N(carboxymethyl)lysine as a biomarker for microvascular complications in type 2 diabetic patients

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SUMMARY

Aims: Hyperglycaemia is linked to vascular dysfunction in patients with diabetes mellitus, either directly or through advanced glycation and product (AGE) formation. Experimental evidence has indicated the possible involvement of AGEs in the genesis of vascular complications. We investigated whether serum levels of AGEs and of the glycoxidation compound carboxymethyl-lysine (CML) were increased and correlated with vascular complications in type II diabetes mellitus.

Methods: Serum levels of AGEs and CML-human serum protein (CML-HSP) were measured by a specific immunoassay in 51 men and 26 women aged 58 ± 6.1 years (mean ± SD) who had been treated for type II diabetes mellitus for 11 ± 8 years, and in a non-diabetic control group consisting of 39 men and 21 women aged 55.5 ± 7.5 years. Patients with macroalbuminuria or abnormal creatinine clearance were excluded from the study.

Results: The serum levels of AGEs were significantly increased in patients with type II diabetes compared to controls (P < 0.001). Blood levels of CML-HSP were significantly increased in diabetic patients compared to normal subjects [35.3 ± 27.4 and 9.3 ± 7.2 (mean ± SD) pmol/mg of protein, respectively; P < 0.0001]. In diabetic patients with retinopathy or microalbuminuria (urinary albumin excretion: UAE > 30 mg/24 h), CML-HSP levels were significantly higher (P < 0.02), and even more elevated in patients with both complications.

Conclusion: In patients with type II diabetes, CML-HSP levels that are at variance with the HbA1c index for blood glucose may be a biomarker of glycoxidation, and related to the development of microvascular complications.

Key-words: Advanced Glycation End Product · Carboxymethyl-Lysine · Microangiopathy · Type II Diabetes.

RéSUMÉ

La N(carboxyméthyl)lysine biomarqueur des complications microvasculaires du diabète de type 2

Objectif : Dans le diabète sucré, les atteintes vasculaires sont corré- lées avec l’hyperglycémie et/ou la formation, de produits de glycation avancée (AGE). Les résultats expérimentaux indiquent que les AGE participent au développement des lésions vasculaires. Nous avons exploré la possible corrélation entre les concentrations d’AGE ou le produit de glycoxydation, la (carboxyméthyl)lysine (CML) et le degré de l’atteinte vasculaire chez des patients atteints de diabète de type II.

Méthodes : Les taux sériques d’AGE et de CML associés aux protéines du sérum (AGE-HSP, CML-HSP) ont été mesurés par une technique immunologique (ELISA) chez 51 hommes et 26 femmes âgés de 58 ± 6.1 ans (moyenne ± écart type) qui avaient une ancienneté de diabète de 11 ± 8 ans et dans un groupe de sujets non diabétiques de 39 hommes et 21 femmes âgées de 55,5 ± 7,5 ans. Les patients ayant une macroalbuminurie ou une clairance de la créatinine anormale ont été exclus de l’étude.

Résultats : Les concentrations d’AGE sériques sont significativement plus élevées chez les patients diabétiques (p < 0.001). Les taux sanguins de CML-HSP sont très augmentés chez les patients comparés à ceux des sujets non diabétiques (35,3 ± 27,5 et 9,3 ± 7,2, p < 0,0001). Les patients diabétiques ayant une rétinopathie et une microalbuminurie ont un taux significativement plus élevé de CML-HSP (p < 0,02).

Conclusion : Les patients ayant un diabète de type II ont un taux de CML-HSP qui à la différence de l’HbA1c, index de l’hyperglycémie, peut être un marqueur biologique de la glycoxydation et est corrélé avec le développement des lésions microvasculaires.

Mots-clés : Produits de glycation avancée · (Carboxyméthyl)lysine · Microangiopathie · Diabète de type II.

