Blood proteome characterization of the interplay between glycation and aspirin-mediated acetylation in vitro and in diabetic patients

Finamore, Francesco

UNIVERSITÉ DE GENÈVE

FACULTÉ DES SCIENCES
Section des Sciences Pharmaceutiques — Prof. Denis Hochstrasser

FACULTÉ DE MÉDICINE
Département de Science des Protéines Humaines — Prof. Jean-Charles Sanchez
Département de Médecine interne des Spécialités — Prof. Pierre Fontana

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THÈSE

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Francesco Finamore

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A mia Madre.

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Table of content

SUMMARY

Chronic hyperglycaemia represents a major risk factor contributing to the development of long-term diabetic complications involving several organs such as the cardiovascular and nervous systems, the eyes or the kidneys. Long-lasting glucose exposure promotes non-enzymatic glycation which was shown to play a pivotal role through the alteration of protein structure/function in a wide variety of tissues and by the enhancement of oxidative stress, inflammation and thrombosis. Beside continuous advances in treatment of those deleterious effects, aspirin was probably the first molecule shown to prevent proteins form excessive glycation. Aspirin is indeed usually prescribed to diabetic patients for the prevention of cardiovascular events mediated through the inhibition of platelet function via the acetylation of the cyclooxygenase (COX)-1 protein. The mechanism of protection by aspirin against glycation has long been assigned to the acetylation of the reactive free amino groups of proteins. However, the exact interplay between these two uncontrolled PTMs and the quantification of their mutual impact was poorly investigated, especially in complex biological samples.

In the first part of this project, we described the application of a qualitative and quantitative method for the analysis of glycated proteins in biological fluids and cell lysates. The dynamism of the each glycated proteome as a function of glucose unbalance, was assessed using a stable isotope labelling approach based on the differential labelling of proteins with isotopic $^{13}C_6$-glucose, followed by two data-dependent MS methods: HCD-MS2 and CID neutral loss triggered MS3. This analytical strategy allowed the confident identification and quantification of novel glycation protein targets in plasma, red blood cells (RBCs) and cerebrospinal fluid (CSF), as well as the accurate detection of the sugar attachment positions for each protein, paving the way towards the use of new potential markers for glycaemic control.

The second main objective of this project relied on the evaluation of the effect of aspirin over protein glycation, through an extensive characterization of the interaction between *in vitro* protein glycation and aspirin-mediated acetylation in the main blood compartments. A middle-down proteomic approach coupled with label-free quantification was performed to provide qualitative data by detecting the preferential glycation/acetylation sites in blood proteins, as well as quantitative information to measure

the extent of the influence between these two PTMs. The results showed that aspirin reduces protein glycation in all the blood compartments including most of the major plasma proteins, different haemoglobin subunits and proteins involved in platelet activation and inflammation. In addition, high glucose level was found also to favour aspirin-acetylation of several other protein sites through a process that might be supported by structural conformational changes. These findings provide a comprehensive profile of several blood proteins that are affected by both aspirin and glucose, and highlight new insights on the potential mechanism by which aspirin-induced acetylation and protein glycation may influence each other at protein level.

These *in vitro* studies were then validated by assessing the impact of chronic hyperglycaemia on *in vitro* aspirin-mediated acetylation of platelet proteins from diabetic patients. A shotgun proteomic strategy allowed the quantification of 1088 acetylated proteins, including COX-1 by which, a total of 7 acetylated sites were specifically detected and quantified by a targeted MS method. Among them, the acetylation level of the catalytic residue of serine 529 was shown to be highly reduced in diabetic platelets compared to controls. Moreover, the lowering of aspirin-induced inhibition of COX-1 activity in presence of increasing glucose concentrations was evidenced as well. These findings suggest that, in diabetes, high glucose levels interfere with the acetylation of serine 529 in COX-1, leading to a less-than-expected inhibition of the enzyme activity by aspirin. Predictive models suggest that glucose may hamper the acetylation process by placing itself at the entrance of the catalytic pocket of COX-1, hindering the subsequent access of aspirin. However this model has to be validated, together with other potential mechanisms that could explain the observed low response of platelet COX-1 inhibition by aspirin in diabetic patients.

RÉSUMÉ

L'hyperglycémie chronique chez les patients diabétiques représente un facteur de risque majeur contribuant sur le long terme au développement de complications au niveau des systèmes cardiovasculaires et nerveux, ou des organes tels que les yeux et les reins. Il a été démontré que l'exposition prolongée du glucose favorise la glycation non-enzymatique et joue un rôle important dans l'altération des protéines en modifiant leur structure et leur fonction via une augmentation du stress oxydatif, de l'inflammation et de la coagulation. L'aspirine est probablement la seule molécule qui peut prévenir une glycation excessive des protéines. L'aspirine est habituellement prescrite aux patients diabétiques à risque pour prévenir les évènements cardiovasculaires et agit par l'inhibition de la fonction des plaquettes via l'acétylation de la cyclooxygénase 1 (COX-1). Le mécanisme de protection de l'aspirine contre la glycation a été longtemps associé à l'acétylation du groupe amine des protéines. Par contre, leur compétition et la quantification de leur impact a été peu étudié, en particulier dans les échantillons biologiques complexes.

Dans la première partie de ce projet, nous avons décrit une méthode qualitative et quantitative pour analyser des protéines glyquées dans des liquides biologiques et des lysats cellulaires. L'évolution de chaque protéome glyqué a été évaluée en fonction de la concentration de glucose, en utilisant un marquage isotopique stable basé sur la différence de marquage des protéines avec un isotope $^{13}C_6$-glucose, suivi par deux méthodes de spectrométrie de masse data-dépendantes HCD-MS2 et CID-MS3. Cette stratégie analytique a permis l'identification et la quantification de nouvelles cibles de protéines glyquées dans le plasma, les globules rouges et dans le liquide céphalorachidien, ainsi que la détection précise de la position des glycations sur chaque protéine, ouvrant la voie vers l'utilisation de nouveaux marqueurs pour le contrôle de la glycémie.

Le deuxième objectif de ce projet est basé sur l'évaluation de l'effet de l'aspirine sur la glycaton des protéines, à travers une large caractérisation de l'interaction entre la glycation des protéines et l'acétylation induite par l'aspirine *in vitro* dans les principaux compartiments sanguins. Une approche protéomique « middle down » couplée à une quantification sans marquage a été réalisée pour fournir des données qualitatives détectant les sites préférentiels de glycation/acétylation dans les protéines

sanguines, ainsi qu'une information quantitative pour évaluer l'influence de ces deux PTMs. Les résultats montrent que l'aspirine réduit la glycation des protéines dans tous les compartiments sanguins incluant la plupart des protéines plasmatiques les plus abondantes, différentes sous-unités de l'hémoglobine et des protéines impliquées dans l'activation plaquettaire ainsi que dans l'inflammation. De plus, il a été trouvé qu'un taux élevé de glucose favorise l'acétylation induite par l'aspirine de plusieurs sites protéiques via un processus qui pourrait être dû à un changement de conformation des protéines. Ces résultats fournissent des informations sur les caractéristique de plusieurs protéines sanguines lorsqu'elles sont exposée à la fois par l'aspirine et le glucose, et mettent en lumière une nouvelle voie sur le mécanisme potentiel par lequel l'acétylation et la glycation peuvent s'influencer réciproquement. Ces études *in vitro* ont été confirmées en évaluant l'impact de l'hyperglycémie chronique chez des patients diabétiques issu de l'acétylation *in vitro* de l'aspirine, sur les protéines plaquettaires. Une stratégie de protéomique « shotgun » a permis la quantification de 1088 protéines acétylées, incluant COX-1, où 7 sites acétylés ont été détectés et quantifiés par une méthode MS ciblée. Parmi ces sites, le niveau d'acétylation de la sérine 529 (la cible spécifique de l'aspirine) s'est montré fortement réduit dans les plaquettes issues de patients diabétiques en comparaison au groupe contrôle. Par ailleurs, la réduction de l'inhibition de COX-1 par l'aspirine augmente proportionnellement avec le taux de glucose. Ces résultats suggèrent qu'un taux élevé de glucose interfère avec l'acétylation de la serine 529 de COX-1, aboutissant à une inhibition de l'activité enzymatique de l'aspirine. Les modèles prédictifs *in silico* suggèrent que le glucose peut entraver le processus d'acétylation en se plaçant à l'entrée de la poche catalytique de la COX-1, ce qui entrave l'accès ultérieur de l'aspirine. Toutefois, ce modèle doit être validé, ainsi que d'autres mécanismes qui pourraient expliquer la réduction de l'inhibition constatée de la COX-1 par l'aspirine chez les patients diabétiques.

Chapter 1

INTRODUCTION

1. Diabetes Mellitus

1.1. Dysregulation of glucose metabolism and hyperglycaemia

Among metabolic disorders, diabetes mellitus represents the most frequent disease throughout the world in the 21st century. According to a global report from World Health Organization (WHO) published in 2016, more than 400 million of adults (8.5%, aged 18 years and older) had diabetes in 2014 with 1.5 million of deaths attributable to diabetes and 2.2 million to high blood glucose in 2012[1]. These numbers are subjected to a rapid and alarming increase due to the growing prevalence of risk factors such as overweight and obesity, genetic predisposition and aging[2]. In general, diabetes mellitus is a group of chronic and progressive disorders characterized by elevated levels of blood glucose, namely hyperglycaemia, mostly due to the impairment of insulin secretion by Langerhans β cells and tissue insulin resistance. The former refers to type 1 diabetes (previously known as insulin-dependent or juvenile diabetes), in which pancreatic β cells are destroyed by autoimmune response leading to a drastic decrease of endogenous insulin production that in turn lead to the increase of glucose in blood and urine[3,4]. This form of diabetes mostly occur in children and adolescent and altogether with the gestational diabetes, it accounts for between 5% and 10% of all the diabetes cases[5]. Despite the exact cause of this form of diabetes is still unknown[6], it is generally agreed that it might result from genetic predispositions and environmental risk factors. Type 2 diabetes (formally called non-insulin-dependent or adult onset diabetes) represents the most common form of the disease with a 90% of all diabetes cases characterized by insulin resistance, β cells dysfunction and high blood glucose[7]. With respect to the type 2, the body is *per se* able to produce insulin but it is unable to fully mediate the effect of this hormone. As insulin is required for an efficient cellular uptake of glucose to convert into energy, the low response to insulin causes a positive feed-back mechanism by pancreatic β cells to enhance insulin production and secretion. Prolonged exposure to high blood insulin levels, in turn induce the down-regulation of glucose transporter GLUT4 isoform on the cell membranes[8] (primarily on skeletal muscle and adipose tissue), contributing to the increase of hematic glucose and insulin as well. However, such an increase has a limit, failing to meet the need for more insulin production[9,10]. Under this condition, β cells die[11], plasmatic levels of insulin decrease and hyperglycaemia develops and progresses[12]. The onset of hyperglycaemia and diabetes is preceded by many years of insulin resistance, often associated to obesity

in a condition also known as metabolic syndrome[13]. Obesity plays a pivotal role in this phenomenon providing a link between type 2 diabetes and fat accumulation. Adipose tissue is an active source of free fatty acids (FFAs) and inflammatory cytokines and it has long been recognized that obese individuals with type 2 diabetes display increased plasma levels of FFAs[14,15], mainly due to the expansion of fat mass and defects in adipocyte metabolism. The obesity-associated increase of FFAs induces insulin resistance through activation of NF-κB and PKC mediators that in turn trigger the up-regulation of IL-6 and TNFα genes and the serine phosphorylation of the insulin receptor substrate (IRS-1), altering the PI3K/Akt signalling (Figure 1). These molecular events result in the down-regulation of GLUT4 and, hence, insulin resistance and hyperglycaemia, which in turn trigger the development of the long-lasting diabetic complications.

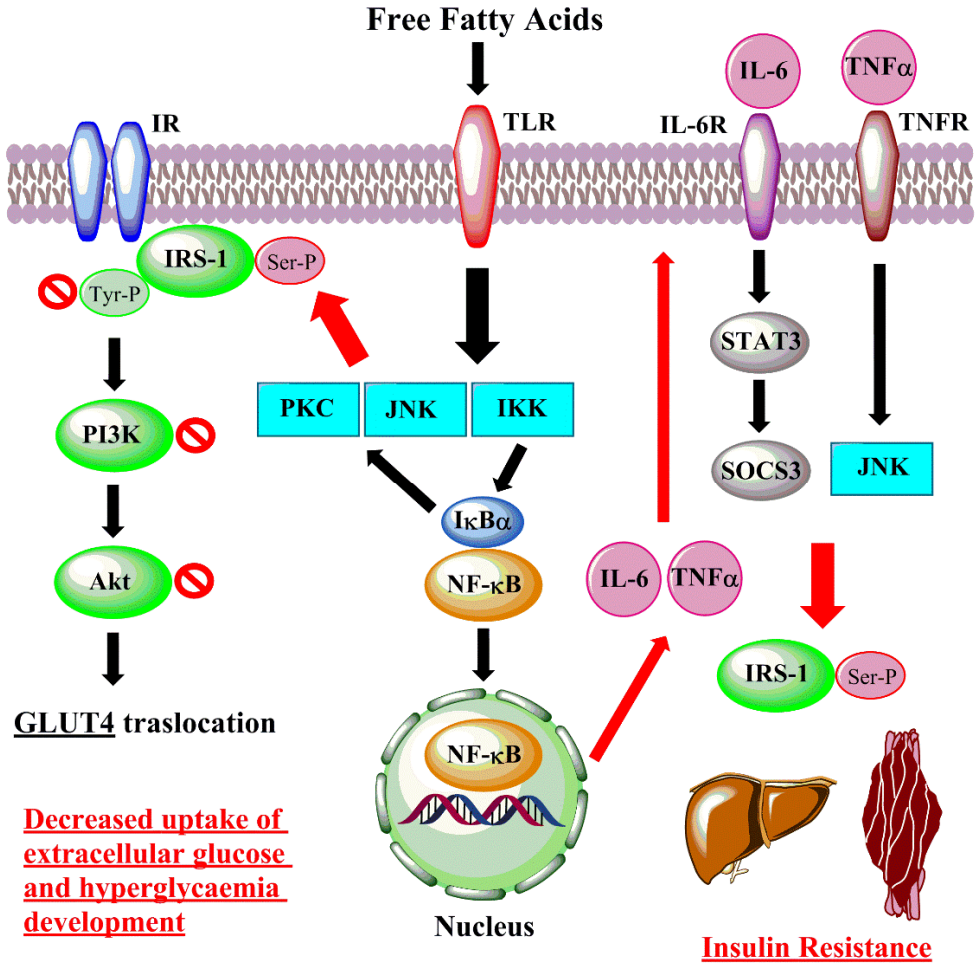

Figure 1. Insulin resistance and hyperglycaemia signalling cascade triggered by obesity. FFA, free fatty acids; IR, insulin receptor; TLR, toll-like receptor; IRS-1, insulin receptor substrate-1; JNK, c-Jun amino-terminal kinase; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha.

1.2. Vascular complications of diabetes and their molecular basis

Regardless of the type of diabetes, it is the persistent level of hyperglycaemia that causes most of the metabolic problems behind diabetic complications. Glucose toxicity can negatively affect a wide range of organs and tissues, mostly through macro- and micro-vascular complications, which symptoms are often observed several years after the development of the illness[16,17]. Macro-vascular complications are mainly related to atherosclerotic disease of large and medium-size arteries (coronary artery disease, peripheral artery disease and stroke), accounting for the increased risk of cardiovascular events and representing the first cause of morbidity and mortality in both type 1 and type 2 diabetes. In addition, diabetic-related micro-vascular disease are ascribed to neuropathy, retinopathy and nephropathy which are the major cause of defects in peripheral nerves (motor neurons, autonomic nervous system and pain fibres), blindness and renal insufficiency, respectively. The alteration in vascular homeostasis due to endothelial cell dysfunction is the main feature of a prolonged exposure to hyperglycaemia, favouring a pro-inflammatory/thrombotic state[18]. Oxidative stress plays a key role in vascular injury observed in type 2 diabetes as well as in the development of β cell dysfunction and insulin resistance, the two hallmarks of diabetes[19]. The mechanism underlying the increased oxidative stress in presence of high glucose concentration relies on the unbalance between nitric oxide (NO) bioavailability and the accumulation of reactive oxygen species (ROS), leading to endothelial cell dysfunction[20,21]. High blood glucose levels induce the body to mobilize all the possible pathways involved in glucose clearance (Figure 2). One of those pathways is the polyol pathway[22], which is usually silent in non-diabetic state but can be enhanced to metabolize up to 30% of glucose in diabetes[23]. In this pathway, glucose is converted into fructose through a two steps process that results in an excess of NADH by consuming NADPH, hence breaking the redox balance between NADH and NAD^+. The excess of NADH can overload the mitochondrial electron transport system and drive overproduction of ROS that can induce protein modifications[24]. Moreover, consumption of NADPH can impair the function of the glutathione reductase that use this metabolite to reduce the oxidized form of glutathione, thus aggravating cellular redox balance[25]. Another major source of ROS in addition to the polyol pathway is represented by the PKC pathway and its downstream targets. Diabetic hyperglycaemia can induce a chronic increase of diacylglycerol in endothelial cells that triggers the activation of PKC and the following increase of

superoxide anion via NADPH oxidase[26]. Accumulated superoxide anions react with NO to generate peroxynitrite ($ONOO^-$), an excellent oxidant that induce nitrosylation of a large number of protein targets including the endothelial NO synthase (eNOS), with the consequent decrease of its activity[27]. The increased oxidative stress associated to the reduced bioavailability of NO contributes to vessel dysfunction in retina and in peripheral nerves and it is a strong predictor of cardiovascular outcomes as well.

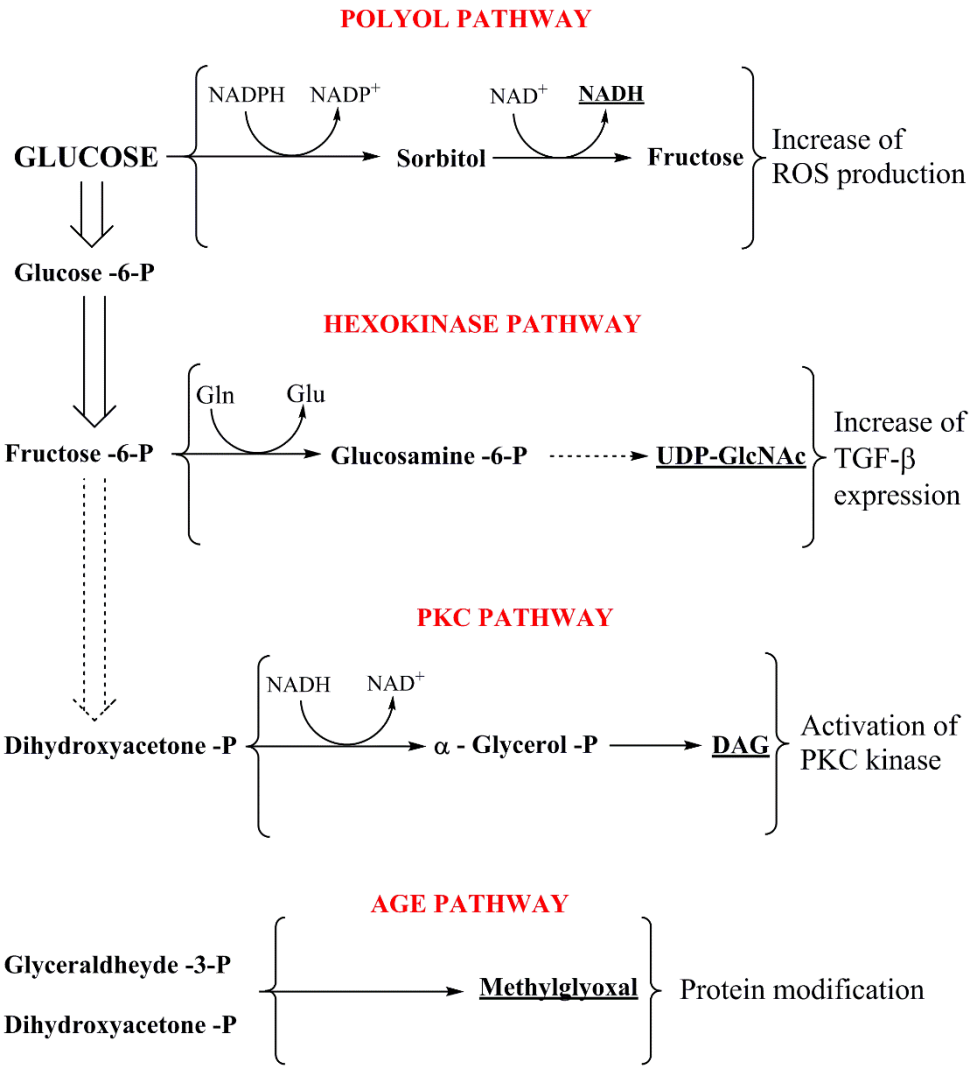

Figure 2. Secondary biochemical pathways induced by hyperglycaemia.

Hyperglycaemia state can also activate other metabolic pathways that lead to extensive protein post-translational modifications (PTMs) which are involved in vascular disease, sclerosis and insulin resistance. In the hyperglycaemia environment, excess of fructose 6-phosphate is diverted from glycolysis path and fluxed into the hexosamine pathway[28], resulting in elevated levels of uridine

diphospho-N-acetylglucosamine that can attach to proteins[29]. Protein O-GlcNAcylation has been found to take part in numerous biological processes, such as transcription, redox signalling, apoptosis and protein degradation[30]. Specifically, O-GlcNAcylation of eNOS leads to the decrease of its activity contributing to endothelial dysfunction[19,31]. Moreover, it was shown that this PTM influences the activity of several transcription factors causing the up-regulation of inflammatory (TGFα-β) and pro-thrombotic (PAI-1) genes representing the main cause of glomerulosclerosis and coronary events, respectively[32]. The development of diabetes and its complications is also associated to a group of PTMs that occur between proteins and high reactive carbonyl compounds derived from glucose autoxidation (glyoxal), lipid peroxidation (3-deoxyglucosone) and from the rearrangement of some intermediates of the glycolytic pathway (methylglyoxal)[33]. The resulting protein adducts are called advanced glycation end products (AGEs), which exert their pathological effect through the enhancement of oxidative stress[34], intensifying the inflammatory response and the alteration of protein structure/function[35] and representing the bridge that correlates hyperglycaemia and diabetic complications[36]. The damaging effect of AGEs results from the direct modification of structural proteins, such as collagen or integrin receptors, impairing matrix-matrix as well as matrix-protein interaction[37,38]. Intracellular non-structural proteins and enzymes are also targets of modification and AGEs formation has been shown to interfere with their function[39,40]. Beside direct changes in protein structure and function, AGEs can mediate cellular dysfunction through the interaction with their specific receptors (RAGEs) on the cell surface, leading to the increase of pro-inflammatory state and enhancing insulin resistance and β cell impairment[41]. Despite carbonyl products of hexose and pentose metabolism are more reactive towards proteins, the most common pathway known to form AGEs is ascribed to a reaction discovered more than one century ago and describe in the next section.

1.3. The role of non-enzymatic glycation

Non-enzymatic glycosylation or glycation is a reaction described for the first time in 1912 by Louis Camille Maillard, who observed rather by chance, that mixtures of amino acids and sugars assumed an intense brown colour upon heating, with the generation of carbon dioxide[42]. In this process di-carbonyl compounds are formed and their rearrangement is responsible for the aroma, taste and the browning

appearance of thermally processed food, as evidenced in later researches. Independently, in 1920, Mario Amadori observed that the condensation reaction between aromatic amines and glucose led to the generation of a glucose-aniline Schiff base, which is subsequent converted to the more stable aniline-deoxy-fructose derivative. It was not until the 1950s that it was recognised that this reaction was not only limited to aromatic amines but could occur also with aliphatic amines, such as those ones present in amino acids. The conversion of the labile N-glycosides was named as the "Amadori rearrangement" and the amino-deoxy-ketoses were later designated as "Amadori products". Formation of the Schiff base from sugar and primary amines of proteins (N-terminal, ε-amine of Lysine and η-amine of Arginine) is relatively fast and highly reversible, while the conversion to the Amadori product is slower but much faster than the reverse reaction, thus the Amadori glycation products tend to accumulate on proteins (Figure 3).

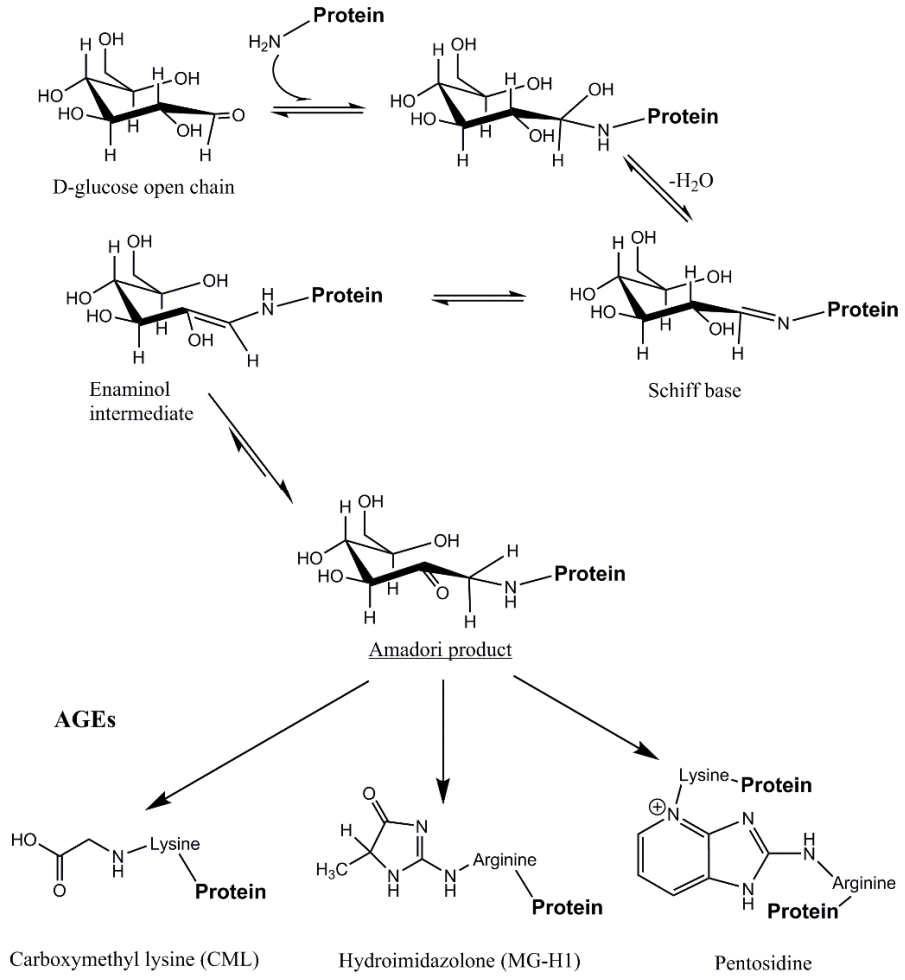

Figure 3. Mechanism of non-enzymatic glycation based on the Maillard reaction. The Amadori compound undergoes to a sequence of oxidations and rearrangements that lead to the formation of the advanced glycation end products (AGEs).

