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Role of HbA1c in identify subjects with prediabetes and preclinical cardiovascular risk.

Tesi di Dottorato

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1 - Prediabetes: definition and clinical characteristics

Pre-diabetes is a general term that refers to an intermediate stage between normal glucose homeostasis and overt type 2 diabetes mellitus. There are three diagnostic criteria for pre-diabetes, according to American Diabetes Association (ADA) (**Table 1**):

- fasting glycemia between 100 and 125 mg/dL (*impaired fasting glucose*, IFG);
- 2-hours glycemia between 140 and 199 mg/dL after oral glucose tolerance test (*impaired glucose tolerance*, IGT);
- glycated hemoglobin A_{1c} (HbA_{1c}) between 5.7 e 6.4%.

Increasing evidence suggests that the agreement between pre-diabetes diagnoses made by IFG, IGT or HbA_{1c} is scarce and, as pointed out by the ADA, the characterization of subjects discordantly categorized by the three tests is, to date, pending [1]. In a study conducted on a large cohort of Caucasian adults the agreement between the three diagnostic criteria was only 10.4% **(Figure 1)**[2].

The discordance in the identification of individuals with pre-diabetes using three different diagnostic tests is not entirely unexpected given that measurements of fasting plasma glucose, 2-hours post OGTT, and HbA_{1c} are likely to reflect different aspects of glucose metabolism, and a diagnosis of pre-diabetes based on IFG, IGT, or HbA_{1c} may represent the different pathophysiologic mechanisms underlying abnormal glucose homeostasis [2]. In fact, subjects with isolated IFG seem to have a moderate liver insulin resistance, impaired first-phase insulin secretion, and normal/near-normal muscle insulin sensitivity, while subjects with IGT should be characterized by nearly normal hepatic insulin sensitivity and marked muscle insulin

Table 1: Diagnostic criteria for categories at increased risk of diabetes.

Category	Marker	Diagnostic Range
IFG	Fasting plasma glycemia	≥ 100 mg/dL (5.6 mmol/L) ≤ 126 mg/dL (6.9 mmol/L)
IGT	2-h post-load glycemia	≥ 140 mg/dL (7.8 mmol/L) ≤ 100 mg/dL (11.0 mmol/L)
HbA_{1c}-Prediabetes	HbA_{1c}	≥ 5.7 % (39 mmol/mol) < 6.5 % (47 mmol/mol)

resistance combined with defective late insulin secretion, thus resulting in prolonged hyperglycaemia after a glucose load [3,4]. All the pathophysiologic abnormalities characterizing IFG and IGT status are showed in **Table 2**. In contrast to the daily glucose picture offered by IFG and IGT, HbA_{1c} is an indicator of the average blood glucose concentrations over the preceding 2–3 months, accounting for chronic exposure to both basal and postprandial hyperglycaemia and, therefore, may reflect a combination of pathophysiologic defects underlying both IFG and IGT. To date, it is still not clear if these aspects that are strictly bound to the physiopathology of pre-diabetes may have a clinical relevance in view of a possible therapeutic intervention.

Cardiovascular disease (CVD) is the leading cause of death among individuals with type 2 diabetes, accounting for 40% to 50% of all deaths [5]. Although type 2 diabetes is frequently associated with other cardiovascular risk factors, such as dyslipidemia and hypertension, it is believed that chronic hyperglycaemia *per se* is an independent risk for macrovascular complications. Currently, it is well established that macrovascular disease starts before the development of diabetes, and the slight increase in plasma glucose levels that characterize pre-diabetes have been shown to be an independent predictor for CVD. Much clinical research has focused on lifestyle

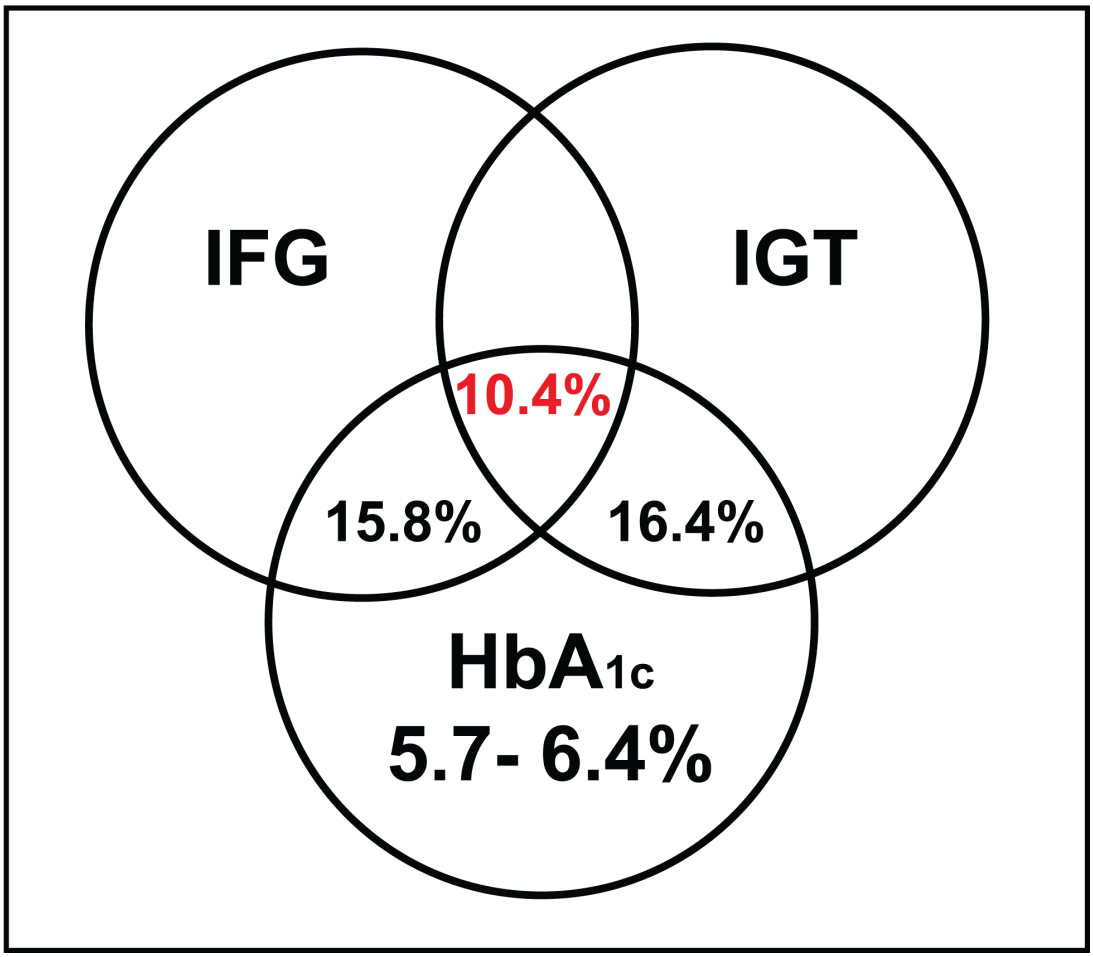

Figure 1: Agreement between HbA_{1c} pre-diabetes, impaired fasting glucose and impaired glucose tolerance [2].

or pharmacological intervention to prevent diabetes in these high risk subjects [6]; however, few studies have been conducted with specific focus on CVD prevention in this population. Since many clinical trials have failed to demonstrated a reduction in cardiovascular risk from glucose-lowering interventions in patients with overt type 2 diabetes [7,8], it is noteworthy that several studies have reported benefits in improving cardiovascular risk factors, as well as absolute CVD event rates, in people with pre-diabetes treated with glucose lowering drugs [9–11].

Since the value of a predictive test for pre-diabetes is also determined by its ability to indicate the risk of macrovascular complication, an important question is whether individuals with pre-diabetes diagnosed by IFG, IGT, or HbA_{1c} are at an equivalent

Table 2. Pathophysiology of IFG and IGT.

Pathophysiology	IFG	IGT	IFG+IGT
Muscle			
Insulin sensitivity	Unaltered	Reduced	Reduced
Liver			
Insulin sensitivity	Reduced	Unaltered	Reduced
Hepatic glucose production	Elevated	Unaltered	Elevated
Pancreas			
First-phase insulin response	Reduced	Unaltered/Reduced	Reduced
Disposition Index	Reduced	Reduced	Reduced
Glucagon Secretion	Elevated	Elevated	Elevated
Gut			
GLP1 secretion	Reduced or elevated	Reduced or unaltered	?
GIP secretion	Unaltered	Reduced or unaltered	?
Adipose Tissue			
Insulin Sensitivity	Reduced	Reduced	Reduced
NEFA release	Unaltered	Elevated	?

risk of CVD. Data on cardiovascular risk comparing IFG, IGT, and HbA_{1c} in the pre-diabetic range are sparse, have been focused on mortality, and have reached controversial results [12–14].

This thesis highlights recent studies and current controversies in the field. In consideration of the expected increased use of HbA_{1c} as a screening tool to identify individuals with alterations of glycaemic homeostasis, we thought that it could be interesting, and relevant from the clinical point of view, to evaluate the evidence regarding the ability of HbA_{1c} to identify patients who have increased cardiovascular risk. With this specific aim we focused our attention on HbA_{1c} as a diagnostic tool to identify subjects with pre-diabetes and early alterations of cardiovascular risk profile.

1.2 - Comparison of IFG, IGT and HbA_{1c}, criteria in predicting type 2 diabetes

Subjects with pre-diabetes have shown a high conversion rate to overt diabetes and much clinical research has focused on lifestyle or pharmacological intervention to prevent diabetes in these high risk subjects [6]. Subjects with an isolated alteration of glucose homeostasis (IFG, IGT or HbA_{1c} 5.7-6.4%) have an incidence of diabetes of 6% per year, a value that is significantly higher compared with subjects with normoglycemia (0.5% per year) [15]. Progression to overt type 2 diabetes is 30-40% in the next 3-8 years, with an increase of 10% when two alterations of glucose homeostasis are present [6].

Changes in diagnostic and screening criteria may have an important impact on the number and characteristics of patients in whom pre-diabetes is diagnosed and, subsequently, on a population that should be considered for a possible therapeutic intervention. According with these considerations, it is important to identify individuals at high risk for type 2 diabetes to prevent or delay the development of the disease and its complications.

In 2011, the ADA revised the criteria for the diagnosis of type 2 diabetes and the categories at increased risk for diabetes. On the basis of an ample analysis performed by an international expert committee, the use of HbA_{1c} measurement was recommended as another diagnostic test option already including IFG and IGT [1]. Specifically for the categories of increased risk for type 2 diabetes, the new ADA recommendations state that an HbA_{1c} from 5.7-6.4% identifies individuals at high risk for diabetes to whom the term pre-diabetes may be applied.

Indeed, both IFG and IGT present some limitations: they require fasting status and are affected by acute perturbation; furthermore, the OGTT is not common in clinical practice because of some practical difficulties: it is time consuming,

costly, and less reproducible than the measurement of fasting plasma glucose (FPG) [16]. HbA_{1c} can indirectly measure mean blood glucose over the previous 2–3 months and is correlated with FPG and 2-h plasma glucose [17]. HbA_{1c} is more reproducible than FPG and within subject coefficients of variation are 1.7 and 5.7%, respectively [17,18]. Moreover, measurement of HbA_{1c} does not require fasting status and could better integrate chronic hyperglycaemia than FPG (**Table 3**). Several prospective studies have reported the utility of HbA_{1c} in predicting type 2 diabetes. Morris *et al.* [19] has shown in a metanalysis conducted on 70 studies that the progression rate to type 2 diabetes of patients with HbA_{1c} pre-diabetes was similar to that for ADA-defined IFG and IFG plus IGT. Furthermore, four prospective studies have reported the utility of HbA_{1c} in predicting type 2 diabetes [20–23]; of these, one assessed the use of two glycemic parameters (in particular IFG and HbA_{1c}) for predicting the incidence of type 2 diabetes; the authors supported the combined measurement of FPG and HbA_{1c} for predicting diabetes incidence in a 4 year follow-up using receiver operating characteristic curve (ROC) analysis. In all participants, the model including both FPG and HbA_{1c} had a greater ROC curve that those including FPG alone (0.853 vs 0.818; $P<0.001$) or HbA_{1c} alone (0.853 vs 0.771; $P<0.001$). Furthermore, the authors reported a weak correlation between HbA_{1c} and FPG at baseline suggesting that HbA_{1c} is not a surrogate marker of FPG [23].

It is necessary to remember that, as far as it is a risk factor for diabetes, HbA_{1c} between 5.7-6.4% seems to identify a much smaller population with pre-diabetes compared with IFG and IGT [24,25]. Conversely, the use of HbA_{1c} may also lead to the reclassification of subjects without IFG or IGT as having pre-diabetes [26]. On the other hand, according to the ADA statement, the lower sensitivity of HbA_{1c} for diagnosing pre-diabetes may be offset by its ability to facilitate establishing a

Table 3 Main points supporting/not supporting the use of HbA_{1c} as diagnostic tool for diagnosis of pre-diabetes.

Supporting	Not Supporting
HbA_{1c} may better integrate chronic hyperglycaemia than fasting and 2-h post-load glycaemia.	HbA_{1c} seems to have a lower sensitivity in pre-diabetes diagnosis.
HbA_{1c} predicts microvascular complications (rethinopathy and nephropathy) similarly to fasting and 2-h post-load glycaemia.	Standardization of HbA_{1c} assay needs to be improved.
HbA_{1c} has a higher predictive value than fasting plasma glucose in predicting cardiovascular disease.	Common, and not always known, clinical conditions (haemoglobinophaties, malaria, anaemia, blood loss) may significantly interfere with HbA_{1c} assay.
HbA_{1c} has a greater pre-analytical stability than blood glucose.	Ethnic differences in HbA_{1c} assay are not well characterized.
HbA_{1c} assay does not need fasting status.	
HbA_{1c} is not affected by acute perturbations (exercize, stress, diet).	The low biological variability of HbA_{1c} provides little information on pathophysiological processes involved in pre-diabetes.
HbA_{1c} biological variability is lower than fasting and 2-h post-load glycemia.	
HbA_{1c} may be an attractive option in settings in which OGTT is not used and rarely repeated.	Glucose assessment is cheaper thant HbA_{1c} assay.

diagnosis [27]. Contrary to these considerations, Rosella *et al.* [28] recently reported that the prevalence of undiagnosed pre-diabetes in a representative sample of Canadians was significantly greater using screening strategies that used HbA_{1c} measures compared with plasma glucose diagnostic criteria. The authors hypothesized that this "reverse association" may be due to a number of factors, such as ethnic differences and the increased prevalence of pre-diabetes from 11.6% in 2003 to 35.3% in 2011 [29]. Accordingly, in a study conducted in the Mexican population, Kumar A *et al.* [30] found a higher prevalence of adults with HbA_{1c} pre-diabetes compared with previous studies conducted in the same population [31]. We reported similar findings in a recent study conducted on 380 subjects attending our out-patients clinic for diabetes and

cardiovascular risk evaluation; although we did not perform an opportunistic procedure during recruitment, the group with high HbA_{1c} and normal fasting glucose and normal glucose tolerance (NFG/NGT) represented, in this study, approximately 30% of the entire population and is, therefore, not a rare subset [32]. These observations may not be surprising; in fact, although subjects with NFG and NGT have a lower relative risk for progression to diabetes than subjects with either IFG or IGT, in several studies 30–40% of all subjects who developed diabetes had NFG and NGT at baseline [33,34]. This indicates that, although subjects with NFG and NGT have a lower risk of developing diabetes compared with IFG and IGT in absolute terms, among these subjects there is also a subgroup at increased risk of developing diabetes and, consequently, cardiovascular diseases. From these considerations stems the need to add HbA_{1c}, as a diagnostic tool to identify a new category of high-risk individuals [35]. Further epidemiological data are needed to characterize the real percentage of this group in the overall pre-diabetic population.

To date, it is unclear why the prevalence of pre-diabetes diagnosed by OGTT and HbA_{1c} criteria is substantially discordant. The concentration of HbA_{1c} depends on glucose concentrations and on factors affecting the glycation rate such as systemic oxidative stress. Previous studies reported that some characteristics, such as obesity, are associated with increased oxidative stress [36]; thus, HbA_{1c} may not reflect the real concentration of glucose and be disproportionately high in obese subjects. Several studies investigated the effects of phenotypic characteristics such as obesity on the agreement between OGTT and HbA_{1c}. Li *et al*. [37] in a recent study conducted on a large cohort of Chinese subjects without a previous diagnosis of diabetes reported a poor agreement between HbA_{1c} criteria and OGTT in patients independently from body mass index. Moreover, different optimal HbA_{1c} cut-off points for pre-diabetes

were reported: 5.6% for normal weight, 5.7% for overweight, and 6% for obese subjects.

Also other studies recommend a different cut-off point of HbA_{1c} for diagnosis of pre-diabetes. In particular, longitudinal epidemiological studies have reported that demographic and ethnic factors may contribute to complications in using HbA_{1c} for the diagnosis of diabetes, and the optimal diagnostic HbA_{1c} value is debated and varies because of genetic and biological differences. Yan ST *et al.* [38] identified optimal HbA_{1c} cut-off points for pre-diabetes in two diverse population-based cohorts with different ages. The optimal HbA_{1c} cut-off point for pre-diabetes diagnosis was 5.6% in the young and middle-aged population, whereas, the optimal cut-off for diagnosing pre-diabetes increased to 5.7%, in the elderly population. Furthermore, many studies have shown that racial disparities affect the performance of HbA_{1c} for diagnosing pre-diabetes [39]. In summary, it is possible that diagnostic tests for glycemic homeostasis should be used and interpreted considering the individual phenotypic characteristics of the patients; further studies are needed to investigate the clinical usefulness of personalized cut-off values.

1.3 - Comparison of IFG, IGT and HbA_{1c}, criteria in predicting cardiovascular risk

The value of a predictive test for pre-diabetes and type 2 diabetes is also determined by its ability to identify the risk of micro- and macro-vascular complications and from this point of view, the high reproducibility and simplicity may make HbA_{1c} dosage an attractive option. Previous observational studies documented that determination of HbA_{1c}, fasting glucose and OGTT significantly predicted the development of retinopathy and nephropathy but no variables had a significant advantage for detecting the incidence or prevalence of either complication [40,41]. However, fasting glycaemia has a low predictive value in terms of cardiovascular disease, while 2-h post-load glycaemia and HbA_{1c} have a higher predictive value for this chronic complication of diabetes [42].

The question whether HbA_{1c} is a better indicator of cardiovascular disease compared with the other glucose homeostasis parameters still remains debated. In a recent work, we showed that arterial stiffness and carotid intima-media thickness were altered in subjects with higher HbA_{1c} levels and increased to a similar extent as that observed in subjects with new onset type 2 diabetes [43]. Furthermore, when we analyzed our population including only subjects with NFG/NGT we found that the NFG/NGT subjects with HbA_{1c} 5.7-6.4% showed an alteration of subclinical markers of cardiovascular risk compared with NFG/NGT with lower HbA_{1c} and there were no significant differences compared with IGT and type 2 diabetic patients (**Figure 2**). According to these data, a reproducible and simple marker such as HbA_{1c} seems to identify subjects at high cardiovascular risk that would be considered normal according to fasting glycaemia and glucose tolerance. Other studies have shown

similar data reporting a positive association between the pre-diabetic stage, echogenic plague and progression of coronary artery calcification [44,45]. A recent study has analysed the routine use of HbA_{1c} for diagnosis of pre-diabetes in patients with ST-segment elevation myocardial infarction. The study showed a similar in-hospital and long-term mortality in these patients with pre-diabetes as those with known diabetes. The authors discussed that the difficulty in performance and the presence of stress hyperglycaemia in an acutely ill patient with myocardial infarction make OGTT a rarely used diagnostic test in this setting. The use of a simple, one–time HbA_{1c} test allowed them to identify a substantial proportion of patients with previously undiagnosed diabetes or pre-diabetes who could be targeted for risk factor modification with lifestyle interventions and tailored medical therapy [46].

The links between alteration of glucose homeostasis and vascular damage in this population is still unclear, however, several studies have emphasized that the interaction of advanced glycation end products (AGE) with their cell-surface receptor (RAGE) is implicated in triggering inflammatory processes strictly connected with cardiovascular disease [47]. A RAGE soluble form termed endogenous secretory RAGE (esRAGE) may contribute to the removal of circulating ligands, thus competing with cell-surface RAGE for ligand binding [48]. Low levels of esRAGE have been associated with cardiovascular disease and, in a recent study, we found that subjects with pre-diabetes showed low esRAGE plasma levels suggesting a decreased scavenger capacity of these subjects (**Figure 2**). Further analysis conducted on mononuclear cells isolated from peripheral blood samples of these patients revealed a decreased esRAGE mRNA expression [32]. The regulatory mechanism for alternative splicing to generate esRAGE remains unclear, and environmental or genetic factors may be involved. Further examinations of the

molecular mechanism underlying esRAGE regulation will provide potential targets for the prevention and/or treatment of cardiovascular disease.

Our research team has further investigated the characterization of the population with HbA_{1c} pre-diabetes (5.7-6.4%) also investigating other markers closely associated with metabolic abnormalities and cardiovascular risk; in a previous study we highlighted a reduced insulin response in combination with impaired suppression of glucagon secretion in subjects with pre-diabetes according to HbA_{1c} undergoing isoglycaemic intravenous glucose infusion [49]. Other data published in 2014 indicated that the presence of pre-diabetes according to HbA_{1c} is associated with hepatic steatosis and with an alteration in the lipid profile known to be predisposing to cardiovascular and liver diseases [50]. Moreover, we showed that the levels of 25 hydroxyvitamin D are reduced and associated with vascular damage in subjects with pre-diabetes by HbA_{1c} with NFG/NGT (**Figure 2**) [51]. Based on these data, we suggest that among subjects with NFG and NGT, HbA_{1c} may identify subjects with different cardiovascular and glycometabolic risks.

These considerations are, furthermore, supported by previous studies. Indeed, it is important to remember that many authors have documented a significant increase in the incidence of cardiovascular events with HbA_{1c} values substantially lower than those used for diagnosis of diabetes [12]. A recent meta-analysis of six prospective cohort studies in subjects without diabetes mellitus showed a linear association of HbA_{1c} levels with primary cardiovascular events. The observed effect estimates for increased HbA_{1c} levels and was strongly attenuated by adjustment for cardiovascular risk factors but remained statistically significant for primary cardiovascular events, cardiovascular mortality and all-cause mortality [52].

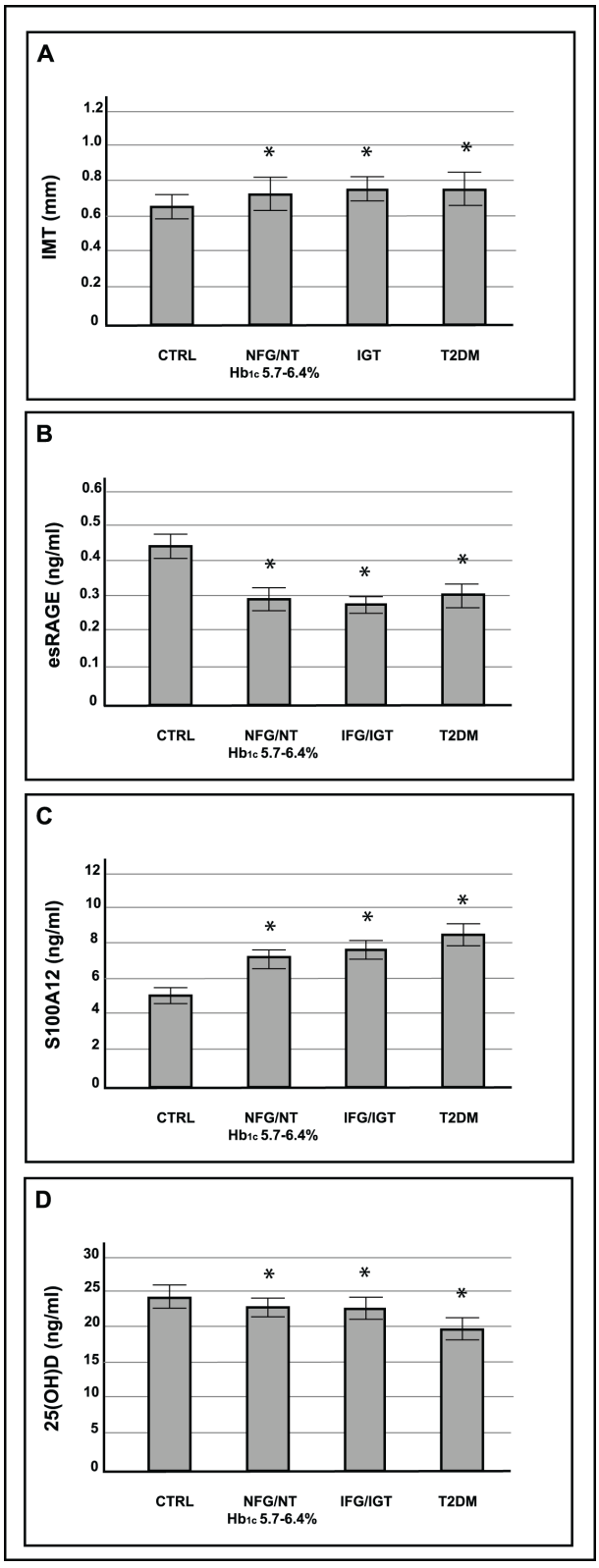

Figure 2: Intima media thickness, esRAGE, S100A12 and 25(OH)D according to glucose tolerance and HbA_{1c} levels. IMT, Intima-media thickness; esRAGE, endogenous receptor for advanced glycation end-products; 25(OH)D, 25-hydroxyvitamin D; NFG, normal fasting glucose; NGT, normal glucose tolerance; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; DT2, type 2 diabetes.

