Glycation of human serum albumin impairs binding to the glucagon-like peptide-1 analogue liraglutide

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ABSTRACT

The long-acting glucagon-like peptide-1 analogue liraglutide has proven efficiency in the management of type 2 diabetes and also has beneficial effects on cardiovascular diseases. Liraglutide’s protracted action highly depends on its capacity to bind to albumin via its palmitic acid part. However, in diabetes, albumin can undergo glycation, resulting in impaired drug binding. Our objective in this study was to assess the impact of human serum albumin (HSA) glycation on liraglutide affinity. Using fluorine labeling of the drug and $^{19}$F NMR, we determined HSA affinity for liraglutide in two glycated albumin models. We either glycated HSA in vitro by incubation with glucose (G25- or G100-HSA) or methylglyoxal (MGO-HSA) or purified in vivo glycated HSA from the plasma of diabetic patients with poor glycemic control. Nonglycated commercial HSA (G0-HSA) and HSA purified from plasma of healthy individuals served as controls. We found that glycation decreases affinity for liraglutide by 7-fold for G100-HSA and by 5-fold for MGO-HSA compared with G0-HSA. A similarly reduced affinity was observed for HSA purified from diabetic individuals compared with HSA from healthy individuals. Our results reveal that glycation significantly impairs HSA affinity to liraglutide and confirm that glycation contributes to liraglutide’s variable therapeutic efficiency depending on diabetes stage. Because diabetes is a progressive disease, the effect of glycated albumin on liraglutide affinity found here is important to consider when diabetes is managed with this drug.

Diabetes is a metabolic disease that is widespread throughout the world, affecting more than 415 million adults in 2015 (1). Diabetes mellitus is characterised by chronic hyperglycemia. Various treatments can be administered, including rapid and long-acting insulin analogue injections, which are used to counteract insulin production deficiency. These insulin analogues include lispro, glargine or detemir. Detemir (Levemir®) is carried in the blood by reversible binding to human serum albumin (HSA) through its myristic acid grafted to LysB29 (2). More recently, another long-acting drug, a glucagon-like peptide 1 analogue called liraglutide, was commercialised as Victoza® for the treatment of type 2 diabetes. Like detemir, liraglutide binds to HSA via a fatty acid chain. Glucagon-like peptide-1 (GLP-1) is a 30-amino acid incretin hormone secreted by intestinal epithelial endocrine L-cells by the differential processing of proglucagon in response to postprandial hyperglycemia (3).

Liraglutide shares 97% homology with human GLP-1, which binds to and activates the GLP-1 receptor. It differs from endogenous GLP-1 by a one amino acid substitution (Lys34Arg) and by the addition of a palmitic acid grafted to Lys26 via a glutamate linker (Fig. 1a). Its slow absorption is allowed due to its transport by albumin as well as due to its auto-association into oligomers (4-6). Moreover, this GLP-1 analogue undergoes much slower degradation by the enzyme dipeptidyl peptidase-4 (DPP-4) than endogenous GLP-1, resulting in a long plasma half-life of 11 to 15 hours. Consequently, liraglutide requires only once-daily subcutaneous injection. Like native GLP-1, liraglutide triggers insulin secretion, inhibits postprandial glucagon secretion, retards gastric emptying and enhances satiety by acting on the brain (7,8).

Liraglutide is increasingly used for the treatment of type 2 diabetes due to its increased efficiency compared to other treatments in controlling and stabilising glucose plasma level and because of its capacity to reduce both cardiovascular risk and HbA1c (hemoglobin A1C) level (9-13).

Nonetheless, diabetes mellitus remains a progressive disease with gradual complications; therefore, treatment has to be closely adapted to patient state evolution. The action of liraglutide is highly dependent on its binding capacity to albumin and to its progressive delivery. This dependent therapeutic action of the drug linked to albumin affinity has already been reported for warfarin. Although a genetic factor has been identified in 30% of cases, the other causes of variability in the therapeutic efficiency of warfarin among patients remain unknown (14-16). In the context of diabetes, hyperglycemia...
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induces albumin biochemical integrity alterations caused by a non-enzymatic process called glycation (17). It has been reported that glycated albumin has a decreased affinity for some fatty acids such as cis-parinaric acid (18). Therefore, in patients with high glycation ratio evaluated according to HbA1c levels (higher than 7%), the action of liraglutide may be compromised.

For diabetic patients, the level of glycated albumin can be two- to three-fold higher compared to in healthy people (19,20). This increased glycation rate of albumin could explain the difference in efficiency of fatty-acid grafted drugs observed in some patients suffering from advanced forms of diabetes (21). In this work, 19F NMR transverse relaxation time experiments were used to investigate how albumin glycation influences its binding properties to liraglutide. Fluorine NMR is one of the best suited techniques for probing protein-ligand interactions (22-27). This method requires either the use of a naturally fluorinated ligand or fluorine labelling of the protein (26), ligand or spy molecules (24). Here, we used a strategy where the peptidic liraglutide ligand was first labelled with a fluorine probe by trifluoroacetylation of the unique N-terminal free amine group using S-ETFA reagent (Fig.1b).

The affinity of albumin to liraglutide was assayed through both in vitro and in vivo glycation models: methylglyoxal- and glucose-induced grafted commercial human serum albumins were used as in vitro models and albumin purified from the plasma of healthy and diabetic subjects as in vivo models. This study shows for the first time the impact of glycation on albumin-liraglutide binding and thereby raises the issue of treatment adaptation to glycation levels in diabetic patients.