Because of their wide distribution in nature, Maillard postulated that the reaction between sugars and amino acids could occur everywhere and at more physiological conditions (37ºC) and that, after sufficient incubation time, the analogous reaction products are ultimately formed as those obtained by strong heating. The first association between glycated proteins and chronic hyperglycaemia was made in 1968 with the discovery of an isoform of human haemoglobin[43] in the erythrocytes of diabetic patients[44]. In this haemoglobin variant, which was later designed as HbA_{1C}, glucose reacts non-enzymatically with the N-terminal valine residue of the β chain, generating the glycated form of this protein. Today, the percentage of HbA_{1C} is used as an important biomarker to retrospectively assess the average blood glucose concentration for the diagnosis and follow-up of diabetes. Glycation of haemoglobin increases its oxygen affinity, thus make it more susceptible to oxidation[45]. However, effects of this type on short-lived proteins like haemoglobin are generally small and limited to the relative number of protein molecules actually glycated, which in turn depend on their half-life. In healthy subjects indeed, only the 2% of body proteins are glycated while in diabetic patients this percentage is 2-3 times higher, because of the increased blood glucose levels[46]. The extent of glycation, and especially AGEs formation, increased with the severity of diabetic complications[47]. During decades of investigation, not only haemoglobin but also a large number of proteins were shown to be affected by glycation under hyperglycaemia conditions, especially those ones with a low "turnover" as collagen in connective tissue and α-crystalline proteins in eye lens, which are prone to glycation for their entire "lifespan". Glycation of collagen type IV in the basement membrane and subsequent AGEs formation and crosslinking, was shown to hamper its attachment with vascular endothelial cells and to induce vascular stiffening, promoting atherosclerosis and coronary disease, respectively[38,48]. Moreover, glycated collagen type I inhibits cell adhesion of cardiac fibroblasts while stimulating myofibroblast differentiation, leading to fibrosis[49], a pathological response that has been associated with myocardial diabetic dysfunction. Glycation of lens crystalline was shown to alter their structure, exposing previously buried sulfhydryl groups, which can auto-oxidize to form intermolecular disulphide bonds. The resulting disulphide linked super aggregates of lens proteins can be of sufficient size to scatter light, impairing the eyesight through clouding of the lens (cataract) that is one of the most common consequences of diabetes[50]. It is worth emphasizing that, although most of the attention has been on

long-lived proteins, even proteins with a rapid turnover like insulin and enzymes are damaged by glycation[51,52]. As aforementioned, in addition to the direct effect of glycation on the protein structure and function, AGEs-modified proteins can bind to RAGE receptors on the surface of many cell types (macrophages, lymphocytes, endothelial cells, mesangial cells), triggering intracellular ROS generation and pro-inflammatory signalling, which contribute to vascular disease[53]. An example is represented by glycated albumin which was shown to interact with RAGE receptor on endothelial cells that lead to the increase of the expression of pro-inflammatory cytokines (TNFα, IL-6, MCP-1) by activation of NF-κB, as well as RAGE itself, thus amplifying the inflammatory response[53]. Furthermore, PKC was reported as a target of glycated albumin, leading to increased serine phosphorylation of IRS receptors, resulting in the suppression of insulin signalling and the consequent development of insulin resistance[54]. Glycated albumin – RAGE interaction has been reported to induce ROS formation in pericytes that surround capillaries in the retina, contributing to their reduced survival and finally to the breakdown of the blood-retina barrier, with consequent micro-aneurysms, macular oedema and vision loss, typical symptoms of diabetic retinopathy[55,56]. Moreover, it has been evidenced that glycated albumin plays also a role in diabetic nephropathy by inducing apoptosis and VEGF expression in mesangial cells[57], resulting in the increased vascular permeability, hyperfiltration and proteinuria in kidney disease. In addition, AGE-induced expression of TGFβ was associated with enhanced synthesis of extra cellular matrix proteins, favouring glomerular hypertrophy[58]. As understanding of the damaging effects of AGEs as potential cause and consequence of diabetes became evident, there has been an increasing interest in protecting proteins against glycation.

2. Protein glycation prevention

2.1. Anti-glycation agents

At the beginning of the 1980s, with the growing number of evidences on the pathophysiological effects of glycation and AGEs accumulation *in vivo*, efforts have been made to find strategies to interfere with any step of this process, helping in the prevention of diabetic complications. Several types of anti-glycating agents have been described[59]: some compete for the amino groups on the protein, some bind to a glycation intermediate to stop the progression up to the AGE formation and some others simply

mop up the open chain form of the glycating sugar. Aminoguanidine was one of the first compound studied *in vitro* and *in vivo*, which was shown to react with dicarbonyl intermediates derived from the Amadori product, blocking further reactions[60]. Thus, this ability make it a potent AGE inhibitor for the prevention of diabetic complications including nephropathy, neuropathy and vasculopathy[61]. Animal studies showed that it was effective in decreasing cataract development[62], preventing albuminuria[63] and reducing carboxymethyl lysine accumulation in heart[64] of diabetic rats. Despite its early success in the protection against glycation, the manifestation of side effects observed during clinical trials has hampered further development[61]. Other compounds that belong to the dicarbonyl scavengers as aminoguanidine and have a strong inhibitory effect on AGE formation are metformin[65], buformin[65], pyridoxamine[66] and thiamine pyrophosphate[67] to name a few. Compounds that react with the open chain of sugars and remove them from reaction with proteins have been proposed as anti-glycation agents as well. These include amino acids[68], polyamines[69] and peptides[70] as glutathione[71] and carnosine[72]. Of course, a treatment that mops up the open chain form of sugars could be harmful since this form is used for vital metabolic processes, thus careful attention must be taken when considering these agents for clinical trials. A further pharmacological strategy emerged 20 years ago with the discovery of "AGE breakers", a class of agents that is presumed to cleave the cross-linked structures in proteins. These molecules contain a thiazolium group that could break α-dicarbonyl compounds by cleaving the carbon-carbon bond between the carbonyls. N-phenacylthiazolium bromide (PTB) was the first cross-link breaker reported to be able to release serum albumin from collagen matrix, to decrease tail tendon collagen cross-linking from diabetic rats, to disaggregate amyloid fibrils and to decrease the binding of IgG to erythrocytes[73]. A derivative of PTB called algebrium (ALT-711) was shown to improve arterial elasticity in diabetic rats and dogs[74,75], showing good promises for clinical trials[76]. Although the potential efficacy of these compounds has been demonstrated, their pharmaceutical inhibition of AGE formation *in vivo* is still under debate[77] and further clinical trials will undoubtedly answer it. Beside all these classes of agents, there is that one including those inhibitors that interfere with the initial attachment of reducing sugars to the amino groups of proteins by competing with it, without affecting the level of glucose. Aspirin was probably the first molecule shown to prevent glycation in that way and one of the most

widely studied drug for the treatment of diabetes, thanks to its numerous beneficial effects at multi-functional levels.

2.2. The beneficial effects of aspirin in diabetes

From its first use from willow leaves by Hippocrates in the 4th century B.C. and the understanding of its action in the early 1970s, aspirin (acetylsalicylic acid or ASA) remains currently one of the most frequently used drugs in the world for the general treatment of pain, inflammation and rheumatic fever[78,79]. Furthermore, due to its anti-thrombotic properties, aspirin represents the medication of choice for the secondary prevention of atherosclerosis such as coronary artery disease (CAD), ischemic cerebrovascular disease and peripheral arterial disease[80]. Several studies also showed its effectiveness for the chemoprevention of certain types of cancer, particularly colorectal cancer[80,81]. In contrast to other nonsteroidal anti-inflammatory drugs (NSAIDs) from which it belongs, aspirin has a different mechanism of action that relies on the irreversible inhibition of cyclooxygenases (COXs). All COXs are homo-dimer membrane-bound heme-containing glycoproteins with a broad tissue distribution[82] and a bi-functional nature that catalyse the first two committed step in prostaglandins (PGs) synthesis. In platelets PGs are further processed by the enzyme thromboxane synthase leading to thromboxane (TXA_2) generation[83]. Platelet TXA_2 production represents a fundamental amplification mechanism of platelet activation, recruitment and aggregation[84], which is vital in healthy subjects to prevent the excessive bleeding during trauma but it becomes highly dangerous if uncontrolled. Chronic hyperglycaemia has been clearly identified as a causal factor for *in vivo* platelet activation and platelet hyper-reactivity in diabetes mellitus[85]. Platelet TXA_2 biosynthesis was shown to be significantly higher in type 2 diabetes and that a tight metabolic control led to a reduction of TXA_2 levels[86]. Moreover, hyperglycaemia-induced oxidative stress is responsible for enhanced lipid peroxidation to form hydro-peroxides and F_2-isoprostanes, which stimulate COXs activity and activate TXA_2 receptor respectively, leading to the pro-inflammatory and pro-thrombotic state characteristic of diabetes, which in turn contributes to the increased risk of cardiovascular events[19]. Aspirin exerts its cardio-protective effect by acetylating the platelet COX-1 active site, thereby blocking the formation of TXA_2 and decreasing platelet activation[87]. Moreover, aspirin-induced acetylation of the COX-2 isoform in

monocyte/macrophage and endothelial cells results in the formation of 15R-hydroxyeicosatetraenoic acid (15R-HETE), preventing the formation of TXA_2. This intermediate is transformed via arachidonate-5-lipoxygenase to generate lipoxins, which have anti-inflammatory effects[88] (Figure 4).

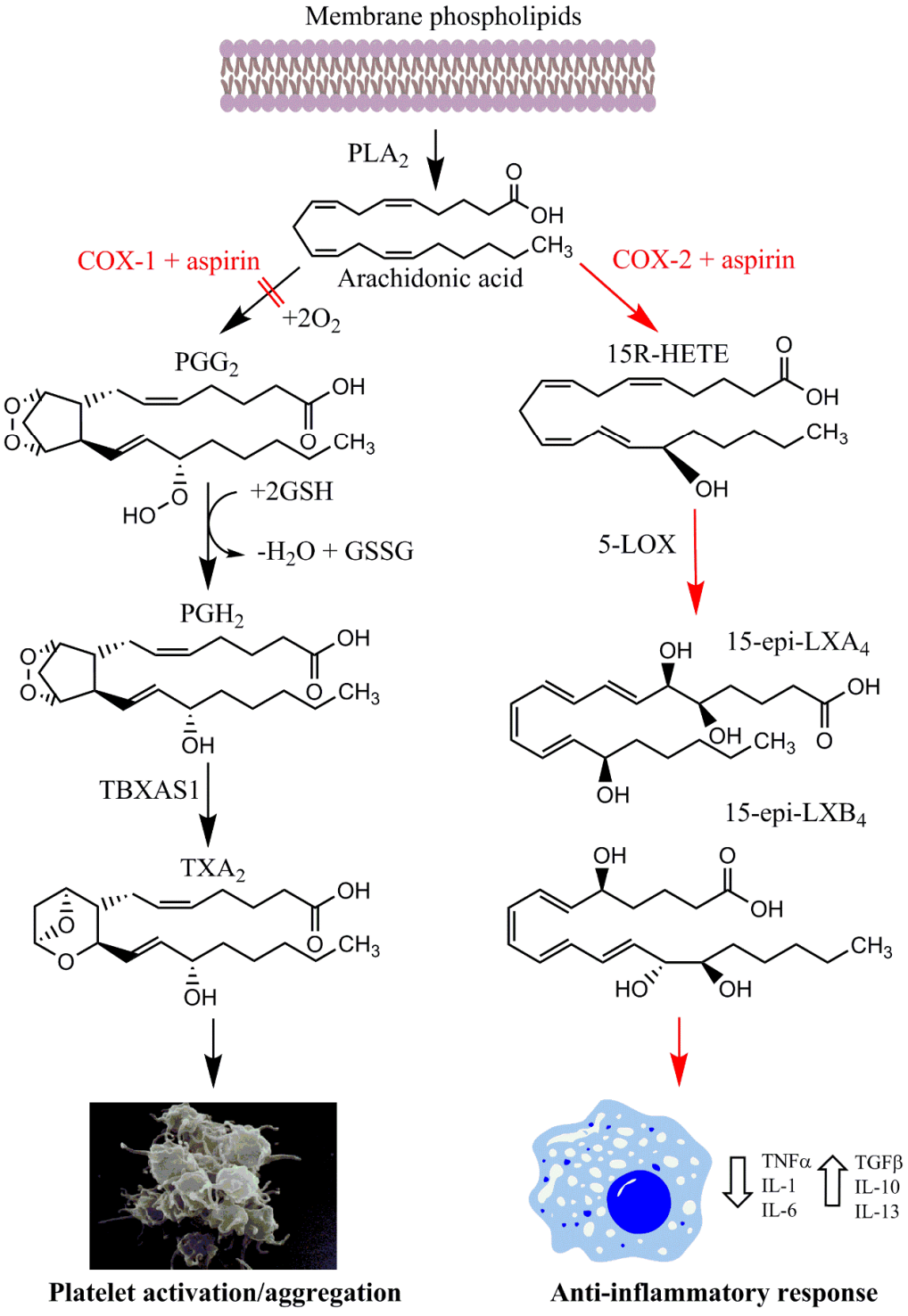

Figure 4. The effect of aspirin on COXs signalling. In platelets, aspirin acetylates and inhibits COX-1 isoform, reducing the generation of TXA_2 and platelet aggregation. In monocytes and endothelial cells, the acetylation of COX-2 isoform by aspirin triggers anti-inflammatory effects and stimulates vasorelaxation.

Beside the prostaglandin signal pathways, the effect of aspirin spans over multiple cellular targets[79] as nitric oxide synthase (cNOS), increasing NO levels and favouring an anti-aggregation platelet phenotype[89]. Aspirin also acetylates and inhibits IKKβ kinase, avoiding the nuclear translocation of NF-κB and the consequent expression of pro-inflammatory cytokines and redox-sensitive genes[90]. The MAPK kinases ERK, JNK and p38 are inhibited by aspirin as well, providing in this way a defence against inflammation and insulin resistance[91,92]. Current guidelines from the American Diabetes Association (ADA) recommend once-daily low dose (75-162 mg/day) aspirin to achieve the maximal anti-thrombotic efficacy with the lowest bleeding risk. This therapeutic regimen has been mainly suggested for primary prevention of cardiovascular disease (CVD) in diabetic patients with a high risk of cardiovascular events (10 years CVD risk over 10%) or intermediate risk (10 years CVD risk between 5-9%) and for secondary prevention in all diabetic patients with history of CVD[93]; whereas aspirin is not recommended for diabetic subjects with low CVD risk (10 years CVD risk under 5%) or those with haemorrhagic events such as intracranial and gastrointestinal bleeding, outweighing its benefits[94,95]. In healthy subjects, inhibition of TXA_2 production by aspirin is cumulative upon repeated daily dosing and saturable at low doses because of its irreversible nature[96]. In diabetic patients, as well in other diseases with a rapid platelet turnover[97,98], newly formed platelets with uninhibited COX-1 enter the blood stream[99] with a rapid recovery of platelet function within 24 hours[100,101]. The low response to aspirin in diabetes has been related to an increased *in vivo* platelet reactivity also due to an up-regulation of other COX-independent signalling pathways, which are not blocked by aspirin[102]. Multiple mechanisms facilitate an on-treatment platelet hyperactivity phenotype in patients with diabetes including an increased activation of PKC associated to an altered intra-platelet Ca^{2+} concentration, the inhibition of the PI3K/Akt pathway and the reduced bioavailability of NO by oxidative stress, enhancing platelet aggregation and thrombus destabilization[103,104]. As mentioned above, an important determinant of platelet hyper-reactivity in diabetic patients treated with aspirin is the increased platelet turnover in which newly generated platelets from bone marrow megakaryocytes provide a replenished cargo of proteins, as uninhibited COX-1 and COX-2, which may be responsible for accelerated recovery of TXA_2 generation in the 24 hours dosing interval. Due to the short half-life of aspirin (15-20 min), one daily dose of low-dose aspirin may be inadequate in this situation because of the large capability of bone

marrow to accelerate platelet production at 10-fold basal rate[105]. However, several groups have reported that twice daily dosing in diabetic patient may overcome this issue[106,107]. A residual TXA_2 generation might also originate from extra-platelet, cellular COX-2 in response to a local inflammatory milieu. Differently from platelets that are persistently inactivated by aspirin, activated vascular cells and macrophages can rapidly (2-4 h) recover the aspirin-dependent irreversible inhibition of COX-2 activity[108]. Moreover, due to the slightly greater volume of its active site that can accommodate larger structures than those able to fit the active site of COX-1[109], COX-2 has a higher threshold of inhibition by aspirin than COX-1, suggesting that this enzyme isoform functions at lower arachidonic acid and hydro-peroxides concentrations and finally contributing to the less-than-expected reaction to aspirin. F_2-isoprotanes, produced by arachidonic acid oxidation and reflecting ongoing *in vivo* oxidative stress, can partially activate the TXA_2 receptor (TP receptor) in a COX-independent way[110-112], bypassing the inhibition of aspirin. Finally, a more diabetes-specific mechanism for on-treatment platelet hyper-reactivity may be related to hyperglycaemia and in particular to the glycation of platelets proteins that may interfere with the acetylation process and lead to an inadequate aspirin-induced effect[113]. Indeed, the interplay between glycation and acetylation has been repeatedly reported on some proteins and cell systems, suggesting that glycation may interfere with aspirin-induced acetylation process and thus explain at least in part the on-treatment platelet hyper-reactivity associated with diabetes.

2.3. Aspirin protection against glycation

The interaction between aspirin-induced acetylation and glycation has been evidenced *in vitro* and *in vivo* on specific subset of proteins. However, the details of this process are still elusive and the nature of the competition between these two PTMs remains unclear. It appeared that the mechanism by which aspirin exerts its protective effect against protein glycation was by acetylation of the reactive free amino groups on proteins (α-NH_2 and ε-NH_2), thus blocking potential active sites where reducing sugars might likely react[114]. One of the pioneering study on the competition between acetylation and glycation showed that aspirin *in vitro* readily acetylates lens crystallins, protecting them from glycation by galactose, glucosamine and glucose[115-117]. Glycation of lens proteins alters its surface charge leading to conformational changes that precede protein unfolding[118], which in turn exposes sulfhydryl groups to

undergo oxidation and subsequent formation of high molecular weight protein aggregates, favouring the development of cataract[50,119]. By inhibiting glycation, a corresponding level of inhibition of protein aggregate formation was evidenced[117]. *In vivo* studies with aspirin fed streptozotocin-diabetic rats also showed decrease in crystallins glycation associated to the reduction of protein aggregates, as well as in plasma glucose levels and HbA_{1C}[117]. Glycation of albumin and haemoglobin was shown to be reduced in presence of aspirin by a very rapid acetylation process, *in vitro* and *in vivo*[120]. Further studies using fluorescence spectroscopy and circular dichroism spectropolarimetry reported that aspirin-mediated acetylation efficiently inhibits secondary structure conformational changes of haemoglobin induced by glycation, preventing the conversion of α-helix to β-sheet[121]. In another *in vivo* study short and long-term aspirin treatment prevented the inactivation of heme pathway enzymes δ-aminolevulinic dehydratase and porphobilinogen deaminase by glycation that would aid in preventing diabetic complications[122]. In corneal and scleral type I collagen, aspirin was not shown to prevent glucose attachment nor the production of reactive intermediates but it was demonstrated to alter the charge distribution along collagen fibrils reducing the interfibrillar spacing seen with glycation upon hydration, highlighting its ability to control corneal oedema in diabetes[123]. The fact that the inhibitory function of aspirin on collagen molecules is likely to occur after Amadori product formation was confirmed by its capability to reduce pentosidine generation in a concentration-dependent way in vitro[124] and *in vivo*[125]. Other interesting studies rely on the effect that glucose and aspirin have on fibrinogen and on the fibrin network structure in diabetes. It has been shown that aspirin-induced acetylation makes the fibrin network more porous[126] and this has been associated with a facilitated fibrinolysis with shorten clot lysis time, which may explain part of aspirin's antithrombotic effects[127]. On the other hand, glycation of fibrinogen has been reported to impair fibrinolysis[128], and an ameliorated glucose haemostasis in diabetes results in increased fibrin gel permeability[129]. Despite a clear effect of low and high dosing aspirin treatment on the increased fibrin network permeability has been reported in type 1 and type 2 diabetes[130,131], no direct competition between aspirin and glucose in binding to fibrinogen was evidenced[132]. The mutual impact between aspirin-induced acetylation and non-enzymatic glycation still remains a field to be vastly characterized, especially in complex biological samples since the extremely variable molecular nature and dynamic range of these two PTMs.

3. Proteomic profiling of glycation and aspirin-mediated acetylation

3.1. Strategies to analyse post-translational modifications (PTMs)

Post-translational modifications (PTMs) represent an important mechanism for diversifying and regulating the cellular proteome. PTMs refer to chemical events that converts a codon coded amino acid residue into a non-standard amino acid residue by the addition of functional groups with a wide range of composition and complexity. The identification of protein substrates and their PTM sites are fundamental to elucidate the intricate process that govern cellular events like cell division, growth and differentiation. PTMs are not mere protein "decorations" but can actively determine the activity state of enzymes, protein turnover, localization and interaction with other proteins. Their role in vital cellular processes is equally compensated by their involvement in a vast set of disorders, mostly associated to alterations of the cellular micro-environment. Given the pivotal role of PTMs in cellular physiology and diseases[133], there is a concomitant growing request of highly sensitive methods for their qualitative and quantitative characterization. Conventional strategies for determination of PTMs in complex protein samples rely on gel- or membrane-based methods, as two-dimensional gel electrophoresis (2-DE) and Western blot. 2-DE separates protein populations according to charge and molecular weight and it often has sufficient resolution to distinguish the modification states of a protein directly. However, this is valid only for those PTMs that alter the protein charge state, like phosphorylation which can be visualized as horizontal trail of protein spots on the 2-DE gel, or PTMs that drastically increase the protein molecular size, like glycosylation that is often indicated by an evident protein mass difference on either 1- or 2-DE gels, before and after glycan motif removal with PNGase F. Protein acetylation, and glycation as well, do not appreciably affect the protein charge state and the relative mass increment is too low to be resolved by classical gel-based methods. Western blot analysis has been successful for identifying candidate substrate proteins for certain types of PTMs, including lysine acetylation[134] and AGE-modified proteins[135]. However, the small size of the structural motif of common PTMs, as acetylation and methylation, makes it difficult to generate specific antibodies that recognize PTM sites independent from its surrounding peptide sequence, with a good affinity. Moreover, although antibody-based Western blot still remain a highly feasible and low-cost method to survey the presence of specific PTMs

in biological samples, more efficient and sensitive analytical technologies are needed to address key bottlenecks in the identification of PTM substrate proteins, in mapping PTM sites and to investigate PTM dynamics. During last decades, mass spectrometry (MS) based proteomics has proven to be an extremely useful and powerful technique in PTMs discovery. The presence of covalent modifications in proteins affects the molecular weight of the modified amino acids, and the mass increment can be detected in the mass spectrum, helping in the determination of the type of PTM. Such studies typically involve three steps: sample preparation, in which proteins are proteolytically digested by a specific protease; chromatographic separation of peptide mixture, to reduce sample complexity; and finally the MS analysis that provides high accuracy and high sensitive means for peptide sequencing to reveal the likely presence of PTMs at individual amino acid residues. Despite the high sensitivity of MS, mapping of PTMs in proteomics is a demanding task because most PTMs exist in low abundance and/or stoichiometry, due to the high dynamic range of protein concentration in complex samples (at least seven order of magnitude)[136]. Indeed, the PTM peptides are present in an ocean of non-PTM peptides, representing the classical "needle in the haystack" in proteomics. Accordingly, MS analysis alone is not sufficient to detect PTMs and methods to enrich modified proteins/peptides are then required to detect those PTMs that modify proteins with low efficiency. This is, particularly, the case of protein glycation which reaction kinetics are much lower than those of other PTMs (glycation reaction takes days compared to the minutes taken by aspirin to induce acetylation) and thus glycated proteins are present at very low concentrations in the human organism as compared to non-glycated proteins. Therefore, an enrichment step is necessary to enable efficient identification of the low-level PTMs prior to MS analysis.

3.2. Enrichment of glycated proteins

Classical enrichment methods for large-scale analysis of PTMs rely on PTM-direct affinity chromatography or immunoprecipitation with PTM-specific antibodies, though only the former approach found a huge utility for the characterization of enzymatic glycoproteins and, to an extent, non-enzymatic glycated proteins. Lectin affinity columns were widely used for the enrichment of glycosylated proteins and peptides[137]. Alternatively, hydrazide affinity matrices may be used to capture

glycosylated peptides following periodate oxidation and, then, captured peptides can be released using glycosidase treatment, as PGNase F[138,139]. However, these methods are not well suited to glycation, because the binding selectivity of lectins relies on specific conformation of oligosaccharides, and periodate fully oxidize the secondary hydroxyl groups of the Amadori compound to carbonyl groups. Fortunately, separation-based assays have been developed for the enrichment of glycated proteins and peptides[140], primarily by boronate affinity chromatography (BAC), which relies on the highly specific and reversible interaction between the tetrahedral anion formed by hydroxylation of coplanar boronic acid at alkaline conditions and the 1, 2-*cis*-diols typically found in sugars[141] (Figure 5).

