keeps increasing as novel treatments are becoming available but still several crucial points need to be addressed.

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GLP-1 modulation by nutrients and single-nutrient foods: monosaccharides

Upon ingestion, digestible carbohydrates undergo enzymatic degradation and are absorbed in the form of glucose, and to a lesser extent in the form of galactose and fructose. Glucose absorption by enterocytes as well as glucose-mediated GLP-1 secretion from L-cells appear to be mediated by the sodium-glucose cotransporter-1 (SGLT-1), a membrane transport protein expressed in the small intestine [247]. Moriya and colleagues [248] investigated the mechanisms underlying glucose-stimulated GLP-1 secretion by administering glucose and Phloritzine, a competitive inhibitor of SGLT-1, in the small intestine of mice. While glucose administration alone acutely increased circulating GLP-1, co-administration of glucose and Phloritzine blocked first-phase glucose-induced GLP- 1 secretion [248]. Similarly, SGLT-1 knockout mice had decreased first-phase GLP-1 secretion and developed glucose and galactose malabsorption compared to controls [249]. This may be explained by the fact that glucose binding to SGLT-1 induces the closure of ATP-sensitive potassium channels, leading to membrane depolarization and to a rise in intracellular calcium concentrations [250]. This work may be of importance as it outlines the implications of SGLT-1 in luminal monosaccharide absorption, as well as glucose-mediated GLP-1 secretion in the proximal small intestine.

The characterization of GLP-1's secretion, which is often considered as being biphasic, led researchers to investigate the prolonged effect the pharmacological inhibition of SGLT-1 in rodents and humans [251-253]. While also confirming an inhibition of first-phase GLP-1 secretion and an increase in luminal glucose concentrations, Powell and al. [252] found an increase in second-phase GLP-1 secretion and a decrease in blood glucose excursions over a 6-hour postprandial period

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in mice. These results were confirmed in two clinical studies conducted in healthy adults, as well as in adults with T2D [253, 254]. The increase in second-phase GLP-1 secretion observed in these studies suggests that SGLT-1 inhibition enhances other mechanisms promoting GLP-1 secretion from the distal part of GI tract. Possible explanations include an enhanced opportunity for interactions with L-cells as carbohydrates move down the GI tract during digestion, as well as an increased fermentation of undigested carbohydrates in the colon which could raise the production of SCFAs [252-254]. Concordant with the latter hypothesis, Powell and al. [252] observed a decrease in the pH of the cecum, which could indeed be an indicator of increased bacterial fermentation, in SGLT-1 knockout mice as well as mice that received a SGLT-1 inhibitor.

In our study, we found a similar initial increase also in GLP-1 levels for both the groups that peaked at 30 minutes after the meals ingestion and then showed a biphasic-like pattern over time too. After the first peak, the HFV group exhibited a more rapid decrease in GLP-1 levels at 60 minutes (the first phase) to slightly increase again at 90 minutes and then reaching the lowest values at 180 minutes (second phase). The MED group maintained significant and consistent higher levels of GLP-1 over time compared to the HFV group and then peaked again at 150 minutes (second phase), delaying the second peak one hour and half after the HFV group.

As summarized in **table 3**, MED meal had lower glycemic content (i.e., carbohydrate and sugar content, as well as lower glycemic index and load) compared with HFV meal. A GLP-1 biphasic secretion pattern has been described before, with an early-phase after 10 to 15 minutes followed by a longer second-phase release after

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30 to 60 minutes upon meal ingestion. The difference in dietary formulation between HFV and MED meal may have played a role in delaying the GLP-1 second-phase release and in inducing the overall higher levels across the 210 minutes observation after the MED meal. Indeed, while the HFV dietary intervention had a higher glycemic profile, which may explain the higher glucose trend seen throughout the trial, as well as more fiber, the MED meal had more protein and higher lipid profile (including SFA, MUFA, and PUFA). In addition, while first-phase has been related primarily to neural signaling, neurotransmitters and gut peptides, second-phase has been related to direct stimulation by L-cells [255, 256]. This may in turn question the importance of gastric emptying as it regulates the entrance of nutrients into the small intestine, whereby direct stimulation occurs.

