Original Article

Study of Aberrant Modifications in Peptides as a Test Bench to Investigate the Immunological Response to Non-Enzymatic Glycation

(protein glycation / immunological response / peptide / β-turn peptide structure / type 1 diabetes / SP-ELISA)

F. NUTI[1], A. GALLO[2], F. REAL-FERNANDEZ[1], C. RENTIER[1,3], G. ROSSI[4,5], F. PIARULLI[2], P. TRALDI[6], S. CARGANICO[1,5], P. ROVERO[4], A. LAPOLLA[2]*, A. M. PAPINI[1,3]*

[1]Laboratory of Peptide and Protein Chemistry and Biology, Department of Chemistry „Ugo Schiff", University of Florence, Sesto Fiorentino, Italy
[2]Diabetology and Dietetics, Department of Medicine (DIMED), University of Padova, Padova, Italy
[3]PeptLab@UCP Platform and Laboratory of Chemical Biology EA4505, University Paris-Seine, Cergy-Pontoise CEDEX, France
[4]Laboratory of Peptide and Protein Chemistry and Biology, Department of NeuroFarBa, University of Florence, Sesto Fiorentino, Italy
[5]Pharma Quality Europe PQE SrL, Località Prulli, Firenze, Italy
[6]Istituto di Ricerca Pediatrica, Città della Speranza, Padova, Italy

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Corresponding authors*: Anna Maria Papini, Laboratory of Peptide and Protein Chemistry and Biology – PeptLab, Department of Chemistry "Ugo Schiff", University of Florence, Via della Lastruccia 13, I-50019 Sesto Fiorentino, Italy. Phone: (+39) 055 457 3561; e-mail: annamaria.papini@unifi.it – Annunziata Lapolla, Diabetology and Dietetics, Department of Medicine (DIMED), University of Padova, Padova, Italy. E-mail: annunziata.lapolla@unipd.it

Abbreviations: 3DG – 3-deoxyglucosone, AGEs – advanced glycation end products, CML – carboxymethyl lysine, CRA – chemical reverse approach, ΔM – mass difference, deoxyFru – 1-deoxyfructopyranosyl moiety, DIC – *N,N′*-diisopropylcarbodiimide, FBS – foetal bovine serum, FL – N^{ϵ}-fructosyl lysine, FPG – fasting plasma glucose, G – glyoxal, HATU – 1-[bis(dimethylamino) methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate, HbA_{1c} – haemoglobin, HAS – human serum albumin, IFCC – International Federation of Clinical Chemistry, LC – liquid chromatography, MALDI – matrix-assisted laser desorption/ionization, MG – methylglyoxal, MG-DH – dihydroxyimidazolidine, MG-HI – hydroimidazolone, MW-SPPS – microwave-assisted solid-phase peptide synthesis, NBD – non-diabetic subject, NMM – *N*-methylmorpholine, PDC-E2 – pyruvate dehydrogenase complex of the E2 component, PTM – post-translational modification, RDCs – dicarbonyls, RIA – radioimmunoassay, SP-ELISA – solid-phase Enzyme-Linked ImmunoSorbent Assays, TD1 – type 1 diabetic, TIS – triisopropyl silane.

Abstract. A side effect of diabetes is formation of glycated proteins and, from them, production of advanced early glycation end products that could determine aberrant immune responses at the systemic level. We investigated a relevant aberrant post-translational modification (PTM) in diabetes based on synthetic peptides modified on the lysine side chain residues with 1-deoxyfructopyranosyl moiety as a possible modification related to glycation. The PTM peptides were used as molecular probes for detection of possible specific autoantibodies developed by diabetic patients. The PDC-E2(167-186) sequence from the pyruvate dehydrogenase complex was selected and tested as a candidate peptide for antibody detection. The structure-based designed type I' β-turn CSF114 peptide was also used as a synthetic scaffold. Twenty-seven consecutive type 1 diabetic patients and 29 healthy controls were recruited for the study. In principle, the 'chemical reverse approach', based on the use of patient sera to screen the synthetic modified peptides, leads to the identification of specific probes able to characterize highly specific autoantibodies as disease biomarkers of autoimmune disorders. Quite surprisingly, both peptides modified with the (1-deoxyfructosyl)-lysine did not lead to significant results. Both IgG and IgM differences between the two populations were not significant. These data can be rationalized considering that i) IgGs in diabetic subjects exhibit a high degree of glycation, leading to decreased functionality; ii) IgGs in diabetic subjects exhibit a privileged response vs proteins

containing advanced glycation products (e.g., methylglyoxal, glyoxal, glucosone, hydroimidazolone, dihydroxyimidazolidine) and only a minor one with respect to (1-deoxyfructosyl)-lysine.