RESULTS

Fluorine labelling, purification and characterisation of liraglutide- The peptidic drug liraglutide was labelled by a nucleophilic acyl substitution reaction between the unique N-terminal amine group of liraglutide and S-ETFA (Fig.1b). During this reaction, the pH had to be carefully controlled (between 9.6 and 10.0) as the amine of liraglutide (pKa ~9.5) has to be deprotonated for the reaction to occur while a too basic medium led to labelled product hydrolysis and potentially altered the protein structure. After fluorine labelling, the purity and biochemical integrity of liraglutide were checked. Anion exchange chromatography profiles exhibited only one peak at 50% of the sodium chloride gradient, corresponding to the purified labelled liraglutide form (Fig. 2a). Liraglutide was labelled with an average final yield of 80% of the initial amount. 1D 1H NMR results confirmed that labelling did not affect liraglutide structure, as demonstrated by the overlaid spectra (Fig. 2b). Furthermore, 1D 19F NMR analysis showed that liraglutide was correctly labelled with a single peak at -74.333 ppm. Labelled degradation product trifluoroacetate (TFA) gave a peak at -74.472 ppm (Fig. 2c). Hydrolysis kinetics of labelled liraglutide was assessed by measuring the 19F NMR signal for liraglutide and trifluoroacetate over a sixty-hour duration. Thereby, a time limitation for experiment was fixed at 6 hours, corresponding to the beginning of the significant detectable hydrolysis of labelled liraglutide into liraglutide and trifluoroacetate (Figure S1, see supplementary data). To also confirm that the fluorine label does not alter the binding of liraglutide to albumin, Isothermal Titration Calorimetry (ITC) experiments were recorded on HSA titrated with either labeled or unlabeled liraglutide. Both labeled and unlabeled peptides yielded similar Kd values, in the micromolar range (unpublished results), demonstrating that the labeling does not influence the drug binding to albumin.

Glucose and methylglyoxal induced different levels of glycation on albumin- After glycation with glucose (100 mM) or methylglyoxal, the commercial fatty acid-free albumin was analysed on SDS PAGE (12% acrylamide) and Coomassie blue staining. Progressively increased molecular weight bands were observed for glycated G100-HSA and MGO-HSA relative to commercial fatty acid-free albumin (66615 Da): G100-HSA (67314 Da) and MGO-HSA (68071 Da). This increase in weight with
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respect to non-glycated samples probably corresponds to the formation of AGE adducts on the native molecule of albumin.

These AGE adducts were further identified as previously described by mass shift determination of AGE-modified peptides resulting from the trypsin digestion of each albumin sample (28). Results from this identification are shown in Tables 1-2. More modification sites have been reported for G100-HSA (21) and MGO-HSA (26) compared to native albumin G0-HSA (14). Only four additional modification sites were common between both in vitro glycated HSA (R98, R145, K262 and K525), eight sites were specifically modified on G100-HSA and eleven were only found for MGO-HSA. Among the glycated sites observed only on in vitro glycated albumins, four residues (R348, K402, R410 and K525) are known to be involved in the binding of palmitic acid (29).

According to the fructosamine assay, the albumin glycation level was about 3-fold higher for G25-HSA (equivalent of 0.30 (±0.02) mol hexose/mol HSA) and 25-fold higher for G100-HSA (equivalent of 2.13 (±0.14) mol hexose/mol HSA) compared to G0-HSA (0.08 (±0.01) mol hexose/mol HSA). Interestingly, MGO-HSA (1.16 (±0.16) mol hexose/mol HSA) was almost 2-fold less glycated than G100-HSA (Fig. 3c). In contrast, MGO-glycated HSA exhibited a five-fold higher AGE levels (6.2×106 CPS.g⁻¹.L⁻¹) compared to G100-HSA (1.2×10⁶ CPS.g⁻¹.L⁻¹) (Fig.3d). The glycated albumin obtained under typical hyperglycemia conditions (G25-HSA) had a lower level of AGE (0.7×10⁶ CPS.g⁻¹.L⁻¹) than the other in vitro glycated albumin models, just slightly higher than G0-HSA (0.6×10⁶ CPS.g⁻¹.L⁻¹).

In vitro glycated albumin exhibit impaired affinity for liraglutide- The NMR 19F Transverse relaxation time T₂ of labelled liraglutide at variable concentrations was measured at a fixed concentration of albumin, and the plot showing [HSA]₀/ΔR₂obs with respect to trifluorinated liraglutide concentration [Lira 19F] shows a linear curve whose horizontal intercept corresponds to K_d (Fig.4a). These plots exhibit a good linearity with correlation coefficients (r²) above 0.976 (mean 0.979 ± 0.016). The dissociation constants K_d featured in Fig. 4b for albumin samples were calculated using these plots.

When albumin is glycated with physiopathological glucose concentration (25 mM), no significant alteration of liraglutide binding was observed as attested by dissociation constant for G25-HSA (42 ±12 µM) quietly similar to control G0-HSA (35 ±8 µM). By contrast, albumin which was glycated following glucose incubation in suprapathological condition (G100-HSA) exhibited an almost seven-fold higher dissociation constant (240 (±10) µM). Methylglyoxal-glycated albumin affinity for liraglutide was also significantly affected with a K_d of 173 (±6) µM, i.e. five-fold higher than G0-HSA (Fig.4b). Unexpectedly, MGO-glycated HSA which underwent a more drastic glycation process compared to G100-HSA presented a quite equivalent impairment of its affinity to liraglutide. Indeed, as seen on SDS PAGE, MGO-HSA sample exhibited a higher molecular weight band, confirmed by measurement of its exact molecular mass (Fig.3a). All these results, obtained by using in vitro glycation models, demonstrate that glycation at suprapathological conditions significantly impaired the albumin-liraglutide interactions. In order to ascertain the relevance of these observations, we investigated this albumin-liraglutide interaction using in vivo-glycated albumin purified from plasma.