One of the recognized GLP-1 effects is indeed slowing gastric emptying, thus decreasing its own secretion upon postprandial activation [250]. As previously reported, the HFV meal displayed higher soluble fiber compared with that of MED meal (4.7g vs 0.8g, **table 3**), whose metabolic effects include delayed gastric emptying and subsequent satiety feeling [257-259]. Yu et al. carried out a clinical trial in which gastric emptying was found to be delayed by soluble dietary fiber administration either in healthy subjects and T2D patients, with higher postprandial glucose levels [259].

Other factors affecting GLP-1 response have been identified in higher weight or BMI, as well as high glucagon concentrations, advocated as lower GLP-1 response predictors; besides, others appear to be determinants of enhanced GLP-1 secretion (e.g., older age, higher fasting NEFA concentrations) [260]. However, we have adjusted for BMI in our AUC and iAUC analysis.

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The ability to predict normal or impaired GLP-1 response in patients with T2D may thus depend on the individual balance of these factors as well as the rate of gastric emptying. Although we did not assess these outcomes so far, the same reasoning may be applied to our study. In another study, T2D patients had high NEFA concentrations as well as elevated glucagon, the influences of which may have balanced each other out, resulting in no net difference in GLP-1 secretory responses [261].

GLP-1 modulation by nutrients and single-nutrient foods: non-digestible carbohydrates and short-chain fatty acids

In the colon, non-digestible carbohydrates undergo fermentation, leading to the production of various amounts of SCFAs according to their type [262-265]. SCFAs are carboxylic acids that contain fewer than 6 carbons, the most abundant ones being acetate, butyrate and propionate [266]. In humans, SCFAs concentrations range from ~130 mmol/L in the caecum to ~80 mmol/L in the descending colon [266, 267]. Acetate appears to be the most abundant SCFA in the colon, followed by propionate and butyrate, with an overall colonic molar ratio of 50-60:15-20:10-20, respectively [266]. Fermentable dietary fiber and their metabolites (SCFAs) seem to promote GLP-1 secretion from L-cells by interacting with the free fatty acid receptors 2 and 3 (FFAR2, FFAR3) [251, 268-273]. An in vitro study showed that propionate was the most potent agonist of both FFAR2 and FFAR3, that acetate was more active and selective on FFAR2, and that butyrate was more active on FFAR3 than FFAR2 [273]. In colonic cell cultures, physiological concentrations of acetate, butyrate and propionate have been shown to stimulate GLP-1 secretion [274]. The role played by FFAR2 and FFAR3 in the SCFA-induced GLP-1 secretion was confirmed by

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demonstrating the loss of postprandial GLP-1 secretion in FFAR2 and FFAR3 knockout intestinal cells [274]. Similar results were found in vivo, where propionate-induced GLP-1 secretion was lost in FFAR2 knockout rodents [275]. Additionally, Chambers and colleagues [276] recently showed that acute targeted delivery of propionate in the colon through an inulin-propionate ester supplement acutely stimulated GLP-1 secretion following a standard breakfast meal and reduced energy intake at a buffet-style lunch in adults with overweight or obesity. Concordant with the acute effects of GLP-1 on appetite sensations and food intake, daily delivery of propionate in the colon over 6 months also reduced body weight, abdominal fat and hepatic lipid accumulation as assessed with magnetic resonance imagery and spectroscopy [276]. The effects of diets rich in fermentable fiber on GLP-1 secretion have also been investigated in animal models and humans [277, 278]. The main way to increase colonic SCFA concentrations in humans is indeed through non-digestible carbohydrate consumption. Bodnaruc et al. have summarized the results from six experimental studies assessing the impact of fiber on GLP-1 secretion in animal models and humans; compared to a standard control diet, the consumption of a diet enriched in fermentable fiber for 50 days increased GLP-1 concentrations in the proximal colon, which was associated with an increased expression of proglucagon in colonic L-cells [246, 279, 280]. Fermentable fiber also decreased food intake, weight gain, as well as blood triglyceride concentrations and triglyceride accumulation in the liver [279]. Similarly, while also increasing GLP-1 blood concentrations and proglucagon expression in the colon, consumption of a diet rich in resistant starch for 10 days increased peptide tyrosine-tyrosine (PYY) blood concentrations and colonic expression in rats [281]. This result is not surprising as the anorectic PYY is also

