AZIENDA OSPEDALIERO---UNIVERSITARIA
"MAGGIORE DELLA CARITÀ" UNIVERSITÀ DEL
PIEMONTE ORIENTALE
SCDU di Endocrinologia

Direttore: Prof. Gianluca Aimaretti

PROTOCOL

NON-PROFIT CLINICAL-DIAGNOSTIC PRACTICE

TITLE

FIGHTING WESTERN DIET-DERIVED AGEs (ADVANCED GLYCATION END PRODUCTS) WITH NATURAL COMPOUNDS TO MITIGATE MUSCLE WASTING IN SARCOBESITY (WESTERNAGE): observational study on the relationship between AGE levels and sarcobesity in an adult population affected by obesity and type 2 diabetes mellitus

FIGHITING AGEs (ADVANCED GLYCATION END PRODUCTS) RESULTING FROM THE WESTERN DIET WITH NATURAL COMPOSITES TO MITIGATE MUSCULAR DECADENCE IN THE SARCOBESITY (WESTERNAGE): observational study on the relationship between AGE levels and sarcobesity in an adult population with obesity and type 2 diabetes mellitus

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STUDY BACKGROUND:

Skeletal muscle is the most abundant tissue in the human body and is critical to the metabolic rate of the entire body, consuming 70% of metabolites and accounting for 80% of total glucose uptake in response to insulin produced (1,2). Therefore, impaired muscle health, particularly loss of muscle mass (atrophy) and strength, known as muscle wasting (MW), can have significant impacts on metabolic health, predisposing to various diseases (3,5). In fact, MW is a common feature that contributes to the adverse outcomes of chronic noncommunicable diseases (NCDs), such as obesity, diabetes, and sarcopenia, which is the specific muscle loss associated with aging (3,5).

Chronic inflammation, both locally and systemically, plays a key role in the onset of MW. It is precisely the circulating cytokines that increase the muscle catabolic state, leading to a promotion of muscle loss through activation of intracellular proteolytic systems (the ubiquitin-proteosome system [UPS] and the autophagic-lysosomal system [ALS]) and reduction in the rate of protein synthesis (3,4,6).

In recent decades, the transition to a "Western diet" known as the "Western diet" (WD) characterized by ultra-processed foods high in sugar, saturated fat, and low in fruits and vegetables has contributed to the rise of NCDs globally. WD, in fact, promotes insulin resistance and metabolic inflexibility, triggering inflammation and oxidative stress (7,11).

One growing NCD is sarcobesity, which is characterized by reduction in muscle mass and increase in total and visceral fat mass, associated with mobility problems and/or the development of adipose-based chronic diseases (ABCD), especially type 2 diabetes, found in 80 to 85 percent of overweight/obese people. The mechanisms underlying the initiation and progression of sarcobesity are still largely unclear.

Advanced glycation end-products (AGEs) contained in foods consumed following WD are considered one of the molecular mediators in the initiation and promotion of metabolic dysfunction and NCD more generally (12). AGEs represent a heterogeneous group of nonenzymatic adducts between reducing sugars and the free amino groups of proteins, nucleic acids, and lipids, usually resulting in fluorescent derivatives (13,14). AGEs can result either from endogenous formation or from exogenous sources, that is, from the diet (dAGEs). Endogenous formation occurs during aging and/or under conditions of oxidative stress and hyperglycemia, whereas exogenous AGEs result from the consumption of foods rich in saturated fats, sugars, meat, and cheese (13). In addition, even the typical WD preparation methods, such as heat and dehydration, and processing methods, such as grilling and frying, induce additional generation of AGEs from the diet.

These, subsequent to consumption of these products thus prepared, can be absorbed by the gastrointestinal system and only in part eliminated in the urine (12), with a significant amount

accumulating in various tissues. The accumulation of AGEs can directly alter the structure and function of cross-link proteins in tissues and can induce tissue injury, leading to the generation of reactive oxygen species (ROS) and amplifying inflammation by interacting with their receptor, RAGE (13). Importantly, diet-derived AGEs remain in contact with tissues for a longer period of time than endogenous AGEs, contributing strongly to tissue damage (12).