The majority of randomized controlled trials in non-diabetic subjects with increased HbA_{1c} failed to observe significant effects when aiming to reduce the cardiovascular risk and mortality of these individuals. In the recent IRIS trial, which involved patients without diabetes but with a recent history of ischemic stroke or transitory ischemic attack and who had insulin resistance, the rate of the primary outcome (fatal or non-fatal stroke or fatal or non-fatal myocardial infarction) was lower in the pioglitazone group compared with placebo [11]. These results, although in contrast, at least in part, with other trials conducted on patients with type 2 diabetes (BARI-2D and Pro-active), are of great interest suggesting a favourable effect of pioglitazone on the progression of subclinical atherosclerosis [53,54]. The mechanism that was responsible for the lower rates of stroke and myocardial infarction in the pioglitazone group remains unclear. A recent meta-analysis of prospective, randomized clinical trials has shown a non-significant trend towards reduced risk of fatal and non-fatal myocardial infarction, and fatal and non-fatal stroke were only reduced to borderline. However, the short average follow-up time of 3.75 years was a limitation of previous trials and further RCTs, with a larger sample size and longer follow-up, are required to explore the efficacy of non-drug and drug based approaches to reduce the cardiovascular risk of non-diabetic subjects with increased HbA_{1c} [55].

These findings are in agreement with other studies and suggest the importance of HbA_{1c} as an early marker of cardiovascular risk, although the metabolic alterations that lead to an increased cardiovascular risk are multiple and complex, and no single laboratory test can adequately identify or classify an individual's cardiovascular risk [56].

1.4 - Treatment of prediabetes

Several studies investigated the efficacy of different interventions (lifestyle or pharmacological) on end-points such as progression to type 2 diabetes and cardiovascular outcome in subjects with prediabetes. In Diabetes Prevention Program trial (DPP) lifestyle intervention decreased the incidence of new diabetes cases by 58% compared to placebo in patients with IGT in 3.2 years follow up. Follow-up of other three large lifestyle intervention studies for has shown similar reduction in the rate of conversion from prediabetes to type 2 diabetes: 43% reduction at 7 years in the Finnish Diabetes Prevention Study; 43% reduction at 20 years in the Da Qing study, and 34% reduction at 10 years in the U.S. Diabetes Prevention Program Outcomes Study [57–59]. In post-hoc analysis of these studies, weight loss was the most important contributor to diabetes prevention. Unfortunately, weigh loss achieved with diet and exercise is difficult to maintain in a long period; patients from DPP trial gained weight after the end of the trial [60].

Several pharmacologic agents have each been shown to decrease incident diabetes to various degrees in patients with prediabetes. Metformin has the strongest evidence base and demonstrated long-term safety as pharmacologic therapy for diabetes prevention and current guidelines recommend metformin therapy for prevention of type 2 diabetes should be considered in those with prediabetes, especially for those with BMI >35 kg/m^2, those aged <60 years, women with prior gestational diabetes mellitus, and/or those with rising HbA_{1c} despite lifestyle intervention [61]. Other drugs have been tested as therapy in patients with prediabetes; in a recent studies Le Roux *et al* reported that Liraglutide, a GLP-1 receptor agonist, might provide health benefits in terms of reduced risk of diabetes in individuals with

prediabetes and obesity (-80% compared with placebo). Furthermore, a high rate of regression from prediabetes to normoglycemia was observed (-66%).

Thiazolidinediones (PPAR-Gamma activators) improve two of the main defects in prediabetes pathophysiology: insulin resistance and beta cell function, so they seem to be an attractive option in prediabetes treatment. In DPP IGT-type 2 diabetes progression was reduced by 23% with troglitazone within 2 years, even if the drug was stopped after ten months. After 1.5 years the incidence of diabetes for every 100 persons/year was reduced compared with placebo (3 vs 12 cases), metformin (3 vs 6.7 cases) and lifestyle intervention (3 vs 5.1 cases) [62]. In Actos Now trials IGT conversion to type 2 diabetes was reduced by 72% in pioglitazone group; similar results were reported in DREAM trial with a reduced conversion rate of 62% in patients in rosiglitazone therapy [63,64].

People with prediabetes often have other cardiovascular risk factors, including hypertension and dyslipidemia, and are at increased risk for cardiovascular disease. According with these considerations, increased vigilance is needed to identify and treat these and other cardiovascular risk factors. In the management of patients with prediabetes is needed to consider and treat aggressively the following cardiovascular risk factors:

Blood pressure <140/85;

LDL-cholesterol <100 mg/dL in patients with prediabetes and no history of cardiovascular disease;

- LDL-cholesterol <70 mg/dL patients with prediabetes and overt cardiovascular disease;
- HDL cholesterol >40 mg/dL in men and >50 mg/dL in women;
- Triglycerides <150 mg/dL;

- Consider aspirin therapy as a primary prevention strategy in patients with high cardiovascular risk and use aspirin therapy as a secondary prevention strategy in those with a history of atherosclerotic cardiovascular disease;

- Smoking cessation.

Despite the elevated CVD risk among people with prediabetes, it has been difficult to document CVD risk reduction as a result of diabetes prevention strategies. The Finnish Diabetes Prevention Study failed to show beneficial effects of lifestyle intervention on CVD outcomes even although a significant reduction in diabetes risk. In the 10-year follow-up of the Finnish Diabetes Prevention Study, total mortality and CVD incidence were not different between the intervention and control groups, but the study participants who had IGT at baseline had lower all-cause mortality and CVD incidence, compared with a Finnish population-based cohort of people with IGT, suggesting that regular follow-up of individuals at high risk may improve long-term outcomes [65]. The Diabetes Prevention Program showed improvements in both lipid levels and blood pressure in prediabetic individuals who regressed to normal glucose tolerance during the study period, and the largest improvements were seen in the intensive lifestyle intervention group [66]

Several pharmacologic trials have shown beneficial effects on diabetes risk reduction in individuals with IGT. The ORIGIN study examined the effect of insulin glargine on CVD outcomes, but also showed no difference compared with placebo treatment in people with IFG and/or IGT [67]. The NAVIGATOR trial did not find any effect of treatment with nateglinide (insulin secretagogue) or valsartan (a blocker of the renin-angiotensin system) on CVD outcomes in individuals with IGT [68]. In STOP-NIDDM

study, a significant 49 % reduction in major CVD events was found in participants with IGT who were randomized to the α- glucosidase inhibitor acarbose and followed for 3–4 years [69]. In IRIS trial involving patients without diabetes who had a recent history of ischemic stroke or transiet ischemic attack and who had insulin resistance, the rate of the primary outcome was lower among patients who received pioglitazone than among those who received placebo [11].

More studies are needed before a hypoglycemic agent should be recommended for primary prevention of CVD.

2 - SCIENTIFIC PROJECTS

2.1 Background

A number of studies reported that the process portending to atherosclerosis begin before of a formal diagnosis of diabetes and from this consideration the value of a predictive test for pre-diabetes and type 2 diabetes is also determined by its ability to identify individuals with subclinical alterations of early markers for cardiovascular risk. Previous observational studies documented that determination of HbA_{1c}, fasting glucose and OGTT significantly predicted the development of retinopathy and nephropathy but no variables had a significant advantage for detecting the incidence or prevalence of either complication. The high reproducibility and simplicity may make HbA_{1c} dosage an attractive option, however, the question whether HbA_{1c} is a better indicator of cardiovascular disease compared with the other glucose homeostasis parameters still remains debated.

The aim of my scientific projects was to study the ability of HbA_{1c} in identify subjects with early alteration of cardiovascular risk that would not be identified with fasting glucose and oral glucose tolerance test. According with these considerations the scientific production was focused on the characterization of the cardiovascular risk profile of subjects with HbA_{1c} in prediabetic range (5.7-6.4%) but with normal glucose tolerance and normal fasting glucose without overt cardiovascular disease.

We studied early markers of subclinical cardiovascular damage:

- Carotid IMT;
- Arterial stiffness;
- Cardiac diastolic dysfunction.

Furthermore, we explored in the same population the other markers of glycation and

inflammation strictly bounded with cardiovascular risk such as:

- Carboxy-methyl-lysine;
- Soluble Receptors for advanced glycation end-products;
- S100A12;
- 25(OH)D.

Finally, according to current guidelines, there are no drugs with a specific indication for prevention of progression to type 2 diabetes and cardiovascular risk in subjects with prediabetes, and the first step in the management of this condition should emphasize therapeutic lifestyle modifications. According with these data we investigate the effect of a controlled dietary intervention that compared the chronic effects of a low dietary AGE (L-dAGEs) with a standard dietary AGE (S-dAGEs) regimen on lipid profile, inflammatory markers and plasma levels of esRAGE in individuals with prediabetes. Furthermore, we examined the effects of an L-dAGE regimen on early markers of cardiovascular disease.

2.2 Cardiovascular Risk Profile in Subjects With Prediabetes and New-Onset Type 2 Diabetes Identified by HbA_{1c} According to American Diabetes Association Criteria

In 2011, the American Diabetes Association (ADA) proposed that glycated hemoglobin A1c (HbA1c) should be used as a diagnostic test for diabetes and prediabetes. An HbA1c value ≥ 6.5% (48 mmol/mol) was recommended for the diagnosis of diabetes, and an HbA1c of 5.7-6.4% (39 to 46 mmol/mol) was identified as a new indicator of prediabetes in addition to impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) [70]. Evidence indicates that subjects who are at high risk for type 2 diabetes also have an increased cardiovascular risk; therefore, the value of a predictive test for type 2 diabetes is also determined by its ability to indicate a high risk of cardiovascular disease (CVD) [71].

The ADA recommendations are mainly based on the relationship between HbA1c and microvascular complications, especially retinopathy [70,72]. The association of macrovascular diseases, which are the primary causes of mortality in patients with type 2 diabetes, with HbA1c compared with other glucose homeostasis parameters (fasting, one-hour and two-hour post oral glucose tolerance tests [OGTT]) is currently less conclusive [73].

Glycosylation of the arterial wall might play a role in vascular damage by affecting arterial stiffness, a progressive process that is accelerated by many age-associated disorders, including diabetes, hypertension and metabolic syndromes. In large arteries, aging is characterized by the decreased turnover of collagen and elastin and increased levels of advanced glycation end products (AGEs) and cross-links. Elastic fibers undergo lysis and disorganization subsequent to their replacement by

collagen and other matrix components [74]. These events cause a loss of elasticity and induce stiffening. Moreover, recent data have shown that the interaction between AGEs and their receptor (RAGE) plays an important role in the development of diabetes complications and accelerated atherosclerosis [75,76]. RAGE is upregulated in the atherosclerotic plaques of diabetic subjects, and interaction with its ligands induces pro-inflammatory gene activation and contributes to tissue injury and arterial stiffening [77].

Our first aim was to investigate the cardiovascular risk profile of subjects with prediabetes (HbA1c 5.7-6.4% [39 to 46 mmol/mol]) and new-onset type 2 diabetes (HbA1c ≥ 6.5 [48 mmol/mol]) according to HbA1c. We studied early markers of atherosclerosis, such as arterial stiffness (pulse wave analysis [PWA] and its central hemodynamic correlates, such as augmentation pressure [Aug], augmentation index [AugI] and pulse wave velocity [PWV]) and intima-media thickness (IMT). In addition, we characterized the association between serum soluble RAGE (sRAGE) and early markers of cardiovascular damage. Our second aim was to investigate whether HbA1c is a better indicator of cardiovascular risk compared with other glucose homeostasis parameters, such as fasting and one- or two-hour post-OGTTs.

Materials and methods

Study subjects

274 subjects with no previous diagnosis of diabetes were recruited from patients attending our University Hospital for diabetes and cardiovascular risk evaluation. The inclusion criteria were an age range of 18-65 years. All patients were Caucasian and underwent a physical examination and review of their clinical history, smoking status and alcohol consumption. None of them had lost weight or changed dietary habits during the three months preceding the study. The exclusion criteria were a previous history of diabetes, previous history of overt cardiovascular events (stroke, ischemic heart disease, chronic obstructive peripheral arteriopathy or heart failure), anemia or hemoglobinopathies, use of medications known to affect glucose metabolism, positivity for antibodies to hepatitis C virus (HCV) or hepatitis B surface antigen (HBsAg), clinical evidence of advanced liver or renal disease, chronic gastrointestinal diseases associated with malabsorption, rheumatic diseases and/or recent history of acute illness, malignant disease, drug or alcohol abuse.

Body weight and height were measured, and BMI was calculated as weight kg/[height (m)]^2. Waist circumference was measured in a standing position at the level of the umbilicus. Blood pressure (BP) was measured with a calibrated sphygmomanometer after the subject had rested in the supine position for 10 min. Venous blood samples were drawn from the antecubital vein on the morning after an overnight fast. Baseline venous blood samples were obtained for the measurement of plasma glucose, total cholesterol, HDL cholesterol, triglycerides, high sensitivity C-reactive protein (hs-CRP) and sRAGE. LDL cholesterol concentrations were estimated using the Friedewald formula. All subjects underwent a 75-g OGTT with 0, 30, 60, 90 and 120 min sampling for plasma and insulin as previously described [78].

According to their two-hour glucose levels, the subjects were classified into the following categories: normal glucose tolerance (NT), IGT or type 2 diabetes. NT was defined as a two-hour plasma glucose level < 140 mg/dl. IGT was defined as a two-hour plasma glucose level of 140 to 200 mg/dl. Type 2 diabetes was defined as a two-hour plasma glucose level ≥ 200 mg/dl [70].

Biochemical analyses

Plasma glucose, serum total cholesterol, triglycerides, HDL cholesterol and hs-CRP were measured using available enzymatic methods as previously described [78]. A commercially available ELISA kit (Human sRAGE ELISA: Biovendor, Brno, Czech Republic) was used according to the manufacturer's instructions to quantify the plasma concentration of sRAGE.

HbA1c was measured via high-performance liquid chromatography using a National Glycohemoglobin Standardization Program (NGSP) and standardized to the Diabetes Control and Complications Trial (DCCT)[79] assay reference. Chromatography was performed using a certified automated analyzer (HPLC; HLC-723G7 hemoglobin HPLC analyzer Tosoh Corp.) (normal range: 4.25-5.9% [23-41 mmol/mol]).

Pulse wave analysis

All measurements were made from the right radial artery by applanation tonometry using a Millar tonometer (SPC-301; Millar Instruments, Houston, TX)[80]. The measurements were performed by a single investigator with the subject in the supine position. The data were collected directly with a desktop computer and processed with SphygmoCorCvMS (AtCor Medical, Sydney, Australia). The aortic waveform

has two systolic pressure peaks, the latter of which is caused by wave reflection from the periphery. With arterial stiffening, both the pulse wave velocity and the amplitude of the reflected wave are increased such that the reflected wave arrives earlier and adds to (or augments) the central systolic pressure. The aortic waveform in pulse wave analysis was subjected to further analysis for the calculation of the aortic Aug, AugI (calculated by dividing augmentation by pulse pressure), central BP, ejection duration (duration of the systolic period in milliseconds) and Buckberg's subendocardial viability ratio (SEVR, area of diastole divided by area of systole during one cardiac cycle in the aorta). Pulse pressure is the difference between the systolic and diastolic blood pressures.

Pulse wave velocity

The SphygmoCor CvMS (AtCor Medical, Sydney, Australia) system was used for the determination of the PWV. This system uses a tonometer and two different pressure waves obtained at the common carotid artery (proximal recording site) and at the femoral artery (distal recording site). The distance between the recording sites and suprasternal notch was measured using a tape measure. An electrocardiogram (ECG) was used to determine the start of the pulse wave. The PWV was determined as the difference in travel time of the pulse wave between the two different recording sites and the heart, divided by the travel distance of the pulse waveform. The PWV was calculated on the mean basis of ten consecutive pressure waveforms to cover a complete respiratory cycle.

Carotid ultrasound examinations

Ultrasound scans were performed using a high-resolution B-mode ultrasound system (MyLab 50 XVision, Esaote Biomedica SpA, Florence, Italy) equipped with a 7.5-MHz linear array transducer. To exclude interobserver variability, all ultrasound examinations were performed by a single physician who was blinded to the clinical and laboratory characteristics of the patients. The subjects were examined in the supine position. Longitudinal images from the angle with the best visibility were displayed bilaterally for the common carotid artery. Scans were performed, and measurements were conducted at a total of six plaque-free sites 1 cm proximal to the carotid bulb. The obtained values were averaged and are presented as the mean of the IMT of the common carotid artery. Plaques, defined as a clearly isolated focal thickening of the intima-media layer with a thickness of 1.4 mm, were not observed in any individuals. All measurements were obtained in diastole, assessed as the phase in which the lumen diameter is at its smallest and the IMT is at its largest.

The study was approved by the local ethics committee. Informed consent was obtained from each participant.

Statistical analyses

The sample size was calculated based on AugI using a level of significance (α) set to 5% and a power (1- β) set to 80%. Statistical comparisons of the clinical and biomedical parameters were performed using Stat View 6.0 for Windows. The data are presented as the means ± SD. The distributional characteristics of each variable, including normality, were assessed by the Kolmogorov-Smirnov test. The statistical analyses were performed with the unpaired *t* test and ANOVA for continuous variables and the χ^2 test for non-continuous variables. A *P* value less than 0.05 was

considered statistically significant. When necessary, numerical variables were logarithmically transformed for statistical analysis to reduce skewness (triglycerides, hsCRP, HOMA-IR, sRAGE, IMT), and values were expressed as median and interquartile range.

Simple regression analysis was performed to relate AugI and IMT to the following variables: HbA1c, fasting glucose, one-hour post-load glucose and two-hour post-load glucose. Subsequently, variables reaching statistical significance were inserted into a multivariate regression model to identify independent relationships between cardiovascular risk factors (age, sex, smoking status, systolic and diastolic BP, total cholesterol, HDL cholesterol, tryglicerides, hs-CRP, HOMA-IR and sRAGE) and variations in AugI and IMT. The variance inflation factor (VIF) was used to check for the problem of multicollinearity among the predictor variables in multiple regression analysis.

Results

In total, 274 subjects participated in the study. The study population was divided into the following three groups (based on HbA1c levels) according to the ADA recommendation [70]: 97 controls (HbA1c < 5.7% [39 mmol/mol]), 117 patients with prediabetes (HbA1c 5.7-6.4% [39 to 46 mmol/mol]) and 60 patients with type 2 diabetes (HbA1c ≥ 6.5% [48 mmol/mol]). Of the subjects who were prediabetic based on the HbA1c levels, 80 (68%) showed NT after the OGTT, and only 8 showed IFG. Therefore, many of these subjects should be classified as normal according to fasting glycemia and OGTT.

As shown in **Table 1**, BMI, waist circumference and total cholesterol were not significantly different among the three groups. Subjects with an HbA1c of 5.7-6.4% (39 to 46 mmol/mol) were older and had higher systolic BP, diastolic BP, fasting glucose, triglycerides, HOMA-IR and hs-CRP but lower HDL cholesterol than the controls. Moreover, these subjects had anthropometric and metabolic characteristics similar to patients with diabetes, except for the higher HDL cholesterol and lower fasting glycemia. No differences were observed in the sRAGE levels among the three groups.

Because a one-hour post-load glucose level ≥ 155 mg/dl has been reported as a key marker for cardiovascular and type 2 diabetes risks [81,82], we also stratified all subjects according to their one-hour glucose plasma levels. We found that 82% of patients with a one-hour glucose ≥ 155 mg/dl presented HbA1c > 5.7%.

Table 1—Clinical and metabolic characteristics of the study population according to HbA_{1c} levels	HbA_{1c} <5.7% (39 mmol/mol) (*n* = 97)	HbA_{1c} 5.7–6.4% (39–46 mmol/mol) (*n* = 117)	HbA_{1c} ≥6.5% (48 mmol/mol) (*n* = 60)
Age (years)	41.5 ± 12.2	48.3 ± 10.7†	51 ± 7.2*
BMI (kg/m^2)	30.6 ± 5.5	31.8 ± 6.1	31.7 ± 5.3
Waist circumference (cm)	102 ± 13.5	103.3 ± 11.9	106.6 ± 11.6
Fasting glucose (mg/dL)	86.4 ± 9.3	93.1 ± 11.6†	114 ± 21.1*‡
1-h glucose post-OGTT (mg/dL)	129.1 ± 33.8	161 ± 40†	206.7 ± 31.8*‡
2-h glucose post-OGTT (mg/dL)	106.2 ± 28.1	133.2 ± 42.3†	204.2 ± 71.4*‡
Total cholesterol (mg/dL)	187 ± 34.2	194.7 ± 34.9	182.1 ± 34.6
HDL cholesterol (mg/dL)	47.3 ± 12.6	44 ± 12.3†	39.5 ± 8*‡
Triglycerides (mg/dL)	86 (138–64)	107 (141–80)†	116 (141.5–87.5)
LDL cholesterol (mg/dL)	117.8 ± 31.6	124.9 ± 26.5	118.3 ± 30
Systolic BP (mmHg)	116 ± 15.8	123.8 ± 14.4†	127 ± 15.3*
Diastolic BP (mmHg)	70.7 ± 10.8	76 ± 10.8†	76 ± 11.7*
hs-CRP (mg/dL)	0.15 (0.3–0.1)	0.27 (0.55–0.12)†	0.36 (0.65–0.17)*
HOMA-IR	1.5 (1.9–1.1)	2 (3–1.4)†	2.7 (3.2–1.4)*
sRAGE (pg/mL)	387.9 (517.8–282.7)	394.4 (475.4–278.4)	391 (502.5–274.6)
1-h glucose post-OGTT ≥155 mg/dL	27 (9%)	70 (59%)	58 (96%)
Active smokers	40 (41%)	57 (48%)	34 (56%)*
Hypertension	22 (22%)	40 (34%)†	22 (37%)*

Table 1: Data are presented as mean±SD or as median (interquartile range) for continuous variables and percentage for the categorical variables: HbA1c,glycated haemoglobin; BP, Blood pressure; Hs-CRP, high sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment-insulin resistance, sRAGE soluble receptor for advanced glycation end-products. Smoking was quantified (number of cigarettes and years smoked) and smoking status was classified in active and nonsmokers Hypertension was defined as systolic blood pressure >135 mmHg or diastolic blood pressure >85 mmHg or taking any hypertension medications.
†P<0.05 HbA1c<5.7% vs HbA1c 5.7-6.4%; *P<0.05, HbA1c <5.7% vs HbA1c ≥6.5%; ‡P<0.05 HbA1c 5.7-6.4% vs HbA1c≥6.5%

Arterial stiffness according to HbA1c levels

Aug and AugI were significantly higher in the prediabetic group (HbA1c 5.7-6.4% [39 to 46 mmol/mol]) than in the control group (HbA1c <5.7% [39 mmol/mol]) (11.6 ± 6.5 vs. 9.5 ± 7.3, P <0.05; 28.8 ± 11 vs. 24.4 ± 13.7, P<0.05; respectively). In contrast, SEVR and PWV were similar between these two groups. In individuals with type 2 diabetes, Aug and AugI were significantly increased compared with the control subjects. No difference was observed between the subjects with prediabetes and with type 2 diabetes (HbA1c ≥ 6.5% [48 mmol/mol]). Furthermore, in the subjects with diabetes, SEVR was impaired, and PWV was significantly increased compared with the normal and prediabetic individuals (**Table 2**).

Table 2—Arterial Stiffness and thickness parameters according to HbA_{1c} levels	HbA_{1c} <5.7% (39 mmol/mol) (*n* = 97)	HbA_{1c} 5.7 to 6.4% (39–46 mmol/mol) (*n* = 117)	HbA_{1c} ≥6.5% (48 mmol/mol) (*n* = 60)
Augmentation pressure (mmHg)	9.5 ± 7.3	11.6 ± 6.5†	12.6 ± 6.1*
AugI (%)	24.4 ± 13.7	28.8 ± 11†	30.5 ± 10.7*
SEVR (%)	156 ± 24.9	157 ± 29.7	144.3 ± 31.6*‡
PWV (m/s)	7.5 ± 2	7.7 ± 1.4	8.8 ± 1.8*‡
IMT (mm)	0.65 (0.7–0.6)	0.73 (0.81–0.64)†	0.79 (0.91–0.69)*

Table 2: Data are presented as mean±SD:
†*P*<0.05 HbA1c<5.7% vs HbA1c 5.7-6.4%; **P*<0.05, HbA1c <5.7% vs HbA1c ≥6.5%; ‡*P*<0.05 HbA1c 5.7-6.4% vs HbA1c≥6.5%

To minimize the impact of glucose tolerance alteration as a confounding factor, we examined the analysis.

As shown in **Figure 1,** AugI remained significantly higher in the NT group with HbA1c > 5.7 (39 mmol/mol) compared with the NT subjects with HbA1c < 5.7% (39 mmol/mol). We found no difference between the NT group with HbA1c > 5.7 (39 mmol/mol) and the subjects with IGT or type 2 diabetes.