Introduction

Chemical modifications of proteins have to be considered as an important aspect in the pathogenesis of a series of diseases, among which diabetes is the most relevant one. In these pathological conditions, an increase of yield of the reaction between glucose and protein amino functions (e.g., *N*-terminal function, lysine, and arginine side-chains), following the pathway originally described by Maillard (1992), is observed. After the sugar is added to the protein, a Schiff base is formed, which in turn rearranges itself, leading to the so-called Amadori product (fructosamine). It is important to emphasize that all the species present in the reaction pathway (sugar, Schiff base, fructosamine) are to be considered responsible as the starting point for production of a wide number of α-oxoaldehydes called advanced glycation end products (AGEs).

Recently, Ahmed and Thornalley (2007) discussed the relevance of AGEs in diabetic complications and they compared the traditional view and new insights into the protein glycation in diabetes, showing that a different panorama has now to be considered (see Table 1). In general, the predominant early glycation adduct is N^{ϵ}-fructosyl-lysine (FL), which degrades slowly to form many different AGEs (Thornalley, 2005), mainly dicarbonyls (RDCs), glyoxal (G), methylglyoxal (MG), and 3-deoxyglucosone (3DG), which are potent glycation agents (as an example, MG is 20,000 more reactive than glucose!). MG primarily reacts with arginine residues to form neutral ring structures: dihydroxyimidazolidine (MG-DH) and hydroimidazolone (MG-HI). These aspects must be necessarily considered in studies related to the monitoring of glycation processes.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Lapolla et al., 2006) was proved to be a valid analytical tool to evaluate the glycation level of circulating proteins. In fact, considering that each glucose molecule linked to a protein leads to a mass increase of 162 Da, the mass difference (ΔM) between the mass values of glycated and unglycated proteins divided by 162 (the molecular mass, in Dalton, of dehydrated glucose) gives directly an evaluation of the number of glucose molecules condensed on the protein (Lapolla et al., 2011). Considering the above-reported view of Ahmed and Thornalley (2007), this number is to be considered a lower limit: in fact, dehydration and oxidation processes present in the Maillard reaction pattern lead to species linked to the protein of lower mass. Furthermore, the same protein can be modified not by reaction with glucose, but also by its above-described reaction with GO and MGO on arginine. The effectiveness of the MALDI approach in the study of proteins modified *in vivo* by reaction with sugars and AGEs was proved by a series of investigations based on the comparison of circulating proteins in healthy subjects and diabetic patients, proving that, as an example, human serum albumin (HSA) is widely glycated in diabetic patients (Lapolla et al., 2011), as shown by a clear mass increase in the case of diabetic patients. This aspect is certainly relevant considering that HSA is the most abundant transport protein and that glycation can somehow inhibit the binding sites responsible for the transport of biologically relevant molecules. But is this the only relevant behaviour?

Alternatively to the MALDI approach, a further view can be considered from both biochemical and biological aspects: the development of an aberrant immune response against the modified proteins originated from the above-described glycation processes could be activated at the systemic level. Then, a study based on the use of synthetic peptides specifically modified on the lysine side chain residues with different biologically relevant aberrant post-translational modifications (PTMs) was undertaken to investigate these aspects.

The aberrant PTM peptides were used as molecular probes for detection of possible specific autoantibodies developed by diabetic patients and, to a lower extent, by control subjects.