AGE accumulation in skeletal muscle, blood, and skin is associated with sarcopenia in patients with diabetes and older adults (15,16), and RAGE signaling induces MW in several conditions, including diabetes and cancer (17,18). This has been demonstrated in some experiments in mice, which were fed a diet enriched with AGEs, reporting elevated levels of carboxymethyl-L-lysine (CML) in muscles, concomitant with low muscle mass and endurance (19). We also note how AGEs derived from fructose, a disaccharide found in abundance in WD, activate lipogenesis, metabolic reconfiguration, and mitochondrial dysfunction in muscles (20). Similarly, prolonged consumption of typical WD foods accelerates age-associated decline in muscles (21), and a short-term high-fat diet promotes denervation-induced MW by impairing mitochondrial function (22).

The direct role of WD and dAGE accumulation in the initiation of sarcobesity and the role of the dAGE/RAGE axis in inducing the WD-dependent metabolic alteration, as well as the role in MW, have not been investigated so far. In recent decades, natural products with traditional values have been tested in vitro and in vivo, demonstrating their medical properties. Recently, natural active metabolites have emerged as promising agents to reduce the harmful accumulation/activity of AGEs in many diseases (23). At the same time, there is a growing interest in the use of natural anti-obesity products to reduce obesity dependent ABCD by increasing energy expenditure and improving glucose homeostasis in muscles (24). Therefore, natural strategies to prevent or reduce the production and/or accumulation of dAGE could be useful in mitigating the impact of WD on metabolic health, preventing the progression of sarcobesity and maintaining muscle function.

These agents could augment the arsenal to enhance the ability to treat sarcobesity and ABCD (25).

STUDY PURPOSE

The study aims to explore whether a high level of diet-derived AGEs may mediate Western diet-related muscle loss, influencing the onset and progression of sarcobesity, predisposing to earlier and more severe metabolic consequences, including type 2 diabetes (T2D).

The primary objective of the study is to investigate how AGEs accumulation correlates with muscle loss in adult patients with obesity and type 2 diabetes or lipodystrophy with the aim of identifying possible targets to mitigate the metabolomic alterations caused by WD. Specifically, the levels of circulating AGEs in the skin and correlated with the stage of sarcopenia in a group of patients with

obesity and a T2D diagnosis will be evaluated. In addition, the relationship between disease duration and AGEs levels will be evaluated.

Secondary objective will be to analyze the obtained clinical data to identify metabolites and metabolic pathways responsible for the WD-induced phenotype.

The final aim of the study is therefore to investigate whether high AGE levels correlate with an earlier and/or more pronounced onset of sarcobesity, in conjunction with increased inflammation and oxidative stress.

STUDY DESIGN

Type of study

The present study is cross-sectional observational. The reference population is defined by patients with obesity and a diagnosis of T2D within 15 years of study entry or patients with type 2 diabetes concomitant with lipodystrophic syndrome. This population was chosen as being at high risk for sarcobesity.

Lipodystrophy includes a heterogeneous spectrum of genetic and acquired diseases, characterized by subcutaneous adipose tissue loss, ectopic fat accumulation, insulin resistance, metabolic and cardiovascular diseases, premature aging, sarcobesity and muscle pain, high-grade inflammation, epigenetic dysregulation, and mitochondrial dysfunction. Therefore, patients with T2D and lipodystrophy are found to be highly inflamed, as they generally have a more severe T2D phenotype, presumed to be sarcopenic, and have a high rate of endogenous AGE production. Therefore, patients with lipodystrophy concomitant with T2D will be enrolled as sarcopenic and obese subjects, which represent an excellent strategy for comparison with the diabetic population without lipodystrophy.