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Glycemic control is considered a major factor in preventing vascular complications. Several clinical investigations have demonstrated a correlation between glycated hemoglobin levels and the development of vascular disease [1-3]. Recent findings have indicated the potential role of advanced glycation end products (AGEs) in the development of vascular dysfunction. First described as Maillard products, AGEs resulting from a reaction between the lysine or arginine free amino groups of proteins and carbohydrates appear to be a complex class of molecules including different products of glycoxidation but also of lipoxidation, usually detected by high performance liquid chromatography and mass spectrometry (MS) [4, 5]. At least two well defined molecules, carboxymethyl-lysine (CML) and pentosidine, are antigenic and can be identified or measured by techniques using specific antibodies [6, 7]. Only a few clinical investigations have been conducted to try to evaluate the relationship between CML protein blood levels and vascular dysfunction [8-10].

HbA1c is an intermediate product of glycoxidation and has been shown to be correlated with the severity of microvascular lesions, but the statistical significance of the correlation between macrovascular lesions and HbA1c is less evident [2]. In different studies, CML protein blood levels or local accumulation have suggested that AGEs may be implicated in the development of retinopathy and nephropathy. Experimental models have shown that the CML protein binds to the AGE receptor, RAGE, and alters endothelial cell functions in diabetic rats [11-14]. Binding of AGE to RAGE is responsible for the increase in vascular permeability. This interaction also results in endothelial cell activation, as demonstrated by vascular cell adhesion molecule (VCAM-1) induction, cytokine production and tissue factor expression [12, 15, 16].

In the present work, we performed an immunoassay to measure blood levels of AGE-human serum protein (AGE-HSP) and a specific immunoassay for CML-human serum protein (CML-HSP) concentrations in a group of type II diabetic patients and in normoglycemic subjects.

### Patients and methods

#### Patients

After obtaining informed written consent, blood samples were taken from normoglycemic controls and patients with type II diabetes mellitus. The investigation protocol was approved by the ethics committees of our respective institutions.

Serum samples were obtained from 77 consecutive type II diabetic patients and 60 age-matched non-diabetic subjects with no vascular complications. The clinical characteristics of these subjects have been included in Tables I and II.

Control subjects were recruited from the medical staff of the Lariboisiere hospital and from blood donors. The patients with type II diabetes were hospitalized for 1 to 5 days for a reason that was unconnected with our study (annual checkup). The usual treatment was administered, i.e. oral antidiabetics or insulin, antihypertensive drugs, and in some cases, antithrombotic treatment. Patients with type I diabetes or abnormal creatinine clearance or urinary albumin excretion (UAE) above 300 mg/24 h were excluded from the study.

#### Table I

Characteristics of non diabetic subjects and of patients with type II diabetes.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Diabetic subjects (n = 77)</th>
<th>Non diabetic subjects (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>58±6.1</td>
<td>55.5±7.5</td>
</tr>
<tr>
<td>Male</td>
<td>51</td>
<td>39</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.2±4.5</td>
<td>23±2.1</td>
</tr>
<tr>
<td>Duration of diabetes (yr)</td>
<td>11±8</td>
<td>0</td>
</tr>
<tr>
<td>Current smoking</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Glycemia (mmol/L)</td>
<td>9.2±0.4</td>
<td>4.3±0.03</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.5±1.8</td>
<td>5.2±0.1</td>
</tr>
<tr>
<td>Hyperlipidemia*</td>
<td>45</td>
<td>6</td>
</tr>
</tbody>
</table>

| Treatment                        |                           |                               |
| Insulin                          | 20                        | 0                             |
| Oral agents                      | 40                        | 0                             |
| Insulin + oral agents            | 10                        | 0                             |
| Diet                             | 7                         | 8                             |
| β-Blockers                       | 19                        | 1                             |
| Calcium antagonists              | 23                        | 0                             |
| ACE inhibitors                   | 21                        | 0                             |
| Lipid-lowering drugs             | 29                        | 3                             |
| Antithrombotic drugs             | 16                        | 0                             |

* LDL cholesterol > 160 mg/dL associated or not with triglycerides > 200 mg/dL.
All patients underwent a complete ophthalmologic examination, including assessment of best-corrected visual acuity, slit-lamp biomicroscopy, direct ophthalmoscopy through dilated pupils and fundus photography. The retinopathy was classified as follows: absence (score 10 — NDR), diabetic retinopathy (DR) and proliferative diabetic retinopathy (PDR). DR (score 20-53) was defined by the presence of neovascularization, vitreous hemorrhage or fibrous proliferation [17]. In the group of diabetic patients 18 had simultaneously a retinopathy and a microalbuminuria. Macrovessel disease was defined as coronary heart disease, stroke, or peripheral vascular disease (Tab II).