Table 1. The relevance of AGEs in diabetic complications

Traditional view of glycation
Glucose-driven reaction forms Amadori products. Advanced glycation end products (AGEs) are formed slowly in post-Amadori pathways under physiological conditions. Chemically stable AGEs accumulate on extracellular proteins. Chemically stable AGEs on long-lived proteins accumulate progressively throughout lifespan. AGEs are lysine residue-derived adducts.
New insights of physiological glycation
AGEs are formed from α-oxoaldehydes produced by the degradation of glycolytic intermediates, lipid peroxidation and Schiff base fragmentation. α-Oxoaldehydes are arginine-directed glycating agents, forming hydroimidazolones – quantitatively important AGEs with relatively short half-lives (12–60 days). Cellular proteins are modified significantly by AGEs – particularly renal glomeruli, retina, and peripheral nerve in diabetes. AGE-modified proteins are degraded by cellular proteolysis to AGE free adducts and excreted in the urine. They accumulate markedly in diabetes and in uraemia. AGEs are lysine residue-derived adducts.

Due to our interest in diabetes, from both medical and analytical point of view (Szymanski and Wren, 2005; Lapolla et al., 2013), we initially focused our attention on glycation. Because of the demonstrated role of mitochondrial machinery in diabetes, the sequence and chemical modification of the E2 component of pyruvate dehydrogenase complex PDC-E2 was considered as an interesting candidate antigen (Van de Water et al., 1988). In fact, this protein plays a key role in the energy metabolism of cells. In particular, PDC-E2 is involved in the oxidative decarboxylation of pyruvates linking the glycolysis metabolic pathway to the citric acid cycle. This aspect was proved during the characterization of the immunodominant epitope of anti-mitochondrial antibodies in a different autoimmune disease, primary biliary cirrhosis, leading to the discovery of the highly conserved domain surrounding a lipoyl-lysine residue in position 173 (K^{173}) within the hPDC-E2(167-186) fragment (Reche and Perham, 1999). In fact, the amino acid linear peptide A^{167}EIETDK^{173}ATIGFEVQEEGYL^{186} is the epitope of the 74-kD mitochondrial autoantigen of primary biliary cirrhosis and corresponds to the functional site of dihydrolipoamide acetyltransferase (Wallis and Perham, 1994). Therefore, K^{173} in the PDC-E2 (167–186) peptide sequence was considered to be an interesting site to investigate for possible aberrant chemical modifications triggering an immune response in diabetes (Fig. 1A).

Consequently, the PDC-E2(167-186) sequence was selected and tested as a candidate peptide for antibody detection because of its strong involvement at the mitochondrial level in the energy cycle of cells and glucose metabolism. On the other hand, the structure-based designed type I' β-turn peptide sequence CSF114 was also used as a synthetic scaffold (Lolli et al., 2005b). Its optimized β-turn conformation was previously developed to maximize the exposure of different PTMs to the external media, in order to assess the hypothetical specificity of the peptide sequence and/or of the chemical modifications introduced towards serum autoantibodies (Carotenuto et al., 2006, 2008; Nuti et al. 2010). Therefore, the type I' β-turn peptide CSF114 sequence was the one selected to assess the role of the specific PDC-E2 (167–186) sequence or of the secondary structure in Ag-Ab recognition in diabetes (Fig. 1B).

Specifically, we decided to investigate the 1-deoxyfructosyl moiety (deoxyFru) as a possible aberrant PTM related to glycation (Fig. 1). DeoxyFru is an intermediate of the Maillard reaction pattern, responsible for production of a high number of α-oxoaldehydes (dicarbonyls (RDCs), glyoxal (G), methylglyoxal (MG), and 3-deoxyglucosone (3DG); however, it degrades slowly (Thornalley, 2005) and consequently could be in principle considered a valid target. The selected peptides were tested in a cohort of type 1 diabetic patient (TD1) sera and non-diabetic subject sera as controls (NBD) to assess their usefulness as molecular probes for the detection of Abs in the sera of different subjects.

Material and Methods

Subjects

Twenty-seven (27) consecutive type 1 diabetic patients (TD1), regularly attending the Diabetology and Dietetics outpatients Clinic of Padova University, and 29 healthy controls (NBD) were recruited for the study. TD1 subjects were treated with insulin (four injections/day). In the morning, after a 12 h overnight fast, blood samples were taken to determine fasting plasma glucose (FPG), HbA_{1c}, and autoantibody levels. After collection, blood was immediately centrifuged at 1,700 *g* and +4 °C for 20 min. Glucose was measured immediately and the rest of the sample was frozen to –80 °C until analysis.

The study protocol complied with the Helsinki Declaration and written informed consent was obtained from all subjects before their participation in the study.