SUBJECTS AND METHODS

A total of 195 consecutive subjects afferent to the SCDU of Endocrinology at Eastern Piedmont University between April 2024 and April 2026 who meet the inclusion criteria will be included in the study.

Inclusion criteria:

1. Patients of both sexes
2. Subjects of legal age

3. BMI compatible with obesity and diagnosis of type 2 diabetes in good metabolic control (HbA1c < 7.5 %) within 15 years of study entry or diagnosis of lipodystrophy (included in the ECLip - Registry (eclip-web.org))

Exclusion criteria

1. Age under 18 years old
2. Secondary obesity or genetic diseases (Prader Willi syndrome, Down syndrome); metabolic and endocrine (Cushing's syndrome, hypothyroidism);
3. Subjects with: Intestine Bowel disease (IBD), cancer.
4. Established or planned pregnancy during the months of study participation

After reading the study information sheet (see Managing Informed Consent), patients will be enrolled only after signing the informed consent form.

Duration of the study:

The study will last two years corresponding to the enrollment period given the cross-sectional nature of the study.

Definition of sarcobesity indices

According to the ESPEN/EASO consensus statement [26], the following aspects will be considered for the diagnosis of sarcobesity:

1. Skeletal muscle functional parameters defined by muscle strength measured by hand grip strenght (Hand grip strenght HGS), chair test (seat-lift test five times; 30-second seat test), balance tests, and walking test and walking speed.
2. Body composition by bio-impedance analysis (BIA).

Specifically, the percentage of fat mass (%MG), detected directly by BIA, and the percentage of skeletal muscle mass relative to body mass will be considered for the definition of sarcobesity

Skeletal mass will be calculated as:

MS(Kg)=[(h^2/(BIA resistance) *0.401)+(sex*3.825)+(age*0.071)]+5.102

Where h indicates height measured in cm, sex is a dichotomous variable that takes value 1 for males and 0 for females, and age is measured in years.

From this measurement, the percentage of skeletal muscle mass (%MMS) will be calculated %MMS=(MS (Kg))/(Body mass (Kg))*100.

According to the guidelines, subjects with impaired musculoskeletal function parameters (HGS ‹16 kg for women and <27 kg for men or chair test >15 seconds for both males and females [27]), impaired %MG (≥25% for males and ≥35% for females [28]) and impaired %MMS (<35.6% (≤28.7% severe sarcopenia) for men, <28.4% (≤23% severe sarcopenia) for females [29]).

Quantification of AGEs and collection of other information

For all patients included in the study afferent from the SCDU of Endocrinology of the AOU

Maggiore della Carità in Novara, following understanding and signing of consent, AGEs levels will be measured by skin fluorescence, using the "AGE reader mu" instrument (DS Medica, granted for free use by the PRIN 2022 project PI Prof. Francesca Riuzzi).

AGEs will also be assessed at the plasma level in free form (ELISA, fluorescence assay) or bound to hemoglobin (HbA1c clinical practice test), discriminating in exogenous (dAGEs) and endogenous AGEs, respectively.

The following information of interest to the conduct of the study will also be collected.

Socio demographic characteristics: gender, date of birth, date of diabetes diagnosis (for diabetic subjects), and date of recruitment, anthropometric measurements, weight, height, calculation of body mass index (BMI), waist, hip, arm, and calf circumference, and body composition analysis through bio-impedance analysis (BIA).

Level of dietary inflammation calculated through the validated Dietary Inflammation Index (DII) and the Alternative Healthy Eating Index-2010 (AHEI-2010) derived from basal metabolic assessment by indirect calorimetry in the morning on an empty stomach.

Level of adherence to the Western diet (via food diary, recall of the last 24 hours, and validated food frequency questionnaire (EPIC)) as potentially related to the level of AGEs.

The level of physical activity, related to sarcopenia, estimated in MET using the IPAQ questionnaire.