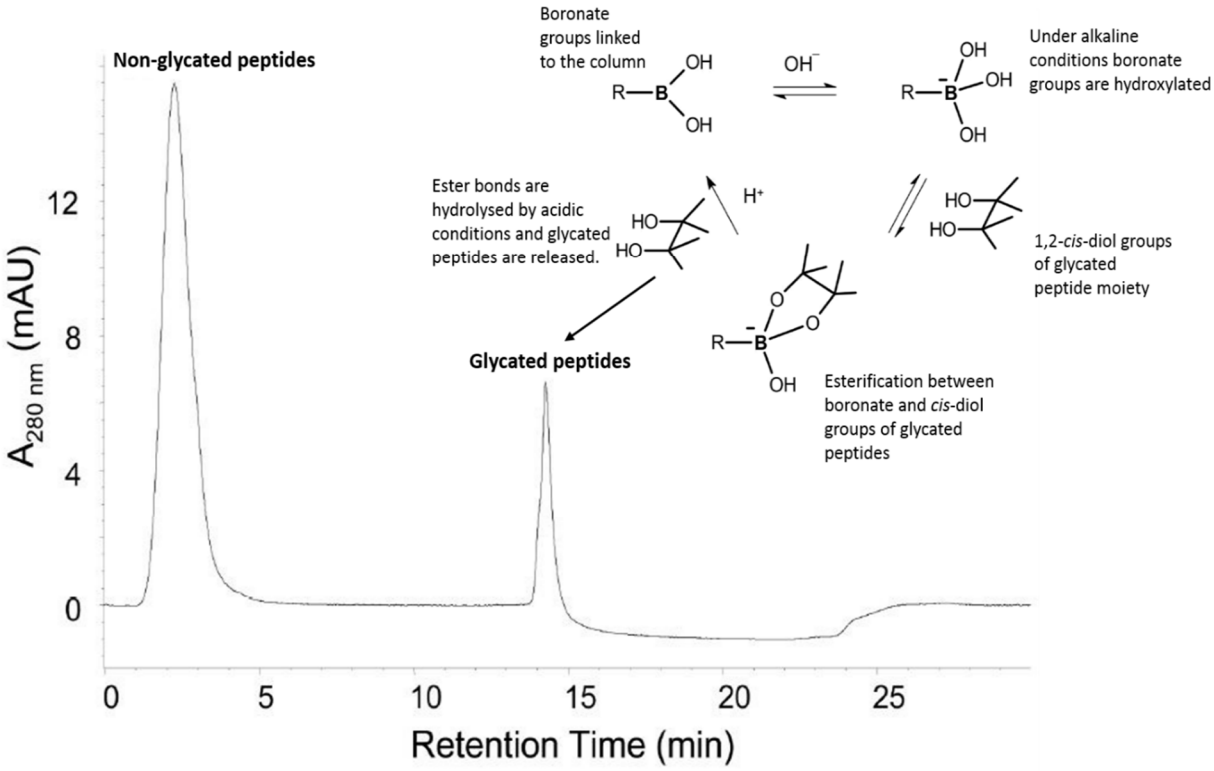

Figure 5. Mechanism of boronate affinity chromatography (BAC) for the enrichment of glycated peptides from non-glycated ones. The inset shows the equilibria between boronic acid and *cis*-diol-containing compounds.

The key interaction in boronate chromatography is the esterification that occurs between boronate ligand and *cis*-diol compounds at basic pH, which in the specific case of protein glycation usually corresponds to the glucose moiety. Ideally, this esterification requires that the two hydroxyl groups of the diol be on adjacent carbon atoms and in approximately coplanar configuration (as the 1, 2-*cis*-diol), to form a reversible 5-member cyclic di-ester that can be hydrolysed under acidic conditions, thus reverting the

reaction[142]. In principle, every compound containing *cis*-diol groups can bind to the boronate stationary phase, therefore BAC is specific for glycated proteins. This selectivity was confirmed by Zhang et al. showing that glycosylated proteins fail to bind to the boronate resin because of the presence of sialic acid at the terminus of the oligosaccharides, which may not contain coplanar cis-diol groups due to O-acetylation[143]. In addition, the low binding efficiency of glycosylated proteins was also ascribed to electrostatic repulsion between boronate anion and negatively charged sialic acid residues, in alkaline conditions, as well as to possible steric hindrance due to the complex structure of larger glycans[143]. Thus, based on the aforementioned mechanism, BAC represents a high throughput method for selective fractionation of glycated proteins from complex samples prior to MS-based approaches. The separation of glycated from non-glycated proteins occurs in a two-step process in which glycated proteins, from a complex protein mixture, are retained on the boronate affinity support of the column whereas the non-glycated species will be flowing through. The bound glycated proteins can then be eluted either by lowering the buffer pH with 0.1 M acetic acid or by using a buffer containing a high concentration of sorbitol, which has higher binding affinity to boronate than glycated proteins. The main restriction in the use of BAC for enrichment of glycated proteins relies on the very limited amount of glycated proteins collected from *in vivo* samples that might undergo to an unavoidable loss during further sample processing (e.g. desalting, digestion, etc.). Glycation, in fact, does not modify every amino acid side chain but just specific sites of proteins, and peptides generated by enzymatic digestion prior to MS analysis increase the sample complexity, counteracting the benefits of enrichment at protein level. Thus, when the initial sample amount is limited, isolation of glycated peptides is preferred over that carried out with proteins[143]. By this way, before enrichment, protein mixtures are cleaved by enzymatic digestion that is one of the most critical steps in proteomics, especially for PTM analysis, where the presence of the modification alters the digestion pattern of the enzymatic reaction. Proteolytic digestion is often carried out in solution with specific endo-proteinases, usually trypsin that cleaves peptide bonds C-terminally at every lysine and/or arginine residues. However, PTMs occurring at those sites, as glycation or acetylation, neutralize the positive charge of those amino acid side chains, blocking the enzymatic cleavage and generating peptides that differ in sequence from their unmodified form. In this sense, other proteases, as Glu-C that cleaves proteins at the C-terminal position of each glutamic acid,

can be used, in order to maintain enzymatic specificity for peptide identification and to obtain orthogonal information to those derived from trypsin digestion. The resulting peptide mixtures are then fractionated and separated by reverse phase liquid chromatography (LC) prior to the introduction to the mass spectrometer, in order to minimize the effects of sample dynamic range and maximize peptide identification rates during the subsequent MS analysis.

3.3. Characterization of glycated and acetylated proteins: the role of mass spectrometry

MS has long been used to map modifications on purified proteins, but only since the beginning of the last decade, with the development of high performance instrumentation, it has extended the range to the large-scale analysis of PTMs in proteomics[144]. This extensive role of MS in PTMs discovery can be attributable to the development of new ionization techniques, the advances in the generation of faster, more sensitive and accurate analyser associated to the development of new fragmentation methods for tandem mass spectrometry (MS/MS or MS2). Generally, MS is an analytical technique that involves the study in the gas phase of ionized molecules, aiming to determine the molecular weight of compounds, to elucidate their structure and to determine their abundance. The MS workflow typically consist of ionization of a sample in an ion source, separation of the ion species according to their mass-to-charge (m/z) ratio in an analyser, detection of the ionized molecules in a detector, and generation of a mass spectrum. Tandem mass spectrometers provide the additional capability of selecting a specific ion from the MS-survey scan (MS1) and fragmenting it in order to obtain structural information on the peptide sequence (MS2). The essential requirements that a mass spectrometer should have for an optimal PTMs analysis include high resolving power and mass accuracy for the unambiguous localization of the modified amino acid and, as well, very high sensitivity to detect sub-stoichiometric PTM peptides[145]. Accurate mass determination is a mandatory step to resolve those PTMs that show a similar peptide mass increment, as acetylation (+42.01 Da) and trimethylation (+42.04 Da) which can be distinguished only by high resolution instruments[146]. Despite recent improvements in resolution, accuracy and speed in all tandem MS platforms (particularly quadrupole – time of flight and ion traps)[147], the Orbitrap mass spectrometer has acquired a prominent role in PTMs characterization thanks to its high resolution, accuracy and wide versatility on fragmentation modes[148-150]. In MS-based PTM analysis, it is

fundamental to generate enough peptide fragmentation information (MS2 scans) for high confidence sequence identification and PTM site localization. Several fragmentation strategies including collision induced dissociation (CID), high-energy collisional dissociation (HCD) and electron transfer dissociation (ETD) have been employed over the past decade for this purpose. The selection of the fragmentation method strongly depends on the nature of PTM, which chemical stability is decisive for its efficient detection. The ergodic nature of CID makes it a suitable technique for those PTMs that remain stable to peptide fragmentation, such as lysine acetylation that can be detected by its characteristic mass shift of +42.01 Da from unmodified forms[146]. However, several common and important PTMs are known to be labile upon CID, and thus neutral loss of the modification can be the predominant dissociation pathway, leading to inadequate peptide fragmentation and diagnostic sequence ion information for the localization of the PTM site.

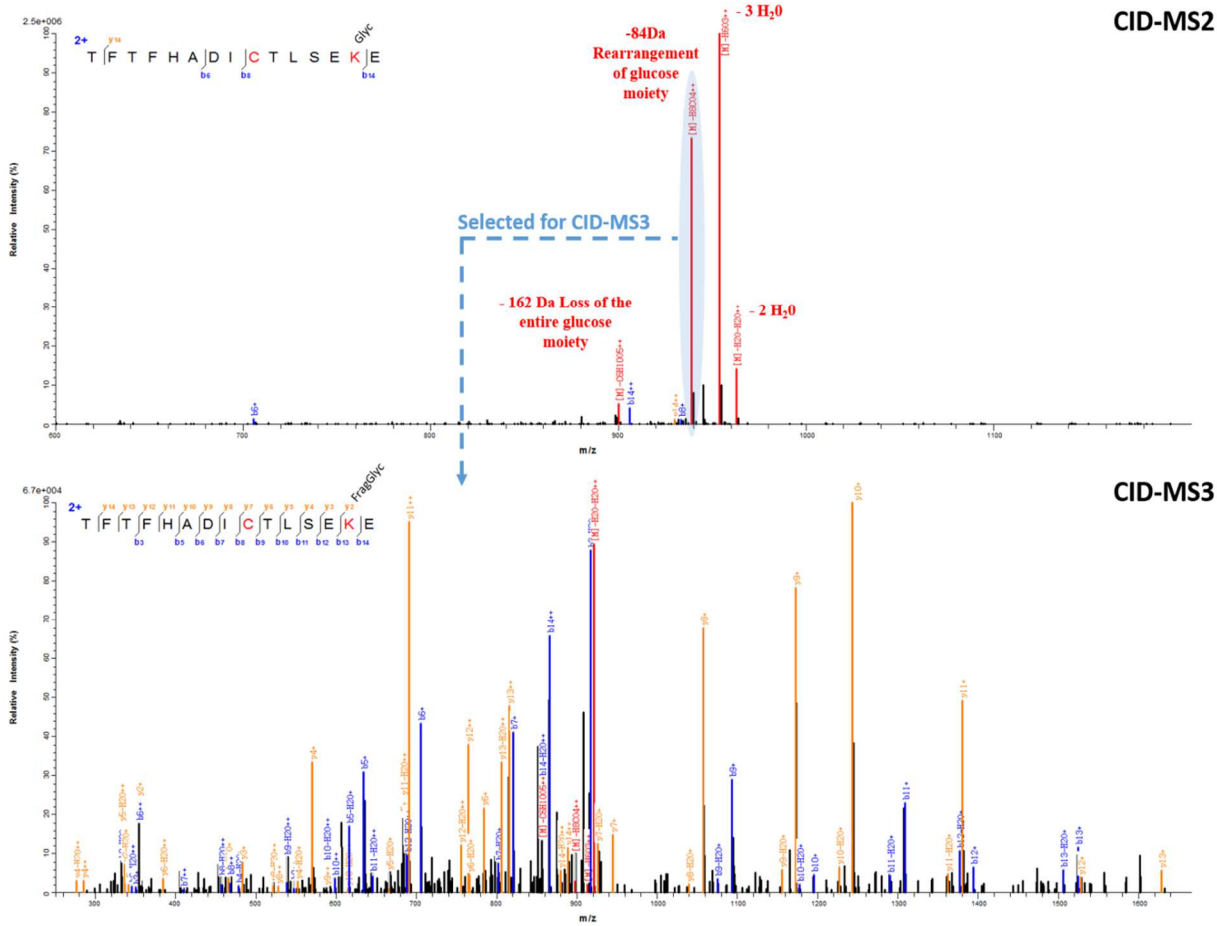

Figure 6. CID triggered neutral loss MS3. At the top, four typical neutral losses of glycation are detected after the first stage of fragmentation (MS2). At the bottom, a second dissociation step allows the sequencing of the glycated peptide (MS3) that has lost part of the glucose moiety (-84 Da).

An example is represented by non-enzymatic glycation, in which the labile Amadori adduct (+162.05 Da) tends to dissociate during CID-based fragmentation of glycated peptides, resulting in low quality spectra because of a poor production of sequence specific ions from the peptide backbone. Consequently, various ion signals derived from the losses of specific neutral fragments preferentially dominate the mass spectrum, hampering the peptide identification and localization of the glycation site[151,152]. This peculiar fragmentation pattern has been used to develop a data-dependent method that benefits from the first step of ion dissociation that promotes the neutral losses to obtain peptide sequence information after a second dissociation step, in the following MS3 stage. This approach, commonly called neutral-loss-triggered MS3, utilize the MS^n capability of ion trap instruments for sequencing peptides containing labile modifications. The typical neutral-losses detected in glycated peptides, such as the loss of the entire Amadori moiety (-162.05 Da), three water molecule (-54.03 Da) and the intermolecular rearrangement of glucose (-84.04 Da), were used to map glycation sites on plasma proteins, demonstrating the feasibility of this method for the characterization of glycation in complex biological samples[153,154] (Figure 6). In addition to CID fragmentation, HCD is an alternative tandem collision induced dissociation method that was originally used in Orbitrap platforms, mainly for the analysis of PTMs[155] (Figure 7). The advantages of HCD for PTMs discovery rely on the fact that the coverage of peptide sequences is higher in HCD compared to CID, improving the identification of PTMs[156]; the high-energy associated to this activation mode lead to a randomization of vibrational energies along peptide backbone that is faster than the dissociation of the target molecular ion, thus the breakage of the weak bond linking PTM to peptide is avoided. Furthermore, HCD fragmentation coupled to Orbitrap detection has the unique advantage to eliminate the low-mass cut-off of fragment ions in ion trap instruments, leading to the production and detection of immonium ions. These immonium ions appear in the lower mass range of the MS2 spectrum (typically below 300 m/z) and pinpoint the presence of modified amino acids, providing diagnostic modification-specific ion signals (Figure 7, insets). Acetylated lysine was shown to generate two specific immonium ions at 143.12 Da and a derivative that has lost ammonia, at 126.09 Da, that are used as marker ions for the validation of the PTM assignment[157]. Similarly, it was observed that glycation at lysine and arginine residues specifically produce two pairs of immonium ions, namely pyrylium ion (after the loss of three water molecules) and furylium ion (after

the additional loss of a formaldehyde molecule), at 192.10 and 162.09 Da for glycated lysine and at 237.14 and 207.12 Da for glycated arginine. The presence of these signals represents a good indicator that the corresponding peptide contains a glycation site[154].

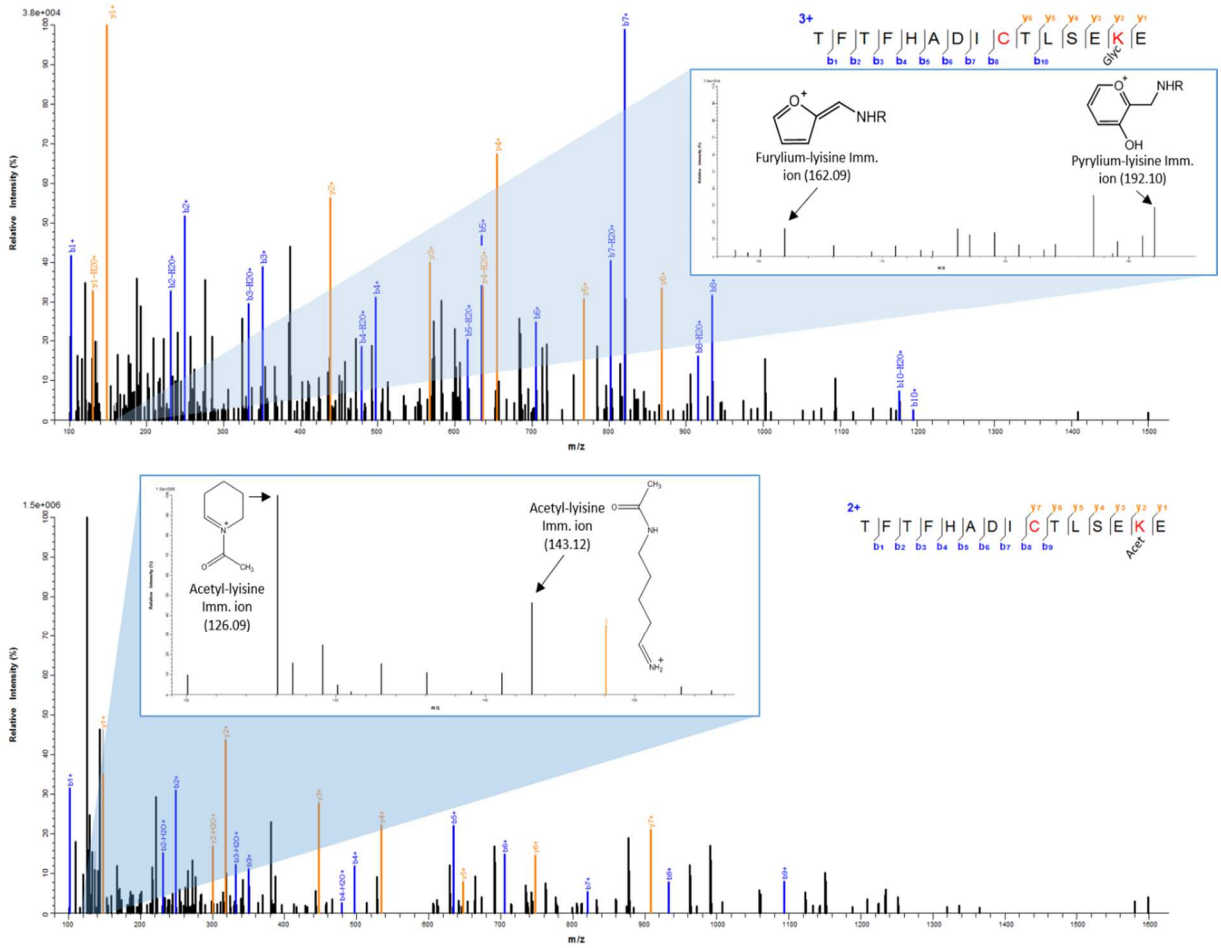

Figure 7. HCD-MS2 spectra of a peptide in its glycated (top) and acetylated (bottom) form. The two magnified regions from the low-mass range of the spectra, indicate the presence of the two typical glycated- (top) and acetylated- (bottom) lysine immonium ions.

The final task in PTMs analysis relies on the quantification of their abundances in order to determine the fine regulation that PTMs exert in different biological contexts. PTM-bearing peptides are quantified in the same way as unmodified peptides. However, it is more challenging because not only encompass the determination of protein abundance, but it is based on the measurement of the occupancy levels at the modification site of single peptides, often of very low concentrations. Stable isotope labelling represents the gold standard for peptide quantification because it minimize the experimental variability sources of errors. It can be achieved *in vivo* (metabolic labelling) through the introduction of heavy

isoforms of nutrient, as amino acids (SILAC), into the cell culture medium[158] or *in vitro* (chemical labelling) by derivatization of peptides after protein extraction and proteolytic digestion with different tags (iTRAQ, TMT)[159,160]. Despite these approaches can be applied to any sample type, they fix a limit to the number of conditions to be simultaneously analysed and, as well, require a large amount of starting sample. Moreover, these methods can be impractical in some cases because of the incompatibility between PTMs and labels. Recent improvements in data quality and algorithms now made label-free quantification very attractive in PTMs field. Although spectral counting is considered a more appropriate approach for relative protein quantification over small dynamic range, methods based on peak area calculation from MS1 seem to be more reliable for the quantification of PTM-modified peptides[161]. In addition to be the most straightforward method operationally, label-free analysis allows direct comparison of MS1 signals between any number of conditions and is applicable to samples of any nature and in PTM screening.

4. Aims of the thesis

Glucose is a fundamental molecule for life, and its combustion represents a predominant source of energy in cells. Its role of universal energy carrier in living systems can be considered as a result of an evolutionary process due to the fact that, among the hexoses, it has the lowest percentage of open-chain form in solution and thus has the lowest reactivity towards macromolecules. However, though glucose is essential for cellular survival, too much of it (as in diabetes) is detrimental and its excess can easily shift the balance from closed- to open-chain states, favouring the reaction with proteins. Over recent years, protein glycation has gradually grown in importance among other PTMs since its clear relation to chronic complications of diabetes. It is well known that the structure, function and turnover of proteins are altered when glycation takes place. The degree of impairment seems to reflect the extent at which the protein is glycated. Hence, the detection of this PTM at the initial stage would be helpful for both diagnostic and prognostic purposes. Moreover, characterizing the profile of glycated proteins in ever more complex biological systems as cells, tissues or bio-fluids, may open the way to a clear understanding of the pathological mechanisms associated to prolonged hyperglycaemia. The first aim of this thesis relies on the large-scale characterization of the glycation pattern of proteins from biological

complex samples including physiological fluids and cell lysates, such as plasma, cerebrospinal fluid and red blood cells. The presented approach, named glycation isotopic labelling (GIL), allowed the qualitative and quantitative deciphering of the glycated proteome dynamism in each biological sample as a function of the unbalanced glucose levels. The application of the method described in chapter 2, revealed the identification of native glycation in a large subset of proteins from the aforementioned samples, with the detection of the preferential sugar attachment sites and their relative quantification.

The next aim represents the fulcrum of the entire thesis discussion, and it is based on the hypothesis that protein glycation can be partially prevented by aspirin-induced acetylation in a competitive interplay that occurs at the reactive amino groups of proteins. In addition, this assumption can be extended as well to elucidate the hampering effect of prior glycation on further protein acetylation, a condition that, to some extent, reflects what occurs *in vivo* in chronic hyperglycaemia. To date, only few studies have brought insights on the interaction between aspirin's effects and glycation, and most of them using single reference proteins. The main goal here was to elucidate and measure the extent of the mutual influence between these two uncontrolled PTMs in samples that span from low (human serum albumin, HSA) to high complexity (protein extracts from the different blood compartments), using an *in vitro* approach based on label-free quantification. The chapter 3 described a preliminary study on the interaction between glycation and aspirin-mediated acetylation in HSA, showing that both PTMs influence each other at protein site level. Then the same qualitative/quantitative strategy was applied for the analysis of glycated and acetylated proteins in whole blood. Chapter 4 showed the results obtained from the analysis of plasma proteins, and gave us information on those target residues that were affected by both PTMs, providing a comprehensive overview of the potential mechanism underlying the mutual influence between glycation and aspirin-induced acetylation. Similarly, chapter 5 focused the attention on the impact of high glucose levels and aspirin on RBC proteins, with a particular emphasis for haemoglobin. Among the results, the analysis showed a possible involvement of aspirin-mediated acetylation in the underestimation of the "real" levels of glycated haemoglobin.

Compelling evidences have shown that extensive glycation of platelets and coagulation factor proteins interferes with their acetylation level. However, the impact of chronic hyperglycaemia on the acetylation of the main target of aspirin in platelets, namely COX-1, has never been reported. The final aim of this

thesis project was to reveal new insights on the effect that high glucose, *in vitro*, and diabetes, *in vivo*, have on the less-than expected response of platelets to aspirin, focusing the attention on the molecular dynamics underlying the influence of glucose on aspirin-mediated acetylation in COX-1. The results presented in chapter 6 show the significant impact of diabetes in lowering the acetylation level of platelet proteins by aspirin, mainly highlighting the role that glucose has in hindering COX-1 acetylation, and consequently the inhibition of the enzyme activity.

Finally, chapter 7 contains the discussion that link the results of the present study each other, and further describes future analytical strategies for validating the proposed molecular mechanisms underlying the interaction between glycation and acetylation, and approaches that can be extended towards clinical applications.

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Chapter 2

QUANTITATIVE ANALYSIS OF GLYCATED PROTEINS

In this chapter, we described the application of a method for large-scale analysis of glycated proteins in heterogeneous biological samples, namely plasma, red blood cells (RBCs) and cerebrospinal fluid (CSF). Among PTMs, non-enzymatic glycation is one of the less understood due to its wide range of targets and the variety of pathological effects to which it is related. Despite a large body of evidences showing accumulation of many glycation adducts, there is a lack of knowledge about which proteins are modified, where those modifications occur, and to what extent. The analytical method presented here was based on the differential labelling of proteins with isotopic $^{13}C_6$-glucose, which were subsequently digested and analysed by using two data-dependent MS approaches, HCD-MS2 and CID-neutral loss MS3, for glycated peptides detection. The doublet signal generated in the MS1 survey scan was representative of the presence of a glycated peptide in its "light" and "heavy" forms, and the ratio between their peak abundances yield semi-quantitative information about the glycated profile for each sample. This approach allowed the identification of new targets of the glycation process among different biological samples and the determination of the dynamism of their glycated proteomes.

For this project, the first two authors carried out the first developments of the method and the analysis of the acquired data, while my contribution was related to further improvement of the methodology and the writing of the part of the manuscript relative to the method section.

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Quantitative Analysis of Glycated Proteins†

Feliciano Priego-Capote,[‡,§,∥] María Ramírez-Boo,[‡] Francesco Finamore,[‡] Florent Gluck,[‡] and Jean-Charles Sanchez*[,‡]

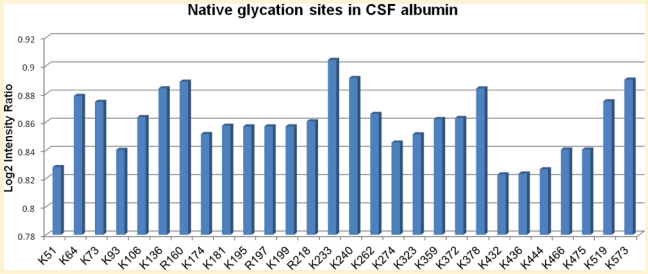
[‡]Translational Biomarker Group (TBG), Department of Human Protein Sciences, University Medical Centre, University of Geneva, 1211 Geneva 4, Switzerland

[§]Department of Analytical Chemistry, Annex C-3 Building, Campus of Rabanales, University of Córdoba, E-14071, Cordoba, Spain

[∥]Maimonides Institute for Research in Biomedicine of Cordoba (IMIBIC), Reina Sofia University Hospital, University of Cordoba, E-14004, Cordoba, Spain

Supporting Information

ABSTRACT: The proposed protocol presents a comprehensive approach for large-scale qualitative and quantitative analysis of glycated proteins (GP) in complex biological samples including biological fluids and cell lysates such as plasma and red blood cells. The method, named glycation isotopic labeling (GIL), is based on the differential labeling of proteins with isotopic [$^{13}C_6$]-glucose, which supports quantitation of the resulting glycated peptides after enzymatic digestion with endoproteinase Glu-C. The key principle of the GIL approach is the detection of doublet signals for each glycated peptide in MS precursor scanning (glycated peptide with *in vivo* [$^{12}C_6$]- and *in vitro* [$^{13}C_6$]-glucose). The mass shift of the doublet signals is +6, +3 or +2 Da depending on the peptide charge state and the number of glycation sites. The intensity ratio between doublet signals generates quantitative information of glycated proteins that can be related to the glycemic state of the studied samples. Tandem mass spectrometry with high-energy collisional dissociation (HCD−MS2) and data-dependent methods with collision-induced dissociation (CID−MS3 neutral loss scan) are used for qualitative analysis.