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synthesized and released from L-cells, and its secretion could be stimulated by similar mechanisms as that of GLP-1. In healthy humans, a crossover pilot study showed that the daily supplementation with 16 g of fermentable fiber for a two-week period increased postprandial satiety, and decreased hunger, prospective food consumption as well as energy intake over 24 hours following the intake of a standard breakfast meal [280]. As GLP-1 blood concentrations were not assessed in this study, it is not known whether these effects were mediated by an increase in its secretion or through other mechanisms [280]. The effects of fermentable fiber in rodent models of diabetes were comparable to those found in healthy animals. Indeed, consumption of a diet enriched with fermentable fiber for 4 to 6 weeks increased GLP-1 concentrations in the proximal colon and portal vein, as well proglucagon expression in the proximal colon [282, 283]. In streptozotocin-treated diabetic rats, the fiber-enriched diet also up-regulated pro-hormone convertase 1 expression in the proximal colon and increased pancreatic β-cell mass which potentially mediated the improvement in glucose tolerance and the observed increase in insulin and C-peptide blood concentrations [282]. In mice with high-fat diet-induced diabetes, these effects were diminished with the use of a GLP-1R antagonist and were completely lost in a GLP-1R knockout group of rats, therefore confirming the importance of GLP-1 interaction with its receptors for exerting its beneficial effects [283]. Overall, results from these studies suggest some pathways linking fermentable fiber to its positive effects on food intake, body weight gain and blood glucose homeostasis via an increased secretion of GLP-1. Fermentable fiber and SCFAs produced by their colonic fermentation appear to increase GLP-1 synthesis by upregulating the expression of proglucagon [280, 282-284], as well as that of pro-hormone convertase 1 [282], which has been related also

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to its cleavage [285]. Cani and colleagues also found an increased proximal colon's L-cell number with fermentable fiber consumption, which could be another possible explanation for the increased GLP-1 synthesis and secretion [284]. The increase in L-cell number was also associated with an enhanced expression of transcription factors that are critical for the differentiation of intestinal stem cells into enteroendocrine cells such as neuregenin-3 and neuro-D [284]. In our study, the effects of SCFA-induced beneficial effects upon GLP-1 release has not been established already. Yet, previous findings from our research group proved a dietary intervention similar to HFV meal to significantly ameliorate gut microbiota health in dysbiotic T2D obese subjects [241]. It is reasonable to perform FFAR2 and FFAR3 receptors assessment in our future studies, considering that little in known regarding short term diet-induced effects upon GLP-1 release and that our dietary interventions differ considerably in terms of insoluble fiber (16.66 g in the HFV meal vs 2.23 g in the MED meal, **table 3**).

GLP-1 modulation by nutrients and single-nutrient foods: free fatty acids

Dietary lipids are often considered as triglycerides, which are composed of a glycerol molecule esterified with three fatty acids [286]. Upon ingestion, triglycerides undergo emulsification by bile salts in the duodenum, hydrolysis by lipases, and are absorbed by enterocytes in the form of glycerol and free fatty acids [286]. Free fatty acids, such as unsaturated long-chain fatty acids, are potent stimulators of GLP-1 release through interactions with free fatty acid receptors 1 and 4 (FFAR1, FFAR4) [287, 288]. Substrate binding to FFAR1 and FFAR4 activate phospholipase C, leading to inositol triphosphate mediated calcium release from the endoplasmic reticulum into the cytosol [289]. The secretion of GLP-1 has been shown to be increased by unsaturated long-

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chain fatty acids. As shown in **table 3**, this is in line with our findings as higher GLP-1 release was assessed in MED meal, whose MUFA and PUFA content was higher than HFV meal (MUFA 15.93g vs 1.02g; PUFA 5.18g vs 1.41g, respectively). Several experimental studies assessing the impact of lipid consumption on GLP-1 secretion and blood concentrations have been conducted. Thomsen and colleagues started assessing GLP-1 responses following a meal containing 80g olive oil 20 years ago, either in healthy adults or adults with T2D [290, 291]. Compared with a meal containing butter, which is high in saturated fatty acids (SFA), the ingestion of the olive oil containing meal resulted in higher postprandial both GLP-1 and GIP blood concentrations, although no significant acute difference in blood glucose, nor insulin blood concentrations was observed [290, 291]. It has to be argued that 80 grams MUFA-rich olive oil is far away the usual daily intake seen in a typical Mediterranean dietary lifestyle, recently estimated to be > 4 tablespoon per day (one tablespoon being equal to 10 grams) [292]. In a subsequent study conducted in rodents, the prolonged consumption of an olive oil-enriched diet resulted in an increased GLP-1 secretion, which coincided with a higher glucose-stimulated insulin secretion, as well as an improved glucose tolerance at the 36th day of the intervention [293]. Comparable results were found in Streptozotocin-treated diabetic rats, where the intake of a diet rich in MUFA from olive oil for 50 days increased GLP-1 blood concentrations, decreased weight gain and improved insulin sensitivity [294]. In humans with abdominal obesity and insulin resistance, Paniagua and colleagues [295] showed that ingestion of a Mediterranean diet rich in olive oil for 28 days resulted in significantly higher postprandial GLP-1 blood concentrations (23% of total daily energy intake). Compared to a diet high in SFA, consumption of the Mediterranean diet also improved