HbA1c plasma levels of diabetic patients correctly matched with the level of glycation in purified albumin samples- Human albumin samples were isolated from the plasma of both healthy subjects and diabetic patients using a differential ammonium sulphate (AS) method based of the difference HSA solubility with other plasmatic proteins. As shown in Figure 5a, the SDS-PAGE pattern showed the major band at around 66 kDa for all purified albumin samples. This pattern attests of the high purity of our human albumin preparations which were obtained with high yields (above 85%, calculated by densitometry) (Fig. 5a).

Human plasma samples used for this study correspond to diabetic patients with a mean HbA1c level of 13.4 (±3.3) %. As clinical observations of drug inefficiency concerned advanced or poorly controlled diabetic patients, our plasma samples were divided into two groups: moderate uncontrolled diabetic
patients named group D (9% < \text{HbA1c} < 11\%) and with a mean of 10.0 (±0.5) \%HbA1c and highly uncontrolled diabetic patients named group D+ (13% < \text{HbA1c} < 18\%) and with a mean of 16.0 (±1.7) \%HbA1c) (fig. 5b). The \text{HbA1c} percentage for each diabetic patient and healthy subject is given in Table S1 (see supplementary data).

The fructosamine assay showed that albumin purified from diabetic groups D and D+ were respectively 1.7 and 3-fold more glycated than albumin purified from non-diabetics (ND) (0.19 ±0.02 and 0.35 ±0.10 versus 0.11 ±0.01 mol hexose/mol HSA for ND) (Fig. 5c) (the fructosamine levels for each diabetic patient and healthy subject are reported in Figure S2, see supplementary data). In parallel, albumin purified from diabetic patients displayed an increase in fluorescent AGE levels although it did not reach the statistical significance (Fig. 5e; p=0.06 for D vs. ND; p=0.12 for D+ vs. ND).

In addition, albumin samples from diabetic patients and non-diabetic individuals showed a significant and positive correlation between \text{Hb1AC}, fructosamine and AGE levels. As shown in Fig. 5d, a strong correlation (r= 0.926, p<0.0001) between \text{Hb1AC} and fructosamine parameters was established. Similarly a significant correlation (r= 0.762, p=0.0104) was obtained between \text{Hb1AC} and AGE levels (Fig. 5f).

Total FFA levels bound to HSA were also determined by GC/MS (Fig. 5g). Albumin purified from diabetic groups D and D+ displayed a tendency to higher FFA levels than albumin purified from non-diabetics (ND) (16.15 ±13.00 and 13.04 ±5.03 versus 8.38 ±3.36 nmol/mg HSA for ND) with a large variability between individuals. No statistical difference was really observed between both diabetic groups and no significant correlation could be established (r= 0.115, p=0.735) between FFA and fructosamine levels (Fig. 5h).

After determination of the glycation level of plasma-extracted albumin samples, their interaction with liraglutide was assessed by the same method used for \textit{in vitro} glycated commercial albumin.

\textit{HSA from highly uncontrolled diabetic patients exhibits an impairment of binding to liraglutide}- HSA from non-diabetic subjects displayed a liraglutide dissociation constant of 48 (±13) \muM, which appears to be quite similar to the value obtained for commercial HSA. As seen in Figure 6, a marked difference was observed between groups of diabetic patients in terms of K_d values (the K_d value of albumin is reported for each diabetic patient and healthy subject in Figure S3, see supplementary data). Indeed, albumin samples from moderately uncontrolled diabetic patients (D group) were found to have an affinity for liraglutide (48 ±2 \muM), similar to control HSA, whereas a significant increase in K_d values was measured for albumin samples of the D+ group (224 ±60 \muM) (Fig. 6). Globally, the affinity of HSA for liraglutide was found to be reduced by almost five-fold for patients with highly uncontrolled diabetes only. These results are consistent with those obtained with \textit{in vitro} models of albumin and attest to the impairment of albumin affinity for liraglutide in the situation of glycation. Finally, these results suggest that a high level of glycation is required to significantly impair the binding of albumin to liraglutide.

**DISCUSSION**

Liraglutide is a recent promising treatment for type 2 diabetes mellitus with a number of benefits relative to existing drugs, especially regarding cardiovascular risk improvement, as described earlier (9-13). The main strategy for the protracted action of liraglutide over the day mainly relies on its transport and progressive release by albumin. Unfortunately, some diabetic patients fail to respond correctly to certain treatments. In particular, this was noticed in clinical observations that glycemia was hardly maintained at acceptable levels for patients treated with long-acting albumin-transported drugs such as insulin detemir (Levemir®) (unpublished data).

Our study aimed to better understand the link between plasmatic oxidative damages and alterations in the treatment efficiency, which remains highly dependent on diabetes evolution and therapeutic adaptation. Notably, GLP-1 analogues are recommended in the “therapeutic climbing” generally in association with a biotherapy or insulin, when first-line oral anti-diabetics, such as metformin or sulfonylureas, are not efficient enough to...
reduce the HbA1c level to the therapeutic target (8).

Chronic hyperglycemia, which characterises diabetes, is the main factor of protein alteration by glycation that is known to be implicated in diabetes aggravation, such as renal failure and atherosclerosis (30). Glycation particularly alters structural and functional properties of albumin (31), including the protein affinity for therapeutic drugs (32). For instance, the affinities of many drugs including warfarin or ketoprofen were found to be affected by albumin glycation, resulting in the variability of therapeutic efficacy for advanced diabetic patients (for warfarin) (32,33). Other studies reported an altered albumin affinity for several sulfonylurea drugs commonly used in the treatment of type 2 diabetes (i.e., acetohexamide, tolbutamide and gliclazide) after glycation (34). In parallel, several studies showed that albumin glycation resulted in an impaired affinity for fatty acids such as lipoic, oleic, linoleic, lauric and caproic acids (35,36).