Clinical examination was performed as previously described [18]. HbA1c was measured using variant II (Biorad-France, Marnes la Coquette, France); creatinine clearance and UAE were routinely determined [19].

<table>
<thead>
<tr>
<th>Micro- and macrovascular lesions</th>
<th>Diabetic subjects (n = 77)</th>
<th>Non diabetic subjects (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>137.2±2.1</td>
<td>129±2.5</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>75±11.6</td>
<td>79±10</td>
</tr>
<tr>
<td>Stroke</td>
<td>3</td>
<td>0</td>
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<tr>
<td>Atherosclerotic lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid artery</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Peripheral vessels</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Coronary vessels</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Retinopathy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>49</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic retinopathy</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Urinary albumin excretion (mg/24 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 30</td>
<td>46</td>
<td>60</td>
</tr>
<tr>
<td>30-300</td>
<td>31</td>
<td>0</td>
</tr>
</tbody>
</table>

Diabetic patients with micro- and macrovascular lesions compared to non-diabetic subjects.

Preparation of glycated proteins

Preparation of AGE-HSA was carried out under sterile conditions by incubation of HSA (25 mg/ml) in PBS containing glucose 6-phosphate (G-6-P, 0.25 M), protease inhibitors (pepsatin A, 0.1 µg/ml; leupeptin, 0.5 µg/ml; aprotinin, 2 µg/ml; and phenylmethyl-sulfonyl fluoride, 0.0015 M), EDTA (0.001 M), and sodium azide (0.001 M) at 37 °C for 60 days in the dark. Control HSA was prepared in an identical manner, except that G-6-P was omitted [20].

To prepare CML-HSA, HSA (50 mg/ml) and sodium cyanoborohydride (0.45 M) were dissolved in sodium phosphate buffer (0.2 M; pH 7.8) [21]. Glyoxylic acid was then added, and the mixture was incubated for 24 h at 37 °C. Control protein was prepared under the same conditions, except that glyoxylic acid was omitted. Preparation of CML-modified proteins was characterized by percentage modification, assessed both via 2, 4, 6-trinitrobenzenesulfonic acid to determine the difference between lysine residues in modified versus unmodified preparations [22] and by gas chromatography-mass spectrometry (GC-MS) [23].

After the incubation period, all glycated and control proteins were extensively dialyzed; the endotoxin levels in all protein preparations were < 3 pg/ml.

Preparation of polyclonal anti-AGE antibodies

Three hens were injected intra-muscularly with glycated proteins (glycated RNase, 500 µg per bird) in complete Freund’s adjuvant for the first injection and in incomplete adjuvant for the other injections at days 10, 20 and 50 (Agrobio, La Ferté St Aubin, France). IgY was prepared by differential polyethylene glycol precipitation using 6 to 8 egg yolks as starting material [24]. IgY was further purified by cryoalcohol treatment [25] and antibody specificity against AGE was assessed by immunoblotting, with a comparison of different glycated and non-glycated proteins [HSA, bovine serum albumin (BSA), ovalbumin, immunoglobulin, RNase] (Fig IA).

Separation of CML and non-CML AGE antibodies from polyclonal AGE antibodies

Antibodies specific for CML or non-CML were isolated from the antiserum by affinity chromatography [26]. A preparation of CML-HSA (10 mg) was coupled to 1 ml of HiTrap NHS-activated column according to the manufacturer’s instructions (Amersham Pharmacia, Orsay, France). Four milliliters of polyclonal anti-AGE (1.5 mg/ml) was then applied to the NHS-activated column coupled with CML-HSA. After extensive washing with PBS, the adsorbed fractions (anti-CML antibodies) were eluted with 0.1 M glycine/HCl buffer, pH 2.8 (Fig IB).