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insulin sensitivity, an effect which could have mediated the observed decrease in insulin secretion as well as fasting and postprandial blood glucose concentrations [295]. It is of interest to notice that these results are related to middle-long term olive oil administration whilst our project deal with acute, short term administration and therefore add knowledge in this field. Furthermore, these works did not address neither oxyntomodulin implications nor hunger/satiety perception in T2D. In addition to the beneficial effects of MUFAs, some studies showed that the colonic administration of the polyunsaturated α-linolenic acid acutely increased GLP- 1 and insulin blood concentrations, and decreased blood glucose concentrations in healthy and diabetic rat models [296]. Similarly, while also resulting in increased GLP-1 blood concentrations, long-term administration of α-linolenic acid has been shown to increase β-cell proliferation through the free fatty acid receptor GPR120 in rodents [297]. As GLP-1 has been shown to decrease apoptosis, as well as to increase neogenesis of pancreatic β-cells, it has been speculated the increased β-cell proliferation may be mediated by the increase in GLP-1 concentrations induced by α-linolenic acid ingestion [297]. A recent study showed that fish oil and flax seed oil (which are a source of α-linolenic acid), taken at a concentration equal to 10% of daily energy intake, increased FFAR4 expression in rodents' colon and decreased the expression of the pro-inflammatory tumor necrosis factor α (TNFα) [298].

Taken together, these studies suggest that, with similar energy contents, diets that are richer in MUFAs or omega-3 PUFA than in SFA could increase GLP-1 secretion from L-cells, which may be a mediator for the increase in insulin secretion, insulin sensitivity, β-cell proliferation, as well as the improved glucose tolerance observed in animal models and in humans. Both PUFA n-3 and PUFA n-6 were

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consistently lower in HFV meal compared with MED meal (PUFA n-3 = 1.38g vs 2.22g; PUFA n-6 = 0.07g vs 2.62g, respectively).

GLP-1 modulation by nutrients and single-nutrient foods: peptides and amino acids

Dietary proteins are generally described as the most satiating nutrient, an effect which may be partly mediated by the stimulation of anorexigenic GI peptides secretion, including that of GLP-1 [299, 300]. Upon ingestion, proteins are broken down by acid hydrolysis and proteases to produce peptones, tripeptides, dipeptides and single amino acids. Specifics mechanisms responsible for protein-induced GLP-1 secretion are relatively poorly understood, and the optimal profile of proteins' breakdown products remains to be elucidated. Nonetheless, two pathways by which products of protein breakdown seem to stimulate GLP-1 secretion is through their binding to the calcium-sensing receptors (CaSR) and the class C, group 6, subtype A GPCR (GPCR-C6A) which are expressed on L-cells. CaSR binds to a variety of amino acids including phenylalanine, tryptophan, asparagine, arginine, glutamine and histidine, as well as to peptones, tri- and dipeptides [301, 302]. Their binding to CaSR stimulated GLP-1 secretion, such effect being abolished with the use of a CaSR inhibitor [301, 302]. Oya and colleagues [303] have shown that GPCR-C6A binds to the basic amino acids arginine, lysine and ornithine. The stimulatory effect of these amino acids on GLP-1 secretion was abolished in GPCR-C6A knockout intestinal cells [303]. Interesting findings in the field of protein and GLP-1 release are those established by Hall et al., in which whey protein has been observed to stimulate GLP-1 release significantly more than casein-derived protein, such findings being corroborated by VAS ratings in

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terms of satiety. Furthermore, plasma amino acid profiles of branched-chain amino acids valine, leucine and isoleucine plus threonine resulted significantly increased following whey preload compared with casein preload (all $P<0.001$) [304]. This group also assessed plasma paracetamol concentrations following whey and casein preload to evaluate gastric emptying, the latter being related to a faster initial rise concentration and then a levelling off compared with the whey preload ($P<0.05$) [304]. It is known that casein differ from whey being a coagulative protein, which results in slower gastric emptying rate (preload X time effect) and lower postprandial plasma amino acids excursions compared with the non-coagulative protein whey [305].