MW-assisted solid phase peptide synthesis

All Fmoc-protected amino acids, Fmoc-Wang resins, DIC (*N,N'*-diisopropylcarbodiimide), and Oxyma were purchased from Iris Biotech GmbH (Marktredwitz,

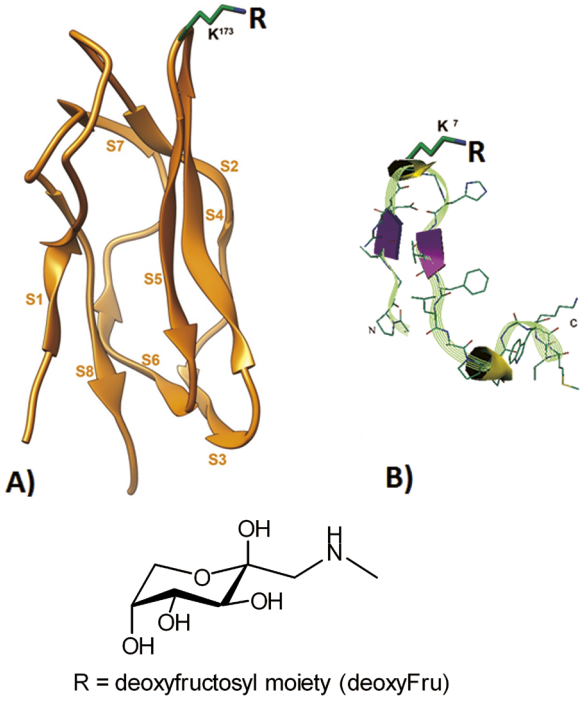

Fig. 1. (**A**) NMR structure of the apo form of PDC-E2 (pdb code 1FYC, orange ribbon) (Pacini et al., 2015). Only Lys^{173} side chain is reported for clarity. Strand numbering of the original work is reported as S1-S8. Structures are shown with the same spatial orientation. (**B**) Hypothetical in-solution structure of the synthetic deoxyfructosylated type I' β-turn peptide CSF114 (Lolli et al., (2005b).
***K^{173}**: lysine: modification site with the deoxyfructosyl moiety **R**; ***K^{7}**: lysine modification site with the deoxyfructosyl moiety **R**.

Germany). Fmoc-Lys(Boc) (2,3:4,5-di-*O*-isopropylidene-1-deoxyfructopyranosyl)-OH was prepared as previously described (Carganico et al., 2009).

[(DeoxyFru)Lys^{173}]PDC-E2(167-186)-KKKK (**1**), [(deoxyFru)Lys^{7}]CSF114 (**2**) were synthesized by microwave-assisted solid-phase peptide synthesis (MW-SPPS) following the Fmoc/tBu strategy, using the Liberty Blue™ automated microwave peptide synthesizer (CEM Corporation, Matthews, NC) (Nuti et al., 2010). The resin used was Fmoc-Lys(Boc)-Wang (loading 0.31 mmol/g). Fmoc-Lys(Boc)(2,3:4,5-di-*O*-isopropylidene-1-deoxyfructopyranosyl)-OH (2.5 equiv.) was coupled for 40 min at room temperature (r.t.) using HATU (2.5 equiv.) and NMM (3.5 equiv.) in DMF. Micro-cleavages, to monitor couplings, were performed with a microwave apparatus CEM Discover™ single-mode MW reactor (CEM Corporation). Final cleavage was performed using a mixture of TFA/TIS/H_2O (95 : 2.5 : 2.5 v : v : v) for 6 h at r.t.

Purification of the synthetic peptides was performed by semi-preparative RP-HPLC in a Waters instrument (Separation Module 2695, detector diode array 2996) using a Phenomenex (Torrance, CA) Jupiter column C18 (10 µm, 250 × 10 mm) at 4 ml/min with solvent systems A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). Characterization of the peptides was performed by analytical UPLC, using a Waters ACQUITY UPLC (Waters Corporation, Milford, MA) coupled to a single quadrupole ESI-MS (Waters 3100 Mass Detector) using a BEH C18 (1.7 µm, 2.1 × 50 mm) column at 35 °C, at 0.6 ml/min with solvent systems A (0.1% TFA in H_2O) and B (0.1% TFA in CH_3CN). The peptides were purified by semi-preparative RP-HPLC and characterized by RP-HPLC ESI-MS, obtaining a final purity ≥ 98 % (Table 2).