In the present study, we showed for the first time that glycation alters the affinity of albumin for liraglutide. $^{19}$F NMR is one of the best validated techniques for monitoring ligand–protein interactions (22-27). This method has already been used to study the binding of various drugs to albumin, either using naturally fluorinated drugs or by competition with fluorinated spy molecules (24,37-42). Although a labelling step was required for liraglutide, the $^{19}$F NMR relaxation time method presents the advantage of specifically targeting the labelled peptide drug signal. Moreover, it requires a small amount of protein, as there is an excess of ligand. The N-terminal amine group was targeted for trifluoroacetylation because it leads to a single substitution on the liraglutide peptide. Moreover, this position is located in an unstructured part of liraglutide far from the palmitoyl fatty acid (d > 40Å on the X-ray structure: PDB ID: 4APD) and does not influence the binding to albumin, as confirmed by ITC measurements (data not shown). Most previous studies on liraglutide and other fatty-acid grafted GLP1 analogues focused on the measure of their binding to the GLP-1 receptor, but rarely on their interaction with albumin (43-45). Recently, a $K_d$ value of about 150 $\mu$M for albumin-liraglutide binding was estimated by fitting data from Small-angle X-ray scattering (SAXS) and static light scattering (SLS) methods (46). This latter value is in good agreement with our results obtained by NMR which present the advantage of giving a direct and accurate measure of the affinity between liraglutide and human serum albumin. Indeed, the measured $K_d$ values obtained for liraglutide in our study (between 35 and 240 $\mu$M) indicate a weak binding to human serum albumin. Similarly, for detemir, which also binds to albumin via a fatty acid chain, a $K_a$ value of $2.4 \times 10^5$ M$^{-1}$ was reported, corresponding to a dissociation constant in the micromolar range (47). These values are much lower than those reported for the binding of free palmitate or FA C16 to albumin which has an approximate $K_a$ of $1.45 \times 10^7$ M$^{-1}$ corresponding to a dissociation constant in the nanomolar range (48). Thus, fatty-acid grafted peptides (detemir and liraglutide) bind weakly to albumin compared to free fatty acids. This may be the consequence of a restricted access to a number of fatty acid binding sites on albumin due to steric hindrance by the peptide.

In our study, in vitro glucose or methylglyoxal glycated commercial albumins appear to mimic the in vivo glycation effects leading to similar alterations of its binding properties. Indeed, $K_d$ values were comparable between G100-HSA or MGO-HSA and HSA from highly uncontrolled diabetic patients. On the other hand, quite similar $K_d$ values were obtained for G0-HSA, G25-HSA and non-diabetic HSA or HSA from moderately uncontrolled diabetic patients.

In vitro glycation with 25 mM of glucose is commonly used for mimicking diabetes conditions. By contrast, in vitro glycation with 100 mM of glucose or methylglyoxal appeared to be more drastic conditions than in vivo glycation; this was confirmed by fructosamine measurements and mass spectrometry analysis. Methylglyoxal is mainly formed as a by-product of glycolysis and its concentration is higher in diabetic patients reaching 1 $\mu$M in blood or plasma relative to 0.55$\mu$M for controls (49); this plays a role and takes part in the exacerbation of protein glycation (50). As confirmed in the present study, MGO-HSA contained more AGE products compared to G100-HSA. Indeed, methylglyoxal is 20,000-fold more reactive than glucose and affects arginine side chains rather than lysines (51), as confirmed by the characterisation and location.
of AGE modifications in albumin molecules. Methylglyoxal is known to induce an important irreversible formation of AGE in glycated proteins (52). Although both in vitro G100- and MGO-glycated HSA models display different biochemical modifications, their binding constants for liraglutide appear quite similar. On the opposite, the G25-HSA model affinity for liraglutide was not affected by the glycation suggesting that a moderate biochemical modification of the protein is not sufficient to alter its drug binding capacity.

In the same manner, the glycation level strongly influenced the binding for in vivo HSA models. Indeed, for healthy subjects and diabetic patients with an HbA1c level up to 10%, the affinity for liraglutide was similar. A decreased affinity was measured for highly uncontrolled diabetic patients only (HbA1c > 13%). This observation suggests that albumin glycation should not affect the therapeutic actions of liraglutide until an advanced stage of chronic hyperglycemia, higher than 300 mg/dL. The altered affinity to albumin could lead to higher free active liraglutide in serum, with altered kinetics and duration of action, which could explain a lower efficacy and unsustained metabolic effects over 24 hours, but also a greater susceptibility to gastrointestinal side effects of the drug due to higher peaks of free GLP1 RA concentrations.

Drug binding to albumin has been extensively characterised. The two most common drug binding sites of albumin are located in the hydrophobic cavities of subdomain IIA and IIIA, known as Sudlow’s sites I and II, respectively (53). Non-esterified medium- and long-chain fatty acids, such as myristic and palmitic acid, can bind to seven sites on HSA, three of which are located on Sudlow’s drug binding sites I and II (54,55). In particular, the albumin region from residues 377-582, including part of domain II and the entire domain III was found to be the primary binding site for long chain fatty acids (56) and, more precisely, the key roles of R410 (Sudlow’s site II) and K525 (domain IIB) residues, which are involved in fatty acid binding, were also glycated in vitro for G100-HSA and may contribute to a reduction in the affinity of albumin for the drug. These data support that glycation reduces albumin affinity for fatty-acid grafted drugs.

Another factor that could influence binding in the in vivo albumin models and contribute to the observed variability between individuals is competition between fatty acids and liraglutide for albumin binding. Such effects at physiological concentrations have been demonstrated on non-glycated and glycated HSA interactions with sulfonylurea drugs (31). Indeed, diabetic patients have higher plasma fatty acid concentration than healthy subjects. This is especially observed in obese patients with increased insulin resistance (59). In our study, total FFA levels bound to HSA appeared to be higher in diabetic patients but without reaching statistical significance. Furthermore, no relevant difference was observed between D and D+ groups suggesting that FFA levels do not significantly contribute to the impairment of liraglutide binding to HSA.