Another important, poorly investigated aspect is protein source in relation to anorexigenic hormonal release and satiety feeling, specifically an assessment of plant-based versus animal protein.

In our study, we compared a vegetarian meal high in complex carbohydrate and fiber with a Mediterranean-like meal, thereby comparing both of these protein sources. As protein content differed considerably (16.3% of total caloric intake in the HFV meal vs. 24% in the MED meal), a future perspective may lie in the assessment of similar quantities of both plat-based and animal-based protein. In addition, MED meal has both protein sources (25.5g animal-derived; 10.21g plant-derived) while HFV had plant-derived protein only (18.33g). This is another topic which deserves further insights, considering that no differences in VAS scores was assessed despite these dietary features – such protein content may be expected to lead to higher satiety feeling during MED meal than that related to HFV meal.

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Incretin release in mixed-nutrient foods

In contrast with single nutrients and single-nutrient foods such as oil, more complex foods containing a mix of nutrients are more representative of what humans consume and could allow targeting a combination of enteroendocrine pathways that would synergistically enhance GLP-1 secretion. In regards to T2D management, clinical guidelines recommend carbohydrate intake from high-fiber foods such as vegetables, fruit, legumes and whole grains, while limiting SFAs intake and promoting that of MUFA and n-3 PUFA [306, 307], all of which have been associated to different extents with an increased GLP-1 secretion. Some experimental studies conducted with healthy individuals have examined the effects of several specific foods on glycemic response, subjective appetite sensations as well as energy intake at a subsequent meal. These include oatmeal [308, 309], dried fruit such as pistachio [310-312], almonds [313-317] and peanuts [318], eggs, either at breakfast [319, 320] and at lunchtime [321, 322], avocado [323], as well as fiber-enriched wheat [324] and barley [325]. Several researchers have investigated the effects of high-fiber grain products, which could enhance GLP-1 secretion by binding to SGLT-1, FFAR2 and FFAR3. In this regard, two recent studies compared appetite responses after isoenergetic breakfast meals containing ready-to-eat breakfast cereals (low-fiber) or oatmeal (high-fiber) among healthy young adults, showing ready-to-eat breakfast cereals to be related to increased satiety and reduced hunger, desire to eat, as well as subjective prospective food intake compared with oatmeal (66.8 g) [308, 309]. Furthermore, oatmeal decreased energy intake at the following meal [309]. Likewise, in adults with insulin resistance, daily consumption of a high wheat-fiber breakfast cereal (test group, 24 g/day of fiber from the cereal) for one year significantly increased colonic SCFA

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production, as well as GLP-1 blood concentrations compared to low-fiber breakfast cereals (control group, 0.5 g/day of fiber from the cereal) [324]. More specifically, at month 9 during the intervention, acetate and butyrate plasma concentrations were already significantly higher in the test group [324]. Along the same lines, Nilsson and colleagues showed that consumption of high-fiber barley kernel-based bread for 3 days was associated with increased fasting GLP-1 blood concentrations, as well as increased postprandial PYY blood concentrations in healthy adults [325]. These changes in GI peptides concentrations were associated with improved insulin sensitivity and decreased post- prandial blood glucose concentrations in healthy individuals [325]. The fact that the authors found an increase in breath hydrogen and SCFAs blood concentrations, suggests that the positive effects of the barley kernel-based bread were mediated by an increased SCFAs production triggered by the colonic of dietary fiber [325]. The addition of foods with a high protein, MUFA and fiber content, such as almonds (30g to 90g) or pistachios (28g to 85g) to a high-carbohydrate meal has also been shown to improve postprandial glycemic responses in a dose-dependent manner [310-317]. Jenkins at al. also found a decrease in insulin secretion in healthy adults and in adults with hyperlipidemia following acute and prolonged almond consumption. Similarly, daily intake of 50g of pistachios for 12 weeks decreased C-reactive protein blood concentrations, systolic blood pressure, and body mass index in adults with T2D [314]. In women with obesity, consumption of either peanuts or peanut butter (42.5g) as part of a standard breakfast meal increased postprandial insulin blood concentrations and decreased desire to eat following a standard lunch meal. Additionally, peanut butter significantly increased postprandial PYY blood concentrations and decreased postprandial blood glucose concentrations