Immunoassays

Immunoassays, to detect IgM or IgG in the sera, were performed by solid-phase Enzyme-Linked Immuno-Sorbent Assays (SP-ELISA), coating the synthetic peptides on 96-well plates Nunc Maxisorp® (Sigma-Aldrich, Milano, Italy). Washing steps were executed with an automatic Hydroflex microplate washer (Tecan Italia, Milano, Italy). Anti-human IgG-alkaline phosphatase conjugates were purchased from Sigma-Aldrich and anti-human IgM-alkaline phosphatase were purchased from Invitrogen (Life Technologies, Monza, Italy). *p*-Nitrophenyl phosphate was purchased from Sigma-Aldrich. Absorbance values were measured in a Sunrise Tecan ELISA plate reader purchased from Tecan.

Activated polystyrene 96-well ELISA plates were coated with 100 µl/well of 1 : 100 diluted synthetic peptides, independently, in 0.05 M pure carbonate buffer (pH 9.6). After overnight incubation at 4 °C, plates were washed three times using washing buffer (0.9% NaCl and 0.05% Tween 20 in Milli-Q water). Nonspecific binding sites were blocked with 100 µl/well of foetal bovine serum (FBS) buffer (10% FBS in washing buffer) at r. t. for 1 h. FBS buffer was removed and plates were incubated overnight with the sera (100 µl/well, diluted 1 : 100 in FBS buffer) at +4 °C. After three washes, plates were treated with 100 µl/well of anti-human IgG or anti-human IgM alkaline phosphatase-conjugated antibody diluted in FBS buffer (1 : 8,000 for anti-human IgG, 1 : 1,200 for anti-human IgM). After 3 h of incubation at r. t. and three washes, 100 µl of substrate buffer (pNPP 1 mg/ml in alkaline substrate buffer pH 9.8, consisting of 10% diethanolamine and 0.1% $MgCl_2$ in Milli-Q water) was added to each well. After 15–30 min incubation at r. t., the absorbance of each plate was read in a multichannel ELISA reader at 405 nm (ref 620 nm). ELISA plates, coating conditions, reagent dilutions, buffers, and incubation times were tested in preliminary experiments. All buffers were brought to room temperature prior to use and filtered through 0.22 µm filters (except FBS buffer). The antibody levels are expressed as absorbance in arbitrary units at 405 nm.

Basic blood chemistry parameters

HbA_{1c} was measured by high-performance liquid chromatography (HPLC, Menarini Akray ADAM A1c HA-8180 v), in line with IFCC standards (International Federation of Clinical Chemistry) (Mosca et al., 2013). Plasma glucose was measured with a glucose-oxidase method (McMillin, 1990).

Statistical analysis

Data are expressed as mean ± SD. Student's t-test for unpaired data was used to compare continuous variables between the groups. Least-square linear regression analysis was used to test any relationship between pairs of variables. Differences were deemed statistically significant when $P < 0.05$ (two-tailored test).

Table 2. Analytical data of the synthetic peptides

N°	Synthetic peptides	Semi-prep HPLC gradient	UPLC/HPLC (R_t, min)	ESI-MS $[M+3H]^{3+}$ (m/z) found (calculated)
1	[(deoxyFru)Lys^{173}]PDC-E2(167-186)-KKKK	20–35% B in 30 min	1.80[a,b]	973.6 (2918.3)
2	[(deoxyFru)Lys^{7}]CSF114	25–40% B in 30 min	3.99[a,c]	874.8 (2620.4)

[a] UPLC performed in Acquity system with 3100 mass detector (Waters); column: BEH C_{18} 1.7 µm, 2.1 × 50 mm (Waters); eluents: A: 0.1% TFA in H_2O, B: 0.1% TFA in CH_3CN; flow: 0,6 ml/min.
[b] Gradient 10–50% B for 3 min. [c] Gradient 20–70% B for 3 min.