Biochemical evaluation by hematochemical sampling and urine samples as per clinical practice (Blood glucose, complete blood count, HbA1c, AST, ALT, GTT, ALP, CPK, Total cholesterol, HDL cholesterol, Triglycerides, Uric acid, Creatinine, Microalbuminuria, urine test, 25-OH-VIt. D, Calcium, Phosphorus and QPE). Additional parameters of scientific interest will also be evaluated (IL-6, IL-10, IL-17, TNF-alpha, INF-gamma, Leptin and Adiponectin) Samples will be stored at the UPO Biobank (https://biobank.uniupo.it/en/). In addition, serum and plasma will be collected for metabolomic analysis, evaluation of cytokine profiles in serum and C-reactive protein, by multiplex assay.

Analysis

Biochemical parameters according to clinical practice will be analyzed at the clinical biochemistry laboratories of the Maggiore della Carità Hospital in Novara. Metabolomic analyses will be performed at the Center for Translational Research on Autoimmune and Allergic Diseases (CAAD) of the University of Eastern Piedmont. Evaluation of exogenous AGEs will be carried out at the laboratories of the University of Perugia.

Sample size

Given a first-type error a of 0.05 and a power of 80 percent, 195 subjects will be required to observe as significant the correlation between AGEs levels and the indices used to assess the level of sarcobesity of at least 0.2. Given the low prevalence of lipodystrophy and considering a one-year recruitment duration, it is planned to include approximately 35 subjects with lipodystrophy. The remaining 160 subjects will have diabetes and obesity. In addition, considering that the duration of diabetes may affect the levels of AGEs and sarcobesity, both subjects with diabetes for a long time (80 patients) and less than one year (80 patients) will be selected who can be considered comparable to pre-diabetes.

Statistical analysis

Descriptive statistics will be used to summarize the sociodemographic, anthropometric, clinical, and lifestyle-related information collected. Categorical variables will be summarized by absolute frequencies and percentages while numerical variables as mean and standard deviation or median and interquartile range if not normally distributed in accordance with the Shapiro-Wilk test and after observation of QQ-plots.

Pearson's correlation coefficient or the corresponding nonparametric Spearman's index and confidence intervals will initially be calculated in order to assess the correlation between the level of individual AGEs and MMS, MMSH, body composition parameters, and skeletal muscle functional parameters. Then linear regression models will be used in order to assess the relationship between AGEs and indices defining sarcobesity adjusted for age, sex, duration of diabetes, and other potential confounding factors such as inflammation, western diet adherence, and physical activity levels. The LASSO method will be used for the selection of variables to be included in the multivariable regression models.

Poisson regression models with univariable and multivariable robust variance will be used to estimate prevalence relative risks for the association between AGEs and patient characteristic with the presence of sarcobesity and corresponding confidence intervals.

An integration of clinical, biochemical data, AGE levels, and patient omics signatures will be performed to develop a multifactorial diagnostic model using multivariate statistical analysis (e.g., factor analysis, principal component analysis, cluster analysis, discriminant analysis, partial principal component analysis, logistic model) and data-driven approaches. Machine learning algorithms will be applied to prioritize and weight risk factors. These analyses will be performed with an in-house statistical consultancy already used by the group.

Expected Results

With this study, we expect to obtain more information and correlations between nutritional assessment and its impact on inflammation, definition, and progression of sarcopenia, obesity, and T2D, based on body measurements and clinical parameters. Through biochemical, hormonal, and metabolomic analyses performed on the biological samples, we expect to identify possible markers related to the presence of AGEs. In conclusion, the primary expected outcome would be to identify a positive correlation between AGE accumulation in at least one compartment (skin plasma, urine) and the severity of sarcopenia status, so that we can obtain a rapid and noninvasive to identify individuals at high risk of developing MW and identify correlations between AGE levels and other metabolic characteristics, including in lipodystrophic disease.

ETHICAL ASPECTS

The experimental protocol for human studies will be submitted to the is Novara Interhospital Ethics Committee, registered on ClinicalTrial.gov before initiation, and conducted in accordance with the Declaration of Helsinki. All enrolled subjects will sign informed consent before participating in the study.