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following the standard lunch meal [325]. Since GLP-1 and PYY are co-released from L-cells, it is plausible that peanut butter may also promote postprandial GLP-1 secretion. Mori et al. found no significant differences in GLP-1 blood concentrations following the addition of 42.5g of almonds to a meal containing carbohydrate, which could be due to a relatively small sample size of 14 IGT patients [316]. Indeed, with a larger sample size, Kendall et al. found higher GLP-1 and glucose insulinotropic peptide blood concentrations following the addition of 85 g of pistachios to a meal containing carbohydrate in adults with metabolic syndrome [311]. As Reis et al. showed no statistically significant difference in GLP-1 blood concentrations with the addition of 42.5g of peanuts or peanut butter to a carbohydrate-containing meal, it is also possible that higher quantities of nuts are necessary to induce a sufficient rise in GI hormone secretion [318]. Consumption of 2 to 3 eggs, which are high in protein and also contain MUFAs, for breakfast or lunch, has been shown to improve subjective postprandial appetite sensations [319-322]. Furthermore, when compared to a bagel breakfast, consumption of a breakfast containing 3 eggs (one egg weighs about 50g to 60g) in adult men was associated with a lower post- prandial blood glucose concentration, decreased hunger, and reduced energy intake in the next 24 hours [160]. Men also reported higher subjective satisfaction after eating eggs [319]. While GLP-1 blood concentrations were not measured in these studies, Liu and al. found a significant increase in postprandial blood concentrations of PYY in adolescents following ingestion of an egg-containing breakfast compared to an isocaloric bagel breakfast [320]. One study also showed that adding avocado (50g to 90g), which is high in fiber and MUFAs, to a high-carbohydrate isocaloric meal improved subjective satiety sensation and satisfaction [323].

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Diet, appetite behavior, and VAS score

Lifestyle interventions are crucial for disease prevention and treatment, including metabolic conditions such as T2D and obesity, such actions being largely mediated by hunger/satiety hormonal release and ultimately by appetite sensation. A recent Australian work has addressed acute GLP-1 response to different macronutrients in adolescents, whose effective weight management and is of paramount importance in reducing disease risk in adulthood, through the administration of a VAS in relation to a high-protein (whey: 55% protein, 30% carbohydrate, 15% fat) or high-carbohydrate (maltodextrin: 79% carbohydrate, 5% protein, 16% fat) liquid breakfast [326]. Whilst orexigenic ghrelin hormone was significantly affected (i.e., diminished) by high-protein meal consumption (P=0.008), with non-significant difference between healthy and obese subjects, GLP-1 and PYY response was similar in both of the test meals. The authors have suggested such smaller ghrelin drop seen in obese adolescents be related to lower baseline levels and therefore to partial release inhibition which may hinder the magnitude of a specific high-protein meal compared with healthy subjects [326]. The lack of significance in GLP-1 response seen by Nguo et al. is in contrast with other studies showing an attenuation in obese children as well as in obese adults [300], despite evidences in GLP-1 release advocating protein to be more effective than carbohydrate exist [327, 328].

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Diet and oxyntomodulin release

Oxyntomodulin is released from the endocrine intestinal cells 5-10 minutes following a meal and proportionally to caloric intake [217, 329], and acts via G-protein coupled receptors (GPCR) either on afferent nerves or directly on hypothalamic arcuate neurons by inhibiting expression and release of orexigenic NPY and AgRP peptides, as described before. It has been established that an impairment in the orexigenic and anorexigenic balance may result in disorders of hunger/satiety circuitry and ultimately in feeding behavior, with subsequent weight gain (obesity) or weight loss (cachexia) [330]. Schjoldager et al. estimated a metabolic clearance rate in humans around 5mL/kg per minute for plasma samples, with a 12-minute half-life as determined after intravenous infusion of synthetic oxyntomodulin to steady state levels [331]. Plasma levels are however quite low, from 0 to 30 pmol/L (0 to 110.1 pg/mL) [230]. As oxyntomodulin may be subjected to DPP-IV mediated degradation, specific resistant analogues have recently been deployed [332, 333].