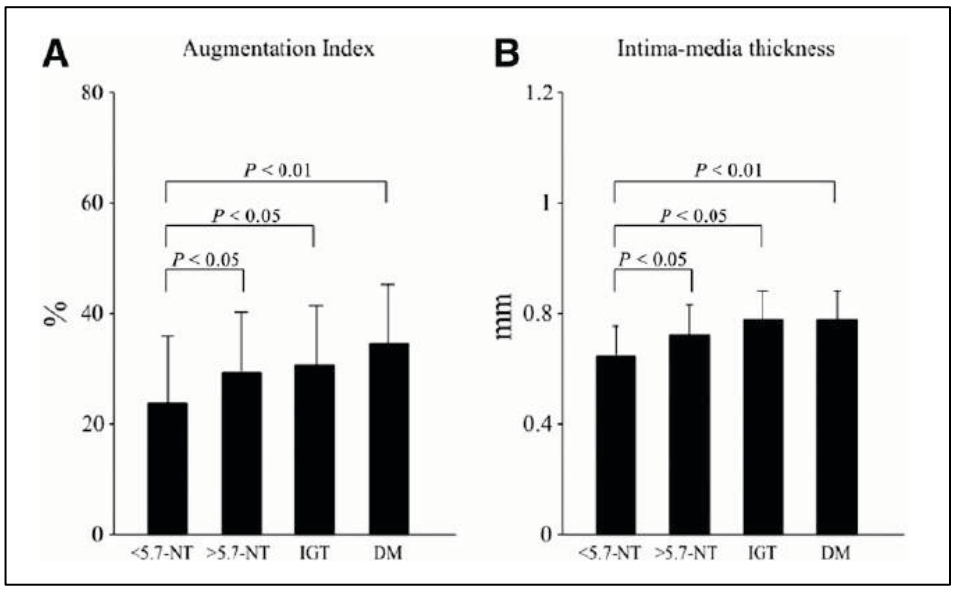

Figure 1: Augmentation index (AugI) (mean±SD) (Panel A) and intima-media thickness (IMT) (mean±SD) (Panel B) by normotolerant (NT) with glycated hemoglogin (HbA1c) <5.7% (n= 86), NT with HbA1c >5.7% (n= 80), impaired glucose tolerance (IGT) (n= 62) and type 2 diabetes (n= 46).

Both simple and multiple regression analyses were performed to evaluate the independent contributions of the glycemic parameters and other cardiovascular risk factors to AugI.

The simple regression analysis included HbA1c, fasting glucose, one-hour post-load glucose and two-hour post-load glucose. AugI was significantly correlated with HbA1c (r=0.15, $P<0.05$), one-hour post-load glucose (r=0.24, $P<0.05$) and two-hour post-load glucose (r=0.19, $P<0.05$). Then, we performed multiple regression analysis using two models. The first model included HbA1c and one- and two-hour post-load plasma glucose and showed a significant ($P<0.05$) correlation between AugI and one-hour post-load glucose. The second model included a variety of atherosclerosis risk factors (age, sex, smoking status, systolic and diastolic BP, total cholesterol, HDL cholesterol, triglycerides, hs-CRP, HOMA-IR and sRAGE) and showed that the only variables that remained significantly associated with AugI were, age ($P<0.0001$) and one-hour post-load glucose ($P<0.05$) **(Table 3)**.

Table 3—Multiple regression analysis evaluating AugI and IMT as dependent variable

	Coefficient β	*P* value
AugI		
Multiple regression: model 1*		
1-h glucose (mg/dL)	0.063	<0.05
Multiple regression: model 2**		
1-h glucose (mg/dL)	0.032	<0.05
Age (years)	0.35	0.0001
IMT		
Multiple regression: model 1*		
HbA_{1c}	0.27	<0.05
Multiple regression: model 2**		
HbA_{1c} (%)	0.016	<0.05
Age (years)	0.02	0.0001
sRAGE (pg/mL)	0.08	<0.05

Table 3: *Model 1 was adjusted for HbA1c, fasting glucose, one-hour and two-hour glucose. **Model 2 was adjusted for age, sex, smoking status, systolic BP diastolic BP, total cholesterol, HDL cholesterol, triglycerides, hs-CRP, HOMA-IR and sRAGE

Intima-media thickness according to HbA1c levels

The IMT was significantly higher (P<0.05) in patients with prediabetes and type 2 diabetes than in subjects with low HbA1c **(Table 2)**. We found that the IMT was higher in the NT subjects with HbA1c 5.7-6.4% than in the NT subjects with HbA1c < 5.7% (39 mmol/mol) (0.73 ± 0.11 vs. 0.68 ± 0.13, P<0.05).

In the simple regression analysis, the IMT was associated with HbA1c (r=0.32, P<0.01), fasting glucose (r=0.29, P<0.01), one-hour post-load glucose (r=0.23, P<0.01) and two-hour post-load glucose (r=0.24, P<0.01). To estimate the independent contributions of the glycemic parameters (HbA1c, one-hour post-load glucose and two-hour post-load plasma glucose), we performed multivariate regression analysis with two models: the first model included the glycemic parameters (HbA1c and one- and two-hour post-load plasma glucose), and the second model included several CVD risk factors (age, sex, smoking status, systolic and diastolic BP, total cholesterol, HDL cholesterol, triglycerides, hs-CRP, HOMA-IR and sRAGE). The first model showed a significant correlation between IMT and HbA1c (P<0.05). In the second model, the only variables that remained significantly associated with IMT were HbA1c (P<0.05), age (P<0.0001) and sRAGE (P<0.05) **(Table 3)**.

Discussion

In this study, we investigated the impact of ADA diagnostic criteria on the cardiovascular risk profile in a population without a previous history of diabetes [83–85]. We evaluated arterial stiffness and arterial thickness, known as early markers of atherosclerosis and predictive of cardiovascular events.

We found that arterial stiffness was altered in subjects with higher HbA1c levels. Both Aug and AugI were significantly increased in the subjects with prediabetes compared with the controls. Additionally, in type 2 diabetes subjects (HbA1c ≥ 6.5% [48 mmol/mol]), Aug and AugI were also increased to a similar extent as that observed in the individuals with prediabetes. Furthermore, the IMT was higher in both prediabetes and type 2 diabetes subjects compared with the controls.

When we divided our study population into NT patients and those with altered glucose tolerance (including subjects with IGT and diabetes), we noted several interesting and original observations. Our data showed that Aug and AugI were increased in the NT subjects with HbA1c 5.7-6.4% compared with their levels in the NT individuals with HbA1c <5.7%; however, these values were similar to those of the IGT and type 2 diabetes patients. Moreover, the NT subjects with HbA1c 5.7-6.4% showed higher IMT values compared with the NT subjects with low HbA1c, and there were no significant differences with respect to the IMT values of the IGT and type 2 diabetes patients. These results suggest that an HbA1c cut-off of 5.7% is more sensitive for the identification of subjects at high cardiovascular risk as many of these subjects (72 out of 117) were classified as NT after the OGTT with a normal fasting glucose level.

The relationship between hyperglycemia and cardiovascular risk in diabetic patients has been well established [73,86,87]. The risk of CVD is increased before glycemia reaches diabetic levels, as previously observed in IFG and IGT subjects and as recently demonstrated in NT individuals with a one-hour post-load glucose >155 mg/dl [88]. However, which among the glucose homeostasis parameters (fasting plasma glucose, one-hour post-load glucose, two-hour post-load glucose and/or HbA1c) could be a better predictor of CVD in prediabetic patients remains unclear. In the present study, HbA1c was initially associated with AugI in an unadjusted model. After adjustment for conventional risk factors, fasting and post-load glucose (including one- and two-hour plasma glucose), the association with HbA1c disappeared, and only one-hour post-load glucose remained significantly associated with AugI, suggesting that one-hour post-load glucose may be a better glycemic marker of vascular damage. This finding thus might highlight the importance of undergoing OGTT and obtaining intermediate values during glucose load, as previously indicated [89]. However, we found that most subjects with a one-hour glucose exceeding 155 mg/dl are included in the group with HbA1c in the 5.7-6.4 (39 to 46 mmol/mol) range, leading to the conclusion that HbA1c could be used to identify subjects at a higher cardiovascular risk. Furthermore, only HbA1c was associated with the IMT after a multiple regression analysis in two models, including glucose homeostasis parameters and multiple atherosclerosis risk factors. These findings are in agreement with previous studies [56] and suggest the importance of HbA1c as an early marker of cardiovascular risk, although the metabolic alterations that lead to an increased cardiovascular risk are multiple and complex, and not a single laboratory test can adequately identify or classify an individual cardiovascular risk.

We did not find any differences in the sRAGE levels among our three groups. However, sRAGE was significantly associated with the IMT in the simple and multiple regression analyses, suggesting the adverse effect of sRAGE on the CV profile.

Previous studies reported that higher sRAGE levels are associated with cardiovascular events and all-cause mortality in subjects with type 1 diabetes; however, the findings regarding the circulating total sRAGE levels in both type 1 and type 2 diabetes are controversial [47,90]. sRAGE reflects the total pool of soluble RAGE in the plasma. Splice variants, including endogenous secretory RAGE (esRAGE), appear to act as decoys, binding inflammatory RAGE ligands such as AGEs. In contrast, cleaved-type soluble RAGE, derived from the cell surface, appears to be modulated by the RAGE-ligand interaction, leading to increased receptor expression [91]. In this work, we measured the total pool of plasma sRAGE; therefore, we could not discern whether the variants of sRAGE might have different associations with the morphological and functional markers of cardiovascular disease.

Our findings are in good agreement with previously published data. In a longitudinal study in a population without diabetes at intermediate-to-high cardiovascular risk, glycated hemoglobin predicted all-cause and cardiovascular mortality independently of fasting glucose [92]. Furthermore, Juan Liang et al. reported that HbA1c was related to high PWV independently of conventional cardiovascular risk factors in a healthy Chinese population [93]. In our study, we found a significant and strong correlation between HbA1c and AugI, Aug and IMT; in contrast, no correlation was observed with the PWV. This discordance could be due, at least in part, to ethnic differences in the study population or differences in the sample size. Accordingly, another study on a Chinese population showed that the AugI and PWV

were differentially affected in subjects with diabetes [94]. Thus, the discordance between PWV and AugI is not surprising. Previous studies have also showed that wave reflection indices and aortic stiffness do not always change in parallel. AugI is primarily determined by the magnitude and timing of the reflected pressure waves, which depend on the tone and elasticity of the small muscular arteries at the major sites of pressure wave reflection; PWV is a measure of elastic-type large artery stiffness and is inversely related to aortic distensibility and compliance [95]. Therefore, AugI may be changed independently of PWV due to alterations in vascular smooth muscle tone that do not affect the elastic aorta. Increases in oxidative stress and reduced endothelial nitric oxide availability may impact the peripheral arteries more than the aorta.

In this study, SEVR, which is an estimate of myocardial perfusion, was not significantly different when we compared prediabetic subjects with controls. These findings are in agreement with those we reported in a recent study [80], in which SEVR was affected by metabolic syndrome but not by altered glucose tolerance.

Our study has several limitations. Our results could be affected by age, although such an effect would not be surprising, considering that aging is strongly correlated with arterial stiffness [96]. However, previous studies have indicated that the deterioration of glucose tolerance is associated with increased arterial stiffness and that many of these changes occurred before the onset of type 2 diabetes [97]. In addition, the OGTT was performed once; thus, intra-individual variation in plasma glucose levels cannot be taken into account.

In conclusion, our data suggest that a simple, reproducible and less expensive marker such as HbA1c may be better able to identify prediabetic subjects at high cardiovascular risk compared with the use of fasting glycemia or OGTT alone.

2.3 Low circulating vitamin D levels are associated with increased arterial stiffness in prediabetic subjects identified according to HbA_{1c}

In 2011, the American Diabetes Association (ADA) recognized a new method to identify prediabetic subjects in addition to impaired fasting glucose (IFG) and impaired glucose tolerance (IGT): a glycated hemoglobin (HbA_{1c}) value between 5.7–6.4% [70].

The clinical relevance of HbA_{1c} and its agreement with fasting plasma glucose and 2-h glucose post-OGTT for the diagnosis of prediabetes remain controversial. In a recent study, we analyzed the cardiovascular risk profile in subjects with prediabetes identified according to HbA_{1c} (5.7-6.4%), normal fasting glycemia and normal glucose tolerance (NFG/NT) after an oral glucose tolerance test (OGTT). These subjects would not have been classified as prediabetic on the basis of fasting or post-OGTT values.

We found that these subjects with prediabetes identified by only HbA_{1c} presented with increased arterial stiffness and carotid intima-media thickness (IMT) in a similar manner to those with new onset type 2 diabetes [98]

The pathogenic mechanism leading to vascular damage in this population is unknown. To further characterize the cardiovascular risk profile of these subjects, we decided to study their vitamin D levels.

Growing evidence indicates that suboptimal vitamin D levels play a role in the development of various clinical conditions, including cardiovascular disease [99–101]. A regulatory role for vitamin D in the cardiovascular system has been clearly demonstrated in animal studies examining activation of the systemic and local cardiac renin-angiotensin system. A study in knockout mice confirmed that the absence of vitamin D receptor activation leads to tonic up-regulation of the renin-

angiotensin system, with the development of hypertension and left ventricular hypertrophy[102,103].

A strong association of vitamin D deficiency with increased all-cause and cardiovascular mortality has been described in the general population [9]. Moreover, suboptimal vitamin D status appears to be involved in impaired glucose homeostasis, insulin resistance and obesity, and this condition may predispose people to type 2 diabetes. Clinical cross-sectional studies have shown a significant inverse relationship between HbA_{1c} and serum 25(OH)D levels in diabetic subjects [104], furthermore, other studies have observed low serum 25(OH)D levels in individuals with prediabetes identified according to ADA recommendations (IFG, IGT and HbA_{1c} 5.7-6.4%) [70]. However, recent literature has indicated that the association between 25(OH)D with cardiovascular and metabolic disease could be weaker in special populations [105,106]. Then, the exact role of vitamin D in these diseases needs to be clarified in randomized clinical trials.

The purpose of this study was to evaluate the levels of vitamin D in patients with prediabetes, particularly in those identified by HbA_{1c} with NFG and NT after OGTT, and whether these values are associated with alterations of arterial stiffness and IMT, early markers of atherosclerosis.

Materials and methods

Study subjects

Subjects (n=286, age range of 18–65 years) with no previous diagnosis of diabetes were consecutively recruited from patients attending our University Hospital for diabetes and cardiovascular risk evaluation during the winter months (November-March). The exclusion criteria were the following: a previous history of diabetes; previous history of overt cardiovascular events (atrial fibrillation, stroke, ischemic heart disease, chronic obstructive peripheral arteriopathy, or heart failure); primary hyperparathyroidism; clinical evidence of advanced liver or renal disease; anemia or hemoglobinopathies; use of medications known to affect glucose metabolism; positivity for antibodies to hepatitis C virus or hepatitis B surface antigen; chronic gastrointestinal diseases associated with malabsorption or chronic pancreatitis; rheumatic diseases; and/or recent history of acute illness, malignant disease, and drug or alcohol abuse. All patients were Caucasian and underwent a physical examination and review of their clinical history and alcohol consumption. Smoking status was assessed in all the patients. During the visit we ask the number of cigarettes and years smoked in order to obtain a categorical variable (active smokers or nonsmokers). Those who reported smoking cigarettes regularly during the year before the exam were considered active smokers. None were taking calcium or vitamin D supplements.

Body weight and height were measured, and BMI was calculated as weight (kg)/[height (m)]^2. Waist circumference was measured in a standing position at the level of the umbilicus. Blood pressure (BP) was measured with a calibrated sphygmomanometer after the subject had rested in the supine position for 10 min. Venous blood samples were drawn from the antecubital vein on the morning after an

overnight fast. All subjects underwent a 75-g OGTT with 0-, 30-, 60-, 90- and 120-min sampling for plasma and insulin as previously described [107]. Glucose tolerance status was definited on the basis of OGTT according to ADA recommendations [70].

The study was approved by the local ethics committee. Informed consent was obtained from each participant.

Biochemical Analyses

Plasma glucose, serum total cholesterol, triglycerides, HDL cholesterol, and hs-CRP were measured using available enzymatic methods as previously described [78] LDL cholesterol concentrations were estimated using the Friedewald formula. Serum calcium and phosphorus levels were measured in all subjects.

Serum 25(OH)D was measured using a chemiluminescent microparticle immunoassay; the interassay and intra-assay coefficient of variation were ≤10% (ARCHITECT; ABBOTT, Wiesbaden, Germany). PTH was measured using a chemiluminescent assay (PTH LIAISON N-TACT; DiaSorin, Saluggia [VC]). Estimated glomerular filtration rate (e-GFR) was calculated with the Cockcroft-Gault formula.

HbA_{1c} was measured via high-performance liquid chromatography using a National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial (DCCT) assay reference[79] Chromatography was performed using a certified automated analyzer (HPLC; HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.) (normal range 4.25–5.9% [23–41 mmol/mol]; intra- and inter-assay CVs were 1.7% and 2.6%, respectively).

Pulse Wave Analysis

Evaluation of arterial stiffness was performed with the patients in fasting status and explicity expressed the recommendation to avoid coffee intake in the morning of the procedure. All measurements were made from the right radial artery by applanation tonometry using a Millar tonometer (SPC-301; Millar Instruments, Houston, TX)[80]. The measurements were performed by a single investigator with the subject in the supine position. The data were collected directly with a desktop computer and processed with SphygmoCorCvMS (AtCor Medical, Sydney, Australia). The aortic waveform in pulse wave analysis was subjected to analysis for the calculation of the aortic Aug and AugI (calculated by dividing augmentation by pulse pressure). Pulse pressure was calculated as the difference between the systolic and diastolic BPs.

Carotid Ultrasound Examinations

Ultrasound scans were performed using a high-resolution B-mode ultrasound system (MyLab 50 XVision; Esaote Biomedica SpA, Florence, Italy) equipped with a 7.5-MHz linear array transducer. All ultrasound examinations were performed by a single physician who was blinded to the clinical and laboratory characteristics of the patients. Scans were performed and measurements were conducted at a total of six plaque-free sites 1 cm proximal to the carotid bulb. The obtained values were averaged and are presented as the means of the IMT of the common carotid artery. All measurements were obtained in diastole, assessed as the phase in which the lumen diameter is at its smallest and the IMT is at its largest.

Statistical Analyses

We based the power calculation on previous studies examining vitamin D differences among patients with cardioscular risk factors and control subjects; the level of significance (α) was set to 5%, and power (1-β) was set to 80%[108]. The estimated sample size was 284 patients. Statistical comparisons of the clinical and biomedical parameters were performed using Stat View 6.0 for Windows. The data are presented as the means ± SD. The distributional characteristics of each variable, including normality, were assessed by the Kolmogorov-Smirnov test. Independent t-test was performed to compare the differences in 25(OH)D levels between men and women. ANOVA for clinical and biological data was performed to test the differences among groups. When ANOVA *P*-value was <0.05 the Bonferroni *post hoc* test for multiple comparisons was further performed. The χ^2 test was used for categorical variables.

A *P* value less than 0.05 was considered statistically significant. If necessary, numerical variables were logarithmically transformed for statistical analysis to reduce skewness [25(OH)D, triglycerides, hs-CRP, homeostasis model assessment of insulin resistance (HOMA-IR) and IMT], and values were expressed as the medians and interquartile ranges.

Simple regression analyses were performed to identify the association between 25(OH)D, AugI, the IMT and the following variables: age, BMI, waist circumference (WC), sex, smoking status, systolic and diastolic BP, total cholesterol, HDL cholesterol, triglycerides, hs-CRP, HbA_{1c}, fasting glycemia, PTH and HOMA-IR. Then, we performed a multiple regression analysis including the variables reaching a *P* value <0.10 in simple regression analysis.

The variance inflation factor (VIF) was used to check for the problem of multicollinearity among the predictor variables in multiple regression analysis. We

used a maximum VIF value of 4 that is recommended in the literature for exclusion of a covariate [18]. Any variable with a VIF that exceeded 4 was excluded from the model (no variable was detected with VIF greater than 4).

Results

In total, 286 subjects participated in the study. The study population was divided into the following four groups based on fasting glucose, OGTT and HbA_{1c} levels, according to the ADA recommendations: 80 control subjects (NFG and NT with HbA_{1c} <5.7%), 83 patients with NFG/NT and HbA_{1c} 5.7–6.4%, 62 patients with IFG/IGT and 61 patients with new onset type 2 diabetes (HbA_{1c} ≥6.5%).

The baseline characteristics of the study subjects are presented in **Table 1**.

Table 1
Clinical and metabolic characteristics of the study population according to fasting glucose, glucose tolerance and HbA_{1c} levels.

	Controls ($n = 80$)	HbA_{1c} 5.7–6.4% NT/NFG ($n = 83$)	IFG/IGT ($n = 62$)	Type 2 diabetes ($n = 61$)	P#
Age (year)	44.8 ± 11.6	49.6 ± 8.9*	52.8 ± 8.8*†	53.8 ± 7.4*†	0.0001
BMI (Kg/m^2)	29.2 ± 4.2	29.3 ± 4.1	30.8 ± 6	29.1 ± 5	0.33
Waist circumference (cm)	99.7 ± 10.5	100.1 ± 9.7	100.6 ± 10.8	99.4 ± 13.2	0.81
Fasting glucose (mg/dL)	87.7 ± 9.2	90.8 ± 7	98.7 ± 6*†	121.4 ± 18*†‡	0.0001
Total cholesterol (mg/dL)	189.9 ± 38.2	212 ± 39.4*	202.6 ± 40	199.5 ± 45.5†	0.008
HDL cholesterol (mg/dL)	47.6 ± 11.9	47.1 ± 12.7	42.6 ± 11*†	38.3 ± 9.2*†	0.003
Triglycerides (mg/dL)	91.5 (66–122)	99 (76–132)	123.5 (82–175)	114 (83–143)	0.15
LDL cholesterol (mg/dL)	121.8 ± 33.1	142.7 ± 35.6	135.3 ± 34	136.3 ± 39.2	0.10
Systolic BP (mmHg)	119 ± 16.4	121.4 ± 14.2	124.4 ± 13.8*	126.1 ± 12.4*	0.05
Diastolic BP (mmHg)	72 ± 10.7	74.4 ± 9.7	74.3 ± 11.5	74.5 ± 10.9	0.59
GFR (mL/min)	113 ± 47.4	103.4 ± 27.3	102.8 ± 28.4	107.5 ± 40.6	0.52
Hs-CRP (mg/dL)	0.13 (0.08–0.28)	0.2 (0.1–0.4)*	0.21 (0.08–0.38)*	0.32 (0.19–0.72)*†‡	0.04
HOMA-IR	1.5 (1–2.1)	1.5 (1–2.1)	2.0 (1.6–3.3)*†	2.3 (1.8–3.8)*†	0.06
25(OH)D (ng/mL)	23.1 (17.1–29.7)	21.7 (15.8–31.1)*	21.3 (17.2–30.3)*	19.4 (13.8–28.5)*	0.01
PTH (pg/mL)	38.6 ± 15	40.5 ± 16.6	41.4 ± 14.2	42.7 ± 16	0.08
Calcium (mg/dL)	9.3 ± 0.6	9.3 ± 0.4	9.2 ± 0.4	9.5 ± 0.4	0.25
Phosphorus (mg/dL)	3.2 ± 0.5	3.2 ± 0.5	3.4 ± 0.7	3.3 ± 0.4	0.21
Hypertension	16%	26%	31%	26%	0.31##
Active smokers	30%	26%	42%	52%*†	0.03##
Sex (M/F)	35/45	44/39	35/27	33/28	0.27##

Table 1: The data are presented as the mean±SD or median(IQR). Continuous and categorical variables were compared by ANOVA and χ^2 test respectively. Bonferroni post hoc test was further performed.
NT/NFG, normotolerant and normal fasting glucose; IFG/IGT, impaired fasting glucose and impaired glucose tolerance; HbA_{1c}, glycated hemoglobin; BP, blood pressure; GFR, glomerular filtration rate; Hs-CRP, high sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment-insulin resistance; 25(OH)D 25-hydroxyvitamin D; PTH, parathyroid hormone.
Smoking was quantified (number of cigarettes and years smoked), and smoking status was classified in active and nonsmokers.
Hypertension was defined as systolic blood pressure ≥135 mmHg, diastolic blood pressure ≥85 mmHg or taking any hypertension medications.
By ANOVA; ## By χ^2; *P<0.05 vs controls; †P<0.05 vs ≥5.7% NT/NFG; ‡P<0.05 vs IFG/IGT

Compared with controls, individuals with NFG/NT and HbA_{1c} 5.7–6.4% were more likely to be older (44±11.6 vs 49±8.9 years, P=0.005), to present higher levels of total cholesterol (189.9±38.2 vs 212±39.9 mg/dL, P=0.001) and to have higher hs-CRP serum levels (0.13 [0.08-028] vs 0.2 [0.1-0.4] mg/dL, P=0.048). These subjects had anthropometric and metabolic characteristics similar to patients with IFG/IGT

and new onset type 2 diabetes, except for lower fasting glucose and HOMA-IR and higher HDL cholesterol. Plasma levels of calcium and phosphorus were similar among the four groups (**Table 1)**.