In advanced diabetic cases, a 50% higher drug dosage is usually required (60), with poorly known effects on health. Dose-dependent detrimental effects concerning liraglutide have been reported, such as the induction of a high risk of pancreatic and thyroid cancer in rats (61-63). Our present work showing impaired liraglutide binding in diabetics raises the need for further research to prevent glycation-mediated alterations of albumin-drug binding capacity. For example, amino acid or fatty acid length modifications in the liraglutide molecule could modulate its affinity for albumin and afford resilience to glycation-induced modifications at specific binding sites. Indeed, this strategy has been explored successfully by researchers from Novo Nordisk who recently developed a GLP-1 analogue called semaglutide (64). This peptide contains a substitution of an alanine residue (A8) by a 2-aminoisobutyric acid for stabilisation against DPP-4 degradation and a
stearic acid instead of a palmitic acid on the same lysine residue (K26). Its longer-chain fatty acid confers a higher affinity for albumin to semaglutide. It would be interesting to test the hypothesis that the higher affinity of semaglutide for albumin could be less susceptible to alterations by glycation for highly uncontrolled diabetic patients. Optimization of GLP-1 analogues under glycation conditions is an interesting alternative to potentially dangerous dose escalation.

EXPERIMENTAL PROCEDURES

Liraglutide fluorine labelling and purification- For liraglutide fluorine labelling, the pH of 1.5 mL of a 6 mg/mL commercial liraglutide pen (Victoza®, Novo Nordisk A/S) was adjusted from the initial value of about 8.3 to 9.6 using 0.01 M NaOH. Every hour, 10μl of 97% S-Ethyltrifluorothioacetate (S-ETFA, Aldrich, 177474) was added to liraglutide for five hours at room temperature. A total of 50 μL of S-ETFA was used for each labelling, corresponding to a substrate/protein ratio of 110. The pH was maintained between 9.6 and 10 throughout the reaction in order to allow the proper reaction to occur. After the labelling step, the reaction mixture was first purified by triple dialysis (Spectra/Por® 2,000 Da MW cut-off) against borate buffer 50 mM pH 7.5. Then, labelled liraglutide was centrifuged at 12,000 g for 15 minutes and purified by anion exchange chromatography on a 5 mL Hi-Trap Q column (GE Healthcare) coupled with AKTA Primeplus apparatus (1 mL/min flow). A 100 mM NaCl gradient from 0.01 to 1 M was then applied to elute the proteins. Elution fractions were further washed and concentrated using a centrifugal filter device (20 mM Vivaspin MW cut-off 3,000 Da). Purified liraglutide concentration was assessed by measuring UV absorbance (ε: 1.99 L.g⁻¹.cm⁻¹) and Bradford assay. Glycated albumin samples were stored at -20°C. Commercial delipidated (or fatty-acid free (FA Free)) HSA was used in order to circumvent eventual competitive effect due to fatty acids which could remain bound to albumin after extraction from human serum.

Albumin extraction from human sera by ammonium sulphate precipitation- Human plasma samples from diabetic patients were obtained from the Centre Hospitalier Gabriel Martin (Saint-Paul, La Réunion). The procedure and the collection of human materials were approved by the local governmental French Ethical Committee and conformed to the standards set by the Declaration of Helsinki. Control bloods samples from healthy individuals were taken from non-diabetic volunteers. An average of about 2 mL of plasma was retrieved from a whole blood sample of 5 mL after two successive centrifugations (2,000 g for 10 min., then 2,500 g for 15 min.). The supernatant was collected and stored at 80°C. A differential ammonium sulphate precipitation two-step protocol described by Mandia’s team (66) and modified in our lab (67) was used to extract HSA from the plasma. The first step consisted of adding 54% of ammonium sulphate (AS) to a plasma sample using a saturated 100% AS solution (540 g/L; pH adjusted to 7.4 with NH₄OH). After stirring for one hour at 4°C, the whole solution was centrifuged (5,000 g for 10 min.) and the
precipitate was discarded. Saturated AS solution was further added to the supernatant under stirring to reach 70% of AS where HSA precipitates. The pellet was collected after centrifugation (10,000 g for 30 min.) and resuspended in 0.1 M sodium phosphate. HSA solution was then washed three times and concentrated by ultrafiltration (Amicon 3K, Millipore). The final concentration of purified albumin was determined using UV absorbance ($\varepsilon = 34,445 \text{ M}^{-1}\text{cm}^{-1}$) and samples were stored at -80°C.

Fructosamine assay on human serum albumin- In order to assess the albumin glycation rate, the fructosamine assay developed by Johnson et al. (68) was performed on HSA samples. 1-Deoxy-1-morpholino-D-fructose (DMF) was used as a fructosamine reference for the standard range. Then, 100 µL of Nitroblue Tetrazolium (NBT) was added to 100 µL of sample or commercial glycated albumin range (Sigma A8301, mean of 2.2 mol of hexose per mol of HSA) on 96-well plates. Samples were incubated at 37°C and absorbance was measured over time at 540 nm using a FLUOstar Omega microplate reader (BMG LABTECH). Kinetic curves were plotted and slopes (AU/min) were calculated using the Omega software. Fructosamine concentrations in samples were expressed as mole of hexose equivalent per mole of albumin.

AGE fluorescence measurement- In order to assess AGE-peptide levels in glycated commercial albumin, fluorescence measurements were made in a 96-well plate with excitation at 370 nm and emission at 440 nm measured on 100 µl samples using a FLUOstar Omega microplate reader. Fluorescent AGE levels were expressed in arbitrary units.