KEYWORDS: *nonenzymatic glycation, glucose, mass spectrometry, glycation isotopic labeling, hyperglycaemia, Amadori product, post-translational modification*

HISTORICAL BACKGROUND

Glycation is a common post-translational modification associated with the prevalence of glucose as a source energy in cells. Glycated proteins are formed by nonenzymatic condensation between reducing carbohydrates (e.g., glucose and fructose as the main sugars) with amino groups predominantly located in lysine residues and, less extensively, in arginine residues or N-terminus positions of proteins. The mechanism of the glycation process (Maillard reaction) is illustrated in Figure 1 with glucose as the sugar. The reaction between the reducing sugar and the amino group is initiated with the reversible formation of an adduct known as Schiff base by conversion of the aldehydic carbon−oxygen double bond of the sugar to a carbon−nitrogen double bound with the amine. The Schiff base is thermodynamically unstable in relation to the equilibrium cycled pyranose or furanose form. Therefore, the Schiff base is rapidly converted into an enaminol intermediate by rearrangement and, subsequently, to a ketoamine compound named Amadori compound. It is worth mentioning the biological differences between glycation and glycosylation, apart from the latter being catalyzed by glycosyl transferase and occurring in specific protein side chains (mainly N-linked asparagine and O-linked serine and threonine). While glycosylation is involved in a diverse variety of essential and nonessential tasks, glycation is an undesired modification that causes impairment of protein function by structural damage or, at long-term, by formation of advanced glycation end-products (AGEs) that alter the structure of proteins. This defective modification is one of the mechanisms involved in hyperglycaemia and derived disorders.[1−3]

A few studies have been reported for analysis of glycated proteins emphasizing the relevance of two particular steps of the analytical process:[4−7] enrichment of glycated peptides and/or proteins and detection/identification based on mass spectrometry methodologies. Enrichment at peptide/protein level is crucial to allow detection in biological samples due to the low concentration of the glycated form of the proteins. Concerning the mass spectrometry analysis, the utilization of ion traps and Orbitrap mass analyzers seems to be the preferred option for characterization of this post-translational modification.

Here, we present the approach developed in our laboratory to analyze glycated proteins with identification of sugar attachment

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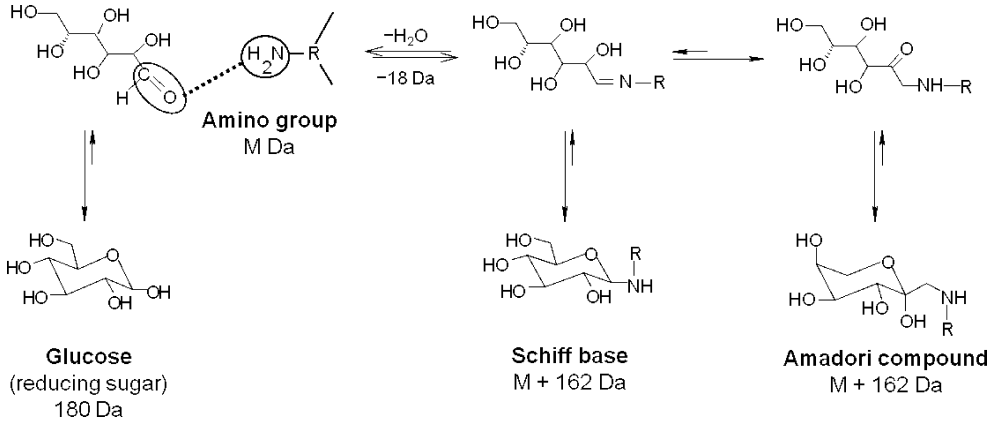

Figure 1. Mechanism of the nonenzymatic glycation of proteins based on the Maillard reaction. The amino group of the protein reacts with the reducing sugar through a reversible process to form the Schiff base, which is thermodynamically unstable. The Schiff base gives rise to a relatively stable ketoamine compound termed Amadori compound. Further, the Amadori compound can lead to a variety of carbonyl compounds, which act as propagators to the formation of a variety of heterogeneous structures irreversibly formed and commonly known as advanced glycation end-products (AGE).

sites in complex biological samples such as cellular extracts or body fluids. Our approach, schemed in Figure 2, is based on a protocol (detailed in Supporting Information) involving *in vitro* labeling of target glycation sites present in a biological sample with an isotopic $^{13}C_6$-sugar (glucose as the main sugar) to pinpoint the detection of native glycated peptides in mass spectrometry precursor scanning. Target glycation sites are labeled through a chemo-selective process under physiological conditions to avoid sample alteration. Once marked proteins are digested, a mixture of peptides is obtained among which glycated peptides are a minor fraction. Isolation of glycated peptides is selectively attained with boronate affinity chromatography (BAC) to eliminate the redundant nonglycated fraction as an enrichment step prior to liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Structural assignment of glycated peptides and identification of modification sites are performed by combination of two MS operational modes: MS2 with high-energy collision dissociation (HCD) and MS3 with collision-induced dissociation (CID) in neutral loss scanning mode so as to enhance the detection coverage. This highly sensitive and selective approach is specially suited to characterize the glycated pattern of proteins, cells, tissues and physiological fluids, allowing not only the identification of glycation sites but also their relative quantitation.

BASIC CONCEPTS

Considerations for Biological Samples

As previously mentioned, the protocol can be applied for analysis of physiological fluids as well as cell lysates such as plasma and red blood cells. In any case, collection, processing and storage of biological material from volunteers and/or patients should be carried out following GLP. In the case of blood samples, the SOP described in the Supporting Information can be used.

In Vitro Labeling of Proteins by Incubation with $^{13}C_6$-Glucose

Direct identification of glycated proteins in biological samples characterized by a complex matrix such as plasma, blood hemolysate, cerebrospinal fluid and cellular and subcellular fractions is a difficult task.[8] Consequently, most glycation studies have been supported on *in vitro* experiments to obtain information about glycation sites and structural modifications of interest for *in vivo* situations.[9−12] In this methodology, labeling with [$^{13}C_6$]-glucose is a crucial step to favor identification of native glycated proteins in experiments involving MS precursor scanning due to the detection of doublet signals for each glycated peptide. Evidently, this labeling is a chemo-selective process; in other words, only potential glycation targets will be marked isotopically. With this hypothesis, each glycated peptide provides a doublet signal in mass precursor scanning, which enhances the detection confidence of the approach. Additionally, the labeling step is a normalization operation that enables relative quantification of the glycation level of biological samples obtained under different conditions. Glycation isotopic labeling is supported on high reproducibility estimated by a low coefficient of variation for analytical replicates (<15%, data not published), which ensures quantitative capability of the overall method.

Labeling with [$^{13}C_6$]-glucose is carried out under physiological conditions (pH and temperature) to avoid sample degradation. After incubation, samples can be stored without chemical alteration for months. This fact has been demonstrated by analysis of proteins from human hemolysate stored after glucose incubation.

Enzymatic Digestion of Glycated Proteins

Similarly to other post-translational modifications, enzymatic digestion is one of the most critical steps to ensure a high selectivity level in the identification of glycation sites. Mass spectrometry-based proteomics most of the time involves enzymatic degradation of proteins to peptides by trypsin hydrolysis. This protease is characterized by a high cleavage specificity and efficiency and is stable under a wide variety of conditions. Most importantly, cleaving C-terminal to arginine or lysine residues leads to peptides in the preferred mass range for effective fragmentation in tandem mass spectrometry (MS/MS) and places the highly basic residues at the C terminus of the peptides. This generally results in informative high mass y-ion series and makes tandem mass spectra more easily interpretable. However, the presence of glycation residues alters the digestion pattern of enzymes cleaving at lysine or arginine residues.[8] For this reason, trypsin is not the suited enzyme for analysis of glycated proteins due to the generation of missed cleaved peptides.

The enzyme used in the protocol described here is endoproteinase Glu-C that cleaves after glutamate residues but also can cleave after aspartate residues which is kinetically less favored than after glutamate residues (~100–300 times). Buffer composition strongly affects the specificity of Glu-C. In phosphate buffers, both glutamate and aspartate residues are cleaved; however, in

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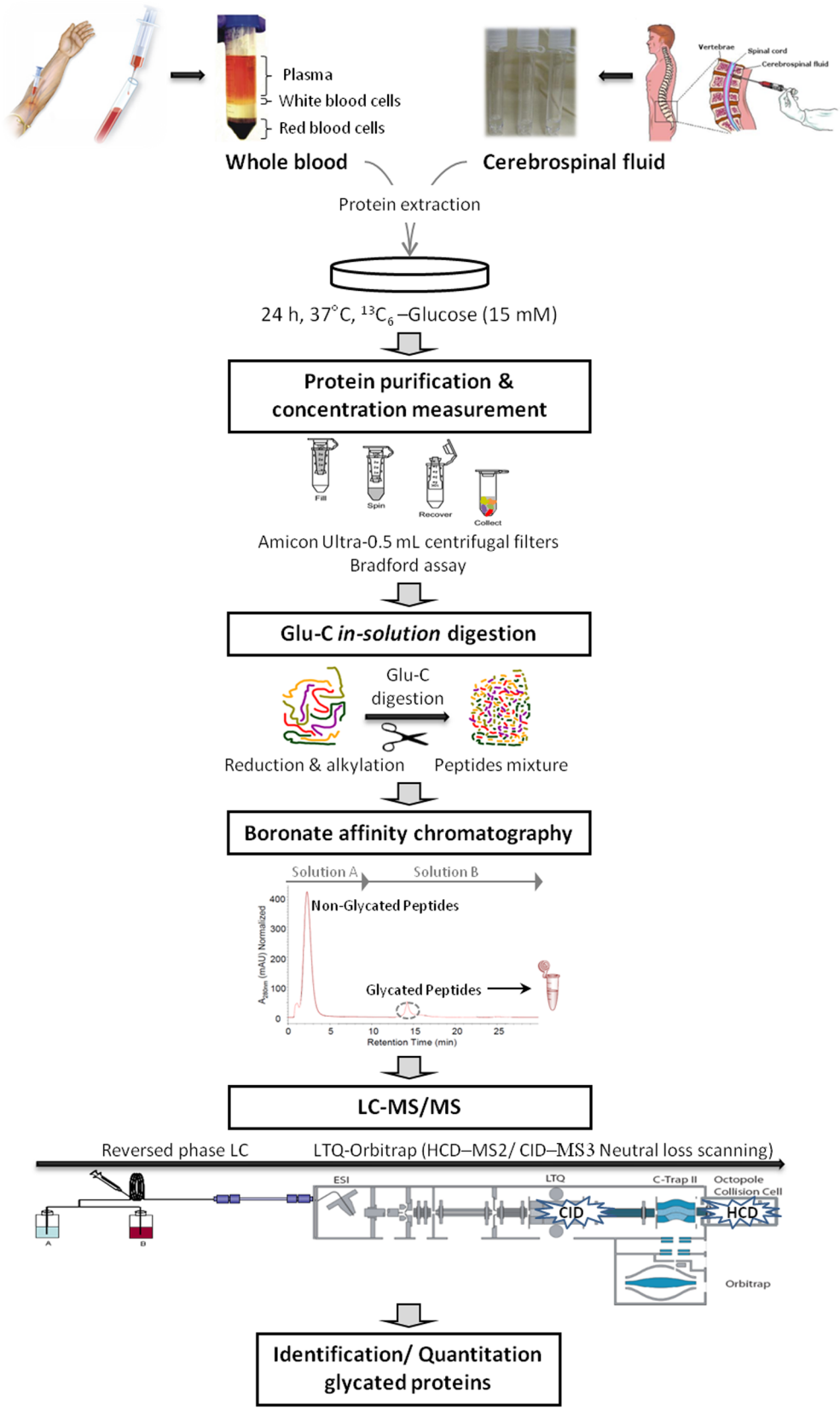

Figure 2. Scheme of the protocol proposed for comprehensive analysis of glycated proteins in human biofluids and cell lysates.

ammonium bicarbonate and ammonium acetate buffers (pH 4.0), only the glutamate residues are cleaved.[13] Buffer selection could be an alternative to control this lack of selectivity. One other option is supported on the in silico detection of half-cleaved peptides or by selection of enzymatic digestion pattern in phosphate buffers.

Since glycation is not affecting this amino acid residue, enzymatic digestion patterns for glycated and nonglycated proteins are very

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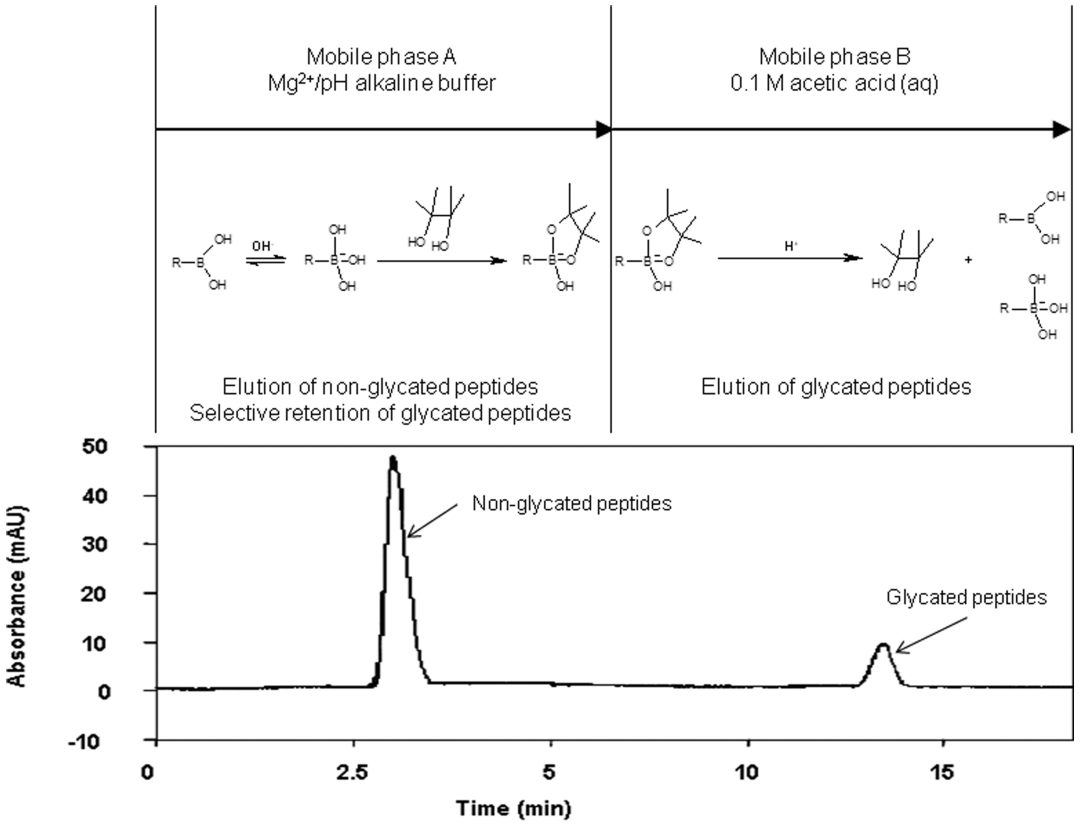

Figure 3. Boronate affinity chromatography separation of glycated peptides released after Glu-C digestion of proteins from euglycemic human serum. The mechanism of the separation process is supported on two steps: (i) retention of glycated peptides in the BAC column using an alkaline solution as initial chromatographic mobile phase while nonglycated peptides are washed out and (ii) elution of glycated peptides using an acid solution as mobile phase B.

similar. Apart from that, this enzyme generates longer peptides when compared to trypsin due to the lower occurrence of glutamate residues in human proteins. These large peptides, detected at high charge states, can be fragmented to generate characteristic fingerprints that can be accurately measured using high-resolution mass spectrometers.[14,15] It is worth mentioning that quantitative recovery of glycated peptides is ensured in the complete protocol since the involvement of peptides labeled with the isotopic [$^{13}C_6$]-glucose standardizes the measurement. The protocol has been preliminary optimized both with synthetic peptides but also with mixtures of model proteins.[16]

Enrichment of Glycated Peptides

There are two main limitations in the analysis of glycated peptides with conventional bottom-up proteomics protocols. The first one is the low ionization capability of glycated peptides in mass spectrometry detection under standard operation conditions. The second limitation is also associated to sensitivity due to the reduced efficiency of the glycation process and, thus, the low concentration of the glycated form of the proteins present in the human organism as compared to nonglycated proteins. Therefore, the application of selective steps for isolation and/or enrichment of glycated peptides is mandatory.

Affinity chromatography based on the utilization of boronic acids as ligands is the technique selected in the proposed protocol to enrich glycated peptides. This alternative, with the acronym BAC, is based on the selective interaction between boronic acids and biomolecules including *cis-diol* structures under alkaline conditions.[17] A glucose moiety possessing this *cis-diol* configuration is responsible for retention of glycated peptides by esterification of the two hydroxyl groups of *diol* with the boronate ligands.

On the basis of the above-mentioned mechanisms, BAC is highly efficient for isolation and enrichment of glycated peptides. The stationary phase usually is composed of phenylboronic acid derivatives covalently attached to an insoluble, inert agarose matrix. When the sample containing glycated peptides is run through the boronate affinity support, they are retained on the gel, whereas the nonglycated species are washed away. For this purpose, UV absorption is measured with a spectrophotometry detection system. The bound glycated peptides can then be collected by elution with an acidic pH mobile phase such as 0.1 M acetic acid and analyzed without the massive interference of nonglycated peptides. As can be seen in Figure 3, the fraction corresponding to glycated peptides is clearly minor as compared to that composed by nonglycated peptides.

Mass Spectrometry Analysis

Mass spectrometry analysis is crucial for detection of post-translational modifications. Concerning mass analyzers, ion trap and Orbitrap analyzers seem to be especially well suited for the characterization of PTMs since they allow the structural elucidation of modified peptides based on the efficiency of a theoretical MS^n process.[15] In the protocol presented here, the Orbitrap analyzer is used to take the benefit from their mass accuracy in the detection of precursor and product ions, which enables the easy detection of glucose cleavage in tandem mass spectrometry analysis. Two complementary approaches are combined to enhance the identification capability of the method. Both approaches are based on tandem mass spectrometry but they use a different activation mode for the peptide

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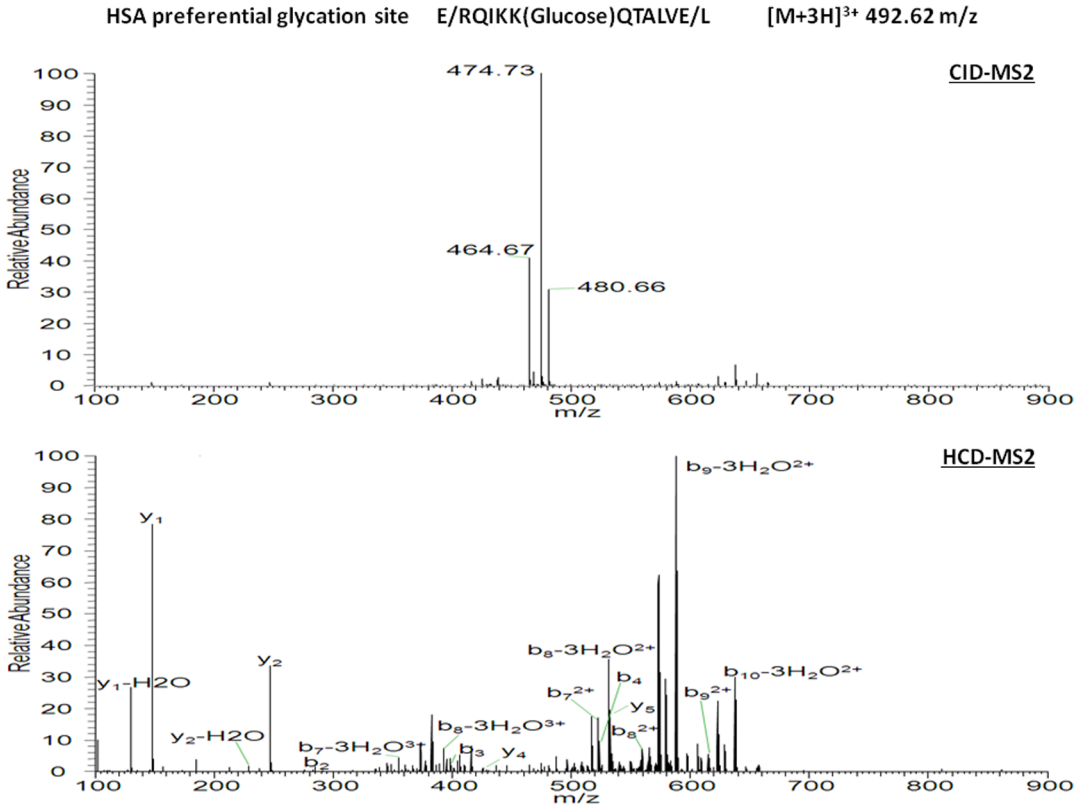

Figure 4. MS2 spectrum obtained by HCD activation of the glycated peptide containing the preferential modification site for human serum albumin. The most significant ions are indicated.

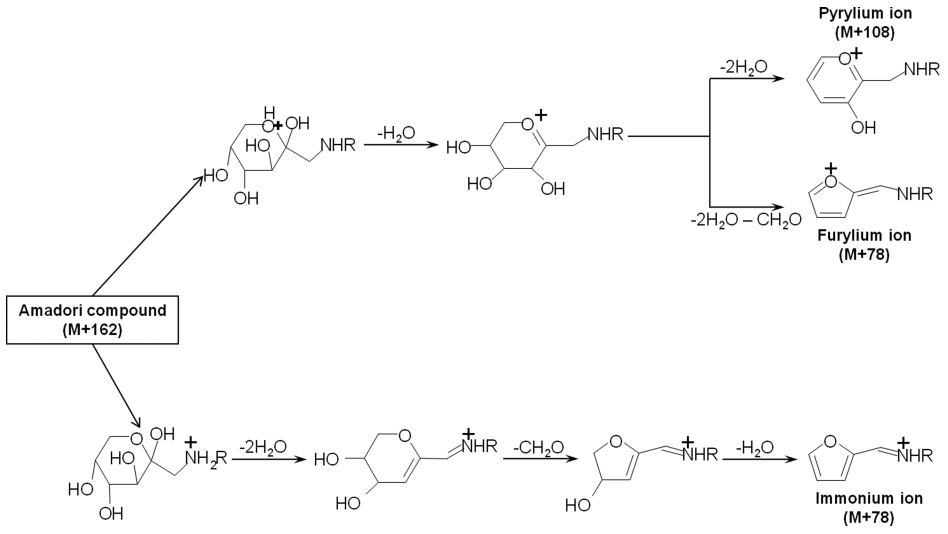

Figure 5. Fragmentation schemes of glycated peptides obtained by tandem mass spectrometry.

fragmentation. These are high-energy collisional dissociation in MS2 and collision-induced dissociation with data-dependent MS3 in neutral loss scan. HCD activation mode is characterized by its occurrence in the octopole collision cell annexed to the C-trap and nitrogen as collision gas. The use of nitrogen results in a more energetic fragmentation than helium-based dissociation occurring in CID. In addition, HCD is a fast activation mode as compared to CID, which may reach high vibrational energies per bond before dissociation of the target molecular ion.[18−20] As a result, high-quality fingerprinting spectra are obtained as shown in Figure 4, which enhances the identification of long glycated peptides.