Table 3. Clinical characteristics of the subjects under study

	Type 1 diabetes	NBD
Number	**27**	29
Age (years)	42.0 ± 10.0	41.0 ± 3.0†
Disease duration (years)	10.0 ± 8.0	–
BMI	25.0 ± 0.8	24.0 ± 1.5†
Fasting plasma glucose (mg/dl)	136.0 ± 30.0	83.5 ± 4.8*
HbA_{1c} (%)	7.3 ± 1.8	5.2 ± 0.2*

* P < 0.05 1 vs 2; † P = 0.05 1 vs 2

Results

The clinical and metabolic characteristics of the patients under study are reported in Table 3 showing that diabetic patients have significantly higher values of fasting plasma glucose and HbA_{1c} with respect to the controls.

Sera from 27 TD1 and 29 NBD subjects were tested by SP-ELISA using the synthetic peptides **1** and **2**. The applied ELISA protocol for detection of IgM and IgG antibodies against [(deoxyFru)Lys^{173}]PDC-E2(167-186)-KKKK and [(deoxyFru)Lys^{7}]CSF114 antigens was previously developed and applied routinely in our laboratory for [Lys^{173}]PDC-E2(167-186)-KKKK and [Lys^{7}]CSF114 antigens (Lolli et al 2005a; Pacini et al. 2015; Nuti et al. 2018). The obtained IgM and IgG data distribution is reported in Fig. 2.

The chemical-physical properties of the peptide antigen do not change by modifying Lys in position 17 of the CSF114 sequence or in position 173 of PDC-E2(167-186)-KKKK.

The synthetic peptides **1** and **2** failed to detect specific antibodies against non-enzymatic glycation products. The mean values (± SD) of IgG were very low (TD1-**1**: 0.02 ± 0.02; TD1-**2**: 0.03 ± 0.06 AU; (NBD-**1**: 0.04 ± 0.02; NBD-**2**: 0.08 ± 0.12 AU). IgM values were slightly higher than the IgG values in both TD1 (TD1-**1**: 0.21 ± 0.11; TD1-**2**: 0.22 ± 0.14 AU) and NBD subjects (NBD-**1**: 0.19 ± 0.11; NBD-**2**: 0.17 ± 0.1 AU), further evidencing no meaningful differences between the two groups, thus allowing us to assume that the 1-deoxyfructosyl moiety (deoxyFru) as a possible aberrant PTM is irrelevant.

No relationships were found between autoantibody levels (either IgG and IgM), fasting plasma glucose, HbA_{1c}, and duration of the disease in the patients evaluated.

Discussion

It has been widely reported that hyperglycaemia can lead to the formation of advanced glycation end products (AGEs), which are part of the normal metabolism, but when expressed in excess (as in the case of hyperglycaemia), become pathological for tissues, altering the cellular structure and function and promoting oxidative stress (Brownlee et al. 1984; Ulrich and Cerami, 2001). Therefore, it appears relevant to investigate the PTMs of proteins possibly present in hyperglycaemia. It has been reported in the literature that humans and animals generate antibodies to modified forms of LDL, either oxidized or glycated (Lopes-Virella et al., 1997, 2007). Both IgG and IgM antibodies have been detected in humans. IgG isolated from the sera of diabetic patients and healthy controls reacts with oxidized human LDL *in vitro*, forming immune complexes (Virella et al., 2008).

In the case of AGE-modified LDL, the main modification recognized by these antibodies was carboxymethyl lysine. As described in the introduction, glycation