25(OH)D according to HbA1c levels

Our data demonstrate lower levels of 25(OH)D in diabetic patients compared to controls **(Table 1).** Accordingly, we found higher PTH levels in the diabetic patients compared to the controls, but PTH levels were not significantly different among the four groups. Although there is no consensus on optimal serum levels of 25(OH)D, vitamin D deficiency is defined by most experts as a 25(OH)D level of less than 20 ng per milliliter [19]. In the control group, the prevalence of 25(OH)D deficiency (≤20 ng/mL) was 32%; in the IFG/IGT group, the prevalence of 25(OH)D deficiency was 44%; similarly, subjects with NFG/NT and prediabetes according to the new ADA criterion (HbA_{1c} 5.7–6.4%), showed a prevalence of 25(OH)D deficiency of 47%. In the new onset type 2 diabetic group, the prevalence of 25(OH)D deficiency 57% **(Figure 1).**

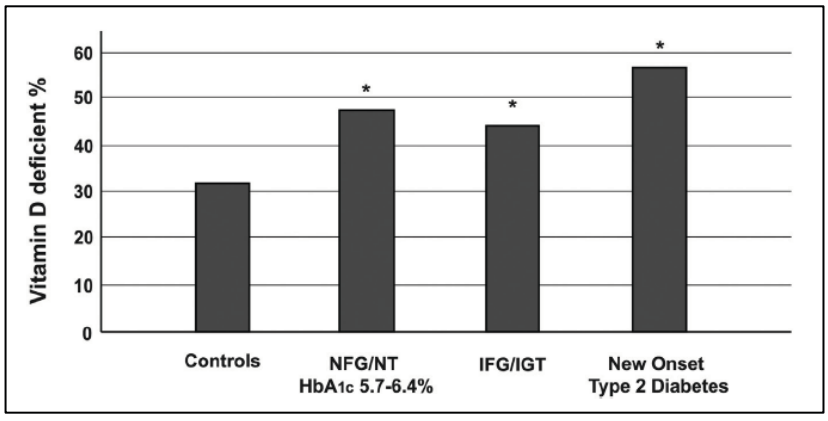

Figura 3: Vitamin D deficiency according to HbA_{1c} and glucose tolerance. Variables were compared by χ^2 test. NGT/NT: normal fasting glycemia and normotolerant; IFG/IGT: impaired fasting glucose and impaired glucose tolerance subjects. *P<0.05 vs Controls.

The independent t-test showed no significant differences between men and women in 25(OH)D plasma levels [24(39.4-17.4) vs 22.5(30.2-17.1) *P*=0.23].

Both simple and multiple regression analyses were performed to evaluate the independent contributions of metabolic parameters and cardiovascular risk factors to vitamin D status.

In the simple regression analysis, 25(OH)D showed a significant correlation with BMI (r=-0.23, *P*=0.001), WC (r=-0.19, *P*=0.04), hs-CRP (r=-0.22, *P*=0.008), HbA_{1c} (r=-0.26, *P*=0.0003), and fasting glucose (r=-0.15, *P*=0.04). Furthermore, univariate analysis showed no significant association between PTH and 25(OH)D levels (r=0.06, *P*=0.35).

To estimate the independent contributions of the latter variables on 25(OH)D levels, we performed a multivariate regression analysis. The model included only variables reaching a *P* value <0.10 in the simple regression analysis (BMI, WC, hs-CRP, HDL cholesterol, LDL cholesterol, fasting glucose and HbA_{1c}) and showed that only HbA_{1c} and BMI remained significantly associated with 25(OH)D **(Table 3)**.

25(OH)D levels, arterial stiffness and intima-media thickness

As previously reported, AugI and the IMT were significantly higher (*P*=0.005) in patients with NFG/NT and HbA_{1c} 5.7–6.4%, IFG/IGT and new onset type 2 diabetes than in control subjects [3] **(Table 2)**. Both simple and multiple regression analyses were performed to evaluate the independent contributions of 25(OH)D on AugI and the IMT in this population.

In the simple regression analysis, AugI was significantly correlated with 25(OH)D (r=-0.25, *P*=0.0004) **(Figure 2)**, age (r=0.56, *P*=0.001), WC (r=0.14, *P*=0.04), systolic BP (r=0.13, *P*=0.04), total cholesterol (r=0.18, *P*=0.008), and HbA_{1c} (r=

0.31, *P*=0.001). Although it is well known that smoking status strongly affect AugI, we found no significant association between these two variables (r=0.13, *P*=0.07). No association was found between AugI and PTH levels (r=0.02, *P*=0.70).

Table 2
Arterial stiffness and thickness parameters according to fasting glucose, glucose tolerance and HbA_{1c} levels.

	Controls ($n = 80$)	HbA_{1c} 5.7–6.4% NT/NFG ($n = 83$)	IFG/IGT ($n = 62$)	Type 2 diabetes ($n = 61$)	*P#*
AugI (%)	24 ± 12.9	29.2 ± 11.2*	31.7 ± 11.5*	29.5 ± 6.5*	0.002
IMT (mm)	0.66 (0.62–0.75)	0.72 (0.65–0.81)*	0.76 (0.68–0.86)*	0.78 (0.7–0.9)*†	0.003

The data are presented as the mean±SD or median(IQR). Variables were compared by ANOVA. Bonferroni post hoc test was further performed.
NT/NFG, normotolerant and normal fasting glucose; IFG/IGT, impaired fasting glucose and impaired glucose tolerance; HbA_{1c}, glycated hemoglobin; AugI, augmentation index; IMT, intima-media thickness.
By ANOVA; **P*<0.05 vs Controls; †*P*<0.05 vs ≥5.7% NT/NFG; ‡*P*<0.05 vs IFG/IGT

Table 3
Multiple regression analysis evaluating 25(OH)D, AugI and IMT as dependent variables.

	Coefficient β	P value
25(OH)D		
Multiple regression		
BMI (Kg/m^2)	−0.70	0.02
WC (cm)	0.08	0.49
hs-CRP (mg/dL)	−0.11	0.96
HDL cholesterol (mg/dL)	−0.10	0.11
LDL cholesterol (mg/dL)	−0.13	0.09
Fasting glucose (mg/dL)	−0.05	0.36
HbA_{1c} (%)	−0.24	0.00
AugI		
Multiple regression		
25(OH)D (ng/mL)	−0.13	0.00
Age (years)	0.31	0.00
WC (cm)	0.12	0.39
Systolic BP (mmHg)	0.08	0.07
Total cholesterol (mg/dL)	−0.03	0.29
HDL cholesterol (mg/dL)	0.09	0.06
LDL cholesterol (mg/dL)	0.00	0.85
HbA_{1c} (%)	0.23	0.02
IMT		
Multiple regression		
25(OH)D (ng/mL)	0.00	0.58
Age (years)	0.00	0.00
WC (cm)	0.00	0.13
Systolic BP (mmHg)	0.01	0.18
Fasting glycemia (mg/dL)	0.00	0.33
HbA_{1c} (%)	0.02	0.02
BMI (Kg/m^2)	−0.01	0.77
PTH (pg/mL)	0.00	0.84

25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; WC, waist circumference; hs-CRP, high sensitivity C-reactive protein; HbA_{1c}, glycated hemoglobin; AugI, Augmentation Index; IMT, intima-media thickness; BP, blood pressure; PTH, parathyroid hormone.

The multivariate analysis, including each of the components reaching a *P* value <0.10 in the simple regression analysis [25(OH)D, age, WC, systolic BP, total cholesterol, HDL cholesterol, LDL cholesterol, HbA_{1c}], showed a correlation only with age, HbA_{1c} and 25(OH)D **(Table 3).**

The IMT was significantly correlated with 25(OH)D (r=-0.16, *P*=0.02) **(Figure 2)**, age (r=0.56, *P*=0.001), WC (r=0.21, *P*=0.001), systolic BP (r=0.25, *P*=0.0004), and HbA_{1c} (r=0.28, *P*=0.0001) in the simple regression analysis. No significant association was found between IMT and PTH levels (r=0.12, *P*=0.08).

25(OH)D was not independently associated with IMT after multivariate analysis [the model included 25(OH)D, age, WC, systolic BP, fasting glycemia, $HbA_{1c,}$ BMI, PTH]; the only major determinants of IMT were age and HbA_{1c} **(Table 3).**

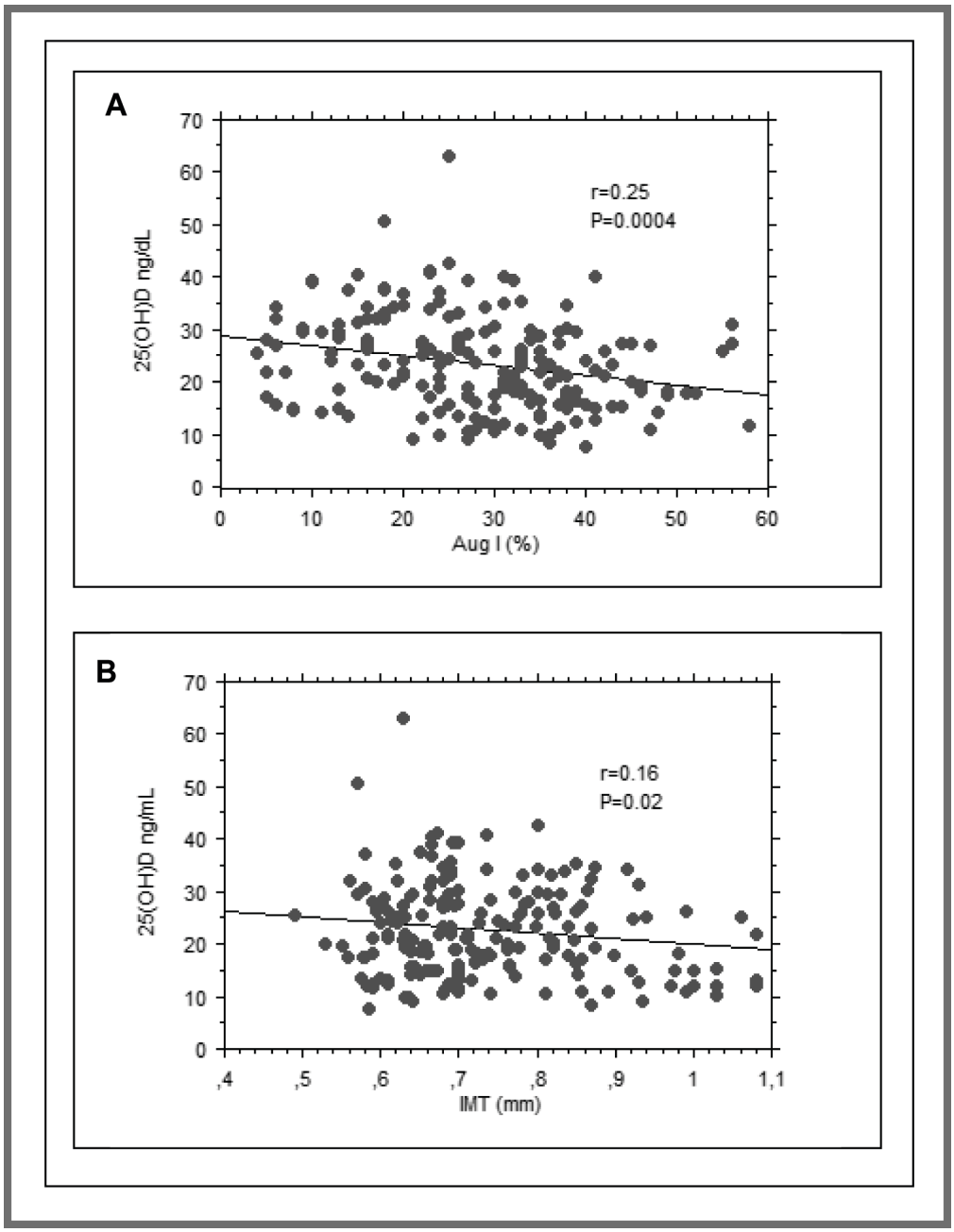

Figura 2: Correlation between Augmentation Index (AugI)(Panel A), intima-media thickness (IMT)(Panel B) and 25-hydroxyvitamin D [25(OH)D].

Discussion

Prediabetes represents a relevant clinical problem because of the substantial number of affected subjects and their increased cardiovascular risk. We recently focused on subjects with an HbA_{1c} value between 5.7–6.4% but with normal fasting plasma glucose and normal glucose tolerance post-OGTT [98].

Recently, some experimental and clinical data suggested the hypothesis that vitamin D deficiency may play a role in metabolic and cardiovascular disease. Thus, we designed this study to evaluate 25(OH)D levels and their possible association with early markers of cardiovascular risk. We found an association between serum 25(OH)D concentrations and arterial stiffness, a marker of preclinical atherosclerosis, independently of various potential confounding factors, including classical cardiovascular risk factors, glycemic homeostasis factors, renal function tests and markers of inflammation. This data are consistent with reports from cross-sectional observational studies, which have shown that lower vitamin D levels are associated with increased arterial stiffness in type 2 diabetic patients[99,100,109], adolescents with type 1 diabetes [110], elderly populations[111] and in nondiabetic women with systemic lupus erythematosus [112].

In this study, we did not confirm the association between IMT and 25(OH)D. The discordance between AugI and IMT is not surprising and the findings regarding the association between 25(OH)D and IMT are currently controversial. The lack of association between vitamin D and carotid IMT was also reported in a large cohort study[113]. Small studies have reported a lack of association of 25(OH)D with carotid IMT in special populations[113–115]. In contrast, other clinical based studies reported positive cross sectional association between lower vitamin D and larger IMT. However, these studies examined selected population (type 2 diabetes and HIV) and

did not exclude participants with known cardiovascular disease, resulting in potential confounding or bias [116,117]. It has been suggested that arterial stiffness and carotid IMT may represent two different and independent markers of subclinical vascular involvement or damage; vitamin D may impact arterial stiffness through altered vascular smooth tone, oxidative stress, reduced endothelial nitric oxide and endothelial dysfunction and that all these factors may affect peripheral arteries, more than carotid, at least in a early stage.

In our study, 25(OH)D was inversely related to fasting glucose and HbA_{1c}, and the inverse correlation with HbA_{1c} persisted in the multivariate analysis. A significant inverse correlation between HbA_{1c} and 25(OH)D levels in diabetic subjects was also reported in previous studies, underlying the important role of glucose control over time, as reflected by the levels of HbA_{1c}. These studies suggested a possible connection between glycemic control and vitamin D metabolism [118,119].

Other cross-sectional studies on humans have demonstrated an independent association of 25(OH)D with both insulin sensitivity and beta-cell function in subjects without diabetes[117]. Furthermore, a population-based prospective study on a large cohort of non-diabetic men and women confirmed the inverse association between baseline serum 25(OH)D, future glycemia and insulin resistance[120]. Contrary to our findings, De Las Heras and associates reported no differences in plasma 25(OH)D concentrations across the glucose tolerance groups; the discordance in the findings between our study and the latter could be due to differences in the mean age (14 vs 48 years) and BMI (35 vs 29) of the study populations [108].

We found a significant correlation between BMI, WC and 25(OH)D in the simple regression analysis; the association with WC disappeared in the multivariate model. Data from literature remarked that obesity is closed associated with vitamin D

deficiency and it has been suggested that this may be due to vitamin D deposition in adipose tissue, resulting in lower circulating 25(OH)D levels in the blood[121,122]. In order to obtain a more clear interpretation of the role of adipose tissue in vitamin D hypovitaminosis bio-impedance analysis could be more sensitive rather than BMI or waist circumferences, surrogate indicators of adiposity.

Although an inverse relationship between 25(OH)D and PTH plasma levels has been often reported, we cannot find a significant correlation between these two variables. According to our data, another recent study conducted on a sample of 585 healthy subjects reported a very weak but significant association between 25(OH)D and PTH (r=-0.08, *P*=0.039); in that study, the two variables did not remain significantly related for 25OHD plasma levels >19 ng/dL in further regression analysis. We found a similar r value (r=0.06) and the lack of significance in our data could be due to the different sample size (286 vs 585) [123].

As shown by previous reports and a recent meta-analysis of randomized controlled trials, evidence of a beneficial effect of vitamin D supplementation on metabolic risk is currently insufficient and controversial[124]. Tabesh *et al.* have shown that vitamin D supplementation improves the glycemic status and lipid profile of vitamin D insufficient people with type 2 diabetes [125]. Conversely, Davidson *et al*. reported that vitamin D supplementation had no effect on insulin secretion, insulin sensitivity or the development of diabetes compared with placebo administration in a double-blind randomized control study[126]. Further research is needed to test the hypothesis that vitamin D supplementation can reduce cardiovascular risk.

A strength of this study is that we assessed PTH levels; thus, we can conclude that our results were not mediated by secondary hyperparathyroidism. Moreover, subjects taking vitamin D supplementation were excluded from the study. This study

has several limitations. First, information on important determinants of serum 25(OH)D concentration, such as dietary intake of vitamin D and calcium, sunlight exposure, skin pigmentation, physical activity and dietary caloric intake, were not collected. Second, because our study used a cross-sectional design, the causative nature of the associations reported cannot be established.

In conclusion, our data show that prediabetic patients, including those identified only according to HbA_{1c}, have significantly lower 25(OH)D concentrations (vs controls), which is associated with early markers of cardiovascular disease independent of classical risk factors, renal function tests and inflammatory markers. Thus, our data suggest that HbA_{1c} can be considered a reliable marker of cardiovascular and metabolic risk independent of fasting and post-load glycemia.

2.4 Low endogenous secretory RAGE levels are associated with inflammation and carotid atherosclerosis in prediabetes

Prediabetes, which is typically defined as blood glucose concentrations higher than normal but lower than the diabetes threshold, is a high-risk state for diabetes and cardiovascular disease development [33]. In 2011, the American Diabetes Association (ADA) revised its criteria for the diagnosis of type 2 diabetes and the categories at increased risk for diabetes, which already included impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). On the basis of a thorough analysis performed by an international expert committee, the use of glycated hemoglobin A_{1c} (HbA_{1c}) measurement was recommended as another diagnostic test option in addition to glucose values. Specifically for the categories of increased risk for type 2 diabetes, the new ADA recommendations state that an HbA_{1c} from 5.7-6.4% identifies individuals at high risk for diabetes to whom the term prediabetes may be applied [70].

Previous studies have confirmed that events portending accelerated atherosclerosis are underway prior to the formal diagnosis of diabetes. According to these reports, prediabetes is associated with more advanced vascular damage compared with normoglycemia[127,128]. In a recent study, we analyzed the cardiovascular risk profile in subjects with prediabetes identified according to HbA_{1c} (5.7-6.4%) and reported an alteration of early markers of cardiovascular damage in subjects with higher HbA_{1c} [43]

To explain the association between hyperglycemia and vascular complications in diabetes, several studies have emphasized the role of advanced glycation end-products (AGE) and their receptors (RAGE). The interaction between RAGE and its ligands (AGEs and other molecules, such as S100A12) effectively modulates several

steps of atherogenesis, triggering an inflammatory-proliferative process and, furthermore, critically contributing to the propagation of vascular perturbation, mainly in diabetes [91]. RAGE has a secretory isoform, which is termed soluble RAGE (sRAGE). sRAGE is primarily formed by the proteolytic cleavage of membrane-bound RAGE and, secondarily, by a secreted, non-membrane-bound form of the receptor resulting from alternative splicing of the RAGE gene, which is known as endogenously secreted RAGE (esRAGE). esRAGE may contribute to the removal/neutralization of circulating ligands, thus functioning as a decoy by competing with cell-surface RAGE for ligand binding [129].

sRAGE has been recently associated with a greater risk of cardiovascular complications. Several studies have demonstrated an inverse cross-sectional association between sRAGE plasma levels and coronary heart disease or atherosclerosis in nondiabetic men [130]. Prospective studies have shown that low levels of sRAGE predict cardiovascular mortality in diabetic and nondiabetic subjects[131]. Moreover, common oral agents such as thiazolidinediones and statins are known to modulate the AGE-RAGE system, even if long-term prospective studies are needed to evaluate whether the modulation of sRAGE can be helpful in preventing macrovascular disease [132,133].

Although numerous data have been reported in subjects with both type 1 and 2 diabetes, to date, very little information is available regarding circulating sRAGE levels in subjects with prediabetes and their possible link with vascular damage in this population.

In this study, we measured sRAGE and esRAGE levels and examined their associations with other pro-inflammatory factors and early markers of atherosclerosis in subjects with prediabetes.

Although different criteria for the diagnosis of prediabetes have good predictive values for the development of type 2 diabetes, there are scarce and conflicting data as to which best predicts cardiovascular disease [134]. Therefore, we studied the AGE/RAGE axis and pro-inflammatory profile, focusing on individuals with prediabetes identified only by HbA_{1c} (5.7-6.4%) who had normal fasting glucose (NFG) and were normotolerant (NT) after oral glucose tolerance test (OGTT).

Materials and methods

Study subjects

Three-hundred-eighty subjects with no previous diagnosis of diabetes who attended our university hospital for diabetes and cardiovascular risk evaluation were consecutively recruited for the study. The inclusion criteria were ages ranging from 18–65 years. All patients were Caucasian and underwent a physical examination and review of their clinical history, smoking status (active or nonsmokers) and alcohol consumption. The exclusion criteria were as follows: a previous history of diabetes, a previous history of overt cardiovascular events (stroke, ischemic heart disease, chronic obstructive peripheral arteriopathy or heart failure), anemia or hemoglobinopathies, the use of medications known to affect glucose metabolism, clinical evidence of advanced liver or renal disease, chronic inflammatory disease or other chronic diseases and/or recent history of acute illness, malignant disease and drug or alcohol abuse.

Body mass index (BMI) was calculated as weight (kg)/[height (m)]^2. Blood pressure (BP) was measured with a calibrated sphygmomanometer after 10 min resting. Venous blood samples were drawn from the antecubital vein on the morning after an overnight fast. Baseline venous blood samples were obtained for the measurement of clinical biochemistry parameters. LDL cholesterol concentrations were estimated using the Friedewald formula. All subjects underwent a 75-g OGTT with 0-, 30-, 60-, 90-, and 120-min sampling for plasma and insulin, as previously described [107]. Glucose tolerance status was defined on the basis of OGTT according to ADA recommendations[70].

Biochemical analyses

Plasma glucose, serum total cholesterol, triglycerides, HDL cholesterol, and high sensitivity C-reactive protein (hs-CRP) were measured using available enzymatic methods, as previously described [49].

To quantify the plasma concentration of sRAGE (Human sRAGE ELISA; Biovendor, Brno, Czech Republic), esRAGE (B-Bridge esRAGE ELISA Kit, Cupertino, California, USA), Carboxymethyl-lysine (CML) (CircuLex, ELISA Kit for CML-Nε_(Carboxymethyl)lysine, Nagano, Japan), and S100A12 (Cloud-Clone Corp., ELISA Kit for S1000A12, Houston, TX, USA), fasting blood samples were collected, and specimens were immediately centrifuged and stored at -80°C. The commercially available ELISA kits were used according to the manufacturer's instructions. The inter-assay and intra-assay coefficients of variation (CVs) ranged respectively from 5.5 to 8.8% and 2.6 to 5.3% for sRAGE; from 5.9 to 7.5% and 0.7 to 1.5% for esRAGE; from 4.7 to 15.2% and 5.2 to 7.4% for CML; <10% and 12% for S100A12.

HbA_{1c} was measured via high performance liquid chromatography using a National Glycohemoglobin Standardization Program and was standardized to the Diabetes Control and Complications Trial (DCCT) (10) assay reference. Chromatography was performed using a certified automated analyzer (HPLC; HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.) (normal range 4.25–5.9%); intra- and inter assay CVs were 1.7% and 2.6%, respectively.

Carotid ultrasound examination

Ultrasound scans were performed using a high-resolution B-mode ultrasound system (MyLab 50 Xvision; Esaote Biomedica SpA, Florence, Italy) equipped with a 7.5-MHz linear array transducer. All ultrasound examinations were performed by a single physician who was blinded to the clinical and laboratory characteristics of the

patients. Scans were performed, and measurements were conducted at a total of six plaque-free sites 1 cm proximal to the carotid bulb. The obtained values were averaged and are presented as the mean of the intima-media thickness (IMT) of the common carotid artery. All measurements were obtained in diastole, assessed as the phase in which the lumen diameter is at its smallest and the IMT is at its largest.

Arterial stiffness evaluation

The SphygmoCor CvMS (AtCor Medical, Sydney, Australia) system was used for the determination of the pulse wave velocity (PWV). This system uses a tonometer, and two different pressure waves obtained at the common carotid artery (proximal recording site) and at the femoral artery (distal recording site). An electrocardiogram was used to determine the start of the pulse wave. The PWV was determined as the difference in travel time of the pulse wave between the two different recording sites and the heart, divided by the travel distance of the pulse waveform. The PWV was calculated on the mean basis of 10 consecutive pressure waveforms to cover a complete respiratory cycle.

Mononuclear cell (MNC) isolation

Blood samples (10-12 ml) were collected in Na-EDTA as an anticoagulant and were carefully layered on lympholyte medium (LymphoprepTM, Stemcells Technologies) according to the manufacturer's instructions. The samples were centrifuged, and two bands separated on the top of the red blood cell pellet. The MNCs were removed at the plasma/LymphoprepTM interface without disturbing the erythrocyte/granulocyte pellet and were washed with 0.9% NaCl.

Total RNA isolation and reverse transcription polymerase chain reaction

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, purified with ribonuclease-free deoxyribonuclease I (Sigma-Aldrich, St. Louis, MO, USA) and quantified by spectrophotometry. First-strand cDNA was produced from 1 μg of total RNA using Superscript II and OligodT primers (Invitrogen, Life Technologies) according to the manufacturer's instructions.

The transcripts of human advanced glycosylation end product-specific receptor (RAGE gene, also termed AGER NM_001136) and its splice variant (esRAGE, NM_001206940) were obtained by polymerase chain reaction (PCR) using the primers 5'-CAGCATCATCGAACCAGGC-3' and 5'-TGGATGGGATCTGTCTGTGG-3'. The forward 5'-CTCTTCCAGCCTTCCTTCCT-3' and reverse 5'-AGCACTGTGTTGGCGTACAG-3' primers specific to the human actin beta (ACTB) sequence (NM_001101.3) were used as the internal control of gene expression. With these primer pairs, the expected bands were 282 bp for the AGER gene and 214 bp for the esRAGE gene; the expected band for the ACTB gene was 116 bp.