An additional benefit of HCD-MS2 is that immonium ions formed by MS2 activation can pinpoint the presence of glycated arginine and lysine.[16] Thus, immonium-derived ions calculated for glycated arginine are at 207.124 and 237.135 Da (the most favored Arg immonium ion provides a signal at 129.114 Da, which is displaced to 291.166 Da with glucose attachment). Similarly, immonium-derived ions for glycated lysine are at 162.091 and 192.102 Da (84.081 Da as the most favored Lys immonium ion signal). The detection of immonium-derived ions for glycated Arg and Lys will aid users to assess the glycation level by observing the ion signals intensity. In addition, the same

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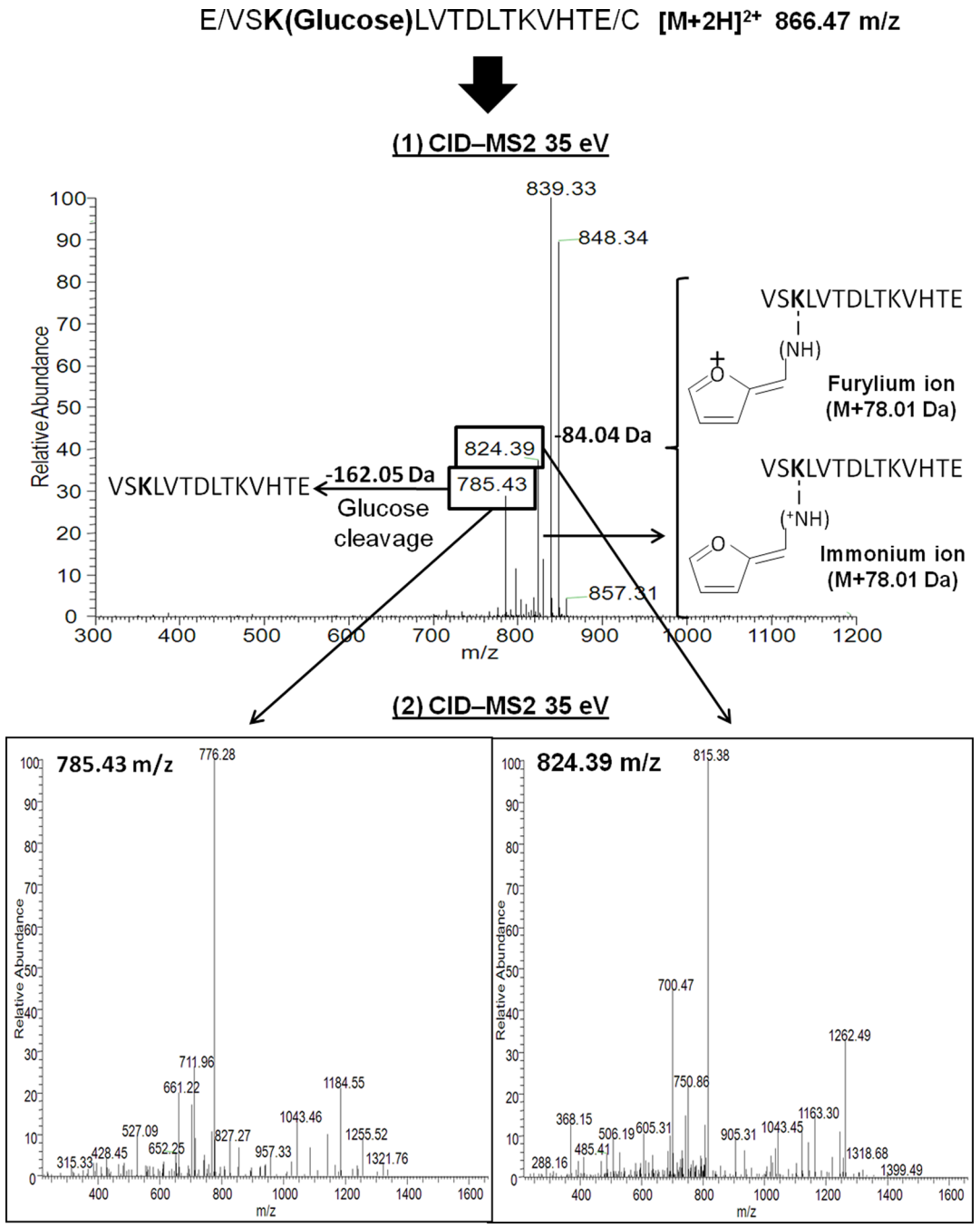

Figure 6. MS2 and MS3 spectra obtained by CID with a data dependent method based on neutral loss scanning. The example corresponds to a glycated peptide detected in human serum albumin.

strategy can be applied to estimate other immonium ions, which correspond to preferential glycation sites in specific proteins.

Analysis in MS2 is complemented by MS3 in neutral loss scanning. This process can be explained with the aid of Figure 5 that shows the different MS fragmentation pathways of glycated peptides as described in the literature.[21,22] The mechanism of the MS3 neutral loss scanning is initiated by activation of the precursor ions in a first step. If the precursor ion corresponds to a glycated peptide, detected neutral losses make reference to the dehydration of up to three water molecules (18.01, 36.02, and 54.03 Da) to generate pyrylium ion, dehydration with additional loss of a formaldehyde to form the furylium and immonium ions (84.04 Da), and the cleavage of the glucose moiety (162.05 Da). After this fragmentation, only ions obtained by loss of 84.04 and 162.05 Da are isolated in the ion trap for a second fragmentation, which provides representative MS3 spectra for identification tasks. Ions formed by the other neutral losses (18.01, 36.02, and 54.03 Da by loss of water molecules) are excluded because they do not generate MS3 spectra with enough quality for identification. Since these ions still present labile parts in their structure, the formed MS3 spectra are similar to CID-MS2 spectra of glycated peptides, as shown in Figure 6.[4,16]

According to the fundamentals of both operational modes, HCD-MS2 seems to be especially suited for detection of long glycated peptides (10−40 residues), most of which are formed by Glu-C enzymatic digestion, while CID-MS3 favors detection of

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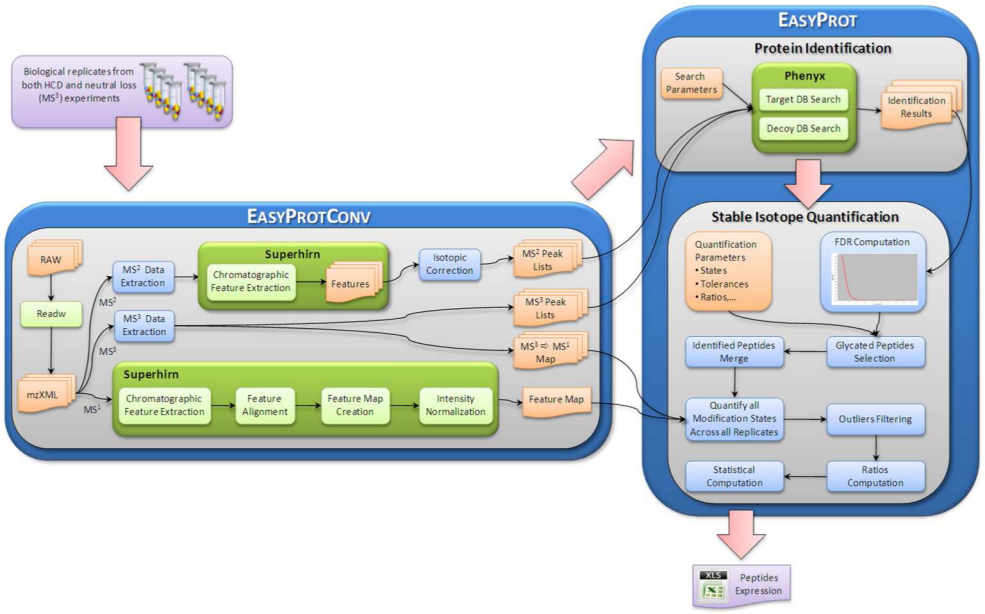

Figure 7. Scheme of the protocol proposed for treatment of raw data files applied to the qualitative and quantitative analysis of glycated proteins.

short glycated peptides. Therefore, the combination of both modes is justified by complementary detection of modified peptides.

Data Analysis: Identification and Quantitation of Glycated Proteins

A new stable isotope quantitation module was implemented in the EasyProt[23] platform to characterize and quantify proteins carrying glycated post-translational modifications. Two different approaches, one dedicated to HCD experiments and one based on neutral loss experiments, were developed and integrated into the EasyProt platform. The quantitation of glycated peptides is based on MS1 data while the identification of peptides is based on MS2 and MS3 data. The whole quantification workflow requires three steps which are described in the next paragraphs and illustrated in Figure 7.

Data File Preparation. The first step is to create the peak lists required by the peptide and protein identification engine. RAW data files are acquired on a Thermo LTQ Orbitrap XL. For HCD experiments, MS2 data originating from multiple replicates are converted to the MGF peak list format and then submitted to EasyProt for protein and peptide identification. Similarly, multiple replicates from neutral loss experiments fragmented in MS3 are converted to MGF and submitted for identification. The next step is to detect, extract and align chromatographic peaks from multiple MS1 replicates in order to create global feature maps. Quantitative values are obtained by integrating ion abundance (through m/z and retention time using the Superhirn[24] software) under the chromatographic curve for a given detected chromatographic peak. These feature maps, one for HCD analyzes and one for neutral loss experiments, contain quantitative data extracted from ion abundance, which is later to be matched to identified peptides. Furthermore, because the neutral loss experiment features peak lists based on MS3 spectra instead of MS2 spectra, an additional step is required. Indeed, later on it is necessary to lookup MS1 data based on peptides identified from MS3 data. To achieve this goal, an indirection map allowing to lookup MS1 data from MS3 spectra is created along the global feature map.

Protein Identification. The second step is the identification of peptides and proteins from peak lists generated in the first step described previously. Each identified peptide is characterized by a sequence, a charge, a mass (m/z) and a retention time according to its elution properties. For a given experiment (HCD or neutral loss), assessing the relative quantification of glycated peptides requires combining or matching identified peptides with the global feature maps generated during the first stage. This matching first requires locating all glycated peptides. To do so, only peptides carrying mass shifts corresponding to light or heavy glycation modification forms are kept. Then, each glycated peptide is looked up in the feature map according to their m/z and charge to locate the corresponding quantitative value (i.e., chromatographic feature). Usually, only one form of a peptide is identified, either the light or the heavy form, but almost never both at the same time. Because of this, it is necessary to find the quantitative value of the missing form. The quantification of the missing peptide is implemented by searching the corresponding m/z at a similar retention time. Because of their physicochemical properties, both forms elute around the same time. Since one peptide of the pair is usually identified, its charge, m/z and retention time are known. It is therefore possible to deduce these very parameters for the missing peptide form since the retention time between both forms is similar and m/z values are shifted by the mass shift of the difference between the light and heavy modifications. In case of neutral loss experiments, an additional lookup through the indirection map generated previously is required to go from MS3 to the global feature map storing MS1 quantitative values. Hence, for a neutral loss experiment, the mapping becomes the following: identified peptide (MS3) to indirection map to MS1 quantitative values (from the global feature map).

Glycated Peptide Quantification. The last step is the combination of quantitative values with regard to assessing the expression of each glycated peptide. Doublet signals representing precursor abundance for light/heavy glycated forms are computed for every peptide identified in at least one of the replicates. To increase the reliability of our LC−MS-based quantitative approach, quantified peptides present in less than two replicates

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are discarded. Outlier removal is based on filtering out values falling outside (typically 95%) the distribution of abundances per peptide. Ratios are computed for each peptide and key statistical metrics are calculated, such as median, min, max, first and third quartiles. These statistical values as well as other relevant information such as the list of all quantified peptides from all replicates are then written to an Excel file.

WORKED EXAMPLES

The main application fields of this approach are to unravel specific aspects of hyperglycaemia effects and to evaluate the efficiency of normoglycaemia treatments. In this sense, the presented approach could be applied as a complementary tool to other studies previously reported for characterization of glycation in different pathologies such as diabetes.[25−27] It is worth taking into account that the glycemic control is currently assessed in individuals with the reference test for measurement of glycated hemoglobin (HbA1c). However, the proposed strategy may also provide semiquantitative information to monitor the glycemic control of samples under different physiological states. Also, a perspective about the glycated profile of any biological sample under certain conditions can be obtained. These profiles can be correlated with the glycemic state of individuals. Recently, we have successfully applied this approach to the analysis of native glycated proteins from different human blood components, including plasma and hemolysates.[16,28] We have also identified native glycation by applying this approach to the human cerebrospinal fluid.[28] The approach has been used to propose a mass spectrometry strategy for the characterization of glycated proteins in the prognosis of diabetes and neurological disorders.[28,29] Thus, this approach can be used to study the glycated proteins encountered in a complex biological medium (e.g., fluid, cells, organ, and tissue).

Assessment of the Native Level of Protein Glycation in Biological Samples

The glycemic state of individuals is currently evaluated with the test for measurement of glycated hemoglobin (HbA1c) concentration, which is a long-term indicator due to the erythrocytes lifespan (~120 days).[30] However, other measurements indicative of short-term glucose perturbations or indicative of glycaemia in other biofluids or tissues are required to establish the biological effect of glycation. More importantly, any protein can be theoretically glycated.

RAW data acquired from MS2-HCD and MS3 neutral loss experiments can give users significant information of the native background glycation in target samples. Due to the selectivity of immonium ions and the high accuracy of MS2 fragments with

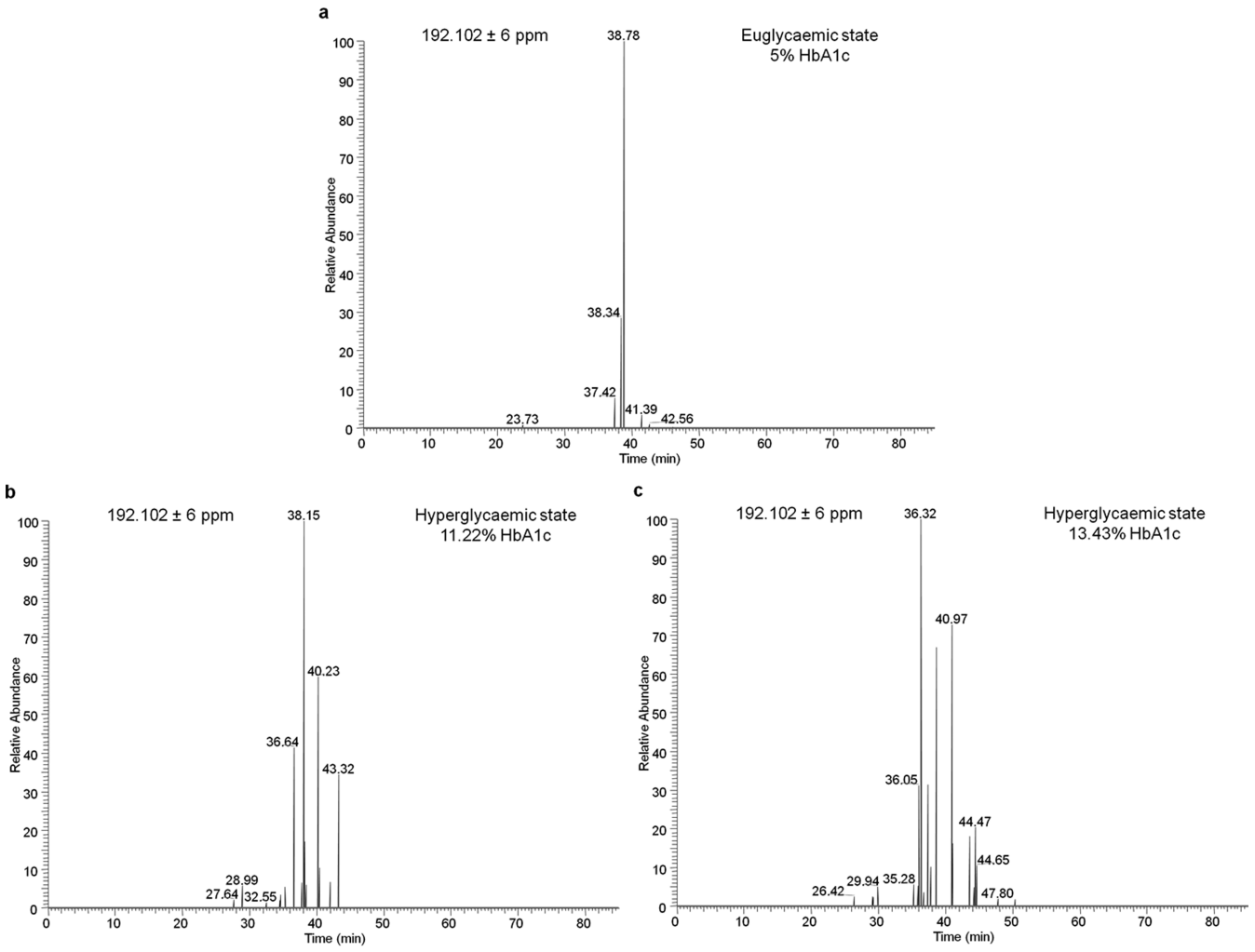

Figure 8. Native glycation profile of blood hemolysates from (a) euglycemic and (b and c) hyperglycemic individuals by monitoring extracted MS2 chromatograms for immonium ions of glycated lysine residues. The value marked for each signal (which should fit with one glycated peptide) corresponds to the chromatographic retention time expressed in min.

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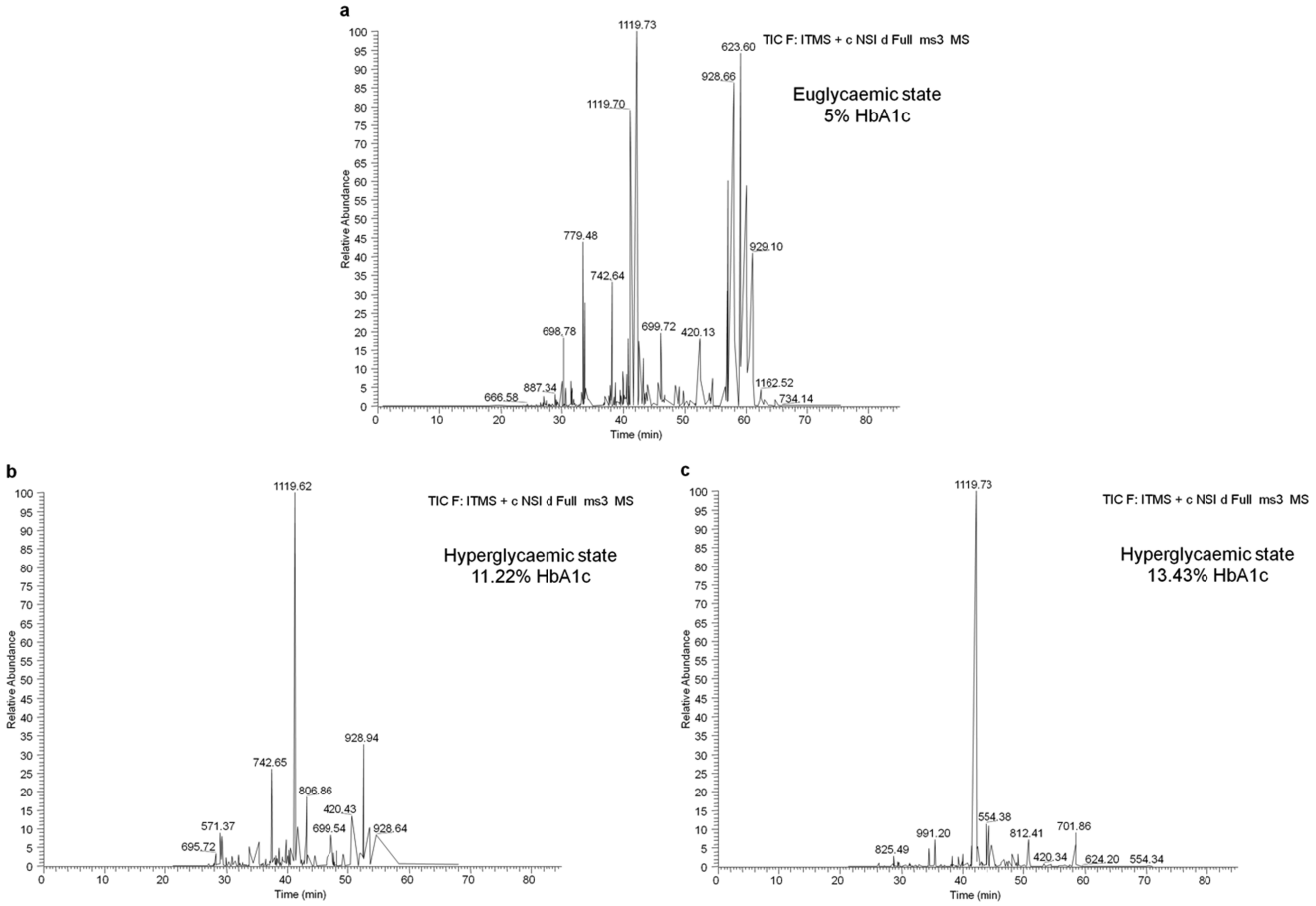

Figure 9. Native glycation profile of blood hemolysates from (a) euglycemic and (b and c) hyperglycemic individuals by monitoring total ion MS3 chromatograms obtained in neutral loss scanning mode. The value labeled for each signal corresponds to the m/z ratio of the base peak obtained in each MS3 spectrum.

Orbitrap detection, glycated peptides may be mapped by extracting ion chromatograms in MS2 scans. Figure 8 shows the extracted ion chromatograms in MS2 for the immonium ion estimated for glycated lysine residues (190.102 Da) provided by the analysis of blood hemolysates from euglycemic (5% HbA1c) and hyperglycemic (11.22 and 13.43% HbA1c) individuals. Each signal, labeled with chromatographic retention time expressed in min, should fit one glycated peptide. As can be seen, the relative abundance of glycated peptides increased considerably in the hemolysates corresponding to hyperglycemic states.

This background glycation can also be deduced by MS3 total ion extracted chromatograms acquired after neutral loss because of the high selectivity of the MS operational mode. The selectivity of this analysis is supported by the fact that only glycated peptides are reisolated in the ion trap for a second fragmentation step. Thus, this analysis can also be used to compare the glycemic state of individuals as shows Figure 9 with MS3 exctracted chromatograms (labeled values correspond to the m/z ratio of the base peak obtained in each MS3 spectrum) provided by the analysis of blood hemolysates from euglycemic and hyperglycemic individuals. As can be seen, the profile of the chromatogram changes considerably for hyperglycemic individuals increasing in relative terms the signal that corresponded to the peptide containing the preferential glycation site of human hemoglobin located at the N-terminus site of the β chain (m/z 1119.7 $\pm$ 0.1 is the base peak obtained by MS3 fragmentation of the cited peptide).

The native glycation can be estimated in relative terms by using the quantitation approach based on the differential labeling with glucose to compare biological states. The estimation of the peak area ratio between the native and the *in vitro* glycated peptides can provide a global view about the glycemic state of a particular biological sample. This analysis also reports information about the preferential glycation sites for each detected protein as shows Figure 10 for human albumin detected in cerebrospinal fluid.

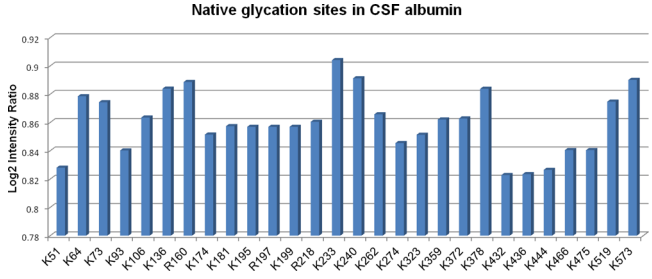

Figure 10. Glycation affinity of the different sites identified in human serum albumin detected in cerebrospinal fluid.

Predictive Analysis of Glycated Proteins

In addition to the analysis of native glycation, predictive analysis about the possible incidence of hyperglycaemia conditions over

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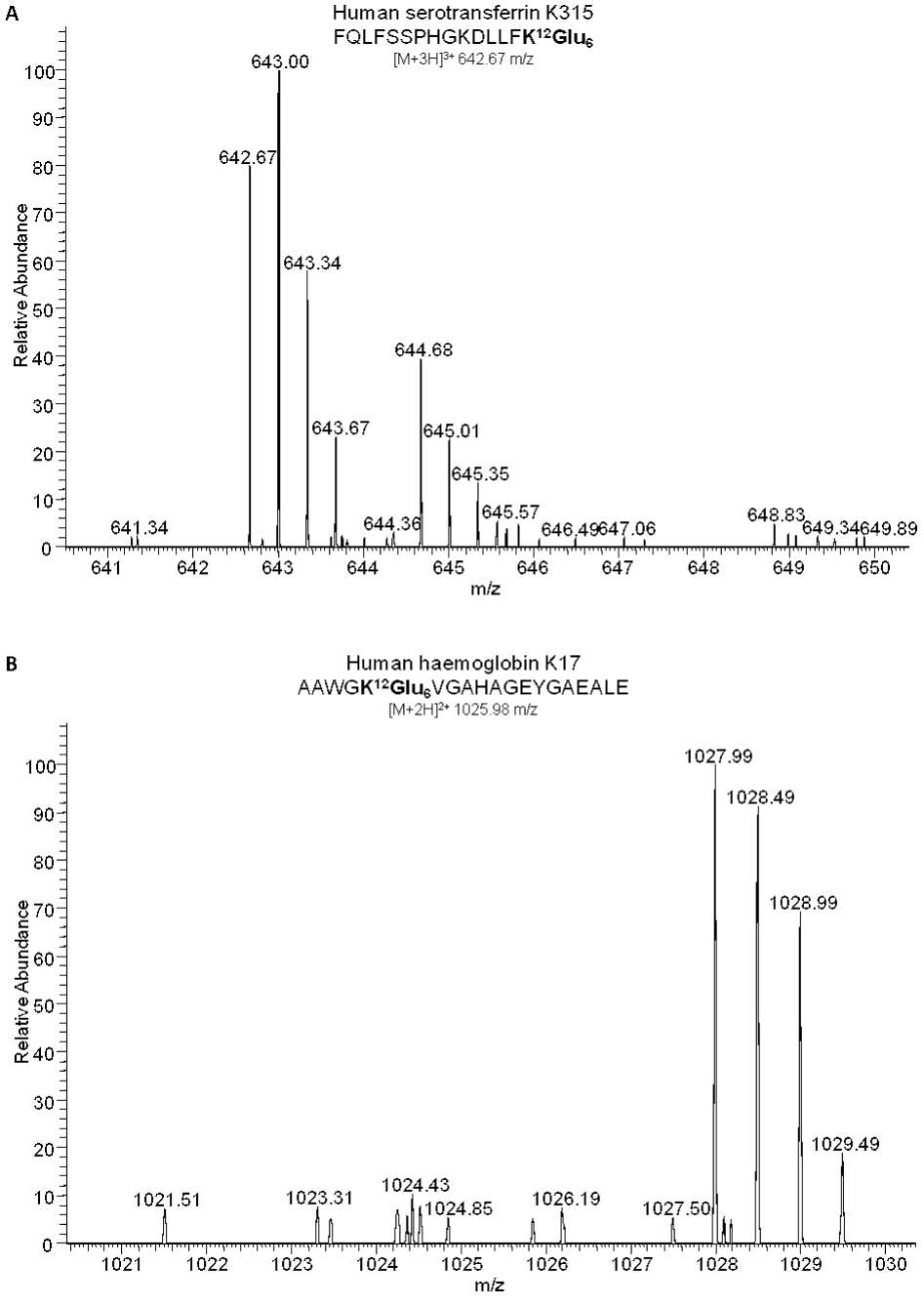

Figure 11. Prediction analysis of two glycated peptides from proteins present in human biofluids. (A) Serotransferrin peptide detected in human serum modified in position K315. (B) Hemoglobin peptide (α chain) detected in blood hemolysate modified in position K17.

target glycation sites can also be determined by current protocol. For this purpose, we have to consider the signals ascribed to peptides labeled with [$^{13}C_6$]-glucose taking as reference in this case the signal corresponding to the native glycated peptide. Additionally, new glycation targets can be identified if only the [$^{13}C_6$]-glucose-labeled peptide is detected and no doublet is found. Figure 11 illustrates a representative example of glycated peptides detected in human body fluids. As can be seen, in the first case (Figure 11A), we can obtain information about the behavior of a glycated peptide under prolonged hyperglycaemia conditions by measuring the signal of the [$^{13}C_6$]-glucose-labeled peptide, which is less intense than that observed for the native [$^{12}C_6$]-glucose labeled peptide. In the second case (Figure 11B), it is a nonglycated peptide in native conditions but the [$^{13}C_6$]-glucose labeled peptide (m/z 1027.99) is detected as a result of the *in vitro* glucose addition.