Enzyme-linked immunosorbent assay

Ligand inhibition and AGE measurements were performed by competitive ELISA, as described elsewhere [7] (Fig IC, D). Ninety-six-well microtiter plates were coated with AGA-HSA or CML-HSA (2 µg/well), and incubated for 18 h at 4 °C. After washing, the wells were blocked with PBS containing 0.2% BSA. After washing with PBS-Tween, 50 µl of competing antigen was added followed by 50 µl of antiserum (final dilution: 1/5,000). The plate was incubated for 2 h at room temperature with gentle shaking. Wells were then washed with PBS-Tween and peroxidase-conjugated
anti-IgY (Agrobio, La Ferté St Aubin, France; final dilution: 1/5,000; 100 µl/well) was added.

Results were expressed as B/B0 × 100 calculated as (experimental OD – background OD)/(total OD – background OD). The immunoreactivity of each fraction was read from the calibration curve (AGE-HSA or CML-HSA), and was expressed as AGE units (U/ml) with 1 U corresponding to the amount of antibody-reactive material found in AGE-HSA at a protein concentration of 1 µg/ml and for CML as pmol CML/mg of protein. The intra-assay CV was 7 ± 2%, and the inter-assay CV was 13 ± 2%. To explore whether other protein than albumin can account for CML-proteins, we have separated the proteins according to their molecular weight by filtration (Centricon 10, AMICON Beverly MA, USA) as previously described [27]. The sera of 5 normal and 5 diabetic subjects were filtered and CML was measured in the sample before filtration and in the filtrate. In 3 out of 5 sera from normal subjects no CML was detected in the filtrate. In 4 of the 5 diabetic sera detectable amount of CML was found. The percentage of low molecular weight CML proteins when present in the filtrate varied between 2 and 25% of the total CML serum proteins measured by ELISA.

Statistical analysis

The results are presented either as individual values, or as mean (SD, SEM, or median and percentile (10-90%, 25-75%, and external values). The statistical analysis was performed using one-way ANOVA followed by the parametric Dunnett’s test, or by the non-parametric Mann-Whitney test. The Pearson coefficient was used as a measure of linear

Figure 1
1A) Immunoblotting of anti-AGE antibodies. Lanes 1, 2, 3: CML-ovalbumin (5, 2.5, 1 µg respectively), lanes 4, 5, 6: ovalbumin (5, 2.5, 1 µg respectively), lane 7: AGE-BSA; lane 8: BSA. Immunopurification of anti-CML antibodies.
1B) Immunoreactivity of anti-CML protein antibodies for CML proteins and structurally related compounds.
1C) The immunoreactivity of CML antibodies with HSA, AGE-HSA, CML-HSA, L-arginine (L-Arg), DL-alanine (DL-Ala), (carboxyethyl) L-arginine (CE-L-Arg), and N-acetylysine (N-AcetylLys) was determined by competitive ELISA, as described in patients and methods.
1D) The immunoreactivity of CML antibodies with HSA, AGE-HSA, CML-HSA, L-arginine (L-Arg), DL-alanine (DL-Ala), (carboxyethyl) L-arginine (CE-L-Arg), and N-acetylysine (N-AcetylLys) was determined by competitive ELISA, as described in patients and methods.
association between two variables, and the Spearman correlation coefficients were calculated.

**Results**

**AGE blood levels**

As expected, HbA1c blood levels were significantly increased in diabetic patients compared to normal controls (8.5 ± 1.6% and 4.3 ± 0.2%, respectively; P < 0.001). Serum levels of AGE-HSP were also elevated in diabetic patients, and the differences compared to those of control subjects were highly significant (P < 0.001) (Fig 2A).