Aliquots of each amplified reaction were analyzed by electrophoresis on 2.5% agarose gels and visualized by SYBR Safe DNA gel stain (Invitrogen).

Statistical analyses

We based the power calculation on previous studies examining sRAGE differences among patients with altered glycemic homeostasis and control subjects to compare the means of 3 groups using ANOVA; the level of significance (α) was set to 5% and

power (1-β) was set to 80%. The rho value selected was 0.25.

Statistical comparisons of clinical and biomedical parameters were performed using Stat View 6.0 for Windows. The data are presented as the mean±SD. Each variable's distributional characteristics, including normality, were assessed by the Kolmogorov–Smirnov test. ANOVA for clinical and biological data was performed to test the differences among groups, and the Bonferroni post hoc test for multiple comparisons was further performed. The χ^2 test was used for categorical variables. A *P* value less than 0.05 was to be considered significant. When necessary, numerical variables were logarithmically transformed to reduce skewedness, and the values are expressed as a median and interquartile range.

Simple regression analysis was performed to relate esRAGE, IMT and PWV to the following variables: age, sex, BMI, systolic and diastolic BP, total cholesterol, HDL cholesterol, triglycerides, LDL cholesterol, non-HDL cholesterol, HbA_{1c}, fasting glycemia and fasting insulin. To identify variables independently associated with variations of esRAGE, IMT and PWV, we performed two multivariate regression models. The first model included several cardiovascular risk factors (age, sex, BMI, smoking status, systolic and diastolic BP, HDL cholesterol, triglycerides, LDL cholesterol, non-HDL cholesterol, statin therapy, HbA_{1c}, fasting glycemia and fasting insulin). Subsequently, variables reaching significance were inserted in a second multiple regression model that included glycation and inflammatory markers (sRAGE, esRAGE, CML, hs-CRP, S100A12). The variance inflation factor (VIF) was used to check for the problem of multicollinearity among the predictor variables in multiple regression analysis. Any variable with a VIF that exceeded 4 was excluded from the model, as recommended in the literature (no variable was detected with VIF greater than 4) (17). The study was approved by the local ethic committee.

Informed consent was obtained from each participant.

Results

In total, 380 subjects participated in the study. The study population was divided into the following three groups based on fasting glucose, OGTT and HbA_{1c} levels according to the ADA recommendations: 99 control subjects (NFG and NT and HbA_{1c}<5.7%), 220 patients with prediabetes (IFG and/or IGT and/or HbA_{1c} 5.7-6.4%) and 61 patients with new onset type 2 diabetes (fasting glucose ≥126 mg/dL and/or 2-h glucose post-OGTT ≥200 mg/dL and/or HbA_{1c} ≥6.5%).

The clinical and biochemical characteristics of the study subjects are presented in **Table 1**.

The subjects with prediabetes were older than the controls but were similar with regard to BMI, plasma levels of total cholesterol, HDL cholesterol, systolic and diastolic BP, and HOMA-IR. There were no significant differences between subjects with prediabetes and new onset type 2 diabetes with respect to anthropometric and metabolic characteristics except for the lower systolic BP, lower fasting glycemia, higher HDL cholesterol and lower HOMA-IR.

The circulating plasma levels of S100A12, hs-CRP and white blood cells (WBC) were higher in the subjects with prediabetes and type 2 diabetes compared with the controls. CML plasma levels were similar among the three groups **(Table 2)**.

Table 1. Clinical and Metabolic Characteristics of the Study Population According to Glucose Tolerance

	Controls (n = 99)	Prediabetes (n = 220)	T2DM (n = 61)	P[c]
Age, y	46 ± 11.2	49.1 ± 9.8[a]	50.9 ± 8[a]	<.0001
BMI, kg/m^2	28.8 ± 4.5	29.4 ± 3.9	30 ± 4.9	.12
Fasting glucose, mg/dL	86.4 ± 8.6	93.4 ± 11[a]	120.2 ± 19[a,b]	<.0001
Fasting insulin, microu/mL	7 (4.9–9.3)	7.6 (5.6–11.7)	8.2 (6.6–11.3)	.53
2-h glucose post-OGTT, mg/dL	117 ± 28.7	150.5 ± 42.6[a]	222.2 ± 39.7[a,b]	<.0001
HbA_{1c}, %	5.3 ± 0.2	6 ± 0.3[a]	7.1 ± 0.5[a,b]	<.0001
Total cholesterol, mg/dL	188.9 ± 36.6	195.5 ± 39.3	189.3 ± 41.5	.1
HDL cholesterol, mg/dL	47.8 ± 12.4	46 ± 11.5	39.1 ± 8.3[a,b]	<.01
Triglycerides, mg/dL	86 (66–122)	109.5 (78–151)[a]	117 (83–146)[a]	.004
LDL cholesterol, mg/dL	120.2 ± 32.8	129.2 ± 35.5[a]	126.2 ± 36	.01
Non-HDL cholesterol, mg/dL	140.4 ± 35.4	154 ± 38.6	150.1 ± 40.7	.09
Systolic BP, mm Hg	119 ± 15.4	122.6 ± 14.2	126.7 ± 15.4[a,b]	.02
Diastolic BP, mm Hg	71.8 ± 10.2	73 ± 10.6	76.2 ± 12.8[a]	.02
HOMA-IR	1.5 (1–2)	1.5 (1.6–3.3)	2.6 (1.9–0.72)[a,b]	<.0001
Hypertension, %	18	24	26	.24[d]
Active smokers, %	30	33	36	.63[d]
Statin therapy, %	20	24	23	.23[d]
Sex, M/F	37/62	97/93	27/34	.34[d]

Table 1: The data are presented as the mean±SD or median (IQR): HbA_{1c}, glycated hemoglobin; BP, blood pressure; HOMA-IR, homeostasis model assessment-insulin resistance.
Smoking was quantified, and smoking status was classified as active smokers or nonsmokers.
Hypertension was defined as systolic blood pressure ≥135 mmHg or diastolic blood pressure ≥85 mmHg or as the prescription of any hypertension medications.
* P<0.05 vs. Controls; # P<0.05 vs. Prediabetes; § by ANOVA; §§ by χ^2.

Table 2. Circulating Levels of sRAGE, esRAGE, Inflammatory Makers, CML, Intima-media Thickness (IMT) and Pulse Wave Velocity (PWV) According to Glucose Tolerance

	Control (n = 99)	Prediabetes (n = 220)	T2DM (n = 61)	P[c]
sRAGE, ng/mL	1.464 ± 596.1	1.328 ± 580.1	1.437 ± 532.3	.56
esRAGE, ng/mL	0.52 ± 0.26	0.32 ± 0.18[a]	0.3 ± 0.19[a]	<.0001
S100A12, ng/mL	5.35 ± 3.38	7.13 ± 5.4[a]	8.41 ± 4.44[a,b]	.04
hs-CRP, mg/dL	0.14 (0.08–0.27)	0.34 (0.11–0.47)[a]	0.45 (0.18–0.72) [a,b]	.001
WBC, $10^3/\mu L$	6.4 ± 1.6	7.1 ± 1.8[a]	7.2 ± 1.8[a]	.03
CML, $\mu g/mL$	2.64 ± 1.18	2.45 ± 1.26	2.46 ± 1.44	.51
IMT, mm	0.67 (0.6–0.73)	0.75 (0.65–0.78)[a]	0.78 (0.7–0.92)[a]	<.0001
PWV, m/sec	7.1 ± 1.7	7.6 ± 1.6[a]	8.6 ± 1.7[a,b]	<.0001

Table 2: The data are presented as the mean±SD or median (IQR).
*P<0.05 vs. Controls; #P<0.05 vs. Prediabetes; § by ANOVA.

Circulating plasma levels and MNC RNA expression of esRAGE in subjects with prediabetes

The plasma levels of esRAGE were significantly lower in the subjects with prediabetes than in the controls (0.32±0.18 vs. 0.52±0.26 ng/mL, P<0.05) but were similar to those with new onset type 2 diabetes. As shown in **Table 2**, no differences were observed in the sRAGE levels among the three groups.

In the simple regression analysis, esRAGE was inversely associated with age (r=-0.19, P<0.05), BMI (r=-0.15, P<0.04), HbA_{1c} (r=-0.18, P<0.01), hs-CRP (r=-

0.17, $P<0.05$) and S100A12 (r=-0.28, $P<0.01$). To estimate the independent contributions of cardiovascular risk factors, glycation and inflammation markers to esRAGE levels, we performed multiple regression analysis using two models. The first model included a variety of cardiovascular risk factors (age, sex, smoking status, BMI, statin therapy, systolic BP, diastolic BP, HDL cholesterol, triglycerides, LDL cholesterol non-HDL cholesterol, HbA_{1c}, fasting glycemia, and fasting insulin), and the second model included variables reaching significance in the first model and several markers of glycation and inflammation (S100A12, hs-CRP, WBC and CML). The first model exhibited a significant correlation between esRAGE and age ($P<0.001$), BMI ($P<0.05$) and HbA_{1c} ($P<0.001$). In the second model, the variables that remained significantly associated with esRAGE were HbA_{1c} ($P<0.05$), hs-CRP ($P<0.05$) and age ($P<0.001$) **(Table 3)**.

Table 3. Multiple Regression Analysis Evaluating esRAGE, IMT and PWV as Dependent Variables

	Coefficient β	P
esRAGE		
Multiple regression—model 1[a]		
Age, y	−0.07	<.001
HbA_{1c}, %	−0.05	.02
BMI, kg/m^2	−0.007	.04
Multiple regression—model 2[b]		
Age, y	−0.005	<.001
HbA_{1c}, %	−0.09	.007
hs-CRP, mg/dL	−0.21	.01
IMT		
Multiple regression—model 1[a]		
Age, y	0.002	<.001
HbA_{1c}, %	0.03	.01
Multiple regression—model 2[b]		
Age, y	0.003	<.001
HbA_{1c}, %	0.04	.03
esRAGE, ng/mL	−0.06	.001
PWV		
Multiple regression—model 1[a]		
Age, y	0.025	<.001
Systolic BP, mm Hg	0.015	.01
Multiple regression—model 2[b]		
Systolic BP, mm Hg	0.02	.001
S100A12, ng/mL	1.1	.02

Table 3: *Model 1 was adjusted for age, sex, smoking status, BMI, systolic BP, diastolic BP, HDL cholesterol, LDL cholesterol, non HDL cholesterol, triglycerides, statin therapy, HbA_{1c}, fasting glycemia and insulin.
**Model 2 was adjusted for sRAGE, esRAGE, WBC, S100A12, hs-CRP and CML.

When the patients with prediabetes were analyzed separately from controls and type 2 diabetes, age (P=0.03), hs-CRP (P=0.01) remained significantly associated with esRAGE plasma levels **(Table 4)**.

Table 4. Multiple Regression Analysis Evaluating esRAGE, IMT, and PWV as Dependent Variables for Prediabetic Group

	Coefficient β	P
esRAGE		
Multiple regression—model 1[a]		
Age, y	−0.07	<.001
BMI, kg/m^2	−0.04	.03
Multiple regression—model 2[b]		
Age, y	−0.006	.03
hs-CRP, mg/dL	−0.16	.01
IMT		
Multiple regression—model 1[a]		
Age, y	0.003	<.001
Systolic BP, mm Hg	0.01	.01
Multiple regression—model 2[b]		
Age, y	0.003	<.001
esRAGE, ng/mL	−0.04	.04
hs-CRP, mg/dL	0.07	.004
PWV		
Multiple regression—model 1[a]		
Age, y	0.025	<.0001
Systolic BP, mm Hg	0.031	.001
Multiple regression—model 2[b]		
S100A12, ng/mL	0.9	.02

Table 4: *Model 1 was adjusted for age, sex, smoking status, BMI, systolic BP, diastolic BP, HDL cholesterol, LDL cholesterol, non HDL cholesterol, triglycerides, statin therapy, HbA_{1c}, fasting glycemia and insulin.
**Model 2 was adjusted for sRAGE, esRAGE, WBC, S100A12, hs-CRP and CML.

esRAGE is produced from the alternative splicing of the RAGE gene and is formed from the inclusion of part of intron 9 and the removal of exon 10, which changes the reading frame sequence of the protein and leads to the loss of both the transmembrane and cytosolic domains (18). To determine whether esRAGE gene expression was changed in our population, we performed an RT-PCR analysis of MNC from prediabetic and new onset type 2 diabetic patients compared with control subjects (n=10 for each group). The mRNA expression level of the esRAGE splice variant progressively decreased in patients with prediabetes and new onset type 2 diabetes with respect to the controls **(Figure 1).**

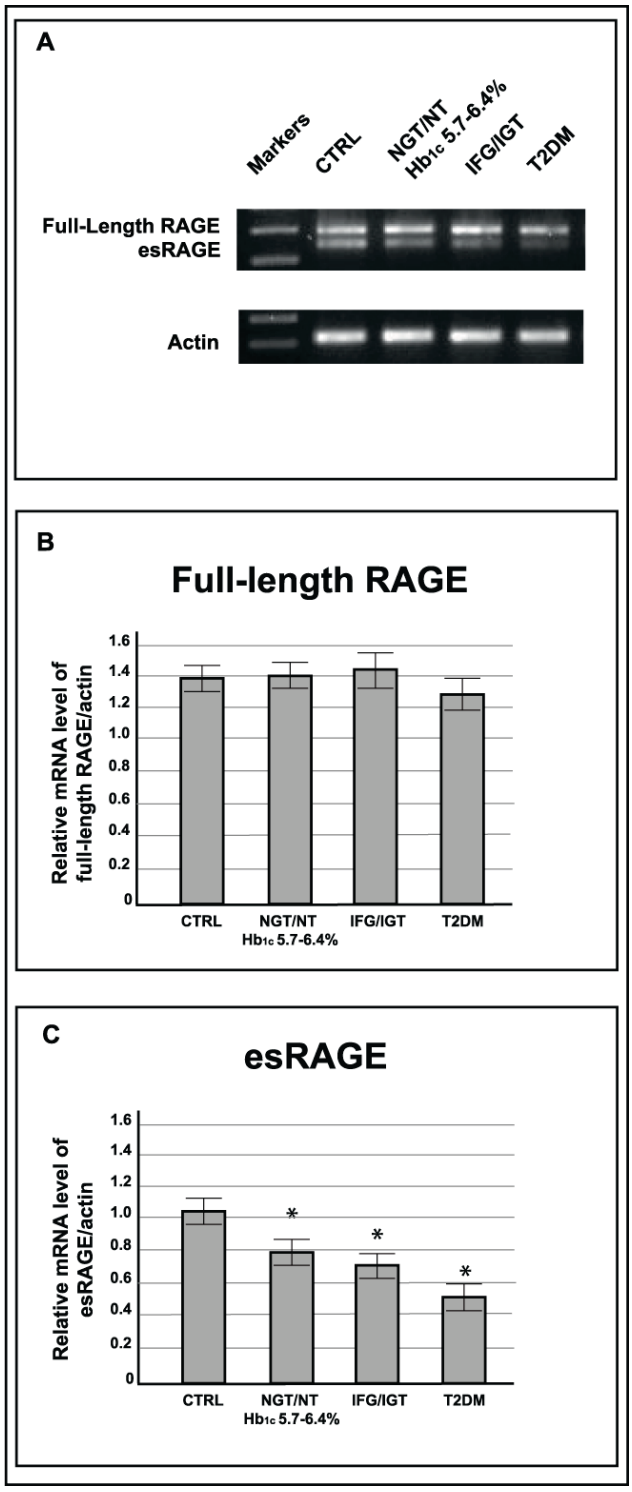

Figure 1: Differential expression of full-length RAGE and esRAGE in peripheral blood mononuclear cells (PBMC) by RT-PCR.

Panel A shows a representative agarose gel image for RT-PCR analysis of full-length RAGE (282 bp) and esRAGE (216 bp) splicing variants in PBMC from controls, HbA1c 5.7-6.4% with NFG/NT, IFG/IGT and type 2 diabetic subjects.

Statistical data showing the mRNA ratio of full-length RAGE/Beta-actin and esRAGE/Beta-actin are presented in Panel B and Panel C, respectively. The data are expressed as the mean±SD from ten different experiments; **P*<0.05.

Intima-media thickness and pulse wave velocity in subjects with prediabetes.

IMT was higher in the patients with prediabetes than in the control group [0.75 (0.65-0.78) vs. 0.67 (0.6-0.73) mm, $P<0.05$]. Furthermore, subjects with new onset type 2 diabetes exhibited no significant difference in IMT with respect to the prediabetic patients **(Table 2)**.

IMT was associated with age (r=0.55, $P<0.0001$), HbA_{1c} (r=0.4, $P<0.001$), fasting glycemia (r=0.34, $P<0.001$), and esRAGE (r=-0.2, $P<0.02$) in the simple regression analysis.

Next, we performed multiple regression analysis using two models. The first model included a variety of cardiovascular risk factors (age, sex, smoking status, statin therapy, BMI, systolic BP, diastolic BP, HDL cholesterol, triglycerides, LDL cholesterol, non-HDL cholesterol, HbA_{1c}, fasting glycemia and fasting insulin), and the second model included variables that reached significance in the first model and several markers of glycation and inflammation (sRAGE, esRAGE, S100A12, hs-CRP, WBC, and CML). The first model exhibited a significant correlation among IMT, age ($P<0.001$) and HbA_{1c} ($P<0.05$). In the second model, the variables that remained significantly associated with IMT were HbA_{1c} ($P<0.05$), age ($P<0.001$) and esRAGE ($P<0.05$) **(Table 3)**.

In the subgroup analysis including only subjects with prediabetes the variables that were significantly associated with IMT were age ($P=0.003$) and esRAGE ($P<0.03$) **(Table 4)**.

The PWV was significantly higher (7.6±1.6 vs. 7.1±1.7 m/sec, $P<0.05$) in the patients with prediabetes than in the control subjects **(Table 2)**. Furthermore, these subjects exhibited lower PWV with respect to new onset type 2 diabetes patients.

PWV was associated with age (r=0.29, *P*<0.0001), HbA_{1c} (r=0.25, *P*<0.001), fasting glycemia (r=0.18, *P*<0.01), systolic BP (r=0.27, *P*<0.0001), diastolic BP (r=0.22, *P*<0.0001), triglycerides (r=0.16, *P*<0.01), HDL cholesterol (r=0.16, *P*<0.01), CML (r=0.17, *P*<0.05), and S100A12 (r=0.14, *P*<0.01) in the simple regression analysis.

Subsequently, we performed multiple regression analysis using two models. The first model included a variety of cardiovascular risk factors (age, sex, smoking status, statin therapy, BMI, systolic BP, diastolic BP, HDL cholesterol, LDL cholesterol, non-HDL cholesterol, triglycerides, HbA_{1c}, fasting glycemia and fasting insulin), and the second model included variables reaching significance in the first model and several markers of glycation and inflammation (sRAGE, esRAGE, S100A12, hs-CRP, WBC and CML). The first model exhibited a significant correlation between PWV and age (*P*<0.01) and systolic BP (*P*<0.05). In the second model, the variables that remained significantly associated with PWV were S100A12 (*P*<0.05) and systolic BP (*P*<0.05) **(Table 3)**. In the subgroup analysis including only subjects with prediabetes the variables that were significantly associated with PWV was S100A12 (*P*=0.02).

Circulating levels of sRAGE, esRAGE splice variant and inflammatory profile in subjects with prediabetes according only to HbA_{1c} with normal fasting glycemia and normal glucose tolerance after OGTT.

As a new approach for prediabetes diagnosis, it is unclear whether HbA_{1c} could provide additional information regarding cardiovascular risk compared with fasting glycemia and glucose tolerance. In line with this question, we focused on individuals with prediabetes identified only by HbA_{1c} (5.7-6.4%) who had NFG and

were NT after OGTT. Thus, we re-examined the prediabetic population separating the patients with prediabetes identified only by HbA_{1c} from all those with IFG and/or IGT after the OGTT. As shown in **Figure 2**, the subgroup with prediabetes identified only by HbA_{1c} (n=127) showed significantly lower plasma esRAGE levels compared with controls (0.29±0.18 vs. 0.45±0.26 ng/mL, $P<0.05$) but similar to those of patients with IFG and/or IGT (n=93) and new onset type 2 diabetes (n=61). Circulating plasma levels of S100A12 were higher in subjects with prediabetes than in controls according only to HbA_{1c} and IFG and/or IGT. sRAGE and CML plasma levels were similar among the four groups (**Figure 2**). The mRNA expression level of the esRAGE splice variant progressively decreased in patients with prediabetes with respect to controls according only to HbA_{1c}, IFG/IGT and new onset type 2 diabetes (**Figure 1).**

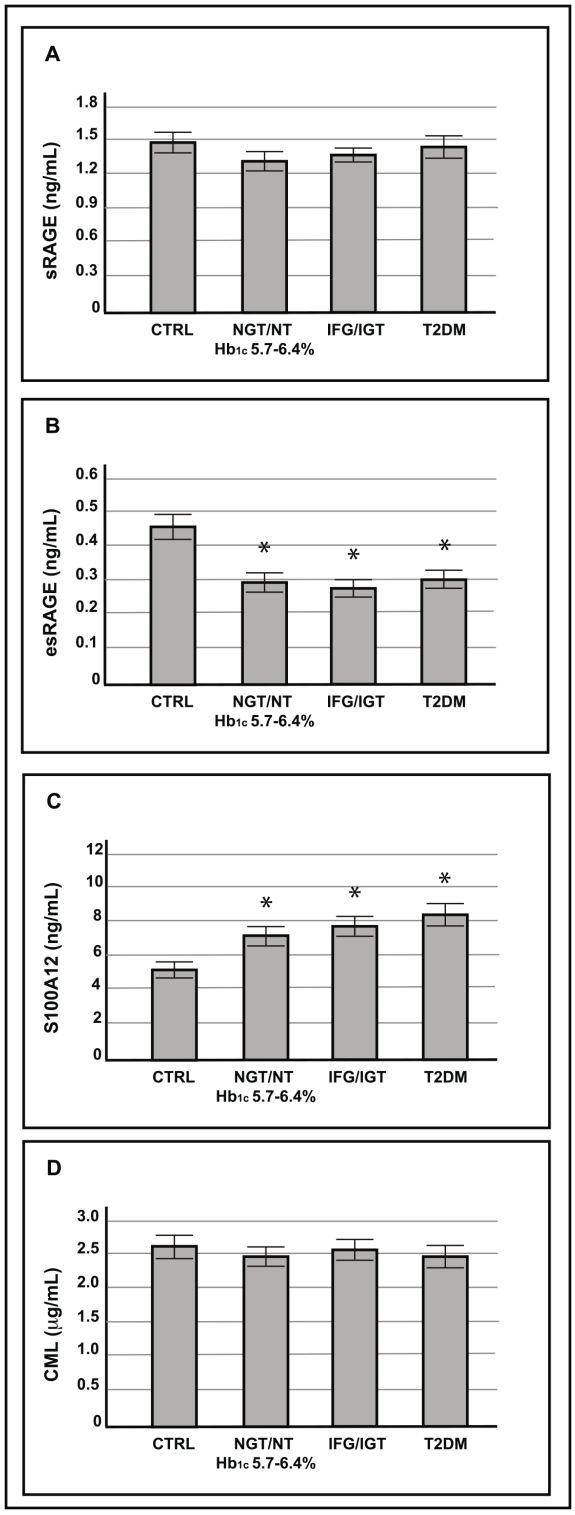

Figura 2: Circulating levels of sRAGE (A), esRAGE (B), S100A12 (C), CML (D) (mean±SD), according to glucose tolerance and HbA_{1c} levels.

Discussion

In this study we measured sRAGE, esRAGE and examined their association with other pro-inflammatory factors and early markers of atherosclerosis in subjects with prediabetes.

We found that prediabetic patients exhibited lower esRAGE plasma levels than controls and that the levels were similar to those of patients with type 2 diabetes. Furthermore, we demonstrated an inverse association between low plasma esRAGE and carotid atherosclerosis in prediabetic patients. In addition, S100A12, a relevant pro-inflammatory molecule strongly linked with RAGE signaling, was increased in prediabetic and diabetic patients with respect to controls.

Although diabetes imparts a two- to three-fold increase in the risk of developing macroangiopathy, the magnitude of the risk for cardiovascular disease associated with prediabetes remains unclear and is dependent on diagnostic criteria. In consideration of the expected increased use of HbA_{1c} as a screening tool to identify individuals with alteration of glycemic homeostasis, it is important to evaluate the ability of HbA_{1c} to identify patients who are at increased risk for cardiovascular disease. With this aim, we specifically focused on individuals with prediabetes identified only by HbA_{1c} (5.7-6.4%) who had NFG and were NT after OGTT. As previously reported [43], we found that these subjects exhibited an alteration of early markers of cardiovascular injury. In addition, we found that NFG/NT subjects with high HbA_{1c} (≥5.7%) showed low esRAGE levels and high S100A12 concentration. These data may indicate that HbA_{1c} can identify subjects with NFG and NT with alteration of early markers of cardiovascular damage. Accordingly, in a recent prospective study, European subjects with prediabetes identified by HbA_{1c} experienced a significant increase in coronary heart and cardiovascular disease with

respect to normoglycemic subjects[135]. All of these observations suggest that HbA_{1c} may be a relevant marker of cardiovascular risk.