Free fatty acids levels determination on purified human serum albumin- Free fatty acids was determined on purified albumin isolated from diabetic and non-diabetic plasma by capillary gas chromatography tandem mass spectrometry (GC-MS) in ICANanalytics lipidomic platform (Paris, France). Purified albumin samples (40 µl) were preliminary supplemented with internal standard mixture of 3 deuterated fatty acids in 500 µl of methanol and 275 µl HCl 0.1N. Free fatty acids (FFA) were successively extracted twice using 1.5 ml isooctane and derivatised for GC/MS analysis using 50 µl of pentafluorobenzyl bromide (PFB Br) (10% in acetonitrile) and 50 µl of 10% disopropylamine (DPIEA) (10% in acetonitrile). Analysis of FFAs in HSA was performed using a ISQ™ LT Single Quadrupole GC-MS System (Thermo Fisher Scientific). Separation of FFAs was performed on a FAST 10mx0.1mm, 0.2µm BPX70 capillary column. The injector temperature was 250 °C, and the samples were injected using the splitless injection mode. Quantification was performed on a single quadrupole using chemical ionisation with methane.

Mass spectroscopy and peptide characterisation- The glycation of serum albumin was analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) for mass shift determination and for AGE-modified peptide exploration, as previously described (69).

NMR analysis of purified $^{19}$F-labelled liraglutide with albumin- Purified protein solution was analysed by 1D $^1$H and $^{19}$F NMR to check for the presence of correctly folded and $^{19}$F-labelled liraglutide. A final volume of 500 µl of liraglutide in borate buffer 50mM, pH 7.5 was used, to which 50 µl of deuterium oxide (D$_2$O) was added. No $^1$H or $^{19}$F reference was used in order to avoid possible influence on the binding. 1D $^1$H experiments were performed using water suppression with gradients (128 scans, relaxation delay of 2s). Fluorinated liraglutide hydrolysis kinetics leading to unlabelled liraglutide and TFA was followed by recording 1D $^{19}$F NMR spectra at 25°C on a 100 µM sample in borate buffer 50 mM, pH 7.5 for 60 hours (512 scans, relaxation delay of 7s and a transmitter frequency of 564.690823 MHz). All NMR experiments were recorded on a 600 MHz Bruker Avance III spectrometer equipped with a triple resonance $^1$H/$^{19}$F/$^{15}$N/$^{13}$C TCI cryoprobe. NMR spectra were processed and analysed with Topspin 2.1.6 software (Bruker).

Transversal relaxation rate analysis for albumin-liraglutide binding $K_d$ determination- To study liraglutide–albumin interaction,
transversal relaxation time \( T_2 \) (or corresponding rate \( R_2 = 1/T_2 \)) was measured by a pseudo 2D \(^{19}\)F CPMG (Carr-Purcell-Meiboom-Gill) pulse program consisting of acquiring signal with increasing relaxation delays (1; 5; 10; 20; 30; 50; 70; 100; 150 and 200 ms). The parameters used for the \(^{19}\)F CPMG experiments were the following: a temperature of 25°C, 512 scans, a relaxation delay of 3.5 s and a transmitter frequency of 564.690823 MHz. A specific concentration of HSA (10, 20 or 30 µM) was added to a range of labelled liraglutide from 50 to 300 µM. For each titration point, the transversal relaxation time \( T_2 \) (\( T_2 = 1/R_2 \)) was calculated with the \( T_2 \) determination menu from Topspin. From these \( T_2 \) values, dissociation constants were calculated by the method developed by the team of J-P. Girault (70): \( [P]_0/\Delta R_{2obs} \) values were plotted as a function of liraglutide concentration in order to obtain a \( K_d \) value from the horizontal intercept. \( [P]_0 \) corresponds to total albumin concentration ([HSA]_0 and \( \Delta R_{2obs} \) is the difference between \( R_2 \) values, in the presence and in the absence of albumin (\( 1/T_{2obs} - 1/T_{2free} \)). Higher \( K_d \) values reflect a lower affinity. All titrations were recorded in triplicate.

**Statistical analysis**- The data are expressed as the means ± standard deviation (SD) from a minimum of three experiments. Statistical significances were determined using one-way ANOVA (followed by the Student’s t test) for multiple comparisons; a \( p \) value of less than 0.05 was considered as significant.

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**Author contributions.** A.G. researched data, wrote and reviewed manuscript. P.G. researched data, reviewed manuscript. A.C. researched data, reviewed manuscript. S.A.K. researched data. A.G.D. contributed discussion, reviewed manuscript. X.D. had the original idea, contributed discussion, and reviewed manuscript. N.L.M. reviewed manuscript. E.B. reviewed manuscript. S.B. researched data and reviewed manuscript. B.P.D. researched data and reviewed manuscript. E.A. researched data, contributed discussion and reviewed manuscript. O.M. reviewed manuscript. P.R. coordinated the study, researched data, wrote, reviewed and edited manuscript. J.C. had the original idea, conceived and coordinated the study, researched data, wrote, reviewed and edited manuscript.

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FOOTNOTES

**Abbreviations used are:** AGE, Advanced glycation-end products; DMF, 1-Deoxy-1-morpholino-D-fructose; D$_2$O, deuterium oxide; DPP4, dipeptidyl peptidase 4; FFA, free fatty acids; GLP-1, glucagon-like peptide-1; HSA, human serum albumin; $K_a$, association constant; $K_d$, dissociation constant; MGO, methylglyoxal; NBT, nitroblue tetrazolium; NMR, nuclear magnetic resonance; S-ETFA, S-Ethyltrifluorothioacetate; TFA, trifluoroacetic acid.
**TABLES:**

**TABLE 1: Glycation-modified peptides identified in native HSA (GO HSA) by MALDI-TOF-MS peptide mapping**

Tryptic peptides of G0-HSA were analysed by MALDI-TOF-MS. By comparing with theoretical digestion of unmodified serum albumin, the presence of glycation adducts on albumin was identified after determining the tryptic peptide ions with mass values, which were only present in the samples. Then, modifications present in these peptides were characterized by comparing the mass shift with a list of shift mass deviations caused by different glycation adducts.