CURRENT LIMITATIONS AND USEFUL WORKING LIMITS

The application of the described methodology provides qualitative and quantitative information in relative terms. Therefore, the main limitation is that no absolute quantitative information can be obtained. For this purpose, selected reaction monitoring (SRM) based methods with synthetic peptides as internal standards should be the preferred option. Nevertheless, absolute quantitation of a target glycated protein also demands for quantitation of the corresponding nonglycated protein, which would complicate development of methods addressing this issue.

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An additional aspect is the absence of approaches coupling affinity chromatography based on boronate ligands and mass spectrometry. BAC separation is currently implemented as preparative chromatography in off-line configurations. Therefore, online separation of glycated peptides with subsequent MS detection would improve analytical features such as sensitivity, selectivity and precision.

FUTURE DEVELOPMENTS

The qualitative/quantitative approach presented here for analysis of glycated proteins in biological samples opens new expectations for characterization of the human glycated proteome. Hyperglycaemia, which causes negative effects on different tissues, is a conditioning factor promoting the nonenzymatic glycation of proteins in those sites kinetically favored. The glycated proteome is characterized by its dynamic profile which evolves qualitatively and quantitatively with unbalanced glucose concentration. With these premises, it is interesting to obtain profiles containing information of glycation sites as a function of hyperglycaemia level. As an example, the application of the method described here has revealed the identification of 50 glycated proteins in normoglycaemic plasma with detection of 161 glycation sites.[16] The dynamism of the glycated proteome occurring under hyperglycaemia justifies the mapping of glycated proteins in different biological samples to understand modifications occurring owing to unbalanced glucose homeostasis.

ASSOCIATED CONTENT

Supporting Information

Supplementary PowerPoint presentation. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +41 (0) 22 379 54 86.Fax: +41 (0) 22 379 55 05. E-mail: jean-charles.sanchez@unige.ch.

Notes

The authors declare no competing financial interest.

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Chapter 3

IMPACT OF HIGH GLUCOSE CONCENTRATION ON ASPIRIN-INDUCED ACETYLATION OF HUMAN SERUM ALBUMIN: AN IN VITRO STUDY

The study presented in this chapter represents the proof-of-concept underlying the core of the entire thesis project. Aspirin-mediated acetylation is a PTM that occurs through the transfer of its acetyl residue to the amino groups of proteins located at lysine and N-terminal residues. This modification plays a key role in medicine, since it decreases the general inflammatory status of patients and partially prevents the risk of cardiovascular ischemic events. The effect of aspirin was demonstrated in several blood proteins including haemoglobin, fibrinogen and serum albumin (HSA). HSA is involved in many physiological processes that range from the regulation of osmotic pressure, the mediation of lipid metabolism, the binding of toxins and free radicals (antioxidant activity) and the transport of a wide variety of solutes such as hormones, fatty acids and drugs. Glycation of HSA was shown to affect its structure/function, compromising its ability to bind molecules, however the mutual impact between aspirin-induced acetylation and glycation was poorly investigated. The MS-label free approach described here allowed characterizing the preferential acetylation sites in HSA from low to high aspirin concentrations, and to determine the extent of the interplay between aspirin-induced acetylation and protein glycation in HSA. The results showed in this study might pave the way to a better understanding of the acetylation-glycation interactions at the protein level in even more complex samples and in diabetic patients.

For this study, I carried out the entire experimental part, optimized the MS method with the quantitative approach, analyzed the data and fully wrote the manuscript, with a strong support of the co-authors.

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Impact of high glucose concentration on aspirin-induced acetylation of human serum albumin: An *in vitro* study

Francesco Finamore[a], *Feliciano Priego-Capote*[b], *Florent Gluck*[a], *Anne Zufferey*[a], *Pierre Fontana*[c], *Jean-Charles Sanchez*[a,*]

[a] *Translational Biomarker Group (TBG), Department of Human Protein Sciences, University Medical Centre, University of Geneva, 1211 Geneva 4, Switzerland*

[b] *Department of Analytical Chemistry, University of Còrdoba, Annex C-3 Building, Campus of Rabanales, Spain*

[c] *Division of Angiology and Haemostasis, Geneva Platelet Group (GPG), Geneva University Hospital, Geneva, Switzerland*

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ABSTRACT

Aspirin (ASA) plays a key role in protecting high risk cardiovascular patients from ischaemic events. The modifications underlying its effects are the results of the trans-acetylation that occurs between ASA and the amino groups made up of lysine and N-terminal residues. ASA's effects have also been demonstrated on several plasma proteins, including human serum albumin (HSA). However, its beneficial effects seem to be lower in diabetic patients, suggesting that protein glycation may impair ASA's acetylation process. Using immunoblotting and mass spectrometry, this study characterized the degree of HSA acetylation mediated by ASA *in vitro*, as well as the impact of high glucose concentrations. Glycation's influence on HSA acetylation might impair the latter's biological functions, leading to a potential failure of ASA to prevent cardiovascular complications in diabetes.

1. Introduction

Since its serendipitous discovery and thanks to mid-20th century improvements, aspirin, also known as acetylsalicylic acid (ASA), has become a landmark drug in the history of medicine. ASA is the world's most well-known non-steroidal anti-inflammatory drug; this is due to its pleiotropic effects, either in the ordinary treatment of pain, fever and inflammation, or in clinical practice [1,2]. Indeed, it is widely used as a preventive agent in atherothrombotic diseases, because of its inhibition of the COX-1 platelet enzyme [3]. It also enhances the resolution of inflammatory states by increasing the activity of the COX-2 vascular enzyme [4], and thus prevents the risk of cardiovascular ischaemic events [5,6]. ASA plays a relevant role in lowering insulin resistance, by reducing levels of pro-inflammatory cytokines [7], and furthermore it exerts its therapeutic action through the transfer of its acetyl moiety to the primary amino groups of proteins located at lysine and N-terminal residues. Under

Abbreviations: ASA, acetyl salicylic acid; HCD, high energy collisional dissociation; HSA, human serum albumin; MS, mass spectrometry.

* *Corresponding author at:* Translational Biomarker Group, Department of Human Protein Sciences, University of Geneva, Rue Michel Servet 1, 1211 Geneva 4, Switzerland. Tel.: +41 022 3795486; fax: +41 022 3795505.
E-mail address: Jean-Charles.Sanchez@unige.ch (J.-C. Sanchez).

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physiological conditions (37 °C, pH 7.4) this reaction is fast and follows a first order kinetic dependent only on the concentration of ASA. After usual therapeutic doses, normal ASA plasma levels range from 0.7 to 2.2 mM, however toxicity can occur when serum concentrations exceed 2.9–3.6 mM and can become fatal beyond 5.4 mM [8]. Several studies have shown the effect of ASA-induced acetylation on different plasma proteins. It increases haemoglobin's affinity to oxygen and consequently reduces the severity of sickle-cell disease [9,10]. Acetylation of fibrinogen by ASA improves the porosity of the fibrin cloth making it more accessible to plasmin for the fibrinolytic process [11]. ASA also acts on low density lipoproteins, reducing their oxidation level and thus exerting a preventive effect against atherosclerosis [12]. ASA-mediated acetylation has also been detected on human serum albumin (HSA) [13]. HSA is known to be the most abundant circulating plasma protein, and because it has multifunctional properties it plays key roles in metabolism. It is the main transporter of drugs, hormones, fatty acids and other small metabolites. It regulates oncotic pressure, prevents oxidative damage and has an intrinsic esterase activity. Previous studies showed the involvement of different amino acidic residues in HSA's reactions with low ASA concentrations (0.1–0.5 mM) [14,15]. It was demonstrated that the acetylation of some of these sites had an impact on HSA's pseudo-esterase catalytic activity, and on its affinity for binding to anionic drugs and metabolites such as bilirubin and prostaglandins. However, HSA's beneficial effects are lower in poorly monitored diabetic patients, *via* a process termed as 'aspirin resistance'. This can be attributed to insufficient doses of ASA, drug interactions, or the genetic polymorphism of genes involved in thromboxane biosynthesis and turnover [16,17]. In addition, because high plasma glucose levels encourage protein glycation, they might have a significant effect on aspirin resistance and other metabolic disorders. Glycation is a non-enzymatic post-translational modification, which is boosted under hyperglycaemic conditions (blood glucose concentration $\geq$ 11 mM). It occurs in a condensation reaction between the carbonyl group in glucose (or any other reducing sugar, such as fructose and derivatives) and a protein's free amino residues (mostly N-terminal and lysine). This process leads to the formation of Amadori compounds which then undergo a series of oxidation, dehydration and fragmentation reactions, finally generating advanced glycation end-products (AGE) [18,19]. AGEs are associated to long term diabetic complications, such as retinopathy [20,21], nephropathy [22–24] and macroangiopathy [25,26]; this is due to the formation of cross-linked structures [27,28] that alter the structure/function of proteins, their turn-over, and tend to accumulate in specific tissues, impairing their homeostasis [29,30].

Apart from its clinical relevance as a more rapid indicator for glycaemic monitoringl than glycated haemoglobin (HbA1c), glycated HSA has also been described as having altered functions in diabetes (glycated albumin levels in plasma are considered to indicate the glycaemic state over the last 3 weeks, while HbA1c indicates the glycaemic state over the last 3 months) [31–33]. Indeed, it was shown that glycation significantly impairs HSA's antioxidant properties [34] and its drug-binding capacity [35]. Furthermore, the alterations to HSA's biophysical properties, induced by glycation, increase its affinity to the receptor for advanced glycation end-products (RAGE) [36]. This leads to several cellular dysfunctions, such as: the intracellular accumulation of lipids in macrophages [37]; the increase of their inflammatory activity by inducing NF-κB [38]; the secretion of IL-6 by adipocytes [34]; and the promotion of neuronal apoptosis in the human brain [39], contributing to the development of Alzheimer's-like diseases.

The potential protective role of ASA in inhibiting the glycation mechanism was previously shown in lens crystallins [40,41], collagen [42], haemoglobin [43] and, most recently, fibrinogen [44]. However, the impact of increased blood glucose concentrations and glycation on ASA-mediated acetylation has been poorly investigated. To date, only Watala et al. have shown the impairment of ASA-induced protein acetylation on platelet membrane proteins in diabetes mellitus, due to an increased level of glycation [45]. Further research is required to determine how high glucose concentrations might hamper the protective action of ASA at the protein level. This study aimed to highlight HSA's acetylation response after incubation, from low to very high ASA concentrations, but also to elucidate which amino acidic residues showed the highest reactivity towards ASA. The study's second aim was to examine the competition between glycation and acetylation in order to demonstrate how these two reactions take place at the protein level. Finally, the characterization of the preferential sites where these two modifications occur was performed using mass spectrometry (MS), and the quantification of glycated and acetylated peptides of HSA was carried out using a label-free approach. This proof-of-concept study lays the foundations for a better understanding of the interaction between the ASA-mediated acetylation and glycation processes on proteins, and gives new insights into their potential antagonist effects in the pathophysiology of diabetes.

2. Materials and methods

2.1. Chemicals

Dulbecco's phosphate-buffered saline (DPBS; 1×, pH 7.4) was from Invitrogen™. Human serum albumin (HSA, solution 20%) was obtained from CSL Behring AG. Aspirin (ASA, acetylsalicylic acid) was purchased from Aspegic Inject®. Anti human Nε-acetyl-lysine monoclonal antibody was from Cell Signaling Technologies®. ECL™ detection reagent was from GE Healthcare. Protein assay dye reagent concentrate (liquid) was from Bio-Rad. D-Glucose [$^{12}C_6$] (lyophilized powder, $\geq$99.5%), Naphtol blue black (lyophilized powder, dye content *ca.* 80%), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP; 0.5 M, pH 7.0), iodoacetamide (IAA, crystalline, $\geq$99%), endoproteinase Glu-C from *Staphylococcus aureus* V8 (lyophilized powder, 500 U), HPLC-grade water (CHROMASOLV®) and HPLC grade acetonitrile (ACN; CHROMASOLV®, $\geq$99.9%) were purchased from Sigma–Aldrich®. Bovine serum albumin (BSA, lyophilized powder, $\geq$96%), triethylammonium hydrogen carbonate buffer (TEAB; 1 M, pH 8.5), ammonium acetate (NH_4Ac, solid, 98.0%), magnesium chloride ($MgCl_2$, solid, $\geq$99.0%), acetic acid (99.5%), and formic acid (FA; 98.0%) were from Fluka.

2.2. ASA and glucose incubation of HSA

To test the detection limit of ASA-mediated acetylation using Western blot and LC–MS approaches, five aliquots of 20 μL HSA were diluted in phosphate buffer up to 200 μL, and subsequently incubated with several ASA concentrations (20, 2, 0.2, 0.02 and 0 mM) for 24 h at 37 °C. To analyze the impact of high glucose concentration on ASA's effects, three aliquots of 20 μL HSA were diluted in phosphate buffer to reach a final volume of 200 μL, and then incubated: (1) alone for 24 h at 37 °C, (2) with 20 mM ASA for 24 h at 37 °C, and (3) sequentially with 10 mM glucose for 24 h and then 20 mM ASA for 24 h at 37 °C. For the third immunoblot experiment, two groups of three aliquots of 20 μL HSA in phosphate buffer (200 μL final volume) were incubated with 20 mM ASA and different glucose concentrations (0, 10, and 625 mM) for 24 h at 37 °C; sequential and simultaneous incubations were performed for the first and the second group, respectively. Finally, four conditions (200 μL final volume) were created: HSA was incubated alone for 24 h at 37 °C, with 20 mM ASA for 24 h at 37 °C, with 10 mM glucose for 24 h at 37 °C, and with 10 mM glucose followed by an incubation with 20 mM ASA for 24 h at 37 °C. After incubation, protein amounts were determined using the Bradford assay, and BSA was used to create a standard calibration curve.

2.3. Western blot analysis of ASA-mediated acetylation

Samples (200 ng) were separated using electrophoresis on an 8%T/2.6%C polyacrylamide gel and protein transfer was carried out for 1 h at 350 mA. Membranes were stained with Amido black [46] to highlight the protein bands, and washed with water to remove the excess of background. Immunoblot was carried out with an anti-human antibody against N ε-acetyl-lysine in a dilution of 1:5000. The acetylation signal intensity of the immunoblot was normalized relative to the corresponding band stained with Amido black. ECL reagents were used to detect the acetylation signal. Each sample was evaluated with four experimental replicates ($n = 4$).

2.4. Protein purification and Glu-C digestion

After incubation, glucose and salts were removed using Amicon Ultra-0.5 mL, Ultracel® 3K membrane (Millipore™) devices to purify the proteins that were reconstituted in 500 mM TEAB, pH 8.5. Protein concentrations were subsequently measured using the Bradford assay, with BSA as the calibration protein. Samples were split into two experimental groups in order to analyze the acetylated and glycated peptides separately for each experimental set. For the acetylation study, 100 μg of protein were diluted in 400 μL of TEAB; while for the glycation study, 1 mg of protein were diluted in 400 μL of TEAB, for subsequent protein digestion. For this purpose, disulfide bonds were reduced using 4 μL of TCEP to reach a final concentration of 5 mM and the reaction mixture was incubated for 1 h at 60 °C. Cysteine groups were alkylated using 10 μL of IAA to reach a final concentration of 20 mM, and incubation was carried out for 30 min at room temperature in dark conditions. Freshly prepared endoproteinase Glu-C (1 mg/mL) was added in a ratio of 1:10 (w/w), and digestion took place for 16 h at 37 °C. For acetylation analysis, digestion mixtures were evaporated under speed vacuum, and directly reconstituted in 5% ACN/0.1% FA for the subsequent desalting procedure. For the glycation analysis, peptide digests were evaporated and reconstituted in mobile phase A (200 mM NH_4Ac, 50 mM $MgCl_2$, pH 8.1) to reach an estimated concentration of 16 mg/mL, for the fractionation of glycated peptides.

2.5. Enrichment of glycated peptides by boronate affinity chromatography

Reconstituted peptides were enriched using boronate affinity chromatography—by interaction between cis-diol groups of glycated peptides, present at low concentration, and boronate groups of the stationary phase, as previously described [47,48]. For this purpose, a 50 μL room temperature sample was injected in a Waters 600E HPLC system equipped with a TSK-Gel boronate affinity column from Tosoh Bioscience (7.5 cm length × 7.5 mm inner diameter, i.d., 10 μm particle size). A chromatographic method based on a gradient with two isocratic steps was used to separate non-glycated from glycated fractions. Step 1 was a 0–10 min, 100% mobile phase A (200 mM ammonium acetate, 50 mM magnesium chloride, pH 8.1) for retention of glycated peptides by esterification of the 1,2 cis-diol group of glucose moieties and the hydroxyl groups of boronate ligands under alkaline conditions. Non-glycated peptides were eluted. Step 2 was a 10–20 min, 100% mobile phase B (100 mM acetic acid) for the elution of glycated peptides under acidic conditions; 20–30 min, 100% mobile phase A to bring the column back to the initial equilibrium conditions. The glycated fraction was collected for subsequent evaporation and reconstitution in 5% ACN/0.1% FA, for the ensuing desalting and concentration.

2.6. LC–MS/MS detection of acetylated and glycated peptides

Samples from acetylated and glycated data sets were desalted and pre-concentrated using a MacroSpin Column™ C_{18} (Harvard Apparatus), according to the manufacturer's protocol. Peptides were eluted in 400 μL of 50% ACN/0.1% FA and then evaporated to dryness before reconstitution in 5% ACN/0.1% FA, prior to LC–MS/MS analysis. Peptide digests were analyzed using electrospray ionization in positive ion mode (1.6 kV ionization voltage), on an Orbitrap hybrid linear ion trap (Thermo Fisher). Nanoflow was carried out using a Waters NanoAquity HPLC system consisting of a pre-column (100 μm inner diameter and 18 mm in length) packed with C_{18} resin, where peptides are initially loaded and trapped at a flow rate of 3 μL/min in water/ACN (95/5, v/v) with 0.1% FA. After retention, peptides were eluted using an ACN gradient developed in an analytical column packed with C_{18} resin, at the flow rate of 200 nL/min with mobile phase A (water, 0.1% FA) and B (ACN, 0.1% FA). The mass spectrometer was operated in data-dependent MS/MS mode with a precursor-ion scan range of 400–1600 m/z and a resolving power of 60,000. Each sample in both data sets was analyzed four times using high energy collisional dissociation (HCD) as the activation mode. The 3 most abundant ions detected from the MS survey scan were selected as precursor ions for fragmentation (normalized collision energy, 40%),

followed by Orbitrap detection with a resolving power of 7500 with a dynamic exclusion of 45 s, to minimize repeated analysis of the same precursor ion. All analyses were carried out using four replicate injections.

2.7. Data analysis

After data-dependent acquisition, peak lists were generated from raw data using EasyProtConv v1.5 software, and the resulting data files were searched for matches against the UniProtKB/Swiss-Prot database (Release June 13, 2012, 647,369 entries) using the EasyProt v2.2 (build 612) tool [49]. Because this study analyzed human albumin, we selected *Homo sapiens* taxonomy for the protein search. The high resolution of the acquired data allowed the scoring model to use an accuracy of 6 ppm on fragments and 10 ppm on precursors. Common amino acid modifications detected were carbamidomethylation of cysteine residues (57.0215 Da) and oxidized methionine (15.9949 Da); these were set as fixed and variable modifications, respectively. Additional variable modifications, for acetylation and glycation, were selected on lysine residues and N-terminal positions. For the analysis of acetylated peptides, the selection of acetylation-induced modification was based on the peptide attachments of the acetyl group (42.0100 Da), while for the selection of glycated peptides, the mass shift was built on the attachment of the entire glucose molecule (162.0523 Da). The number of modifications per peptide was set to a maximum of 2 in order to reduce the search space of all possible combinations of variable modifications. Endoproteinase Glu-C was selected as the cleavage enzyme, with three potential missed cleavages allowed. The minimum peptide length was 8, with a minimum peptide z-score of 4. Four analytical injections per sample, each analyzed in independently, provided replicate data values. The peak list files obtained for each technical replicate were merged and submitted for the database search. For the significance of peptide identification we used a false-discovery rate of 5%. Peptides were considered as identified if they were present 2 times out of 4, but the majority of them were found at least 3 times out of 4.

2.8. Peptide quantification

Label-free quantitation of glycated and acetylated peptides from HSA was done using Nonlinear Dynamics' Progenesis software. This relies on the accurate alignment of *m*/z and Rt features for every chromatographic run in each group. A global peak list file, containing all the features detected, was used to query the database as above and, from the identification results, a pepXML file was exported to Progenesis. All the identified peptides were then matched using their relative features. Quantitation is carried out using the normalized abundance for each peak corresponding to features and comparing it to the same peak for all the conditions analyzed. The normalization of peptide abundance was performed automatically by Progenesis. Briefly, the software selects one technical replicate as a reference; after calculating the raw intensities for each peptide on each replicate, it determines the ratio between the replicate being normalized and the reference replicate. Then, a distribution of log (ratios) is calculated and a global scaling factor (the anti-log of the average of the log (ratios)) is determined. The raw intensities for each peptide among different replicates are multiplied by the global scaling factor.

3. Results and discussion

For several decades, ASA and ASA derivatives have been shown to have a significant biological and clinical effects. One of these effects relies on ASA's protective action against protein glycation, and has been clearly documented using *in vivo* and *in vitro* models [50,51]. Indeed, modifications in both environments occur at the same functional groups on proteins, but it is not clear whether acetylation and glycation share the same target sites, and are thus in direct competition, or whether they target different sites and thus induce potential conformational changes in protein structures. This study's purpose was to characterize the effects of glucose on ASA-mediated acetylation using an *in vitro* model based on HSA. Only one previous study demonstrated an association between glycation and ASA on HSA [50], but it reported no particular information regarding the exact sites at which these modifications were involved. The current study established a relationship between ASA-induced acetylation and protein glycation, starting from the analysis of the preferential acetylated sites on HSA and moving on to the influence that high glucose concentration might have on the acetylation process, and *vice versa*, through label-free quantification.

3.1. Qualitative and quantitative analysis of acetylation and glycation on HSA

The present study used a proteomic workflow for the analysis of acetylation and glycation on HSA; this comprised both immunoblot and tandem mass spectrometry (MS) approaches. These gave qualitative and quantitative estimations of the extent of these two modifications at the protein level (Fig. 1). Following exposure to ASA, and after glucose and ASA co-incubation, the acetylation level of native HSA was first measured by a Western blot test using an anti-acetyl lysine antibody, as previously mentioned. The immunoreaction signal was normalized on the corresponding Amido-black stained band on the same blot. Intensity values were measured using TotalLab Quant software. For the MS analysis, a new condition was created in which HSA was incubated with glucose alone, in addition to the other 3 used for immunoblot. Samples were purified from glucose and ASA, and then digested with endoproteinase Glu-C. The choice of this enzyme is supported by the fact that acetyl groups and glucose attachments are selective for lysine residues, and for this reason the trypsin digestion pattern can be affected by an increase of missed cleavage sites. The influence of these two modifications is less dramatic for endoproteinase Glu-C, and the identification of missed cleavage sites might be considerably reduced. After digestion, two distinct data sets were created: one for acetylated peptide analysis and one for glycated peptide analysis.

Concerning mass spectrometry, electron transfer dissociation (ETD) [52] and collision induced dissociation (CID) in

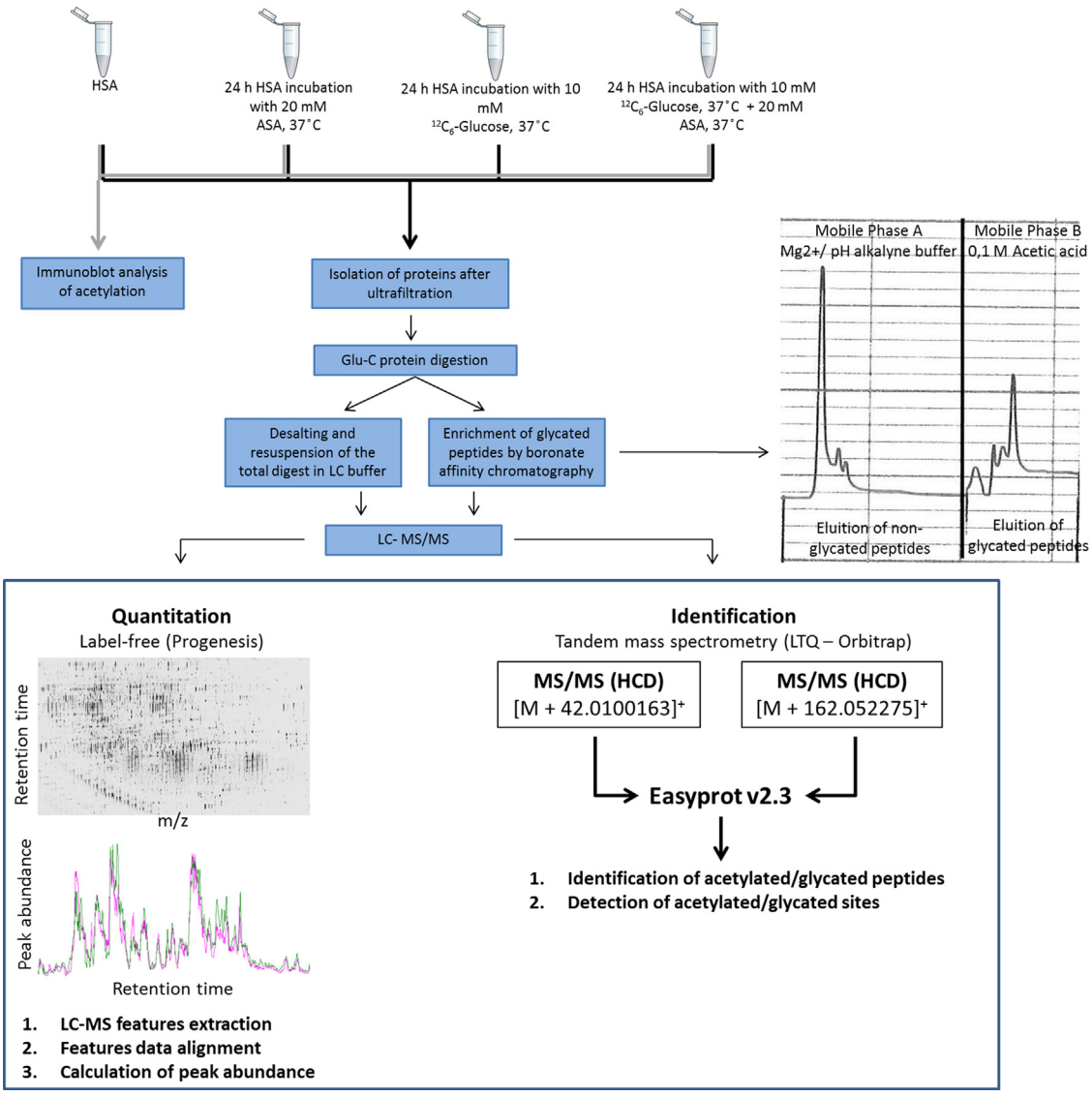

Fig. 1 – Proteomic workflow for the characterization of acetylation and glycation on HSA. The schema shows the different phases of the analysis from sample preparation to the data processing for the identification and quantification of acetylated and glycated peptides.

neutral loss scanning and multistage activation [53] with an ion trap mass analyzer have successfully proved their efficiency in data-dependent methods for the identification of glycated peptides. Nevertheless, coupling HCD fragmentation with Orbitrap detection also allowed the obtainment of high quality MS/MS spectra, which enhanced the identification of acetylated and glycated peptides. HCD employs higher dissociation energies by using nitrogen as the collision gas; this results in more efficient fragmentation than with the helium-based dissociation occurring in CID. Moreover, HCD activation mode is faster than with CID, leading to high vibrational energies before dissociation of the molecular ion. This is fundamental for the analysis of modified peptides in which functional groups are retained on the peptide backbone during the fragmentation process [54]. The present study used HCD fragmentation, and two data files were created for the subsequent identification and quantification by label-free quantification of acetylated and glycated peptides, with localization of the target sites where these two modifications occurs.