In our study, carotid IMT, which is a good predictor of cardiovascular disease, was independently associated with low esRAGE plasma levels, HbA_{1c} and age. Recent clinical reports have focused on the potential significance of circulating esRAGE and sRAGE in a variety of pathophysiological conditions. Studies that used a detection system to specifically measure human esRAGE cross-sectionally reported its significant and inverse association with atherosclerosis in metabolic syndrome and type 1 and type 2 diabetes [136]. In agreement with our data, these findings suggest that esRAGE may represent a potential protective factor for carotid atherosclerosis and suggest that the inverse correlation between esRAGE and cardiovascular risk is not confined to diabetes but is extended to nondiabetic populations, as well.

esRAGE plasma levels were tightly and inversely correlated with HbA_{1c} and hs-CRP in multiple regression analysis after adjusting for other risk factors. The inverse association between esRAGE and glycemic control is unsurprising. Katakami *et al.* have reported an inverse and significant association between esRAGE and HbA_{1c} in type 1 diabetic patients[90]. Furthermore, Koyama *et al*. found that esRAGE is significantly and inversely correlated with HbA_{1c} and components of metabolic syndrome in type 2 diabetic and nondiabetic subjects[76]. Because a number of factors, such as glycemic control, inflammation, underlying disease, renal function and drugs, appear to affect plasma esRAGE levels, we evaluated esRAGE mRNA expression in MNCs isolated from peripheral blood samples. We observed decreased mRNA expression of full-length RAGE and esRAGE in patients with prediabetes and type 2 diabetes with respect to controls. The regulatory mechanism for alternative splicing to generate esRAGE remains unclear, and environmental or

genetic factors may be involved. Other studies have reported the ability of some oral agents such as statins to modulate AGE/RAGE axis increasing circulating esRAGE levels in type 2 diabetic patients; in this study, we did not exclude patients who were on lipid lowering therapy, however, we found no difference in the percentage of patients in statin therapy in each group and further statistical analysis evidenced that our results are not affected by statin therapy. Another possibility is that esRAGE production may be down-regulated via hyperglycemia-induced oxidative stress directly or via increased cytokines.

Shemitani *et al.*, in a recent molecular study on K562 cells, supported this hypothesis and revealed a significant decrease in sRAGE levels following treatments with high glucose[137]. In Chinese patients with type 2 diabetes mellitus, an association between RAGE gene polymorphisms and circulating levels of esRAGE has been reported [138]. Other studies are needed to explain the precise mechanism underlying the decrease in sRAGE in diabetic and non-diabetic humans.

Inflammation may play a role in linking the RAGE system with atherosclerosis because RAGE ligands include proinflammatory proteins. In the current study, esRAGE was initially associated with hs-CRP and S100A12 in an unadjusted model. After adjustment for conventional risk factors and inflammatory markers, only hs-CRP remained significantly associated with esRAGE, and the association with S100A12 disappeared.

In our study, we found no significant differences in sRAGE plasma levels among the three groups. However, to date, the data regarding circulating total sRAGE levels remain controversial. Falcone *et al*. reported that low levels of sRAGE in plasma are independently associated with coronary heart disease in non-diabetic men [139]. Furthermore, Basta *et al*. reported that plasma sRAGE levels were lower in

diabetic patients than in controls [47]. Low levels of sRAGE have been indicated as being associated with the risk of diabetes, coronary heart disease and mortality in humans, and the administration of sRAGE suppressed accelerated diabetic atherosclerosis in animal models [140]. In subjects with type 1 diabetes, higher sRAGE levels are reportedly associated with cardiovascular events and all-cause mortality [68]. Accordingly, Nakamura *et al*. demonstrated that serum sRAGE levels are significantly higher in type 2 diabetic patients than in non-diabetic subjects and are positively associated with the presence of coronary artery disease[136]. The disagreement among these studies may be the result of the use of a detection system that was unable to discriminate between specific sRAGE variants.

We also found high levels of S100A12 in subjects with prediabetes and a tight correlation between S100A12 and PWV, an early marker of cardiovascular disease. Many studies have reported that elevated concentrations of acute phase reactants, such as S100A12, IL-6 and TNF-alpha, are found in patients with atherosclerosis-related complications. However, to date, little is known regarding the modulation of S100A12 in patients with alterations of glucose homeostasis and its potential association with cardiovascular disease. Basta *et al*. observed higher plasma S100A12 levels in a group of diabetic patients than in age-matched controls and a strong association with increased cardiovascular risk (Framingham score) [47]. Previous studies have reported that S100A12 was independently associated with major cardiovascular events in patients with chronic heart failure, highlighting the potential role of S100A12 as a biomarker for cardiovascular disease.

There were several limitations of this study. First, this was a cross-sectional study, and a longitudinal causal relationship cannot be established between changes in plasma esRAGE and the IMT. Furthermore, sRAGE gene expression should be

better characterized using quantitative analyses on larger groups of patients. Finally, although we did not perform an opportunistic procedure during recruitment, the group with high HbA_{1c} and NGT/NT represents, in this study, approximately 30% of the entire population and is, therefore, not a rare subset. In line with these finding, Rosella *et al.* have recently reported that the prevalence of undiagnosed prediabetes in a representative sample of Canadians was significantly greater when using screening strategies that used HbA_{1c} measures compared with plasma glucose diagnostic criteria. Further epidemiological data are needed to characterize the actual percentage of this group in the overall prediabetes population[28].

In conclusion, subjects with prediabetes exhibit low esRAGE plasma levels and increased levels of markers of inflammation. These findings indicate that esRAGE may play an important role in activation and progression of atherosclerotic disease in this population.

The further examination of the molecular mechanism underlying esRAGE regulation will provide potential targets for the prevention or treatment of cardiovascular disease.

2.5 HbA_{1c} identifies subjects with prediabetes and subclinical left ventricular diastolic dysfunction

Type 2 diabetes mellitus is an established risk factor for heart failure development, independently from other conditions such as hypertension and coronary artery disease, and diastolic dysfunction represents one of the first manifestations of diabetic cardiomyopathy [141,142]. Previous data recognized that early detection of diabetic heart disease should be a clinical priority, because timely prevention programs or medical intervention may be helpful to avoid or delay the development of heart failure, which is a major cause of morbidity and mortality in these patients [143].

Events portending cardiovascular disease are under way prior to the formal diagnosis of diabetes and a consistent body of studies has demonstrated the association between changes of cardiac function and early alteration of glucose homeostasis such as prediabetes [144]. According to these data, patients with prediabetes may represent a specific population in which prevention programs should be applied [145].

Previous evidences showed that subjects with prediabetes or with normal fasting glycemia and normal glucose tolerance (NFG/NGT) are not a homogenous group and they may present different degrees of cardiometabolic risk [33]. In recent studies, we analyzed the metabolic profile of subjects with prediabetes identified according to HbA_{1c} (5.7-6.4%) but NFG/NGT after an oral glucose tolerance test (OGTT). We found alterations of early markers of cardiovascular risk, an impaired inflammatory profile and low vitamin D levels [32,49,51,98], therefore suggesting an impaired cardiometabolic profile in these subjects, that would not have been classified as having prediabetes on the basis of fasting or post-OGTT values.

The pathogenic mechanism underlying the myocardium impairment in patients with alterations of glucose homeostasis is likely to be linked to multiple and complex metabolic reactions including insulin signaling, lipotoxicity and increased inflammatory state [146]. Several studies have shown a relevant role of advanced glycation end-products (AGEs) and their receptors (RAGE) in the pathogenesis of type 2 diabetes complications in various organs, including the heart [147]. RAGE is also found in a soluble form (sRAGE) primarily formed by the proteolytic cleavage of membrane-bound RAGE and, secondarily, by a secreted, non membrane-bound form of the receptor resulting from alternative splicing of the *RAGE* gene, which is known as endogenously secreted RAGE (esRAGE). esRAGE that may contribute to the removal/neutralization of circulating ligands thus functioning as a decoy by competing with cell-surface RAGE for ligand binding [129]. A critical role of AGE/RAGE axis has been explored in diabetic cardiomyopathy, cardiac ischemia damage and accelerated atherosclerosis [148]; furthermore, in animal models, RAGE blockade protected from the development of diastolic dysfunction, attenuating myocardial collagen expression [149].

In consideration of the expected increased use of HbA_{1c} as a screening tool to identify individuals with alterations of glycemic homeostasis, it is clinically important to evaluate the ability of HbA_{1c} to identify patients who have early alterations of myocardial function. Based on these considerations, in this study we evaluated the diastolic function and examined its association with markers of glycation and inflammation strictly related with cardiovascular disease in patients with prediabetes identified only with HbA_{1c}.

Materials and methods

Study subjects

The study group included 167 subjects (age range 18-65 years) with no previous diagnosis of diabetes attending our University Hospital for diabetes and cardiovascular risk evaluation. All patients were Caucasian and underwent a physical examination and review of their clinical history, smoking status, and alcohol consumption. The exclusion criteria were: a previous history of diabetes, impaired fasting glucose (IFG), impaired glucose tolerance (IGF), new onset type 2 diabetes according to fasting glucose, OGTT or HbA_{1c}, previous history of overt cardiovascular events (stroke, ischemic heart disease, chronic obstructive peripheral arteriopathy, or heart failure), anemia, or hemoglobinopathies, use of medications known to affect glucose metabolism, malignant disease, and drug or alcohol abuse.

Body weight and height were measured, and BMI was calculated as weight (kg)/[height (m)]^2. Blood pressure (BP) was measured with a calibrated sphygmomanometer after the subject had rested in the supine position for 10 min. Venous blood samples were drawn from the antecubital vein on the morning after an overnight fast. Baseline venous blood samples were obtained for the measurement of plasma glucose, total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, high sensitivity C-reactive protein (hs-CRP), sRAGE, esRAGE, and S100A12. Low density lipoprotein (LDL) cholesterol concentrations were estimated using the Friedewald formula. All subjects underwent a 75 g OGTT with 0, 30, 60, 90, and 120 min sampling for plasma and insulin as previously described [107]. Glucose tolerance status was defined on the basis of OGTT, fasting glycemia and HbA_{1c} according to the American Diabetes Association (ADA) recommendations [27].

Biochemical Analyses

Plasma glucose, serum total cholesterol, triglycerides, HDL cholesterol, and hs-CRP were measured using available enzymatic methods as previously described [50].

To quantify the plasma concentration of sRAGE (Human sRAGE ELISA; Biovendor, Brno, Czech Republic), esRAGE (B-Bridge esRAGE ELISA Kit, Cupertino, USA), S100A12 (Cloud-clone corp., ELISA Kit for S1000A12, Houston, USA) fasting blood samples were collected and specimens were immediately centrifuged and stored at -80°. Commercially available ELISA kits were used according to the manufacturer's instructions. The inter-assay and intra-assay coefficients of variation (CVs) ranged respectively from 5.5 to 8.8% and 2.6 to 5.3% for sRAGE; from 5.9 to 7.5% and 0.7 to 1.5% for esRAGE; <10% and 12% for S100A12.

HbA_{1c} was measured via high performance liquid chromatography using a National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial (DCCT) assay reference [79]. Chromatography was performed using a certified automated analyzer (HPLC; HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.) [normal range 4.25–5.9% (23–41 mmol/mol)].

Cardiac ultrasound examination

Echocardiographic examination was performed with all subjects in left lateral decubitus using a GE Vivid 9 Ultrasound system (GE Healthcare, Horten, Norway) equipped with multi-frequency MS5 probe. 2D guided M-mode parameters were

measured by a single operator, blinded to clinical data, according to the recommendations of the American Society of Echocardiography [150].

LV mass was calculate using the Devereux formula and normalized by body surface area (LVM index (LVMI) Partition value for LVH were taken with the cut – off value of 115 g/m2 for men and 95 g/m2 for women according to the American Society of Echocardiography Recommendation. Finally, calculation of relative wall thickness (RWT) with the formula (2 LV posterior wall thickness)/(LV internal diameter at end-diastole) permits categorization of an increase in LV mass as either concentric (RWT>0.42) or eccentric (RWT ≤0.42) hypertrophy and allows the identification of concentric remodelling (normal LV mass with increased RWT).

From the parasternal long axis view, the following parameters were obtained: LV end-diastolic thickness of inter-ventricular septum (LV IVSd) and posterior wall (LV PWd), LV end-diastolic and end-systolic diameters (LV Dd and LV Sd, respectively). Relative wall thickness (RWT) was calculated as in the formula: RWT = 2 LV PWd /LV Dd.

For the assessment of mitral inflow velocities the sampling volume of pulsed-wave Doppler was placed at the tip level of the mitral leaflets from the apical 4-chamber view. The following parameters were measured: the peak mitral inflow in early diastole (E wave), the late diastolic atrial filling velocity (A wave), and their ratio (E to A ratio).

Tissue Doppler Imaging in the pulsed wave Doppler modality (PW-TDI) was used for the assessment of myocardial velocities and was applied at the septal and lateral sides of the mitral annulus obtaining: systolic myocardial velocities (S' wave), early and late diastolic myocardial velocities (E' and A' waves, respectively). All the TDI measurements were obtained by averaging the values at the septal and lateral mitral

annulus. Timings of LV isovolumic contraction (LV IVCT), ejection (LV ET) and isovolumic relaxation (LV IVRT) were measured form the PW-TDI traces obtaining the Myocardial Performance Index (MPI) as in formula: LV MPI= (LV IVCT+LV IVRT)/LV ET [151]. Finally, the ratio between E and E' (E/e' ratio) was calculated and considered as an index of LV filling pressures [152].

Mitral regurgitation (if present) was evaluated and graded according to the American Society of Echocardiography criteria [153]. Echocardiographic data were distinctly examined by a team of two expert echocardiologists and reconsidered by a third reader for consensus in cases of disagreement.

The sphericity index (SI) was calculated as the ratio between the greater cross-sectional diameter and the greater longitudinal diameter of the LV in end-diastolic apical four-chamber view. This index was used as an indicator of geometry change.

Statistical analyses

The sample size was calculated based on the E/A Ratio using a level of significance (α) set to 5% and power (1-β**)** set to 80%. We based the power calculation on previous studies examining the E/A Ratio among patients with early alteration of glucose homeostasis and controls [154]. The estimated sample size was 50 patients per group. Statistical comparisons of clinical and biomedical parameters were performed using Stat View 6.0 for Windows. Data are given as means ± SD or median (IQR). Each variable's distributional characteristics including normality were assessed by Kolmogorov-Smirnov test. Statistical analyses included the unpaired *t* test for continued variables and χ^2 test for non-continuous variables. A *P* value less than 0.05 was considered statistically significant. When necessary, numerical variables were

logarithmically transformed to reduce skewness, and values are expressed as median and interquartile range.

Simple regression analysis was performed to relate E/A Ratio to the clinical and metabolic characteristics of the patients (age, BMI, sex, smoking status, systolic and diastolic BP, total cholesterol, HDL cholesterol, triglycerides, LDL cholesterol, ApoA1/ApoB Ratio, HOMA-IR, HbA_{1c}, fasting glycemia, uric acid, hs-CRP, S100A12, sRAGE and esRAGE). In order to identify variables independently associated with variations of E/A Ratio, LAV and SI, we performed a multiple regression analysis including the variables reaching statistical significance in simple regression analysis. The variance inflation factor was used to check for the problem of multi-collinearity among the predictor variables in multiple regression analysis.

The local ethics committee approved the study. Informed consent was obtained from each participant.

Results

In total, 167 subjects participated in the study. The study population was divided into the following two groups based on fasting glucose, OGTT and HbA_{1c} levels according to the ADA recommendations: 61 control subjects (HbA_{1c} <5.7% and NFG/NGT) and 106 patients with HbA_{1c} prediabetes (HbA_{1c} 5.7-6.4% and NFG/NGT).

The clinical and biochemical characteristics of the study subjects are presented in **Table 1**. Although in the range of normal fasting glycemia and normal glucose tolerance, subjects with HbA_{1c} prediabetes showed higher fasting and 2 h postload glycemia (89.2±6.2 vs 85.3±8 mg/dL, P<0.05 and 112.3±7.7 vs 121.1±8.9 mg/dL, P<0.05 respectively) compared with controls. Furthermore, they presented higher uric acid plasma levels (5.1±1.5 vs 4.4±1.1 mg/dL, P<0.05) and ApoB/ApoA1 Ratio (0.73±0.22 vs 0.63±0.24, P<0.05). There were no significant differences between subjects with HbA_{1c} prediabetes and controls with respect to the other anthropometric and metabolic characteristics.

Table 1 – Clinical characteristics of the study population according to HbA_{1c} levels.

	Controls (HbA_{1c} <5.7%and NFG/NGT) (*n*=61)	**HbA_{1c} prediabetes (HbA_{1c} 5.7-6.4% and NFG/NGT) (*n*=106)**	***P value***
Clinical Variables			
Age (year)	48±8.4	50.4±8.9	0.1
BMI (Kg/m^2)	28.4±3.1	28.7±3.9	0.63
Fasting glucose (mg/dL)	85.3±8	89.2±6.2	0.00
2-h Postload glucose (mg/dL)	112.3±7.7	121.1±8.9	0.01
Fasting Insulin (microu/mL)	5.8(4.1-9.6)	6.7(4.8-10)	0.22
Total cholesterol (mg/dL)	201.1±41.5	207.7±43	0.25
HDL cholesterol (mg/dL)	49.7±11.5	47.6±37.7	0.31
Triglycerides (mg/dL)	101(67-118)	102(71-160)	0.28
LDL cholesterol (mg/dL)	130.3±38	135±37.7	0.47
Systolic BP (mmHg)	119±13.6	122.4±13	0.78
Diastolic BP (mmHg)	70.1±9.9	72.2±9.8	0.29
ApoA1 (mg/dL)	143.5±22.3	138.3±24.7	0.31
ApoB (mg/dL)	89.4±28.5	96.1±23.3	0.22
ApoB/ApoA1 Ratio	0.63±0.24	0.73±0.22	0.04
Uric Acid (mg/dL)	4.4±1.1	5.1±1.5	0.01
HOMA-IR	1.5(0.81-2.3)	1.9(1-2.5)	0.16
Active smokers	33%	33%	0.45
Hypertension	32%	34%	0.53
Sex (M/F)	35%	38%	0.35

Tabella 1: Data are presented as mean±SD or median (IQR): HbA_{1c},glycated hemoglobin; NFG, normal fasting glucose; NGT, normal glucose tolerance; BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B; HOMA-IR, homeostasis model assessment-insulin resistance; BP, Blood pressure; hs-CRP, high sensitivity C-reactive protein; sRAGE, soluble receptor for advanced glycation end-products; esRAGE, endogenous secretory RAGE.

Smoking was quantified (number of cigarettes and years smoked) and smoking status was classified in active and non-smokers

Hypertension was defined as systolic blood pressure >135 mmHg or diastolic blood pressure >85 mmHg or taking any hypertension medications.

The circulating levels of hs-CRP and S100A12 were higher in subjects with HbA_{1c} prediabetes compared with controls [0.18(0.08-0.39) vs 0.14(0.07-0.15) mg/dL, *P*<0.05; 6.9±5.2 vs 4.7±2.9 ng/mL, *P*<0.05, respectively] **(Table 2)**. esRAGE plasma levels were significantly lower in the subjects with HbA_{1c} prediabetes compared with controls (0.41±0.18 vs 0.56±0.23 ng/mL, *P*<0.05). As shown in **Table 2**, sRAGE plasma levels were slightly lower in the group with prediabetes without statistical significance (1.30±0.53 vs 1.45±0.62 ng/mL, *P*<0.07).

Table 2. Inflammatory variables of the study population according to HbA_{1c} levels.

	Controls (HbA_{1c} <5.7%and NFG/NGT) (*n*=61)	**HbA_{1c} prediabetes (HbA_{1c} 5.7-6.4% and NFG/NGT) (*n*=106)**	***P value***
Inflammatory Markers			
hs-CRP (mg/dL)	0.14(0.07-0.15)	0.18(0.08-0.39)	0.04
S100A12 (ng/mL)	4.7± 2.9	6.9±5.2	0.05
sRAGE (ng/mL)	1.45±0.62	1.30±0.53	0.07
esRAGE (ng/mL)	0.56±0.23	0.41±0.18	0.00

Table 2: Data are presented as mean±SD or median (IQR): HbA_{1c},glycated hemoglobin; NFG, normal fasting glucose; NGT, normal glucose tolerance; hs-CRP, high sensitivity C-reactive protein; sRAGE, soluble receptor for advanced glycation end-products; esRAGE, endogenous secretory RAGE.

Echocardiographic parameters for the study population, according to HbA_{1c} levels, are reported in **Table 3**. Patients with HbA_{1c} prediabetes exhibited a lower E/A Ratio compared with controls (1.10±0.24 vs 1.18±0.23, *P*<0.05). Furthermore, they showed a higher left atrium area and volume (28±5 vs 22±3, *P*<0.05 and 15.5±3.6 vs 13.2±2.5, *P*<0.05, respectively). E/e' Ratio significantly increased from controls to patients with prediabetes (7.2±2.5 vs 7.8±2.2, *P*<0.05). Interestingly, SI was significantly higher in patients with prediabetes (0.6±0.06 vs 0.5± 0.05, *P*<0.05).

Table 2 – Echocardiographic variables of the study population according to HbA_{1c} levels.

	Controls (HbA_{1c} <5.7%and NFG/NGT) (*n*=61)	**HbA_{1c} prediabetes (HbA_{1c} 5.7-6.4% and NFG/NGT) (*n*=106)**	***P value***
Echo Doppler parameters			
E/A ratio	1.18 ±0.23	1.10±0.24	0.04
DT (m/s)	181±14	188±15	0.21
IVRT (m/s)	74±7	70±8	0.35
Tissue Doppler parametrs			
Septal e' (cm/sec)	12.2±2.0	11.3±1.8	0.45
Lateral e'	18.8±1.6	16.8±1.8	0.32
E/e' ratio	7.2±2.2	7.8±2.5	0.04
Echo M-mode 2D Parameters			
IVS Diastolic dimension (cm)	1.02±0.15	1.07±0.18	0.45
PW Diastolic dimension (cm)	1.08±0.12	1.09±0.19	0.71
LVM (g)	143±14	153±16	0.32
RWT	0.40±0.6	0.41±1.1	0.50
LV internal dimension			
Diastolic dimension (mm)	46±3.1	47.1±3.7	0.6
Systolic dimension (mm) LV volumes (biplane)	27.1±4.3	28.3±4.7	0.7
LV EDV (mL)	82±8.0	87±7.5	0.36
EF (%)	61±6	63±5	0.5
FS (%)	38±3	40±2	0.48
LA volume/BSA (mL/m2)	22±3	28±5	0.01
LA Area (mm)	13.2±2.5	15.5±3.6	0.02
Sphericity Index	0.5±0.05	0.6±0.06	0.01
MAPSE mm	15±1	15±2	0.6
TAPSE mm	22±3	21±3	0.2

Table 3 - Data are presented as mean±SD: DT, Deceleration Time; IVRT, Isovolumetric relaxation time; IVS Interventricular Septal thickness; PW, Pulse Wave; LVM Left Ventricular Mass; RWT Relative Wall Thickness ; LV Left Ventricular; LV EDV, Left Ventricular End-Diastolic Volume; EF, Ejection fraction; FS, Fractional Shortening, MAPSE, Mitral annular plane systolic excursion; TAPSE, Tricuspid annular plane systolic excursion.

A simple regression analysis was performed to test the relationship between echocardiographic parameters and different clinical variables.

E/A Ratio was associated with age (r=-0.22, *P*<0.005), BMI (r=0.33, *P*<0.0001), total cholesterol (r=0.18, *P*<0.02), LDL (r=0.15, *P*<0.05), HbA_{1c} (r=0.2, *P*<0.02), uric acid (r=0.24, *P*<0.01), HOMA-IR (r=0.18, *P*<0.02), hs-CRP (r=0.35, *P*<0.001), S100A12 (r=0.23, *P*<0.01), sRAGE (r=-0.28, *P*<0.01) and esRAGE (r=-0.22, *P*<0.02). To identify variables independently associated with variations of E/A Ratio, variables reaching statistical significance were inserted in a multiple regression model. To avoid the problem of multi-collinearity between variables (total cholesterol, non HDL-cholesterol and LDL cholesterol) only LDL cholesterol was used as an independent variable in the model. The multivariate analysis including each of the components reaching a *P* value <0.05 in the simple regression analysis, showed a correlation only with age, HbA_{1c}, and esRAGE (**Table 4)**.

Simple regression analysis showed a significant relationship between LAV and BMI (r=0.22, *P*<0.005), HDL cholesterol (r=-0.15, *P*<0.05) ApoB/ApoA1 Ratio (r=0.25, *P*<0.02), HbA_{1c} (r=0.18, *P*<0.02) and sRAGE (r=-0.21, *P*<0.05). Multiple regression analysis including significant variables showed an independent association between LAV, $HbA_{1c,}$ and sRAGE (**Table 4)**.

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To identify variables independently associated with variations of E/A Ratio, variables reaching statistical significance were inserted in a multiple regression model. To avoid the problem of multi-collinearity between variables (total cholesterol, non HDL-cholesterol and LDL cholesterol) only LDL cholesterol was used as an independent variable in the model. The multivariate analysis including each of the components reaching a *P* value <0.05 in the simple regression analysis, showed a correlation only with age, HbA_{1c}, and esRAGE (**Table 4)**.