*Both modifications are possible on lysine and they are indistinguishable by the mass.*

| Position | Modified residues | Peptide sequence | Peptide mass | Modification | Measured mass | Modification |
|----------|-------------------|-----------------|--------------|--------------|--------------|--------------|
| 1-15     | K        | (GDAHSLERAVHR) | 1143.58      | 182.85       | 1311.83      | Fructose-lysine |
| 99-114   | K,K    | (KGKEDPLHRDDINXKLPL) | 1855.83      | 72.02       | 2678.95      | Ne-Carboxymethyllysine |
| 99-114   | K,K    | (KGKEDPLHRDDINXKLPL) | 1855.83      | 124.83      | 2122.68      | Pyrrolidone-2-Lys |
| 176-180  | K       | (KIAACLNTKDLQ) | 1503.78      | 124.83      | 1628.61      | Ne-Carboxymethyllysine |
| 186-205  | K       | (KIAACLNTKDLQ) | 1503.78      | 104.83      | 1654.61      | Pyrrolidone |
| 219-224  | R       | (KIAACLNTKDLQ) | 1503.78      | 92.83       | 1595.84      | Argpyrrolidone |
| 219-224  | R       | (KIAACLNTKDLQ) | 1503.78      | 122.83      | 1625.60      | Argpyrrolidone |
| 263-276  | K,L    | (KCHGNDVASLKD) | 1838.82      | 56.01       | 1742.83      | Ne-Carboxymethyllysine-Pentaosiduronic acid |
| 329-337  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 142.83      | 1627.59      | Ne-Carboxymethyllysine |
| 345-359  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 72.02       | 1860.93      | Ne-Carboxymethyllysine |
| 414-428  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 92.02       | 1880.94      | Ne-Carboxymethyllysine-Pentaosiduronic acid |
| 473-484  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 56.01       | 1742.83      | Ne-Carboxymethyllysine-Pentaosiduronic acid |
| 533-545  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 56.01       | 1742.83      | Ne-Carboxymethyllysine-Pentaosiduronic acid |
| 542-557  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 56.01       | 1742.83      | Ne-Carboxymethyllysine-Pentaosiduronic acid |
| 547-557  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 56.01       | 1742.83      | Ne-Carboxymethyllysine-Pentaosiduronic acid |
| 548-557  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 172.02      | 1960.94      | Fructose-lysine-2-Lys |
| 548-557  | R       | (RLEFYHPLSLVSPHR) | 1788.92      | 172.02      | 1960.94      | Fructose-lysine-2-Lys |
| 565-573  | K       | (KETFAPKMKK) | 1199.84      | 202.11      | 1402.95      | Ne-Carboxymethyllysine |

$^*$Both modifications are possible on lysine and they are indistinguishable by the mass.
TABLE 2: Glycation-modified peptides identified in glucose-glycated HSA (G100-HSA) and methylglyoxal-glycated HSA (MGO-HSA) by MALDI-TOF-MS peptide mapping

Tryptic peptides of G100-HSA and MGO-HSA were analysed by MALDI-TOF-MS. By comparing with theoretical digestion of unmodified serum albumin, the presence of glycation adducts on glycated albumin was identified after determining the tryptic peptide ions with mass values, which were only present in the glycated samples. Then, modifications present in these peptides were characterized by comparing the mass shift with a list of shift mass deviations caused by different glycation adducts.

### G100 HSA

| Position of Sequence | Modified masses | Peptide Sequence | Peptide mass | Modification | Measured mass | Modification | Modification |
|----------------------|-----------------|------------------|--------------|--------------|---------------|--------------|--------------|
| 1-10                 | K4              | (RDKASISVEAVRSSPNT) | 1149.8       | 182.09       | 1331.98       | Fucosyl-lysine |
| 93-108               | K3              | (REIYEGMAACCMQGPIH) | 3072.06      | 257.11       | 3329.14       | Glu-lysine   |
| 94-115               | R50             | (KGDPEPHERCLWG)   | 1745.02      | 142.02       | 1887.04       | Imidazole-lysine |
| 95-114               | R50             | (KGDPEPHERCLWG)   | 1905.04      | 144.04       | 2049.07       | Fucosyl-lysine-1H_3PO |
| 100-159              | R14             | (KDFRLLPEQFETDNFSETED) | 1899.00    | 270.07       | 2169.07       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 175-188              | K131            | (KAAEMHKLMDPVE)   | 1349.78      | 126.03       | 1475.81       | Fucosyl-lysine-2H_2PO |
| 319-324              | R222            | (KLSPRFFAA)       | 875.51       | 80.03        | 955.54        | Arginyl-lysine |
| 319-324              | R222            | (KLSPRFFAA)       | 875.51       | 142.09       | 1017.59       | Imidazole-lysine |
| 356-374              | K263            | (RNLAYLICCDKSSK)  | 1841.02      | 125.03       | 1966.05       | Fucosyl-lysine-2H_2PO |
| 283-297              | K274            | (KVIYCENSDISILKE) | 1644.62      | 99.01        | 1743.63       | Ne-Carboxy-methyl-lysine-Pentosidine |
| 319-318              | L128            | (KADKGLYFTLYGAAYTF) | 2370.16     | 126.03       | 2496.19       | Fucosyl-lysine-2H_2PO |
| 334-347              | R158            | (KVDLTLFKVLQYKVR) | 1770.09      | 142.09       | 1912.02       | Imidazole-lysine |
| 334-347              | R158            | (KVDLTLFKVLQYKVR) | 1770.09      | 270.07       | 2040.07       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 386-321              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 80.03        | 1703.08       | Arginyl-lysine |
| 386-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 270.07       | 1893.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 125.03       | 1748.06       | Fucosyl-lysine-2H_2PO |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 370.07       | 2013.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 540.07       | 2163.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 125.03       | 1748.06       | Fucosyl-lysine-2H_2PO |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 270.07       | 1893.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 450.07       | 2073.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 540.07       | 2163.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 125.03       | 1748.06       | Fucosyl-lysine-2H_2PO |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 270.07       | 1893.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 450.07       | 2073.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 540.07       | 2163.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 125.03       | 1748.06       | Fucosyl-lysine-2H_2PO |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 270.07       | 1893.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 450.07       | 2073.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |
| 394-395              | R148            | (RTHFDSKYLRIK)    | 1623.08      | 540.07       | 2163.05       | 1-Aryl-2-formyl-3-A-glycosyl-lysine |