3.2. *Analysis of the detection limit of ASA-mediated acetylation on HSA*

Acetylation levels of HSA exposed to decreasing concentrations of ASA were analyzed. Immunoblot analysis clearly revealed a strong acetylation reaction when HSA was incubated with 20 mM ASA; this decreased considerably (−85%) with 2 mM ASA incubation. No immunoreactivity was detected after 0.2 and 0.02 mM ASA exposure (Fig. 2), confirming the inability of this analytical technique to detect low acetylation levels on proteins. On the other hand, the high sensitivity of MS analysis allowed the obtainment of more informative results. The degree of acetylation was directly

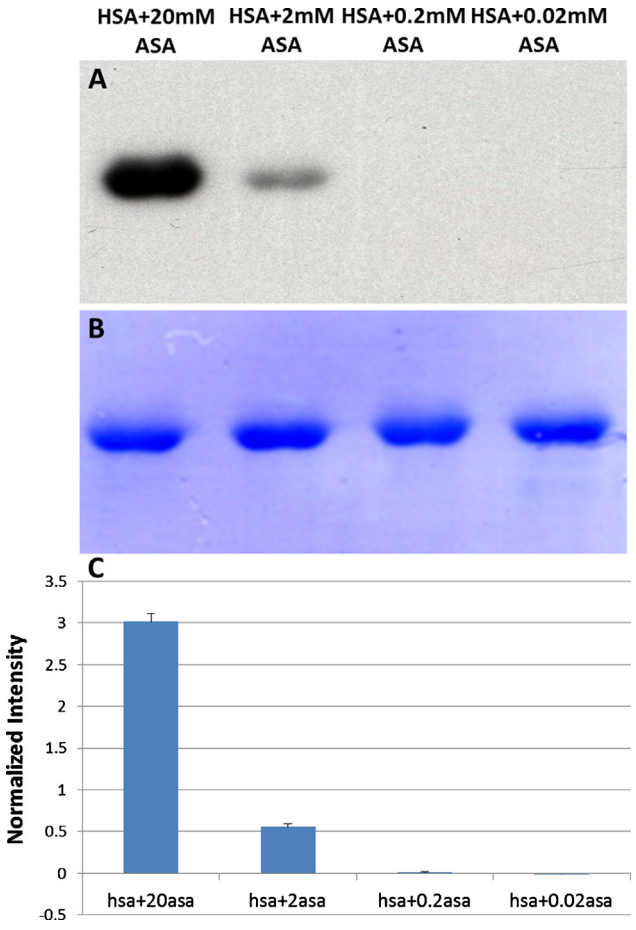

Fig. 2 – Acetylation of HSA as a function of ASA concentration. (A) A scalar concentration of 20, 2, 0.2 and 0.02 mM of ASA was used to assess the detection limit of acetylation on immunoblot. (B) Amido black staining. (C) Bar plot of the normalized intensities among N = 4 replicates.

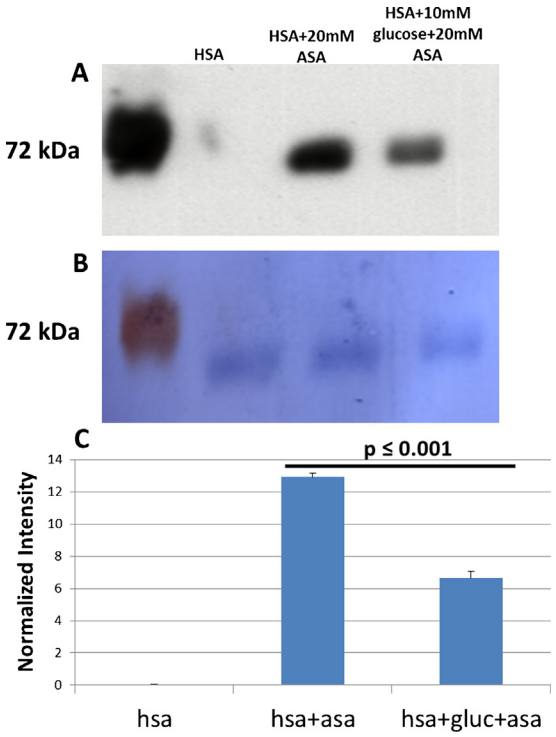

Fig. 4 – Impact of high glucose concentration on ASA acetylation. Western blot analysis of HSA (lane 1), HSA + 20 mM ASA (lane 2), HSA + 10 mM glucose + 20 mM ASA (lane 3). (A) Immunoblot with an anti-acetyl lysine specific Ab. (B) Amido black staining. (C) Bar plot of the normalized intensities of N = 4 replicates.

proportional to the ASA concentration used (from 20 to 0.02 mM); Fig. 3A and B shows the decrease in the total number of unique acetylated peptides and sites, respectively. It should be noted that the higher number of acetylated sites, compared to the number of acetylated peptides can be explained by the low digestive efficiency of Glu-C; because it produces longer peptides than those generated by trypsin digestion, this increases the number of sites modified per peptide sequence. However, when HSA was incubated with 0.02 mM ASA, the reverse situation was found, with a total of 7 acetylated peptides and 6 acetylated sites. This can be explained by the presence of two unique peptides, which modify the same Lys residue, but that differ in the presence of one missed cleavage site. The detailed list of acetylated peptides and their corresponding identified sites are shown in Table 1. MS analysis enabled the

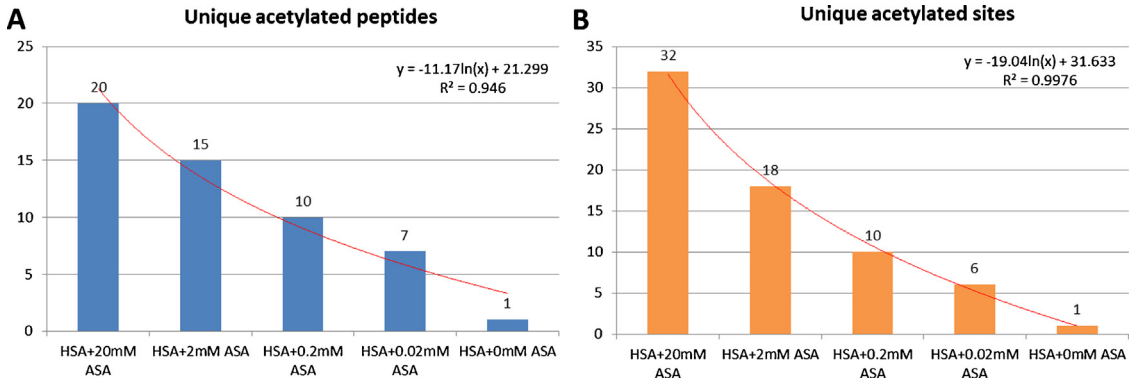

Fig. 3 – Analysis of the acetylation detection limit with LC–MS/MS. Correlation between the concentration of ASA, and (A) the total number of unique acetylated peptides, and (B) unique acetylated sites.

Table 1 – Acetylated peptides and sites identified at decreasing ASA concentration. The precise site of modification is shown for each condition.

Peptide	Position	HSA + 20 mM ASA	HSA + 2 mM ASA	HSA + 200 μM ASA	HSA + 20 μM ASA	HSA
AKRMPCAEDYLSV- VLNQLCVLHE	443–465				444	
CCHGDLLECADDRAD- LAKYICENQDSISSKLKE	245–277	257				
FAKTCVADESAENCDKS- LHTLFGDKLCTVATLRE	49–82	51,64,73	51,64	51,64		
FAKTCVADESAENCDKSLH- TLFGDKLCTVATLRETYGE	49–86		51,64	51,64	51,64	
GKKLVAASQAALGL	572–585	573,574	573,574			
GKASSAKQRLKCASL- QKFGE	189–208		197			
KCCAAADPHECYAKVFDE	359–376	359				
KTPVSDRVTKCCTE	466–479	466,472,475				
KCCKADDKETCFAEE	557–571	557,564	557,564	560,564		
KERQIKKQTALVE	519–531	521,525				
LLFFAKRYKAAFTE	154–167	159				
LRDEGKASSAKQRLKC- ASLQKFGE	185–208	186,190,195,197,199	186,195,199	199		
LVKHKPKATKEQLKAV- MDDFAAFVE	532–556	536			545	
MADCCAKQEPERNECFLQH- KDDNPNLPRLVRPE	87–119		93	93	93	
NCDKSLHTLFGDKLCTVATLRET- YGEMADCCAKQEPERNE	61–100		81		81	
NQDSISSKLKE	267–277	274,276	276			
QLKAVMDDFAAFVEKCCKADDKE	543–565	545		545		
RQIKKQTALVE	521–531	524,525	524,525	524,525		
SLVNRRPCFSALEVDETYV- PKEFNAE	480–505	500				
TFTFHADICTLSEKE	506–520	519	519	519		
TYGEMADCCAKQEPERNE	83–100	93	93	93	93	93
TYGEMADCCAKQEPERNECFL- QHKDDNPNLPRLVRPE	83–119	93	93	93	93	
VAHRFKDLGEE	7–17	12				
VSRNLGKVGSKCCKHPE	426–442	432,439	432,439			
VTEFAKTCVADESAENCDKSLH- TLFGDKLCTVATLRE	46–82		51			
YARRHPDYSVVLLLRLAKTYE	334–354	351				

characterization of acetylation even when HSA was exposed to low doses of ASA (0.2 and 0.02 mM), levels at which detection using immunoblot was impossible. Indeed, identifying sites that showed a high tendency to be acetylated by ASA was an important aspect of analysis. HSA's reaction with 0.02 mM ASA yielded 6 modified sites: Lys 51, 64, 93, 444, 545 and Arg 81. This is the first time that the identity of these sites has been reported at such a low ASA concentration. Furthermore, this method revealed native acetylation, which allowed the identification of Lys 93 residue. The high tendency of these sites to be acetylated by ASA might be attributable to their location at the protein surface, or to the surrounding basic residues that impede their protonation. With 0.2 mM ASA, the number of modified sites increased and it was possible to detect the two known preferential acetylation sites in Lys 199 and 519, as previously reported [15,55]. Differently from Lys 519, which is an external site, Lys 199 is present in an internal pocket of the protein, and owes its high reactivity to the low pK_a of its side chain. A further increase of acetylated residues was found after incubation with 2 mM and 20 mM ASA, but this would be less likely to occur *in vivo* since these two concentrations are much higher than ASA's therapeutic range (0.1–0.5 mM) [14].

3.3. *Glycation effects on HSA's ASA-mediated acetylation*

This study investigated the influence of glycation on HSA's acetylation as induced by ASA. The degree of acetylation of native HSA was compared with those of HSA after 24 h ASA incubation, both with and without exposure to glucose. The immunoblot showed a visible difference in the reactions under the different conditions tested. The acetylation signal in HSA incubated with ASA alone (Fig. 4, lane 2) was clearly lower than the signal in HSA incubated with ASA and glucose (Fig. 4, lane 3), and no signal was detected in native HSA (Fig. 4, lane 1). These results convincingly showed that glycation significantly affects protein acetylation when HSA is incubated with 10 mM of glucose and 20 mM ASA; this suggested that high glucose concentrations had a direct impact, even when the ASA concentration was far beyond the normal therapeutic level in blood. Indeed, the concentration of ASA used in this study was higher than the therapeutic plasma range precisely to enhance the acetylation process and to reach a quasi-saturated state of albumin. Because the physiological plasma levels of ASA are around 0.2–1.5 mM, we assumed that by using

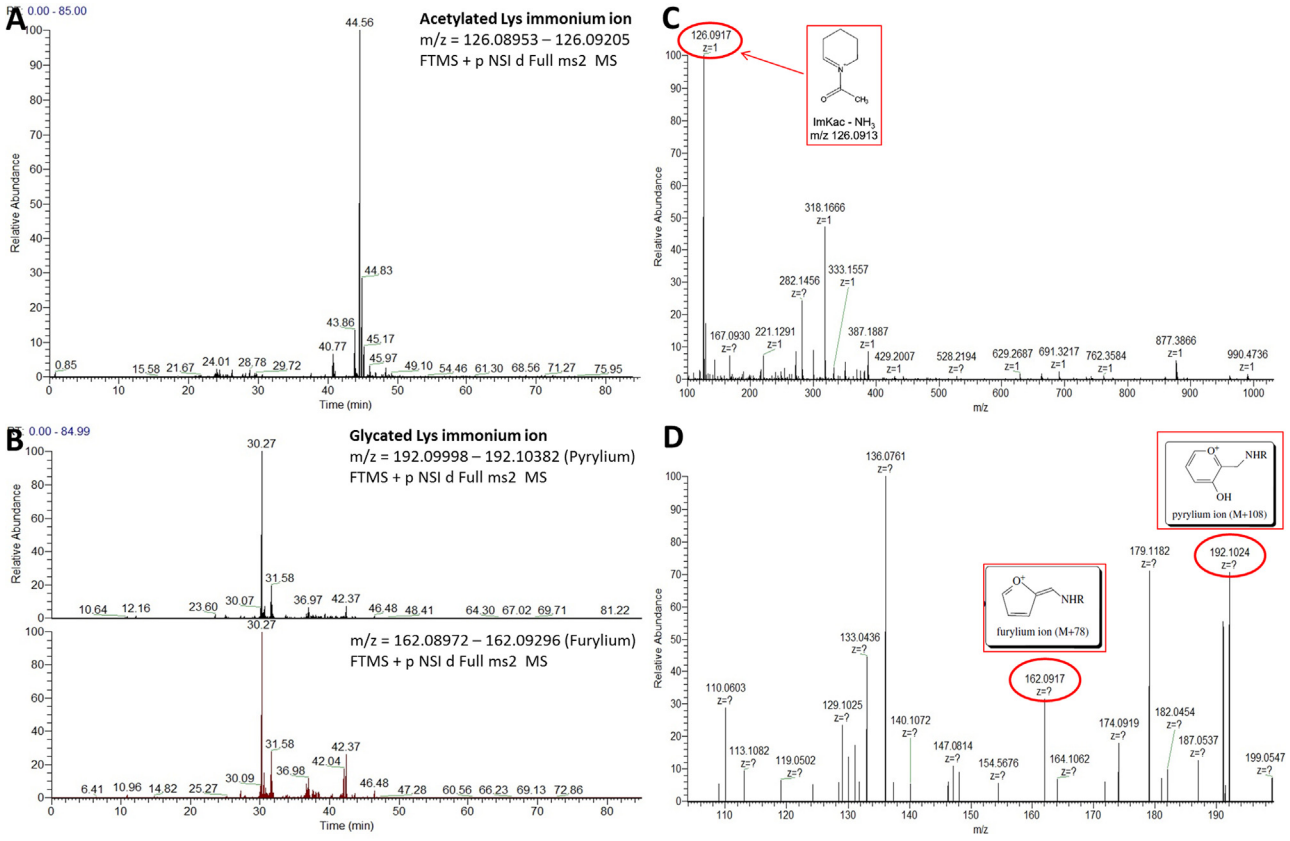

Fig. 5 – Extracted ion chromatograms in MS2 of immonium ions calculated for (A) acetylated, and (B) glycated Lys in HSA after 20 mM ASA and 10 mM glucose incubation, respectively. Tandem mass spectra highlighting the presence of (C) acetylated immonium ions, and (D) glycated immonium ions.

an ASA concentration 20 times higher, the level of occupancy of the lysine sites would be near to completeness; any variation of the degree of acetylation, therefore, would be due to the glucose effect. Moreover, another of the study's aims was to characterize the higher number of preferential acetylation sites on HSA.

The degree to which acetylation is inhibited is dependent on the extent of glycation. Indeed, as the glucose

Table 2 – Acetylated peptides and sites identified. The precise site of modification is shwon for each condition.

Peptide	Position	HSA	HSA+20 mM ASA	HSA+10 mM glucose	HSA+20 mM ASA+10 mM glucose
FAKTCVADESAENCDKSLHTLFGDKLCTVATLRE	49–82		51,64,73		51,64,73
GKASSAKQRLKCASLQKFGE	189–208				190
GKKLVAASQAALGL	572–585		573,574		573,574
KCCAAADPHECYAKVFDE	359–376		359		
KERQIKKQTALVE	519–531		519,521,525		521,525
KCCKADDKETCFAEE	557–571		557,564		557,560
LLFFAKRYKAAFTE	154–167		159		
LRDEGKASSAKQRLKCASLQKFGE	185–208		186,190,195,199		199
LVKHKPKATKEQLKAVMDDFAAFVE	532–556		536,545		536,545
MADCCAKQEPERNECFLQHKDDNPNLPRLVRPE	87–119		93		
NQDSISSKLKE	267–277		274,276		
RQIKKQTALVE	521–531		524,525		
SLVNRRPCFSALEVDETYVPKEFNAE	480–505		500		
TFTFHADICTLSEKE	506–520		519		519
TFTFHADICTLSEKERQIKKQTALVE	506–531		519		519
TYGEMADCCAKQEPERNE	83–100	93	93	93	93
TYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPE	83–119		93		93
VSRNLGKVGSKCCKHPE	426–442		432,439		
YARRHPDYSVVLLLRLAKTYE	334–354		351		351

Table 3 – Glycated peptides and sites identified. The precise site of modification is shown for each condition.

Peptide	Position	HSA	HSA + 20 mM ASA	HSA + 10 mM glucose	HSA + 20 mM ASA + 10 mM glucose
CADDRADLAKYICE	253–266	262	262	262	262
CCHGDLLECADDRADLAKYICE	245–266	257,262	257,262	257,262	
CCQAADKAACLLPKLDE	168–184		174	174	
CFLQHKDDNPNLPRLVRPE	101–119			106	106
FAEVSKLVTDLTKVHTE	228–244	233	233		233,240
FAKTCVADESAENCDKSLHTLFGDKLCTVATLRE	49–82	64	64	51,64	51,64
FKPLVEEPQNLIKQNCE	377–393			378	378
GKASSAKQRLKCASLQKFGE	189–208		195	197	
GKKLVAASQAALGL	572–585		574	574	574
KCCAAADPHECYAKVFDE	359–376	372	372	372	372
KCCKADDKETCFAEE	557–571			564	564
KERQIKKQTALVE	519–531	525	525	525	
KTPVSDRVTKCCTE	466–479	472,475	475	466,472,475	
LRDEGKASSAKQRLKCASLQKFGE	185–208	197,199	195,197,199	195,197,199	195,197,199,205
LVKHKPKATKE	532–542		536	534	
MADCCAKQEPERNE	87–100	93	93	93	
NCDKSLHTLFGDKLCTVATLRE	61–82	64,73	64,73	64,73	64,73
NQDSISSKLKECCEKPLLE	267–285		274		
RAFKAWAVARLSQRFPKAE	209–227			209,218	
RNECFLQHKDDNPNLPRLVRPE	98–119				106
RQIKKQTALVE	521–531	524,525	525	524,525	525
SAENCDKSLHTLFGDKLCTVATLRE	58–82	64,73	64,73	64,73	64,73
SLVNRRPCFSALEVDETYVPKEFNAE	480–505				500
TFTFHADICTLSEKE	506–520	519	519		519
TTLEKCCAAADPHECYAKVFDE	355–376	372			
TYGEMADCCAKQEPERNE	83–100	93	93	93	93
VSKLVTDLTKVHTE	231–244	233	233	233	233
VSRNLGKVGSKCCKHPE	426–442	436	432,436,439	432,436,439	432,436

concentration increased, a significant decrease of the acetylation immunoreaction signal was detected across the different conditions tested (Supplementary data Fig. S1). The level of acetylation was significantly lower after incubation with 625 mM glucose, showing that inhibition of the ASA reaction is directly correlated to the glucose concentration.

3.4. *Detection of modified immonium ions to confirm the presence of modified peptides*

The high fragmentation efficiency of HCD, coupled with its high resolution, high accuracy and the no low mass cut-off of Orbitrap detection, revealed the presence of immonium ions that were clearly visualized in the low mass range, confirming peptide identification. These immonium ions made it possible to pinpoint the presence of modified amino acids, such as acetylated and glycated lysine residues. The most frequent Lys immonium ion provided a signal at 84.081 Da, which shifted to 126.091 Da after acetyl group attachment and to 246.134 Da after glucose attachment. However, in the latter case, the typical neutral losses detected in glycated peptides should be taken into account, such as the loss of three water molecules (−54.031 Da), and the intramolecular rearrangement of the glucose moiety (−84.042 Da), which generated signals at 192.102 and 162.091 Da, respectively. Because of the selectivity of these ions, acetylated and glycated peptides can be localized by extracting ion chromatograms in MS/MS (Fig. 5A and B). These were monitored at 126.09 *m/z* for the acetyl-lysine immonium ion (Fig. 5C) and at 162.09 and 192.10 *m/z* for the furylium- and pyrylium-lysine immonium ions, respectively (Fig. 5D). These fingerprints provided additional evidence for the assignment of acetylated and glycated peptides, respectively [47]. Due to the high accuracy of the instrument used, it was possible to precisely select the *m/z* of either the lysine acetylated or glycated immonium ion which defined the presence of a modified residue in the MS/MS spectrum.

3.5. *Quantitation of acetylated and glycated peptides on HSA*

The application of this protocol provided profiles of the acetylated and glycated peptides including their modification sites on HSA. From a qualitative point of view, the total number of unique acetylated peptides was considered for each condition. Only one unique acetylated peptide was found in native HSA and in HSA after glucose exposure. However, this number increased to 12 peptides after simultaneous incubation with glucose and ASA, and up to 18 after ASA incubation alone (Table 2). It was observed that most of the sequences of acetylated peptides contained at least two modified sites; this was ascribed to the low efficiency of Glu-C, as mentioned above. On the glycation side, 17, 21, 22 and 18 unique glycated peptides were found in native HSA, HSA after incubation with 20 mM ASA, HSA after incubation with 10 mM glucose, and HSA after simultaneous glucose and ASA incubation, respectively (Table 3). The level of glycation and acetylation on HSA was measured using label-free quantitation. This method is based on the precise alignment of chromatographic peaks derived from the Rt and *m/z* features of each peptide identified. Raw LC–MS data were imported into Nonlinear Dynamics'

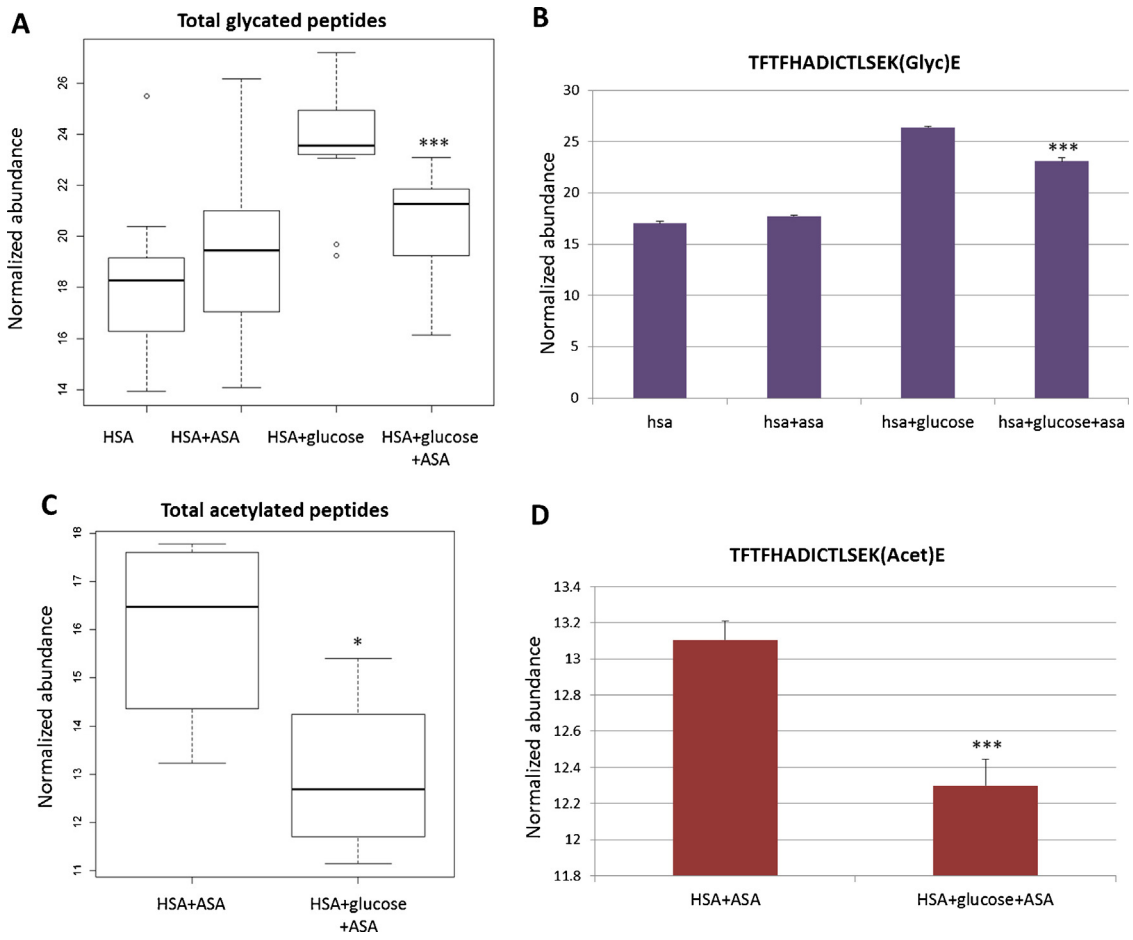

Fig. 6 – Quantification of glycation and acetylation on HSA. (A) Abundance distribution for all glycated peptides for each condition. (B) Peak abundance for a representative glycated peptide. (C) Abundance distribution for all acetylated peptides for both conditions. (D) Peak abundance for a representative acetylated peptide. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.005$.