A wealth of evidence is available regarding oxyntomodulin release and decrease in hunger score and food intake [220, 230, 330, 334, 335], with endocrine effects that include suppression of ghrelin plasma levels [330], increase in energy expenditure [223, 335], and inhibitory control on gastropancreatic functions [230, 336]. An elegant investigation performed by ElHindawy et al. compared dietary starch digestion products with malt-oligosaccharides (MOS) upon L-cells activation, proving the latter to be more effective in GLP-1 release [337]. These findings may be related to previous works led by our research group, in which obese T2D patients ameliorated their

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glycemic response following a high-carbohydrate, high-fiber diet compared with a standard control diet, although these study did not involve GLP-1 nor oxyntomodulin levels, hindering the hypothesis of such relationship [173, 174]. However, the findings from ElHindawi et al. are of usefulness as, at present, very scarce is the evidence addressing nutrients upon oxyntomodulin release in a dose-dependent manner and with a causal effect relating diet and satiety. The levels of oxyntomodulin seen at fasting in our study are a little bit higher than those stated by Holst et al., which may be related to the fact that we enrolled diabetic obese patients with a history of T2D of at least 2 years. This may be of interest as data on the progression of the disease and oxyntomodulin arousal are not available to date. It may be speculated such increase in plasma oxyntomodulin be related to a compensation to mitigate the excess of calories often seen in these subjects. Future studies are needed to clarify and provide novel insights into oxyntomodulin and diabetes progression and management. Future investigations are also required to provide further data on postprandial oxyntomodulin trend. In our study, plasma oxyntomodulin levels do not decrease as quick as Holst et al. have established [230]. Specifically, the composition of the MED control meal we deployed may have significantly stimulated oxyntomodulin release over the following 150 minutes (**figure 7**). Since no data are available on oxyntomodulin trend following dietary approaches different in macronutrient composition, we can speculate the higher protein and fat content be responsible for higher postprandial plasma oxyntomodulin levels.

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CONCLUSIONS AND FUTURE PERSPECTIVES

With this PhD thesis, we aimed to broaden current scientific knowledge regarding the field of T2D and T2D-related hormonal release, with a focus on the evaluation of hunger/satiety circuitry derangement. This project presents several limitations and strengths that should be acknowledged. We provided novel insights into oxyntomodulin release and trend over time, plus new data on GLP-1 release and trend over time, with two different dietary interventions being a) a high-fiber vegetarian meal whose evidence in literature is increasing, and b) the renowned Mediterranean diet.

Manipulating the composition of the diet in order to promote GLP-1 secretion represents a promising lifestyle strategy for obesity and T2D management. The therapeutic potential of GLP-1 has already been established as several pharmaceutical agents promoting its effects are successfully used for blood glucose management in individuals with T2D, and for body weight management in individuals with obesity [338, 339]. In comparison with the use of GLP-1RA and DPP-IVi, targeting endogenous pathways through dietary modifications has the added benefits of potentially stimulating the release of other anorexigenic gut peptides (e.g., oxyntomodulin and PYY), promoting beneficial changes in several markers of cardiometabolic health such as helping normalize blood lipid profile and blood pressure, as well as avoiding side effects associated with medication. Furthermore, as DPP-IV pharmacological action depends on the bioavailability of endogenous GLP-1 into the bloodstream, combining the use of this drug type with a diet promoting GLP-1 secretion could promote DPP- IV inhibitors' action and potentially allow the use of lower doses and/or a synergistic effect. Therefore, it would be worthwhile to

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investigate the relationship between diet composition and DPP-IV inhibitors' efficacy in future studies.

With this thesis is being written in June 2019, a manuscript on these data will be submitted to an international scientific journal for peer-review and publication. The overall scientific production during this PhD course is shown below.

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SCIENTIFIC PRODUCTION

- **Di Mauro A**, Tuccinardi D, Del Toro R, Buzzetti R, Campagna G, Pozzilli F, Pianesi M and Pozzilli P (abstract). Use of Diabetes Risk Score in the evaluation of type-2 diabetes risk following Ma-Pi macrobiotic and other diets. 2017 IDF Annual Congress

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