*Both modifications are possible on lysine and they are indistinguishable by the mass.
FIGURE LEGENDS

FIGURE 1. Liraglutide structure (a) and fluorine labelling reaction (b)

FIGURE 2. Labelled liraglutide analysis
(a) Chromatography UV profile indicates that protein is present in fractions 36 to 42. UV: absorbance at 280 nm; Cond: conductivity; Conc: concentration of NaCl from 0 to 1M in 50mM borate buffer, pH 7.5. (b) Identical 1D 1H spectra for unlabelled and labelled liraglutide show evidence for no significant structural alteration of the protein due to labelling process (top red spectrum: labelled and ultrafiltrated liraglutide, bottom blue spectrum: unlabelled and dialysed liraglutide). (c) 1D 19F spectrum of purified labelled liraglutide presents a main peak at -74.333 ppm extending between -74.302 ppm and -74.369 ppm and a degradation peak at -74.472 ppm corresponding to TFA.

FIGURE 3. In vitro glycated albumin characterization
(a) SDS PAGE analysis of commercial (A1887-HSA), G0-, G100- and MGO-HSA. (b) Mass-to-charge ratio (m/z) vs. the percentage intensity plot for A1887-HSA, G0-, G100- and MGO-HSA. (c) Fructosamine levels of G0-, G25- G100- and MGO-HSA. All data are expressed as means ± SD of three independent experiments. ***Unpaired t-test compared to G0-HSA. G100-HSA: p-value<0.0001; MGO-HSA: p-value=0.007. ##Unpaired t-test comparison between G100- and MGO-HSA, p-value=0.003; (d) AGE fluorescence measurement on G0-, G25- G100- and MGO-HSA. **Unpaired t-test compared to G0-HSA: p-value<0.01; ***Unpaired t-test compared to G0-HSA: p-value<0.001; ####Unpaired t-test comparison : p-value<0.0001.

FIGURE 4. NMR experiments for in vitro glycated albumin interaction with liraglutide
(a) [HSA]₀/ΔR₂obs is plotted as a function of liraglutide concentration. A linear regression is calculated and Kd is obtained from the vertical intercept. G0-HSA: Kd = 35 µM (± 8 µM) n=3 r²=0.989±0.009; G25-HSA: Kd = 40 µM (± 12 µM) n=3 r²=0.955±0.037; G100-HSA: Kd = 240 µM (± 10 µM) n=3 r²=0.974±0.028; MGO-HSA: Kd = 173 µM (± 7 µM) n=3 r²=0.976±0.014 (b) Dissociation constant comparison for non-glycated and in vitro glycated commercial albumin. All data are expressed as means ± SD of three independent experiments. ***Unpaired t-test compared to G0-HSA p-value<0.0001.

FIGURE 5. Plasma-extracted albumin analysis
(a) SDS PAGE analysis of albumin sample purified from plasma of patients divided into three groups (ND, D and D+). (b) HbA1c levels (%) of patients divided into three groups (ND, D and D+). ***Unpaired t-test comparison between D and D+, p-value=0.0041; (c) Fructosamine levels of purified albumin from healthy (ND) and diabetic (D and D+) patients. **Unpaired t-test compared to HSA from ND: p-value=0.0016 and 0.0062 respectively for D and D+ groups. #Unpaired t-test comparison between D and D+ groups: p-value=0.0277; (d) Correlation between %HbA1c and albumin fructosamine levels. (e) AGE fluorescence measurement on HSA from ND, D and D+ groups (n=3). Unpaired t-test compared to HSA from ND: p-value=0.0621 and 0.1282 respectively for D and D+ groups; (f) Correlation between %HbA1c and albumin-AGE levels. (g) Free fatty acid levels (FFA) bound to purified albumin from healthy (ND) and diabetic (D and D+) patients. (h) Correlation between Free fatty acid levels (FFA) bound to purified albumin and albumin fructosamine levels.

FIGURE 6. NMR results for plasma-extracted albumin interaction with liraglutide
Albumin-liraglutide Kd values for non-diabetics (ND), diabetics below 11% HbA1c (D) and diabetics above 11% HbA1c (D+). **Unpaired t-test comparison between ND and D+ groups, p-value=0.0017. #Unpaired t-test comparison between D and D+ groups, p-value=0.0057.
FIGURE 1. Liraglutide structure (a) and fluorine labelling reaction (b)
FIGURE 2. Labelled liraglutide analysis
FIGURE 3. *In vitro* glycated albumin characterization
FIGURE 4. NMR experiments for *in vitro* glycated albumin interaction with liraglutide
FIGURE 5. Plasma-extracted albumin analysis
FIGURE 6. NMR results for plasma-extracted albumin interaction with liraglutide
Glycation of human serum albumin impairs binding to the glucagon-like peptide-1 analogue liraglutide

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