- *Ethical principles for medical research involving human subjects* (Declaration of Helsinki - World Medical Association, current version);
- European Union *Standards of Good Clinical Practice* (ICH/GCP);
- *Convention on Human Rights and Biomedicine* (Oviedo Convention of 04/04/1997);
- Italian codes of ethics for health professions and specific current national regulations on clinical trials.

Management of informed consent

An information sheet explaining the type of study, purpose, procedures, sample and data collection, and possible benefits and risks of the research is available. This information will be presented to interested persons, who will have the freedom to give consent or to be able to withdraw it when the study has already begun.

Management of sensitive data

The following European and national regulations are kept in mind:

- *Regulation (EU) 2016/679* of the European Parliament and of the Council of April 27, 2016 and subsequent amendments published in the Official Journal of the European Union 127 of May 23, 2018 (GDPR);
- *Legislative Decree No. 196 of June 30, 2003* (Personal Data Protection Code), as amended by Legislative Decree No. 101 of August 10, 2018 on "Provisions for the adaptation of national legislation to the provisions of Regulation (EU) 2016/679."
- *Privacy Guarantor, Provision setting out the requirements for the processing of special categories of data, pursuant to Article 21(1) of Legislative Decree No. 101 of August 10, 2018*.

DATA PROCESSING METHODS AND BIOLOGICAL SAMPLES

The study involves the collection of information related to lifestyles and diseases of the subjects involved. The biological samples and the attached personal/sensitive data collected will be immediately *pseudonymized* by random assignment of alphanumeric codes associated with the study and will be entered into the REDCap Management Software. The decoding of said alphanumeric codes will be in the possession of the responsible research investigator. Therefore, it will only be possible for the responsible research investigator to link the information and data obtained from the searches to a specific subject.

Personal data will be processed for the purposes set out in the project, in accordance with the principles of lawfulness, fairness, transparency, purpose limitation, minimization and accuracy of data (Art. 5 GDPR) in paper and electronic form by individuals authorized to process the data. The availability, management, access, storage, and usability of data is guaranteed by the adoption of technical and organizational measures to ensure adequate levels of security (Articles 25 and 32 GDPR). Only researchers involved in the study and authorized by the responsible research investigator will have access to the data.

Biological samples will be stored at UPO Biobank according to certified quality and security standards. The biological samples will be pseudonymized, and only UPO Biobank officials authorized to process the data, and only in cases of necessity, will be able to link the pseudonymization code of the samples to the sensitive data of the study participant. Researchers who will perform analysis on the collected samples will, therefore, have no way of associating them with the participant's identity. Aliquots of the biological samples, in pseudonymized form, may be released to the managers of the facilities that will perform the laboratory investigations. Any remainder will be returned to UPO Biobank.

Participants will also be offered, completely freely and optionally and through the signing of an appropriate informed consent (UPO Biobank Participation Pact), to provide consent for the use of biological samples taken for research purposes to UPO Biobank population studies.

Personal data collected within the scope of this project will be kept at the experimental center, for a period of 15 years after the conclusion of the research or for a longer period, if necessary, in compliance with legal obligations, to which the Owner is bound. The results of the research will be made public or used for scientific communications/publications, only in anonymous and aggregated form.

The data controllers, each for their respective areas of responsibility, are the A.O.U Maggiore della Carità di Novara, DPO: Dr. Alessandra Gaetano (dpo@innovasrl.it; privacy@maggioreosp.novara.it) and Università del Piemonte Orientale, DPO: lawyer Stefano Ricci (dpo@uniupo.it)

COSTS

The funds available to the Investigator are derived from the Ministry of University and Research (MIUR) under the PRIN: Research Projects of Significant National Interest-Call 2022, Prot. P2022Z4EB5 so no additional costs are expected for AOU Maggiore della Carità.

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