Serum levels of CML-HSP were increased in most but not all patients with type II diabetes. However, their CML-HSP blood levels were very different from those of normal controls (P < 0.0001) (Fig 2B). Serum levels of HbA1c were not correlated with those of AGE-HSP or CML-HSP, indicating that these molecules have a different metabolism and/or turnover rate (Fig 3A, B). Serum levels of CML-HSP were more elevated in patients with microalbuminuria (Fig 4A), and were 4- to 5-fold higher in patients with renal failure (not shown). Serum levels of CML-HSP may vary during the day, and could be influenced by the diet. In order to minimize these confounding factors, the samples were taken before breakfast, and we excluded patients with abnormal creatinine clearance and an UAE above 300 mg/24 h.

Serum levels of AGE-HSP and CML-HSP were not correlated with the subjects’ age or sex (results not shown), but the age range in this study did not allow a correct analysis of this variable. The duration of disease in the diabetic patient group did not affect the levels of AGE-HSP and CML-HSP.

**AGE and microangiopathy**

Diabetic patients with microalbuminuria had significantly higher levels of CML-HSP compared to patients with normal renal function (44.5 ± 5.4 and 28.4 ± 3.5 pmol/mg protein, respectively; P < 0.01) (Fig 4A). Serum levels of CML-HSP were higher in patients with retinal complications (P < 0.001) (Fig 4B).
Patients with no retinopathy and normal UAE had significantly lower CML-HSP levels than subjects with NDR and UAE in excess of 30 mg/24 h (26 ± 4.1 and 38 ± 2.9 pmol/mg protein, respectively; P < 0.02). Serum levels of CML-HSP were higher in patients with retinopathy and elevated UAE than in subjects with retinopathy and normal UAE (P = 0.05) (Fig 4C). These results suggest that CML-HSP is a potentially better marker for renal alteration than for retinopathy, but in patients with both complications CML-HSA levels are increased to a greater extent.

AGE and macroangiopathy

The group of diabetic patients with macrovascular complications (coronary heart disease, stroke, lower limb vascular disease) was relatively small (n = 23), and we found no significant difference in CML blood levels in the group of diabetic patients with macroangiopathy compared to the macroangiopathy-free group (39 ± 6.5 versus 34 ± 5.25 pmol/mg protein). However, patients with macrovascular complications affecting two different sites had higher CML blood levels.

We found no difference in AGE-HSP serum levels in relation to the type of macrovascular complication (coronary ischemia, stroke, peripheral vascular disease), but this study included only an assessment of clinical events and it is possible that a more sophisticated means of estimating atherosclerosis, such as the determination of arterial resistance or media thickening could lead to other conclusions.

Discussion

In type II diabetic patients, CML-HSP levels were found to be elevated. CML-HSP concentrations were increased in patients with microangiopathy compared to subjects without retinopathy or nephropathy. It has been observed that CML-HSP is a preferential RAGE ligand, and in animal experiments, the blockade of AGE-RAGE interaction corrected the vascular hyperpermeability observed in rats with streptozotocin-induced diabetes [15]. The correlation between CML-HSP and microvascular complications in diabetic patients further suggests a possible pathophysiological role for AGEs. The production of CML may be connected not only with the glycoxidation reaction, but could also originate from oxidative compounds. The modification regarding CML formation in proteins is irreversible and may be due to AGE activity, as can be demonstrated by the fructose lysine model, but could also be induced by the metal-catalyzed oxidation of polyunsaturated fatty acids [10]. Specific and selective electrospray ionization MS has been utilized for the simultaneous measurement of different AGEs in human serum. With this technique, the authors found that AGE values in normal controls varied between 25 and 40 pmol/mg of protein according to the compound measured [23, 28]. Several studies have used ELISA to determine AGE and/or CML concentrations, but in only a few of them has CML been expressed in conventional units; arbitrary units are frequently used, but do not allow a direct comparison.
Figure 4

4A) CML-HSP serum levels in diabetic patients with normal UAE or with microalbuminuria (UAE: 30-300 mg/24 h).

4B) CML-HSP serum levels in controls, in diabetic patients with NDR and in subjects with DR and PDR.