Simple regression analysis showed a significant relationship between LAV and BMI (r=0.22, *P*<0.005), HDL cholesterol (r=-0.15, *P*<0.05) ApoB/ApoA1 Ratio (r=0.25, *P*<0.02), HbA_{1c} (r=0.18, *P*<0.02) and sRAGE (r=-0.21, *P*<0.05). Multiple regression analysis including significant variables showed an independent association between LAV, $HbA_{1c,}$ and sRAGE (**Table 4)**.

In the simple regression analysis, SI was significantly related with total cholesterol (r=0.28, *P*<0.005), LDL (r=0.29, *P*<0.004), non-HDL (r=0.29, *P*<0.004), HbA_{1c} (r=0.20, *P*<0.01) and fasting glycemia (r=0.16, *P*<0.04). To identify variables independently associated with variations of SI, variables reaching statistical significance were inserted in a multiple regression model. To avoid the problem of multi-collinearity between variables (total cholesterol, LDL cholesterol and non HDL cholesterol) only LDL cholesterol was used as an independent variable in the model. Multiple regression analysis showed that SI was independently associated only with HbA_{1c} and LDL cholesterol **(Table 3).**

Table 4 – Multiple regression analysis evaluating E/A ratio, left atrium volume and sphericity index as dependent variable.

	Coefficient β	*P value*
E/A Ratio		
Age (years)	-0.03	0.26
BMI (Kg/m^2)	-0.036	0.10
LDL (mg/dL)	-0.04	0.19
HbA_{1c} (%)	-0.39	0.04
Uric Acid (mg/dL)	-0.43	0.22
HOMA-IR	0.12	0.49
hs-CRP (mg/dL)	0.09	0.48
S100A12 (ng/mL)	-2.7	0.41
sRAGE (ng/mL)	0.39	0.07
esRAGE (ng/mL)	-0.43	0.02
Left atrium volume/BSA		
BMI	0.4	0.43
HDL	0.24	0.1
ApoB/ApoA1	0.03	0.31
HbA_{1c}	0.67	0.01
sRAGE (ng/mL)	-0.2	0.03
Sphericity Index		
LDL cholesterol (mg/dL)	1.2	0.04
Fasting glycemia (mg/dL)	0.01	0.16
HbA_{1c} (%)	0.21	0.02

Table 4: HbA_{1c},glycated hemoglobin; BMI, body mass index; LDL, low density lipoprotein; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B; HOMA-IR, homeostasis model assessment-insulin resistance; hs-CRP, high sensitivity C-reactive protein; sRAGE, soluble receptor for advanced glycation end-products; esRAGE, endogenous secretory RAGE.

Discussion

Subjects with NFG/NGT are a heterogeneous group of patients with different degrees of cardiometabolic risk. In this study, we examined selected echocardiographic parameters and their association with inflammatory factors strictly bounded with cardiovascular risk in subjects with prediabetes according only with HbA_{1c} (HbA_{1c} 5.7-6.4% and NFG/NGT).

The main finding of this study is that subjects with HbA_{1c} prediabetes exhibited a lower E/A ratio compared with controls; moreover, we found that the relationship between HbA_{1c} and E/A Ratio is independent from other confounding factors. These data confirm a consistent body of previous studies demonstrating the relationship between early alteration of glucose homeostasis and subclinical alterations of cardiac function in different populations with early metabolic alterations such as insulin resistance, high one-hour postload glycemia, and new onset and overt type 2 diabetes [89,144,145]. Furthermore, this result is clinically relevant and endorses the role of HbA_{1c} in the identification of patients with prediabetes and higher cardiovascular risk. Accordingly, in recent studies, we found an alteration of subclinical markers of cardiovascular risk in the same population (HbA_{1c} 5.7-6.4% and NFG/NGT) with no significant differences compared with IFG/IGT and new onset type 2 diabetes [98]. The role of HbA_{1c} in predicting cardiovascular disease in subjects without diabetes has been supported by other authors. Selvin E *et al.* found a significant increase in the incidence of cardiovascular events in subjects with an HbA_{1c} value substantially lower than those used for the diagnosis of diabetes [12]. Moreover, a recent meta-analysis of six prospective cohort studies on subjects without diabetes mellitus showed a linear association of HbA_{1c} levels with primary cardiovascular events [52]. Finally, a recent prospective study showed a significant

increase in coronary heart and cardiovascular disease in subjects with prediabetes identified by HbA_{1c} with respect to normoglycemic subjects [135].

Interestingly, in this study, subjects with HbA_{1c} prediabetes exhibited a higher LAV (another parameter strictly bounded with diastolic function) with respect to controls. In agreement with our data Dinh *et al.* showed an increase in LAV from NGT to IGT and type 2 diabetes; furthermore, the study reports that HbA_{1c} was significantly correlated with LAV and E/e' Ratio, parameters indicative of left ventricular diastolic dysfunction with elevated filling pressure, even in subjects without a history of diabetes. The authors discussed that, LAV reflects a cumulative effect of long duration different contributors to left ventricular diastolic function and is less vulnerable to acute changes in preload and afterload, which might have an acute impact on diastolic function. Therefore, the LAV could be labeled as the "HbA_{1c}" of diastolic dysfunction abnormalities [144].

We recently reported that patients with HbA_{1c} prediabetes showed lower esRAGE plasma levels with respect to controls, and these levels were independently related with early markers of cardiovascular disease [32]. However, the relationship between diastolic function and AGE/RAGE axis in patients with prediabetes was not explored. In this study, we found an independent relationship between low esRAGE plasma levels and E/A Ratio, suggesting that glycosylation and inflammation may play a role in heart damage. Multiple studies have reported the measurement of plasma or serum levels of soluble forms RAGE in human patients, in different diseases, including heart failure, with controversial findings. Rasposeiras-Roubin *et al*. examined the correlation between total sRAGE and severity of heart failure; they found that sRAGE levels were higher with increasing degrees of heart failure (New York Heart Association functional class) [155]. Other authors reported higher total

levels of sRAGE in patients with heart failure, however, in agreement with our data, they found lower esRAGE levels in patients with impaired cardiac function versus controls [94]. Conversely, Falcone *et al.* reported an independent association between low levels of sRAGE and coronary heart disease (CHD) in men without diabetes and low levels of sRAGE have been associated with risk of diabetes, CHD and mortality [130,139]. The mechanisms underlying these controversial results regarding the different trends between total and esRAGE levels have yet to be determined. However, it is essential to note that all these findings may be affected by the recruitment modalities of the different studies; indeed, plasma levels of all soluble forms of RAGE may be modulated by renal function and common drug treatments such as statins, angiotensin receptor blockers and angiotensin-converting enzyme inhibitors.

To the best of our knowledge, this is the first study showing a higher SI in patients with HbA_{1c} prediabetes; furthermore, SI was independently related with HbA_{1c} levels. These data may be relevant from the clinical point of view; indeed, adverse remodeling of the left ventricle has been associated with worse prognostic outcome [156]. In particular, left ventricular sphericity has also been associated with decreased survival after acute myocardial infarction [157]. Another recent study conducted on a multiethnic population, free of cardiovascular disease at baseline, showed that a higher sphericity was a strong predictor of incident heart failure and atrial fibrillation [158].

Based on these considerations, it could be of clinical interest to explore these data in studies with larger populations since therapies that aim to retard the development of increased sphericity should be prospectively evaluated in patients with prediabetes.

Several potential limitations of this study need to be highlighted. First, although our patients were clinically free from coronary heart disease (CAD), we did

not screen for the presence of CAD using coronary angiography. Since CAD has been associated with left ventricular diastolic dysfunction, the lack of information on coronary status may be a potential source of bias. Second, the cross sectional design of this study does not permit any conclusions on causality. Third, although E/A ratio has important diagnostic and prognostic implications, it presents some limitations and should be interpreted in conjunction with clinical characteristics and other echocardiographic parameters to describe diastolic function and guide patient's management.

In conclusion, subjects with HbA_{1c} prediabetes exhibited early subclinical cardiac alterations such as lower E/A Ratio, higher LAV and impaired SI. In consideration of the expected increased use of HbA_{1c} as a screening tool to identify individuals with alteration of glycemic homeostasis, these data may have important clinical implications to identify patients who are at increased risk for cardiovascular disease.

2.6 Low advanced glycation end-product diet improves the lipid and inflammatory profiles of prediabetic subjects

Prediabetes, a common disorder of glucose homeostasis, is considered a state associated with increased risk of diabetes and cardiovascular disease [159,160]. As prediabetes does not typically present with clinical symptoms, measurement of its prevalence has been largely underestimated and, according to current guidelines, the first step in the management of this condition should emphasize therapeutic lifestyle modifications; however, successful management of prediabetes requires an approach that involves screening and treatment of modifiable risk factors for cardiovascular disease such as dyslipidemia, and hypertension [161].

It is well-known that lifestyle or pharmacologic interventions can prevent diabetes in high risk subjects; however, the evidence regarding the prevention of cardiovascular disease is less clear [58,162,163].

The role of nutrition in the prevention of type 2 diabetes and cardiovascular disease has been extensively investigated and, specifically designed regimens based on targeted food properties have attracted attention as potentially useful in reducing the risk of developing macrovascular disease and promoting lifelong health [163,164]. In clinical practice and in most clinical studies, dietary strategies have often been centered on nutrients or caloric restriction, but not on risk-associated processing methods; however, previous study have shown that processed foods and dietary fat are high in glycotoxins known as advanced glycation end-products (AGEs).

AGEs form in common foods during spontaneous reactions between reducing sugars and proteins or lipids, and their potential role in promoting inflammation and

atherosclerotic risk has been explored in several studies [165,166]. AGEs are naturally present in uncooked animal-derived foods, and cooking results in the formation of new AGEs within these foods. The fact that the modern diet is a large source of AGEs is now well-documented [167]: grilling, broiling, roasting, searing, and frying propagate and accelerate new AGE formation. In the past few years, the potential role of dietary AGEs in human health has largely been ignored, however, recent studies with the oral administration of a single AGE-rich meal to human beings as well as labelled single protein-AGEs or diets enriched with specific AGEs such as carboxy-methyl-lysine (CML) and methyl-glyoxal (MG) to mice, have clearly shown that dietary AGEs are absorbed and contribute significantly to the body's AGE pool [168]. In line with these considerations, previous studies showed that restriction of dietary AGEs directly correlates with lower circulating AGEs, such as CML and MG, as well as with markers of inflammation and oxidative stress in patients with diabetes or kidney disease as well as in healthy subjects [169–172]. AGEs play a role in different diseases increasing inflammation through a specific receptor (receptor for AGEs, RAGE). RAGE is also found in the circulation in a spliced form called endogenous secretory RAGE (esRAGE) that may contribute to the removal/neutralization of circulating ligands thus functioning as a decoy by competing with cell-surface RAGE for ligand binding [129].

The aim of this work was to investigate the effect of a controlled dietary intervention that compared the chronic effects of a low dietary AGE (L-dAGEs) with a standard dietary AGE (S-dAGEs) regimen on lipid profile, inflammatory markers and plasma levels of esRAGE in individuals with prediabetes. Furthermore, we examined the effects of an L-dAGE regimen on early markers of cardiovascular disease.

Materials and methods

Study design and participants.

The study was a randomized, controlled, 24 week dietary observational perspective trial involving 62 adults with prediabetes attending our University Hospital for diabetes and cardiovascular risk evaluation. The inclusion criteria were the following: age range between 35 and 65 years; body mass index (BMI) between 18.5 and 40 Kg/m^2; prediabetes identified according to the American Diabetes Association (ADA) recommendation [impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) and/or glycated hemoglobin (HbA_{1c}) between 5.7-6.4%], and Caucasian race. The exclusion criteria were the following: LDL cholesterol serum levels ≥190 mg/dL at baseline; previous history of diabetes; previous history of overt cardiovascular events (atrial fibrillation, stroke, ischemic heart disease, chronic obstructive peripheral arteriopathy, or heart failure); active smoking; clinical evidence of advanced liver or renal disease; anemia or hemoglobinopathies; use of medications known to affect glucose metabolism or lipid profile; use of vitamin supplements; major food allergies; history of eating disorders or dietary pattern different from the typical Mediterranean diet; significant weight loss or change in dietary habits in the previous three months; chronic gastrointestinal diseases associated with malabsorption or chronic pancreatitis; rheumatic diseases; and/or recent history of acute illness, malignant disease, and drug or alcohol abuse.

Participants who met the inclusion criteria and gave informed consent returned on a subsequent morning for screening tests including a physical examination and review of their clinical history, smoking status and alcohol consumption.

Inclusion visit

Anthropometric characteristics were registered and cardiovascular risk evaluation was performed [arterial stiffness and intima-media thickness (IMT)]. Body weight and height were measured, and BMI was calculated as weight (kg)/[height (m)]^2. Blood pressure (BP) was measured with a calibrated sphygmomanometer after the subject had rested in the supine position for 10 min. Venous blood samples were withdrawn from the antecubital vein on the morning after an overnight fast. Baseline venous blood samples were obtained for the measurement of plasma glucose, HbA_{1c}, total cholesterol, high density lipoprotein (HDL) cholesterol, triglycerides, Apolipoprotein B (ApoB), Apolipoprotein A (ApoA), esRAGE and high sensitivity C-reactive protein (hs-CRP). LDL cholesterol concentrations were estimated using the Friedewald formula. All subjects underwent a 75-g oral glucose tolerance test (OGTT) with 0-, 30-, 60-, 90-, and 120-min sampling for plasma and insulin as previously described [78]. Glucose tolerance status was defined on the basis of OGTT according to ADA recommendations [70].

Experimental diet

The study design is shown in **Figure 1**. The patients were randomized using a computerized random number generator for each subject after acceptance of inclusion to either a low-AGE (L-dAGEs) (n = 31) or standard-AGE (S-dAGEs) (n = 31) diet. A physician blinded for the group of study carried out the clinical evaluation of patients. Both diets were compatible with the ADA recommendation and contained 50–55% carbohydrate, 20% protein, <30% fat. The AGE content of each food item was measured according to the database of 500 foods reported by Uribarri *et al*. [168]. Using this reference database for AGE content in foods two study diets

were designed to have a similar content of calories, protein, carbohydrate, and fat but differ by 5-fold in AGEs. The volunteers of the L-AGE group received written indications for food preparation, appropriate cooking times and temperatures. They also received instructions to boil or steam the food, to avoid fried entrees and reheat food indirectly using steam in a double boiler.

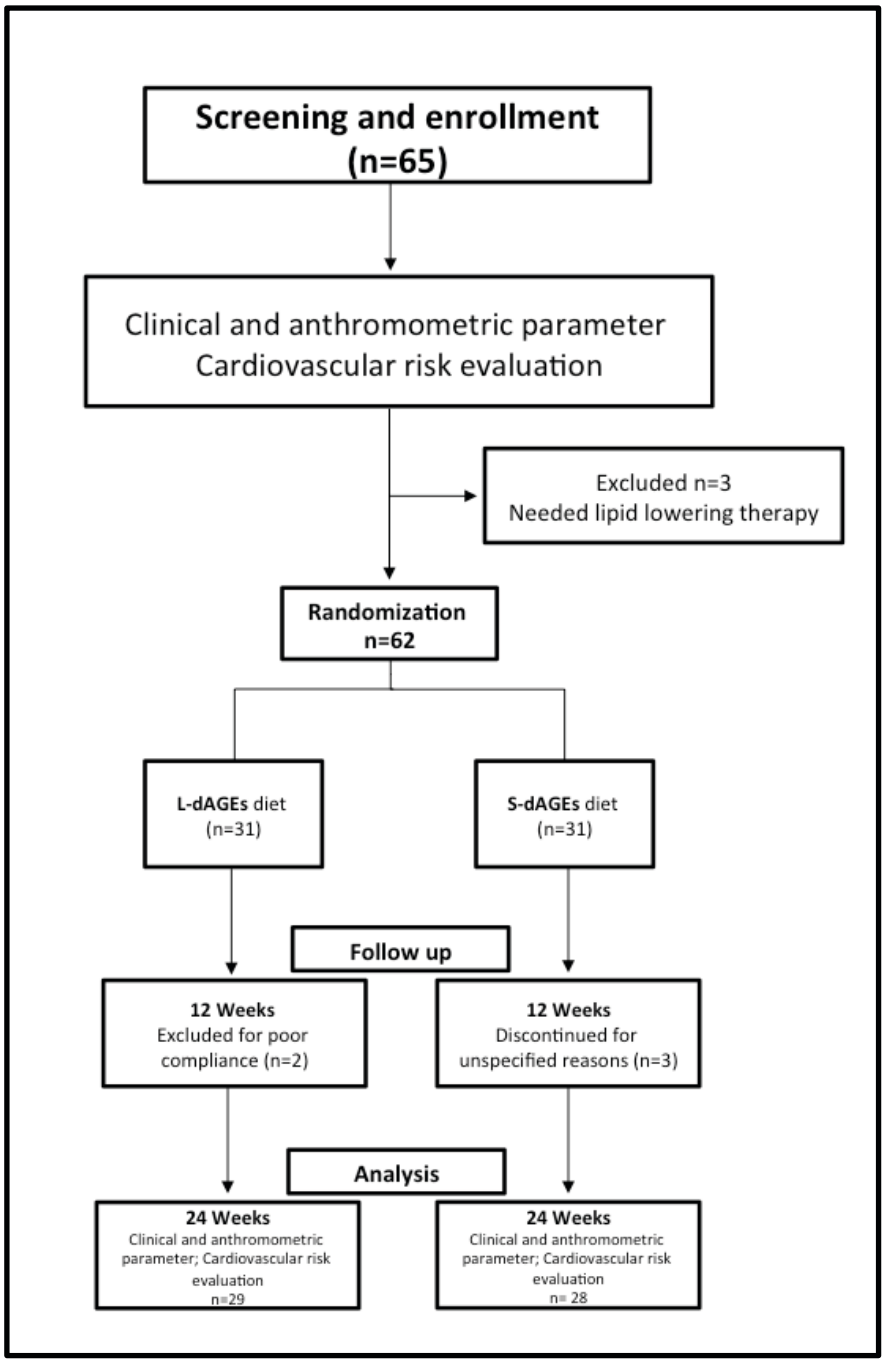

Figure 1: Flow diagram of enrollment, randomization, withdrawals and follow-ups of the study subjects. L-dAGEs, low dietary AGEs, S-dAGEs, standard AGEs.

Clinical and laboratory parameters: weight, BMI, waist circumference, systolic and diastolic blood pressure, total cholesterol, HDL cholesterol, triglycerides, LDL cholesterol, hs-CRP. Cardiovascular risk evaluation: Intima-Media thickness, Augmentation Index, SEVR, Pulse Wave Velocity.

Follow-up

Adherence was monitored by a review of daily food records and weekly meetings to consider eating behaviors and food records. Basiotis *et al.* demonstrated that nutrient intake can be accurately estimated from food intake records [173].

After three months body weight was registered, a plasma sample was collected for assessment of fasting glycemia, lipid profile and hs-CRP. After six months anthropometric characteristics were registered, a plasma sample was collected for assessment of fasting glycemia, lipid profile, hs-CRP, ApoB, ApoA, esRAGE and cardiovascular risk evaluation (arterial stiffness and intima-media thickness [IMT]) (**Figure 1**).

Biochemical analysis.

Plasma glucose, serum total cholesterol, triglycerides, HDL cholesterol and hs-CRP were measured using available enzymatic methods as previously described [107]. Plasma samples were assayed for apolipoprotein using a nefelometer assay (Siemens AG Heathcare Sector, Erlagen, Germany)[50]. LDL cholesterol concentrations were estimated using the Friedewald formula. HbA_{1c} was measured via high-performance liquid chromatography using a National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial (DCCT) assay reference [79]. Chromatography was performed using a certified automated analyzer (HPLC; HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.) (normal range 4.25–5.9% [23–41 mmol/mol]; intra- and inter-assay CVs were 1.7% and 2.6%, respectively). Plasma samples were assayed for apolipoprotein using a nefelometer assay (Siemens AG Healthcare Sector,

Erlagen, Germany).

To quantify the plasma concentration of esRAGE (B-Bridge esRAGE ELISA Kit, Cupertino, USA) fasting blood samples were collected, and specimens were immediately centrifuged and stored at -80°. Commercially available ELISA kits were used according to the manufacturer's instructions.

Cardiovascular risk evaluation

Pulse wave analysis

All measurements were made from the right radial artery by applanation tonometry using a Millar tonometer (SPC-301; Millar Instruments, Houston, TX, USA), as previously described [51]. The data were collected directly on a desktop computer and processed with SphygmoCorCvMS (AtCor Medical, Sydney, Australia). The aortic waveform has two systolic pressure peaks, the latter of which is caused by wave reflection from the periphery. With arterial stiffening, both the pulse wave velocity (PWV) and the amplitude of the reflected wave are increased such that the reflected wave arrives earlier and adds to (or augments) the central systolic pressure. The aortic waveform in pulse wave analysis was subjected to further analysis for the calculation of the aortic Augmentation, Augmentation Index (AugI) (calculated by dividing augmentation by pulse pressure), central BP, ejection duration (duration of the systolic period in milliseconds), and Buckberg subendocardial viability ratio (SEVR; area of diastole divided by area of systole during one cardiac cycle in the aorta). Pulse pressure is the difference between the systolic and diastolic BPs.

Pulse Wave Velocity

The SphygmoCor CvMS (AtCor Medical, Sydney, Australia) system was used for

the determination of the PWV. This system uses a tonometer and two different pressure waves obtained at the common carotid artery (proximal recording site) and at the femoral artery (distal recording site). The distance between the recording sites and suprasternal notch was measured using a tape measure. An electrocardiogram was used to determine the start of the pulse wave. The PWV was determined as the difference in travel time of the pulse wave between the two different recording sites and the heart, divided by the travel distance of the pulse waveform. The PWV was calculated on the mean of 10 consecutive pressure waveforms to cover a complete respiratory cycle.

Carotid Ultrasound Examinations

Ultrasound scans were performed using a high-resolution B-mode ultrasound system (MyLab 50 XVision; Esaote Biomedica SpA, Florence, Italy) equipped with a 7.5-MHz linear array transducer. A single experienced physician who was blinded to the clinical and laboratory characteristics of the patients performed all the ultrasound examinations. Scans were performed and measurements were conducted at a total of six plaque-free sites 1 cm proximal to the carotid bulb. The obtained values were averaged and are presented as the means of the IMT of the common carotid artery. All measurements were obtained in diastole, assessed as the phase in which the lumen diameter is at its smallest and the IMT is at its largest.

Statistical Analysis

We based the power calculation on previous studies examining differences among LDL cholesterol plasma levels in dietary group [174]. We used a level of significance

(α) set to 5% and a power (1-β) set to 80%. The estimated sample size was 25 patients for group.

Data are presented as means±SD for normally distributed variables, as percentages for categorical variables, or as median (interquartile range 25th–75th percentile) for non-normal continuous variables. The distributional characteristics of each variable, including normality, were assessed by the Kolmogorov-Smirnov test. Repeated measures ANOVA was used to compare changes in subject characteristics (baseline versus the 12 week and 24 week follow-ups) considering the confounding effect of weight change in the model. When analysis was significant, Bonferroni *post hoc* tests were performed to determine differences between pairs of means. Comparisons between groups were performed using the unpaired *t* test and Fisher's exact test, when appropriate, for categorical variables. A *P* value less than 0.05 was considered statistically significant. If necessary, numerical variables were logarithmically transformed for statistical analysis to reduce skewness. All analyses were performed using Stat View 6.0 for Windows.

The local ethics committee approved the study. Informed consent was obtained from each participant.

Results

Clinical and biochemical characteristics

Subjects were recruited between January and March 2015. Sixty-five patients with prediabetes accepted to join the study. After clinical and biochemical evaluation three patients were excluded because they needed lipid-lowering therapy. Sixty-two patients were randomly allocated to an L-dAGE or an S-dAGE diet. From the L-dAGE group, two patients were excluded for poor compliance to the dietary regimen

during the first month; from the S-dAGE group three patients discontinued intervention for unspecified reasons. Twenty-nine subjects on the L-dAGE diet group and twenty-eight on the S-dAGE diet group completed the dietary intervention. Thus, results from fifty-seven completers were analyzed (**Figure 1**). **Table 1** shows the comparison of clinical and metabolic characteristics for both groups at baseline. There were no significant differences between the participants in the two dietary groups by age, sex, BMI, systolic and diastolic BP at baseline, lipid profile and glucose homeostasis parameters (fasting glycemia and HbA_{1c}).