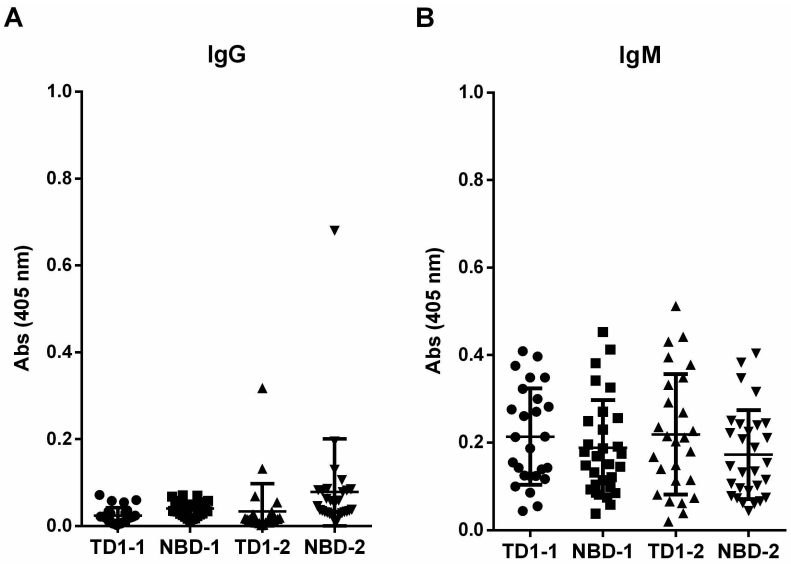

Fig. 2. Total IgG (**A**) and total IgM (**B**) titres in 27 TD1 and 29 NBD subjects, detected by SP-ELISA using the synthetic deoxyfructosylated antigens: [(deoxyFru)Lys^{173}]PDC-E2(167-186)-KKKK (**1**) and [(deoxyFru)Lys^{7}]CSF114 (**2**).

of proteins occurs through spontaneous and reversible condensation of a reducing sugar on a free amino group of a protein, to form a Schiff base. This then undergoes rearrangement into a more stable ketoamine, also known as the Amadori product. In the case of glucose, the initially formed Schiff base rearranges into the more stable 1-deoxyfructosyl moiety (see lower part of Fig. 1). Subsequent dehydration, fragmentation, oxidation, and cyclization reactions lead to the irreversible formation and release of AGEs (Brownlee et al. 1984; Ulrich and Cerami, 2001). Different methods have been utilized until now to estimate the amount of glycation products, e.g., spectroscopic methods (Maillard, 1912; Lapolla et al., 2005), showing however severe limitations in the evaluation of the general level of glycation and unable to identify the structure of the glycated protein. Recently, more accurate methods have been employed, such as liquid chromatography (LC), radioimmunoassay (RIA), and mass spectrometry (MALDI, ESI/MS, LC/MS) (Brownlee et al. 1984; Lapolla et al. 2005, 2006, 2011, 2013, Szymanski and Wren, 2005; Vlassara, and Uribarri, 2014). In this context, methods able to evaluate specific antibodies against non-enzymatic glycation products represent a challenge, especially when these antibodies exhibit high specificity.

We previously proposed to use peptides as synthetic antigens for efficient detection, including the aforementioned chemical modifications in the strategic positions in selected sequences. In particular, we employed CSF114 as an optimally designed β-turn conformation, and the PDC-E2(167-186) fragment, which is a mitochondrial protein component of the pyruvate dehydrogenase complex (Lolli et al 2005b; Pacini et al. 2015; Nuti et al. 2018).

The relevant part of the antigen derived from the native protein sequence and reproducing specific epitopes, or mimicking immunogenic modifications, has been synthetically reproduced. This approach can be even more effective and specific than using the native proteins to detect antibodies. In fact, its principal advantage is the complete control of the synthetic molecules, either of the sequence or of the insertion of the correct aberrant PTM at a suitable position. The folding issue can also be partly reproduced: in fact, synthetic peptides, through sequence optimization, can adopt specific conformations, e.g., β-turns. This approach could have some limits due to the choice of the aberrant PTM peptides that surely can exhibit only one structural change and that, anyway, unlikely reproduce the 3D structure and distribution of the charged peptides around the modified lysine groups in the actual protein. As an example, this view is supported by literature data (Virella et al., 2005) proving that there is very little cross-reactivity of isolated human antibodies to AGE-LDL with CML-modified human serum albumin. However, following this view, we synthesized peptides **1** and **2** (Table 2) that were tested by SP-ELISA of type-1 diabetes sera and NBD sera as controls to assess their usefulness as molecular probes for detection of antibodies. We are conscious that in the case of glycation processes, the antibodies would be developed not only vs fructosyl-lysine, but also vs modified proteins originating by the reactions with GO and MGO described above, leading to HI and DHI species.

It has been previously successfully demonstrated that synthetic peptides exposing an aberrant PTM are valid tools to detect antibodies in autoimmune diseases, like in primary biliary cirrhosis and multiple sclerosis (Lolli et al., 2005a,b; Pacini et al., 2015; Walvoort et al., 2016; Nuti et al., 2018). These tools can be generated by the so-called 'chemical reverse approach' (CRA), based on the use of patient sera to screen focused libraries of synthetic modified peptides. These peptides can allow effective identification of specific probes for the characterization of autoimmune disorders. Considering that the antibodies can be directed to the protein domain exposing a PTM residue through a key-lock interaction, the CRA would be focused on: (a) identification of the side-chain modification involved in the pathogenetic mechanism of a specific autoimmune disease, by screening peptide libraries based on PTM diversity; (b) structure-activity relationship studies for the selection of the best peptide sequence by screening peptide libraries.