4C) CML-HSP levels in patients with no microvascular complications, or in those with only one or two microvascular complications. The unbroken line within the box represents the median, the dotted line the mean. The vertical lines extending beyond the boxes indicate the 25% and 75% percentiles, while the horizontal bars outside the boxes represent the 10% and 90% percentiles. The open circles indicate the values outside this range. The comparisons between control subjects and diabetic patients with retinopathy or microalbuminuria and between diabetic patients with no or two complications were statistically significant (P < 0.02).
comparison to be made. In a group of normal subjects, CML measured by ELISA varied between less than 1 to 12 pmol/mg of protein. In age- and sex-matched diabetic patients, the CML values varied between 6 and above 20 pmol/mg of protein. According to our results, values found in the control subjects were in the same range; but we observed higher values in the group of diabetic patients.

Non CML-AGEs as measured by ELISA are probably a heterogeneous class of molecules and the significance of their negative potential is difficult to determine. In our study, CML-HSP and HbA1c concentrations were both correlated with microangiopathy, but were independent variables. Controversial results have been reported concerning AGE blood levels and HbA1c [26, 27]. Another study reported that in the circulation, CML-HAS had a shorter half-life than hemoglobin present in the red blood cells (RBCs). Imidazole levels were increased in the erythrocytes of diabetic patients compared to normal subjects (8.07 ± 0.2 and 4.8 ± 0.2 arbitrary units, respectively) but were not correlated either with HbA1c or serum fructosamine levels [29]. Since RBC adhesion to the endothelium is increased in diabetic patients, mediated by RAGE present on the endothelium and is correlated to HbA1c levels, it can be speculated that AGEs may have a deleterious effect on the microvessels, either by their presence on RBCs or on plasma proteins [16, 30]. A possible role for CML adducts is also supported by the presence of CML protein in excess in the eyes and kidneys of patients with diabetic retinopathy or nephropathy respectively [31–34].

In this study, we provide evidence that CML-HSP, an AGE-adduct, is correlated with microvascular complications and we know from in vitro studies and from animal experiments that it can induce microvascular complications [35]. Oxidatively formed CML is a regular constituent present in the retina of non-diabetic subjects, but increased levels are found both in the neuroglial and vascular components of diabetic patients. The prevalence of the allele 249C of the RAGE gene is significantly increased in patients with type II diabetes and retinopathy [36], further indicating the potential role for CML, the high-affinity ligand to RAGE, in the development of the latter. In addition to RAGE, other molecules, such as galectin 3 (AGE-R3), may act as receptors for AGEs. Animals deficient in AGE-R3 (knockout mice) develop an accelerated diabetic glomerulopathy, which indicates that when AGE removal is reduced, microvascular injury is amplified [37]. In atherosclerosis, CML staining by immuno-histochemical techniques is enhanced in areas of intimal fibrosis and atheromatous plaque formation when compared to the vascular segment, where less severe alterations are observed [38]. However, atherosclerotic lesions in diabetics are similar to those observed in non-diabetic subjects. CML-HSP was increased in patients with macroangiopathy but was not statistically different from those free of this complication. CML-HSP blood level as other glycated serum proteins was not dependent of age but CML accumulates in aorta, tendon human skin collagen and human lens protein. The low turnover of tissue AGE may explain that a transversal study of CML-HSP level was not directly correlated to macrovascular disease. In addition, antiatherosclerotic treatment may have influenced the development of macrovascular lesions. The role of AGEs in macrovascular complications is evidenced by genetically modified diabetic models (ApoE-null-diabetic mice), since these animals develop complex atherosclerotic lesions which can be prevented by intraperitoneal injection of recombinant soluble RAGE [14]. In the development of nephropathy, AGE-RAGE interaction appears to be crucial, as shown by the accelerated progression of the lesions observed in mice over-expressing RAGE compared to their littermates [13]. The identification of individual AGE products has led to the characterization of chemically defined products such as the fluorescent compounds pentosidine and pyralline, N(carboxymethyl)lysine and erythronic acid result from the cleavage of fructolysine [34]. Since the formation of CML modifications in proteins is irreversible, it has been suggested that CML may be an integrative biomarker for the accumulated oxidative stress the proteins had been exposed. The CML-HSP could be an interesting marker for glycoxidation and more directly related to microangiopathy via a receptor mediated mechanism [11].

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References