Table 1 Clinical and biochemical characteristics, receptors for AGEs, arterial stiffness, and carotid atherosclerosis and dietary variables of the patients at baseline

Variables	All patients (n = 62)	L-dAGEs (*n* = 31)	S-dAGEs (*n* = 31)	*P*
Age (y)	54.8 ± 8.4	55.9 ± 8.8	53.4 ± 7.7	.38
BMI (kg/m^2)	32.5 ± 5.3	32.4 ± 6.2	32.9 ± 4.1	.78
Fasting glucose (mmol/L)	5.24 ± 0.51	5.23 ± 0.48	5.26 ± 0.56	.70
HbA_{1c} (mmol/mol)	42 ± 2.1	42 ± 2.1	42 ± 2.1	.93
Total cholesterol (mmol/L)	5.48 ± 1.09	5.54 ± 0.86	5.41 ± 0.61	.70
HDL cholesterol (mmol/L)	1.29 ± 0.31	1.35 ± 0.31	1.21 ± 0.3	.19
Triglycerides (mmol/L)	1.28 (0.86–1.6)	1.28 (0.82–1.6)	1.21 (0.84–1.61)	.53
LDL cholesterol (mmol/L)	3.59 ± 1.05	3.67 ± 0.83	3.49 ± 0.47	.63
Non-HDL (mmol/L)	4.29 ± 1.03	4.3 ± 1.3	4.28 ± 0.72	.94
ApoB (g/L)	1.17 ± 0.24	1.16 ± 0.27	1.18 ± 0.2	.9
ApoA (g/L)	1.59 ± 0.21	1.54 ± 0.13	1.65 ± 0.26	.18
ApoB-to-ApoA ratio	0.68 ± 0.22	0.66 ± 0.21	0.71 ± 0.23	.49
Systolic BP (mm Hg)	125.2 ± 12.9	123.5 ± 10.4	127.5 ± 15	.36
Diastolic BP (mm Hg)	78.1 ± 9.4	78.5 ± 9.3	77.7 ± 9.8	.81
hs-CRP (mg/dL)	0.23 (0.12–0.44)	0.21 (0.11–0.69)	0.23 (0.15–0.42)	.41
esRAGE (ng/dL)	0.31 ± 0.15	0.28 ± 0.14	0.30 ± 0.15	.68
Aug (mm Hg)	12.2 ± 5.9	11.8 ± 5.2	12.8 ± 6.7	.63
AugI (%)	30.9 ± 10.8	32.1 ± 10.6	29.5 ± 11.2	.49
SEVR (%)	154.6 ± 30.2	160.2 ± 32.3	147.6 ± 26.6	.22
IMT (mm)	0.77 (0.68–0.85)	0.77 (0.73–0.81)	0.78 (0.68–0.85)	.41
PWV (m/s)	7.8 ± 1.6	8.1 ± 1.7	7.5 ± 1.5	.51
Gender (M/F)	25/37	12/19	13/18	.52
Kcal/day	1930.9 ± 370.5	1889.2 ± 355.1	1973.8 ± 376.9	.49
Carbohydrates (g/d)	306.4 ± 87	302.2 ± 86.6	313.2 ± 90.1	.63
Fats (g/d)	60.3 ± 17.2	58.8 ± 17.1	62.5 ± 18.2	.35
Proteins (g/d)	57.1 ± 11	56.7 ± 10.6	59.2 ± 11.3	.41

Table 1: Data are presented as the mean±SD or median (IQR); L-dAGEs, Low dietary AGEs; S-dAGE Standard dietary AGES; HbA_{1c}, glycated hemoglobin; BP blood pressure; hs-CRP high sensitivity C reactive protein; esRAGE endogenous secretory soluble receptor for advanced glycation end-products; Aug, Augmentation Pressure; AugI, Augmentation Index; SEVR, subendocardial viability ratio; IMT, intima-media thickness.

12 week follow-up

Clinical characteristics of the study subjects at the 12 week follow-up are shown in **Table 2.** BMI was lower between baseline and the 12 week follow-up visit in both groups but with no significant difference between dietary groups. The L-dAGE

group showed a significant reduction of total cholesterol, LDL cholesterol and non-HDL cholesterol compared to baseline and to the S-dAGE group (4.85±1.09 vs 5.59±0.78 mmol/L $P<0.05$, 3.17±0.43 vs 3.67±0.53 mmol/L $P<0.05$ and 3.71±1.17 vs 4.2±0.61 mmol/L, $P<0.05$ respectively). In contrast, HDL cholesterol in the L-dAGE group was lower with respect to S-dAGE patients. Additionally, a significant decrease of triglycerides was found only in the S-dAGE group with respect to baseline.

Hs-CRP levels were reduced (without statistical significance) in both groups with respect to baseline and no difference was found between the groups. There were no significant changes in any of the other measures from baseline to follow-up within each dietary group.

Table 2 Clinical and biochemical characteristics of the patients at baseline and at the 12-wk follow-up

Variables	L-dAGEs (n = 29)	L-dAGEs P baseline to 12 wk*	S-dAGEs (n = 28)	S-dAGEs P baseline to 12 wk	P Between diet groups*
BMI (kg/m^2)		.03		.01	.3
Baseline	32.4 ± 6.2		32.9 ± 4.1		
12 wk	31.9 ± 6.7		32.5 ± 3.7		
Fasting glucose (mmol/L)		.4		.6	.4
Baseline	5.23 ± 0.48		5.26 ± 0.56		
12 wk	5.18 ± 0.42		5.19 ± 0.46		
Total cholesterol (mmol/L)		.01		.39	.02
Baseline	5.54 ± 0.86		5.41 ± 0.61		
12 wk	4.85 ± 1.09		5.59 ± 0.78		
HDL cholesterol (mmol/L)		.06		.02	.04
Baseline	1.35 ± 0.31		1.21 ± 0.3		
12 wk	1.22 ± 0.32		1.39 ± 0.26		
Triglycerides (mmol/L)		.01		.005	.8
Baseline	1.28 (0.82–1.6)		1.21 (0.84–1.61)		
12 wk	1.07 (0.88–1.17)		1.04(0.91–1.2)		
LDL cholesterol (mmol/L)		.04		.2	.04
Baseline	3.67 ± 0.83		3.49 ± 0.47		
12 wk	3.17 ± 0.43		3.67 ± 0.53		
Non-HDL cholesterol (mmol/L)		.001		.43	.94
Baseline	4.3 ± 1.3		4.28 ± 0.72		
12 wk	3.71 ± 1.17		4.2 ± 0.61		
Systolic BP (mm Hg)		.5		.09	.3
Baseline	123.5 ± 10.4		127.5 ± 15		
12 wk	125.4 ± 13.8		124 ± 14.6		
Diastolic BP (mmHg)		.5		.3	.45
Baseline	78.5 ± 9.3		77.7 ± 9.8		
12 wk	81.2 ± 6.8		76.1 ± 11.9		
hs-CRP (mg/dL)		.31		.62	.15
Baseline	0.21 (0.11–0.69)		0.23 (0.15–0.42)		
12 wk	0.15 (0.10–0.69)		0.20 (0.11–0.41)		

Table 2: Data are presented as the mean±SD or median (IQR); BP blood pressure; hs-CRP high sensitivity C reactive protein.

*P value for the comparison adjusted for weight change.

24 week analysis

Clinical characteristics of the study subjects at the 24 week follow-up are shown in **Table 3.** BMI was lower between baseline and the 24 week follow-up visit in both groups but with no significant difference between dietary groups. At the end of the dietary interventions subjects on the L-dAGE diet presented lower plasma levels of total cholesterol, LDL cholesterol and non-HDL cholesterol (5.26±1.09 vs 5.53±0.87 mmol/L $P<0.05$, 3.53±0.93 vs 3.68±0.7 mmol/L $P<0.05$ and 3.85±1.14 vs 4.34±0.7 mmol/L $P<0.05$, respectively). ApoB and ApoB/ApoA ratio were decreased in subjects in the L-dAGE group compared with the S-dAGE patients (0.77±0.25 vs 0.16±0.13 g/L, $P<0.05$ and 0.58±0.19 vs 0.68±0.13, $P<0.05$, respectively).

With respect to baseline, hs-CRP levels were significantly reduced only in the L-dAGE group [0.21(0.11-0.69) vs 0.12(0.08-0.48) mg/dL, $P<0.05$], however, no difference was found between the groups [0.12(0.08-0.48) vs 0.19(0.15-0.51) mg/dL, $P=0.15$]. Plasma levels of esRAGE were similar in both groups at baseline and at the 24 week follow-up visit (0.27±0.15 vs 0.31±0.15 ng/dL, $P=0.8$).

There was a small but significant decrease of IMT in the L-dAGE group ($P<0.05$), but not in the S-dAGE ($P=0.25$) group, in comparison with baseline. However, after 24 weeks no difference was found in IMT values between the two groups. There were no significant differences in Aug, AugI, SEVR and PWV between the L-dAGE and S-dAGE diet groups at baseline and at the 24 week follow-up visit (**Table 3**).

Table 3 Clinical and biochemical characteristics, receptors for AGEs, arterial stiffness, and carotid atherosclerosis of the patients at the 24-week follow-up

Variables	L-dAGEs (n = 29)	L-dAGEs P baseline to 24 wk	S-dAGEs (n = 28)	S-dAGEs P baseline to 24 wk	P Between diet groups
BMI (kg/m^2)		.01		.01	.72
Baseline	32.4 ± 6.2		32.9 ± 4.1		
24 wk	31.7 ± 6.3		32.2 ± 4.2		
Fasting glucose (mmol/L)		.2		.2	.3
Baseline	5.23 ± 0.48		5.26 ± 0.56		
24 wk	5.17 ± 0.41		5.22 ± 0.17		
HbA_{1c} (mmol/mol)		.9		.8	.4
Baseline	42 ± 2.1		42 ± 2.1		
24 wk	42 ± 2.1		42 ± 2.1		
Total cholesterol (mmol/L)		.01		.39	.02
Baseline	5.54 ± 0.86		5.41 ± 0.61		
24 wk	5.26 ± 1.09		5.53 ± 0.87		
HDL cholesterol (mmol/L)		.06		.02	.05
Baseline	1.35 ± 0.31		1.21 ± 0.3		
24 wk	1.27 ± 0.32		1.26 ± 0.26		
Triglycerides (mmol/L)		.01		.01	.8
Baseline	1.28 (0.82–1.6)		1.21 (0.84–1.61)		
24 wk	0.98 (0.91–1.23)		1.03 (0.93–1.2)		
LDL cholesterol (mmol/L)		.04		.2	.04
Baseline	3.67 ± 0.83		3.49 ± 0.47		
24 wk	3.53 ± 0.93		3.68 ± 0.7		
Non-HDL cholesterol (mmol/L)		.03		.6	.05
Baseline	4.3 ± 1.3		4.28 ± 0.72		
24 wk	3.85 ± 1.14		4.34 ± 0.7		
ApoB (mg/dL)		.001		.3	.001
Baseline	1.16 ± 0.27		1.18 ± 0.2		
24 wk	0.77 ± 0.25		1.16 ± 0.13		
ApoA (mg/dL)		.01		.05	.03
Baseline	1.54 ± 0.13		1.65 ± 0.26		
24 wk	1.34 ± 0.18		1.5 ± 0.19		
ApoB-to-ApoA ratio		.02		.34	.03
Baseline	0.66 ± 0.21		0.71 ± 0.23		
24 wk	0.58 ± 0.19		0.68 ± 0.13		
Systolic BP (mm Hg)		.5		.07	.3
Baseline	123.5 ± 10.4		127.5 ± 15		
24 wk	124.7 ± 11.6		122 ± 15.6		
Diastolic BP (mm Hg)		.5		.3	.45
Baseline	78.5 ± 9.3		77.7 ± 9.8		
24 wk	81.2 ± 6.8		76.1 ± 11.9		
hs-CRP (mg/dL)		.01		.53	.15
Baseline	0.21 (0.11–0.69)		0.23 (0.15–0.42)		
24 wk	0.12 (0.08–0.48)		0.19 (0.15–0.51)		
esRAGE (ng/dL)		.32		.43	.80
Baseline	0.28 ± 0.14		0.30 ± 0.15		
24 wk	0.27 ± 0.15		0.31 ± 0.15		
Aug (mm Hg)		.57		.8	.41
Basal	11.8 ± 5.2		12.8 ± 6.7		
24 wk	12.3 ± 4.8		13.6 ± 6.1		
AugI (%)		.7		.6	.34
Basal	32.1 ± 10.6		29.5 ± 11.2		
24 wk	33.2 ± 7.1		29.9 ± 11.4		
SEVR (%)		.8		.2	.56
Basal	160.2 ± 32.3		147.6 ± 26.6		
24 wk	161.1 ± 37		153.3 ± 34.1		

Table 3 (*continued*)

Variables	L-dAGEs (n = 29)	L-dAGEs P baseline to 24 wk	S-dAGEs (n = 28)	S-dAGEs P baseline to 24 wk	P Between diet groups
IMT (mm)		.05		.2	.25
Basal	0.77 (0.73–0.81)		0.78 (0.68–0.85)		
24 wk	0.73 (0.70–0.75)		0.76 (0.67–0.84)		
PWV (m/s)		.6		.8	.56
Basal	8.1 ± 1.7		7.5 ± 1.5		
24 wk	8.1 ± 1.6		7.5 ± 1.4		

Table 3: Data are presented as the mean±SD or median (IQR); HbA_{1c}, glycated hemoglobin; BP blood pressure; hs-CRP high sensitivity C reactive protein; esRAGE endogenous secretory soluble receptor for advanced glycation end-products; Aug, Augmentation Pressure; AugI, Augmentation Index; SEVR, subendocardial viability ratio; IMT, intima-media thickness; PWV, pulse wave velocity.

Discussion

In this study, we examined the effects of a six months low AGE vs standard dietary regimen in subjects with prediabetes. Our purpose was to test the effect of a diet prepared by using low temperature cooking techniques (which results in a low dAGE content) on clinical indicators of inflammation and cardiovascular risk, with respect to a diet that is based on standard temperature cooking methods (which results in a high AGE content). Although some studies have dealt with such questions in other populations such as type 2 diabetes or healthy subjects, the existing data are either controversial or not fully convincing.

In our study, both the groups experienced a slight but similar weight reduction during the trial, with a 2-2.5 kg decrease after 12 weeks and 3-3.5 kg total weight loss at the end of the study. This result may suggest an analogous slimming effect of the two dietetic regimen, as suggested in the literature [175]. Moreover, the weight loss may be considered a marker of the patient's compliance to the diet during the six months' trial.

A particularly significant finding of our work is the large reduction observed in total cholesterol, LDL cholesterol, ApoB serum levels and in the ApoB to ApoA ratio in the L-dAGE group, all in accordance with previous observations made during other trials conducted on healthy subjects with low AGE or other dietary regimens [176,177]. Managing risk related to LDL is an important topic for patients at risk for cardiovascular disease and although multiple approaches to lowering LDL cholesterol, a limited number of patients receiving standard of care for lipid lowering therapy reach therapeutic goals. Furthermore, the reductions of LDL, non-HDL cholesterol, and ApoB levels achieved with standard therapy displayed large inter-

individual variation; thus, an effective dietary intervention may be a valid addition in compliant patients.

The relationship between lipid abnormalities and AGEs was previously investigated in patients with diabetes and end stage renal disease. Bucala *et al*. reported that modifications of LDL induced by AGEs may impair the receptor-mediated clearance of LDL contributing to an elevation of LDL serum levels. The authors have further supported this hypothesis by observing that the administration of the AGE inhibitor aminoguanidine to diabetic patients significantly decreased circulating LDL levels [178]. These findings also reflected that habitual or long-term high AGE intake might worsen hypercholesterolemia and promote LDL-associated pathological conditions such as macrovascular complications.

In this study, hs-CRP, a marker of inflammation, decreased under an L-dAGE diet. These data are consistent with previous reports exploring the relationship between dietary age, oxidative stress and inflammation in diabetic and non-diabetic subjects [172,179]. Uribarri *et al.* reported a positive correlation of dietary AGE intake estimated with 3-day food records with serum levels of hs-CRP, IL-1, IL-6 and TNF-α in 172 young healthy individuals [180]. Similar results were reported in a randomized 6 week prospective study conducted on patients with type 2 diabetes who underwent a dietary AGE restriction; the group with L-dAGEs experienced a decreasing trend in hs-CRP values compared with the S-dAGE group, without statistical significance [181]. Finally, Vlassara *et al.* reported a decline of 20% in hs-CRP values in 13 diabetic subjects who underwent a 6-week low AGEs diet [170]. From these reports, it is possible to hypothesize that a dietary regimen with a high AGE content may promote a sustained low-grade inflammatory state. In fact, AGEs may induce the expression of inflammation markers such as cytokines and adhesion molecules via reactive

oxygen species production and nuclear factor κB activation, properties which are also exhibited by dietary AGEs [182,183]. These highly reactive molecules, could, together with hyperglycemia, contribute to the inflammatory state associated with prediabetes and related cardiovascular disease.

In a recent study we found that prediabetic patients exhibited lower esRAGE plasma levels than controls but similar to those with type 2 diabetes [32]. The present study showed that esRAGE levels were not affected by a diet that was standard or low in AGEs in subjects with prediabetes. The regulatory mechanism for alternative splicing to generate esRAGE remains unclear, and environmental or genetic factors may be involved. An association between soluble RAGE and glycemic control has recently been reported and some studies show that oral agents such as thiazolidinediones and statins may modulate the AGE-RAGE system [47,133,184]. Moreover, the association between RAGE gene polymorphisms and circulating levels of esRAGE has been reported. Since the major changes of our study involved plasma lipids, our data suggest that these factors are not involved in esRAGE control. Further studies are needed to explain the precise mechanism underlying the decrease in sRAGE in diabetic and non-diabetic humans [138,185].

In this cohort of prediabetic subjects we found a reduction trend in IMT that is significant with respect to baseline only in the L-dAGE dietary group. Although IMT provide an accurate measurement of atherosclerotic burden and have a high degree of reproducibility, our results in a short-term follow up may be surprising and need to be confirmed in a longer follow-up on a larger cohort of patients [186]. Moreover this data may be affected by a sample size that is not adeguate to evaluate changes in IMT. However, this is not the first study showing the beneficial effects of lifestyle intervention on carotid atherosclerotic burden; other lifestyle intervention

studies with a short follow-up have shown similar results with significant IMT regression observed in the treatment group [187–189]. Moreover, another diet-only intervention study showed that a Mediterranean-style diet rich in fruit and vegetables and poor in red meat and monounsaturated and polyunsaturated fat significantly reduced carotid IMT progression compared with the control diet in elderly men with hyperlipidemia [190] and a recent meta-analysis of randomized controlled trials confirmed that intensive lifestyle modifications are associated with a decrease in carotid atherosclerotic burden [191].

Finally, in the present study, we cannot demonstrate a reduction in arterial stiffness parameters after dietary intervention on both groups. These data are, at least in part, in contrast with other short-term controlled studies in which a hypocaloric diet intervention (5% to 10% weight reduction) was associated with a significant reduction of PWV [192,193]. This discordance could be due to the different weight reduction experienced in our study. The magnitude of arterial stiffness improvement is related to the magnitude in weight reduction and abdominal obesity. In our study both groups experienced a slight but similar weight reduction during the trial, with a 2-2.5 kg decrease after 12 weeks and 3-3.5 kg total weight loss at the end of the study, however, a further 10% weight loss was necessary to reduce the risk of cardiovascular disease [194].

Several potential limitations of this study need to be highlighted. First, a limitation of the current study is the small sample size. A larger sample size would have increased the statistical power to detect changes in the variables measured. Second, we cannot exclude the possibility that the observed reductions in IMT may be, at least in part, effects of weight loss. Third, although adherence to the diet was

monitored by a review of daily food records, a recall bias may persist and the food intake might not have been exact.

In conclusion, an L-dAGE diet produced an improved lipid profile, decreased hs-CRP concentrations and regression of carotid IMT. Presumably, simple dietetic intervention, which does not necessarily mean deprivation of certain foods, but only the preferred use of low AGE culinary techniques, may improve serological and clinical markers strictly bound to cardiovascular risk in subjects with prediabetes. Currently, there are not enough data to determine the impact of the observed variations on cardiovascular outcome.

3 – Conclusions

In 2011, the American Diabetes Association proposed that HbA_{1c} should be used as a diagnostic test for prediabetes. An HbA_{1c} of 5.7–6.4% was identified as a new indicator of prediabetes in addition to impaired fasting glucose (IFG) and impaired glucose tolerance (IGT). Evidence indicates that subjects who are at high risk for type 2 diabetes also have an increased cardiovascular risk; therefore, the value of a predictive test for type 2 diabetes is also determined by its ability to indicate a high risk of cardiovascular disease.

The scientific production has evidenced the role of HbA_{1c} in identify subjects with subclinical alteration of cardiovascular risk markers who were classified as normal fasting glucose and normal glucose tolerance. In particular, we found that subjects with HbA_{1c} prediabetes showed:

- Increased IMT;
- Increased arterial stiffness;
- Decreased E/A ratio;

Furthermore, these subjects seemed to present a negative inflammatory profile; indeed, they showed an alteration of other markers of glycation and inflammation strictly bounded with cardiovascular risk, in the same population.

- Low esRAGE plasma levels;
- High S100A12 plasma levels;
- High hs-CRP plasma levels;
- Low 25(OH)D plasma levels;

Based on these data, we suggest that HbA_{1c} may identify subjects with different

cardiovascular and glycol-metabolic risks among subjects with normal fasting glucose and normal glucose tolerance. It is pertinent to recognize that the determinants of cardiovascular risk in subjects with metabolic alterations are complex and multiple, and individual's cardiovascular risk can't be identified by a single laboratory test.

However, the measurement of HbA_{1c} appears to be a reliable diagnostic approach to identify patients at high risk for diabetes and cardiovascular disease; it seems to provide several advantages, especially in settings where OGTT is rarely used and never repeated as a confirmatory test, and eliminates a long series of biological and analytical limits. In most conditions HbA_{1c} could became the reference method, provided that its assay is aligned with international standards. However, the budget/cost benefit of replacing glucose measurements and OGTT with HbA_{1c} in clinical settings remains unclear and further studies are needed to acquire additional information.

4 – Scientific production

Publications - original articles

1. Scicali R, Di Pino A, Platania R, Purrazzo G, Ferrara V, Giannone A, Urbano F, Filippello A, Rapisarda V, Farruggia E, Piro S, Rabuazzo AM, Purrello F. Detercting familial hypercholesterolemia by serum lipid profile screening in a hospital setting: clinical, genetic and atherosclerotic burden profile ***Nutr Metab Cardiovasc Dis (Accepted Manuscript)***

2. Di Pino A, Currenti W; Urbano F, Scicali R, Piro S, Purrello F, Rabuazzo AM. High intake of dietary advanced glycation end-products is associated with increased arterial stiffness and inflammation in subjects with type 2 diabetes. ***Nutr Metab Cardiovasci Dis 2017 (Accepted Manuscript)***, DOI: http://dx.doi.org/10-1016/j.numecd.2017.06.014 Published online: July 7, 2017.

3. Scicali R, Giral P, Gallo A, **Di Pino A**, Rabuazzo AM, Purrello F, Cluzel P, Redheuil A, Bruckert E, Rosenbaum D HbA_{1c} increase is associated with higher coronary and peripheral atherosclerotic burden in non diabetic patients. **Atherosclerosis** 2016 Dec; 255:102-108. doi: 10.1016/j.atherosclerosis.2016.11.003. PMID: 27870948

4. Di Mauro S, Ragusa M, Urbano F, Filippello A, **Di Pino A**, Scamporrino A, Pulvirenti A, Ferro A, Rabuazzo AM, Purrello M, Purrello F, Piro S. Intracellular and extracellular miRNome deregulation in cellular models of NAFLD or NASH: Clinical implications. **Nutr Metab Cardiovasc Dis.** 2016 Dec;26(12):1129-1139. PMID: 27756518

5. **Di Pino A**, Currenti W, Urbano F, Mantegna C, Purrazzo G, Piro S, Purrello F, Rabuazzo MA. Low advanced glycation end product diet improves the lipid and inflammatory profiles of prediabetic subjects ***J Clin Lipidol.*** 2016 Sep-Oct;10(5): 1098-108. Doi 10.1016/j.jacl.2016.07.001. Epub 2016 Jul 9. PMID: 27678426

6. **Di Pino A**, Urbano F, Zagami RM, Filippello A, Di Mauro S, Piro S, Purrello F, Rabuazzo MA. Low endogenous secretory RAGE levels are associated with inflammation and carotid atherosclerosis in pre-diabetes. ***J Clin Endocrinol Meta*b.** 2016 Apr;101(4):1701-9. doi: 10.1210/jc.2015-4069. Epub 2016 Feb 17. PMID: 26885882

7. Urbano F, Filippello A, **Di Pino A**, Barbagallo D, Di Mauro S, Pappalardo A, Rabuazzo AM, Purrello M, Purrello S, Piro S. Altered Expression of Uncoupling Protein 2 in GLP-1-producing Cells after Chronic High Glucose Exposure: Implications for the Pathogenesis of Diabetes Mellitus. ***Am J Cell Physiol.*** 2016 Jan. PMID: 26739488

8. Zagami RM, **Di Pino A,** Urbano F, Piro S, Purrello F; Rabuazzo AM**.** Low circulating vitamin D levels are associated with increased arterial stiffness in prediabetic subjects identified according to HbA_{1c}. ***Atherosclerosis.*** 293 (2015) 395-401. PMID: 26520892

9. **Di Pino A,** Scicali R, Calanna S, Urbano F, Mantegna C, Rabuazzo AM, Purrello F, Piro S. Cardiovascular risk profile in subjects with prediabetes and new-onset type 2 diabetes identified by HbA(1c) according to American Diabetes Association criteria. ***Diabetes Care.*** 2014 May;37(5):1447-53. PMID: 24574348

Other pubblications

1. Scicali R, Platania R, Purrazzo G, Ferrara V, Giannone A, **Di Pino A**, Urbano F, Filippello A, Piro S, Rabuazzo AM, Purrello F. Trattamento dell'ipercolesterolemia nel soggetto con diabete mellito: aggiornamento sugli approcci terapeutici. *G It Diabetol Metab* 2017;37:27-34
2. **Di Pino A**. Urbano F. Piro S. Purrello F. Rabuazzo MA. Prediabete, Criteri diagnostici e rischio cardiovascolare. *G It Diabetol Metab* 2016;36:134-143.
3. **Di Pino A**. Urbano F. Piro S. Purrello F. Rabuazzo MA. Update on pre-diabetes: Focus on diagnostic criteria and cardiovascular risk *World J Diabetes* 2016 October 15; 7(18).

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