In the present investigation, the CSF114 β-turn structure was tested as a synthetic scaffold for its intrinsic characteristic of best exposing the (1-deoxyfructosyl)-lysine moiety as an aberrant modification.

Quite surprisingly, as shown by the data reported in Fig. 2, the relatively low titres observed for both antigens **1** and **2** indicate neither specificity nor any meaningful difference in antibody response (either IgM or IgG titre) between the patients and controls.

Then, at first sight, this result seems to indicate complete ineffectiveness of the CRA method in characterizing the immune response in diabetes. It must be stressed that this view is just addressed to the protein glycation phenomenon, and a different explanation can be given to rationalize these data. First of all, our results can be compared with those obtained in previous studies of antibody reactive AGEs (Turk et al., 2001) and to carboxymethyl lysine (CML), considered an enzymatic digestion product of glycated proteins (Vay et al., 2000). In the latter case, differences between patient and control sera were found. However, anti-CML IgGs in diabetic patient sera were unrelated to the disease duration and metabolic control (Vay et al., 2000). Moreover, lower anti-AGE antibody titres were observed in the diabetic patients vs controls. This unexpected result was justified by Turk et al. (2001) with formation of an *in vivo* immune complex exhibiting the effective key-lock interaction between the antibodies and antigen. In principle, these results give us an answer to the lack of reactivity observed in our case. However, alternatively to the Turk's proposal, a possible explanation can be related to structural changes of the antibodies more than of the antigen.

IgGs in diabetic subjects exhibit a high degree of glycation similarly as other circulating proteins, necessarily

leading to decreased functionality (Lapolla et al., 1997, 2000). The significant increase in IgG glycation levels has been studied (Lapolla et al., 1997, 2000). When applied to three different populations (healthy subjects, well controlled, and poorly controlled diabetic patients), MALDI mass spectrometry found mean ΔM values of 581 Da for healthy subjects, 1,128 Da for well controlled, and 2,556 Da for poorly controlled diabetic patients, corresponding respectively to at least 4, 7, and 16 glucose molecules condensed on the protein. Although MALDI MS can determine the mean total number of glucoses condensed on IgGs, this technology cannot provide information related to the changes in activity of the Fab and Fc portions. To investigate this aspect, *in vitro* experiments were carried out, proving that the Fab moiety is the most prone to glycation. In fact, enzymatic digestion and MALDI analysis of the digestion products proved the presence of 20 glucose molecules condensed on the Fab portion and 10 on the Fc moiety. These findings were also confirmed by computer molecular modelling. The solvent-accessible surfaces were calculated for each lysine residue, confirming that these are more exposed in the Fab moiety, and that consequently Fab fragments are more prone to the glucose attack, particularly in their light and heavy variable regions (VL and CL). These structural moieties are highly important in the process of molecular recognition in the key-lock interaction between antibody and antigen. It is reasonable to assume that the same behaviour is displayed by IgMs.

The above-described extensive glycation of immunoglobulins in the presence of high glucose levels necessarily leads to the molecular architecture different from the unglycated one, and consequently exhibiting different reactivity. This can be the rationale of the new data described here pertaining to the use of CRA in diabetes monitoring. The lack of antibody recognition by glycated peptides can be due to the ineffectiveness of glycated antibodies present during the diabetic disease.

A further explanation of the negative results obtained by CRA in the present study is that, differently to what is observed in primary biliary cirrhosis, in *in vivo* samples from diabetic subjects the DeoxyFru moiety could be a minor component due to its degradation leading to AGEs. In other words, it must be considered that IgGs in diabetic subjects could exhibit a privileged response vs proteins containing advanced glycation products (methylglyoxal, glyoxal, glucosone, hydroimidazolone, dihydroxyimidazolidine) and only a minor one with respect to (1-deoxyfructosyl)-lysine. Works are now in progress for the synthesis of HI- and DHI-containing model peptides to verify the validity of this hypothesis.

Conflict of interest

Authors declare no conflict of interest.

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