Progenesis analysis software where the feature extraction and alignment processes take place across all the replicates for each condition. As previously explained, the identification results derived from all the peak lists were associated to their respective features and the peak totals for each peptide was calculated. Using this approach, 18, 18, 21 and 20 unique glycated peptides were quantified for native HSA, HSA after incubation with 20 mM ASA, HSA after incubation with 10 mM glucose, and HSA after simultaneous incubation of 10 mM glucose and 20 mM ASA, respectively. Taking all the glycated peptide totals measured in each condition, a significant decrease was observed between when ASA and glucose are incubated together and when glucose was incubated alone (Fig. 6A). Across all 4 conditions, 11 glycated peptides were found to be in common in all 4 conditions and all of them showed a significant decrease in glycation levels when ASA was incubated with glucose. Fig. 6B shows data from a representative glycated peptide (TFTFHADICTLSEKE) of HSA in order to highlight ASA's protective effects against glycation. Fig. 6C represents the total abundance of the acetylated peptides. No acetylated peptides were quantified for either native HSA or HSA with 10 mM glucose. However, the peak abundance of 12 and 9 unique acetylated peptides was measured for HSA after 20 mM ASA incubation and after glucose and ASA co-incubation, respectively. This lends further evidence to the theory of mutual competition between these two uncontrolled post-translational modifications. Across both conditions in which the acetylation level was measured, only 5 quantified peptides were found in common. Fig. 6D shows HSA's TFTFHADICTLSEKE peptide (also found to be glycated) which better illustrates the blocking effect of glucose on ASA-mediated acetylation at the protein level. Taking into account the number of modified peptides (from the acetylation and glycation data sets), and the unmodified form of the same peptides, the extents of acetylation and glycation in HSA were calculated at 42% and 13%, respectively. Taken together these results demonstrate a relevant interaction between these two modifications: from one side ASA tends to reduce the glycation level through the acetylation of preferential sites, and on the other, glucose hampers the ASA effect by decreasing the acetylation content of the protein, as also confirmed by Western blot. These results represent the first proof of the interplay between ASA and glucose at the protein level, suggesting a potential competition effect induced by glucose reducing the content of acetylation, and on the other side, induced by ASA, protecting the protein from further glycation.

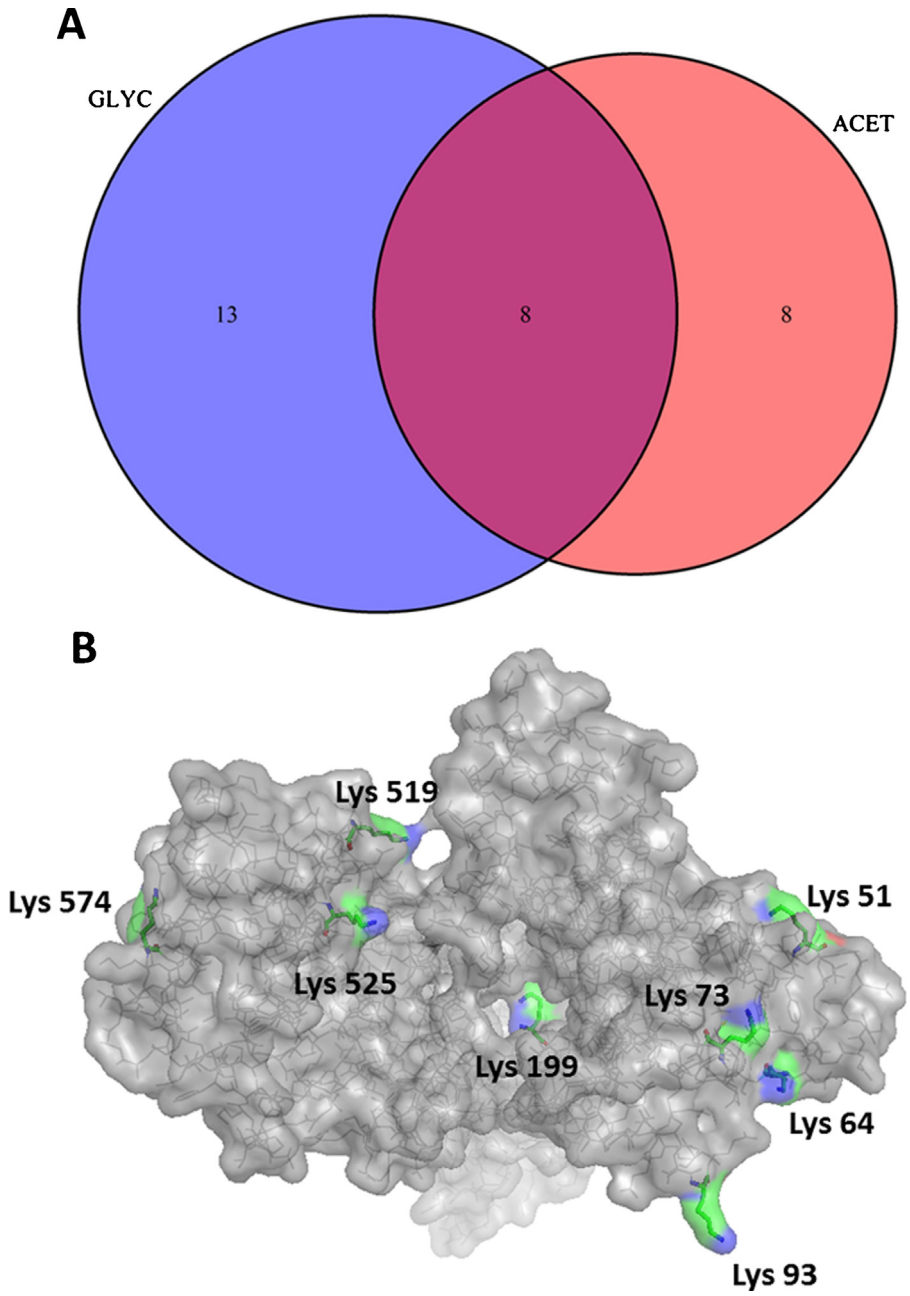

Fig. 7 – Analysis of glycated and acetylated sites on HSA. (A) Venn diagram between glycated and acetylated sites on HSA after glucose and aspirin incubation. (B) Solvent accessibility surface area of the crystal structure of HSA (pdb: 1UOR) with the 8 common modified lysine residues.

3.6. *Localization of glycation and acetylation sites on HSA*

The precise sites of glycation and acetylation modifications on HSA were analyzed in order to characterize the potential amino acid targets. A unique acetylated site (Lys 93) was found both in native HSA and HSA after glucose incubation; while HSA after ASA incubation identified 26 unique acetylated sites, and HSA incubated with glucose and ASA identified 15 unique acetylated sites (Table 2). Lys 199 and Lys 519 were found to be common to both types of incubations and had been reported as preferential acetylation sites in a recent study [15].

Glycation analysis yielded 15 unique glycated sites endogenous in native HSA, of which Lys 525 had been previously reported as a highly reactive glycation site [56,57]. In addition, glycation on native HSA was also detected at Lys 64, 73, 93, 199, 233, 262, 372, 436, 475, 519 and 524. Most of these residues were located at the protein surface exposed to the solvent or close to basic amino acids in the tertiary structure, thus making them more likely to the attach to glucose. The number of glycated sites increased to 27 after glucose incubation and to 21 when glucose and ASA were co-incubated (Table 2). Most of the sites identified were consistent with those reported by previous works in which high glycation levels were analyzed

[19,37]. To assess which sites were shared by acetylation and glycation modifications, the data sets of acetylated and glycated sites obtained from the sequential incubation of glucose and ASA were compared. Eight sites (Lys 51, 64, 73, 93, 199, 519, 525 and 574) were found to be in common (Fig. 7A). Of these, Lys 93 was detected as a native HSA acetylated and glycated site, Lys 525 was previously shown to be a major glycation site, and residues Lys 199 and 519 were found to be highly responsive to ASA. Fig. 7B shows the crystal structure of HSA, with its solvent accessible surface area, and the 8 modified lysines are represented by coloured sticks (Fig. 7B). With the exception of Lys 199, which is present in an internal pocket, the spatial distribution of the other sites' modified residues shows them to be on surfaces of the protein, readily accessible to the solvent, thus explaining their high tendency to acetylation and glycation. Surface solvent accessibility is defined as the surface traced by the centre of an ideal sphere with a radius of 1.4 Å as it rolls across the protein's van der Walls surface, and it is calculated using the rolling ball algorithm present in PyMOL v1.6 software.

Looking at the MS/MS spectrum for each of these sites, it was possible to highlight the peptide backbone, with identification of its *y* and *b* ions, together with the relative mass displacements due to the modifications. As an example, Fig. S2A and B in the Supplementary data shows the MS/MS spectrum for the acetylated and glycated forms of HSA's TFTFHADICTLSEKE peptide at Lys 519. The elucidation of new molecular targets for both ASA-induced acetylation and glycation could have important consequences on the biological functions of HSA. The presence of residues modified by an acetyl group or a glucose attachment could induce conformational changes of different protein regions, thus influencing HSA's affinity for binding to metabolites, drugs and fatty acids. Indeed, most of these interactions occur in pockets on the protein's surface, which is where this study identified the majority of acetylated and glycated sites. Furthermore, many other molecular processes could be affected by this interplay between acetylation and glycation, such as HSA's aforementioned antioxidant activity or its pseudo-esterase activity. Interestingly, one of the residues this study found to be acetylated (Arg 472) is very close to Tyr 411 (11.67 Å) (data not shown), HSA's central site of the esterase activity [58]; it is also close to Lys 199, which was seen to be both acetylated and glycated. Thus, depending on the extent to which these two non-enzymatic, post-translational modifications occur, and their competition for the same chemical groups, their roles in protein structures and functions could be different, leading to significantly altered protein responses.

4. Conclusion

The present work aimed to evaluate the extent of HSA acetylation using a range of ASA doses, and to demonstrate the influence of glycation on the acetylation process. A dose-dependent increase in the number of acetylated peptides and sites was observed after *in vitro* incubation with ASA. The significant impact that high glucose levels have on limiting protein acetylation was also demonstrated, and the favoured acetylation and glycation sites on HSA were characterized. Finally, a label-free quantitation was performed to better confirm the interplay and the reciprocal competition between glycation and ASA acetylation. Our pilot study has laid down the foundations for a more extensive future qualitative and quantitative analysis of acetylated and glycated proteins in clinical blood samples. This will help better evaluate whether these phenomena occur *in vivo* and to define their relevance to the pathophysiological mechanisms related to diabetes.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.euprot.2014.02.001.

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Chapter 4

CHARACTERISATION OF THE INFLUENCES OF ASPIRIN-ACETYLATION AND GLYCATION ON HUMAN PLASMA PROTEINS

This study corresponds to the application of the method described in the previous chapter, for the analysis of the interplay between aspirin-induced acetylation and protein glycation in human plasma proteins. Despite evidences demonstrating the protective role of aspirin over non-enzymatic glycation in a few sub-set of proteins, few information are available in more complex samples. Moreover, the impact of prior protein glycation on the acetylating effect of aspirin has never been demonstrated so far. The qualitative and quantitative analysis showed in this report allowed the identification of those plasma proteins that were both affected by glycation and acetylation, the mapping of the favored modification sites and the quantification of their glycated and acetylated state. In addition, our results indicate that the mutual interaction between aspirin-mediated acetylation and protein glycation might occur through a more complex mechanism compared to that one based on a simple direct competition, and that may involve structural conformational changes induced by glucose and acetyl-groups attachment to the proteins.

This article represents the first part of a comprehensive *in vitro* study carried out on human plasma. For this work, I performed the whole proteomics and MS experiments, data analysis, and I wrote the manuscript.

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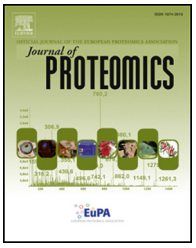

Characterisation of the influences of aspirin-acetylation and glycation on human plasma proteins

Francesco Finamore[a], *Feliciano Priego-Capote*[b], *Severine Nolli*[c], *Anne Zufferey*[c], *Pierre Fontana*[c], *Jean-Charles Sanchez*[a,*]

[a]*Translational Biomarker Group (TBG), Department of Human Protein Sciences, University Medical Centre, University of Geneva, 1211 Geneva 4, Switzerland*
[b]*Department of Analytical Chemistry, Annex C-3 Building, Campus of Rabanales, University of Còrdoba, Spain*
[c]*Division of Angiology and Haemostasis, University Hospitals of Geneva, Geneva Platelet Group, Faculty of Medicine, Geneva, Switzerland*

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ABSTRACT

The competition effect between aspirin-mediated acetylation and protein glycation has been a matter of concern for decades. However, the exact interactions between these two post-translational modifications are still not well understood. Several efforts have been made to explain how aspirin prevents glycation, but the influence of prior protein glycation on the action of aspirin has never been investigated. This study involved qualitative and quantitative analyses to: 1) identify acetylated and glycated proteins; 2) quantify rates of acetylation and glycation; and 3) elucidate the common modification sites. Human plasma was incubated with 30 mM glucose and then 500 μM aspirin. A label-free mass spectrometry approach indicated an increase in the acetylation level after this sequential glucose-then-aspirin incubation; these results were also confirmed by Western blot. Interestingly, for several proteins, decreases in glycation levels were evidenced after aspirin incubation. The common modification sites, where both acetylation and glycation took place, were also identified. The influence that glycation and acetylation processes have on each other could reflect conformational changes induced by glucose and aspirin. In future studies, in order to better understand the interactions between these two PTMs, we intend to apply this strategy to other blood compartments and to diabetic patients.

Biological significance
Non-enzymatic glycation represents an early stage in the development of the long-lasting complications that are associated with diabetes. Aspirin has been shown to prevent this process in a few reference proteins, but how the two post-translational modifications (PTMs) of aspirin-mediated acetylation and protein glycation interact with each other remains poorly investigated. This study used a label-free quantitative proteomic approach to characterise the extent of aspirin-induced acetylation and protein glycation in human plasma. The results clearly supported a mutual influence between these PTMs, which lead us to propose a potential model based on structural conformational changes.

* *Corresponding author.*
E-mail address: Jean-Charles.Sanchez@unige.ch (J.-C. Sanchez).

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1. Introduction

Perturbations to glucose homeostasis, observed in type 1 and type 2 diabetes, develop in situations of hyperglycaemia. Specific perturbations not only determine the disease type, but they are also the cause of long-term complications, which include diabetic neuropathy [1], nephropathy [2], retinopathy [3] and cardiovascular diseases [4,5]. Circulating glucose reacts with proteins through a spontaneous, non-enzymatic post-translational modification (PTM), the rate of which is principally governed by blood glucose concentrations; this PTM is enhanced under hyperglycaemic conditions. The glycation process is the first step in a series of reactions that culminate in the development of the long-lasting deleterious effects typical of diabetes [6–9]. Most of these disorders are correlated to protein structure/function alterations. These include impairment of human albumin's drug-binding capacity [10,11] or its antioxidant activity [12,13]; the glycation of apolipoproteins and extracellular matrix proteins, which have been shown to be correlated to the pathogenesis of atherosclerosis [14–17] and an increased risk of cardiovascular diseases [18]; and the greatly enhanced toxicity of β-amyloid by glycation modification, that in turn increases the rate of protein aggregation into fibrils typical of neurodegenerative diseases [19,20].

Several efforts have been made to prevent excessive human protein glycation and most of these have concentrated on using compounds that act at different stages of the glycation process [21]. Among these, aspirin was probably the first agent shown to protect against protein glycation and to be associated with a decrease in cardiovascular events [22,23]. Aspirin acts through the acetylation of the reactive amino groups on proteins, i.e. the functional groups that are also the targets for glycation. Up until now, the aspirin's impact on protein glycation has been assessed using single reference proteins such as lens crystallins [24,25], collagen [26,27], haemoglobin [28] and fibrinogen [29], as

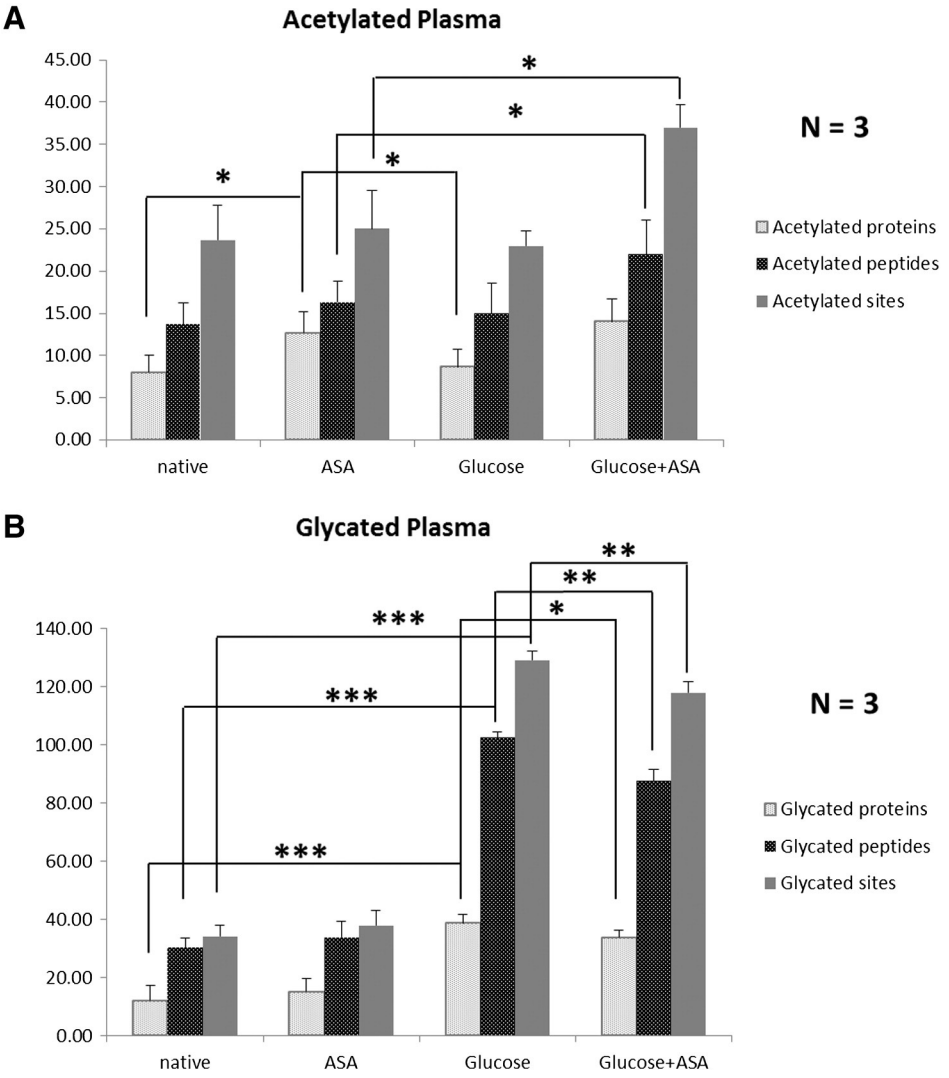

Fig. 1 – The mean number of unique proteins, peptides and sites detected on the acetylation (A) and glycation (B) data sets in native plasma, after 30 min 500 μM aspirin, after 24 h 30 mM glucose and after a sequential incubation of glucose followed by aspirin exposition at the same conditions (N = 3). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.005$.

well in cell systems such as endothelial cells [30] and human platelets [31]. In the latter case, a decrease in the aspirin's ability to inhibit platelet aggregation and to activate the nitric oxide pathway was observed in the presence of high glucose levels, confirming the evidence of aspirin's resistance to hyperglycaemic conditions [32]. Despite the clinical relevance of these studies, few of them offer a global view of the potential mechanisms underlying the mutual influences of the aspirin's effects and glycation. Aspirin-acetylation and protein glycation influence each other at the protein level, but the exact interaction through which these two PTMs exert their action remains poorly understood, especially in complex biological samples.

The present study presents an analysis of aspirin-mediated acetylation and non-enzymatic glycation on human plasma proteins, which was conducted in order to determine the mutual influence exerted by these PTMs. The same experimental approach was previously applied to a single reference protein (human serum albumin) [33]. Here, it is used to obtain qualitative information via the detection of acetylation and glycation sites, as well as quantitative data to determine the levels of these modifications.

2. Material and methods

2.1. Chemicals

EDTA (10.8 mg)-Vacutainer tubes were from BD Vacutainer®. Dulbecco's phosphate-buffered saline (DPBS; 1×, pH 7.4) was from Invitrogen™. Aspirin was purchased from Aspegic Inject®. EDTA-free protease inhibitor cocktail (PIC) tablets were purchased from Roche. Anti-human Nε-acetyl-lysine monoclonal antibody was from Cell Signaling Technology®. ECL™ detection reagent was from GE Healthcare. Protein assay dye reagent concentrate (liquid) was from Bio-Rad. D-Glucose [$^{12}C_6$] (lyophilised powder, ≥99.5%), Naphtol blue black (lyophilised powder, dye content ca 80%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 0.5 M, pH 7.0), iodoacetamide (IAA, crystalline, ≥99%), endoproteinase Glu-C from *Staphylococcus aureus* V8 (lyophilised powder, 500 U), high-performance liquid chromatography (HPLC)-grade water (CHROMASOLV®) and HPLC-grade acetonitrile (ACN; CHROMASOLV®, ≥99.9%) were purchased from Sigma-Aldrich®. Bovine serum albumin (BSA, lyophilised powder, ≥96%), triethylammonium hydrogen carbonate buffer (TEAB; 1 M, pH 8.5), ammonium acetate (NH_4Ac, solid, 98.0%), magnesium chloride ($MgCl_2$, solid, ≥99.0%), acetic acid (99.5%) and formic acid (FA; 98.0%) were from Fluka.

2.2. Glucose and aspirin incubation in human plasma

Human plasma was obtained from three male donors using an inclusion criteria of HbA1c <6%. Briefly, EDTA-Vacutainer tubes containing blood were centrifuged at 2350 × *g* to separate plasma from the red blood cell (RBC) fraction. Plasma samples were pooled. Protease inhibitors were added to plasma at a dilution of 1:7. Four 225 μL aliquots of plasma were diluted in phosphate buffer to reach a final volume of 400 μL and were subsequently incubated with: 1) 500 μM aspirin for 30 min at 37 °C; 2) 30 mM glucose for 24 h at 37 °C; or 3) sequentially with 30 mM glucose for 24 h followed by 500 μM aspirin for 30 min at 37 °C. The final aliquot, without glucose or aspirin, was incubated as a negative control. After incubation, protein amounts were determined using the Bradford assay, and BSA was used to create a standard calibration curve.

2.3. Western blot analysis of aspirin-mediated acetylation

The samples of 5 μg were separated using electrophoresis on a 10%T/2.6%C polyacrylamide gel and protein transfer was carried out for 1 h at 350 mA. Membranes were stained with Amido black to highlight the protein bands [34] and washed with water to remove the excess of a background. A Western blot analysis was carried out with an anti-human antibody directed to Nε-acetyl-lysine in a dilution of 1:5000. The intensity of the Western blot's acetylation signal was normalised relative to the corresponding band stained with Amido black. ECL reagents were used to detect the acetylation signal. Each sample was evaluated using three experimental replicates (N = 3).

2.4. Protein purification and Glu-C digestion

After incubation, glucose and salts were removed using Amicon Ultra-0.5 mL, Ultracel® 3K membrane (Millipore™) devices to purify the proteins. These proteins were reconstituted in 500 mM TEAB, pH 8.5 and their concentrations were subsequently measured using the Bradford assay and BSA as the calibration protein. Samples were split into two experimental groups in order to analyse the acetylated and glycated peptides separately in each. For the acetylation study, 200 μg of proteins was diluted in 400 μL of TEAB; for the glycation study, 1 mg of proteins was diluted in 400 μL of TEAB, for subsequent protein digestion. In order to do this, disulphide bonds were reduced using 4 μL of TCEP to reach a final concentration of 5 mM and the reaction mixture was incubated for 1 h at 60 °C. Cysteine groups were alkylated using 10 μL of IAA to reach a final concentration of 20 mM, and incubation was carried out for 30 min at room temperature in dark conditions. Freshly prepared endoproteinase Glu-C (1 mg/mL) was added at a ratio of 1:10 (w/w), and digestion took place for 16 h at 37 °C. For acetylation analysis, digestion mixtures were evaporated using a vacuum centrifuge, and directly reconstituted in 5% ACN/0.1% FA for the subsequent desalting procedure. For the glycation analysis, peptide digests were evaporated and reconstituted in mobile phase A (200 mM NH_4Ac, 50 mM $MgCl_2$, pH 8.1) to reach an estimated concentration of 16 mg/mL, for the fractionation of glycated peptides.

2.5. Enrichment of glycated peptides using boronate affinity chromatography

Reconstituted peptides were enriched using boronate affinity chromatography—by interaction between cis-diol groups of glycated peptides, present at low concentrations, and boronate groups in the stationary phase, as previously described [35,36]. For this purpose, a 50 μL sample was injected at room temperature in a Waters 600E HPLC system equipped with a TSK-Gel boronate affinity column from Tosoh Bioscience (7.5 cm length × 7.5 mm inner diameter, i.d., 10 μm particle size). A chromatographic method based on a gradient with two

isocratic steps was used to separate non-glycated from glycated fractions. Step 1 was a 0–10 min, 100% mobile phase A (200 mM ammonium acetate, 50 mM magnesium chloride, pH 8.1) for the retention of glycated peptides using esterification of the 1,2 cis-diol group of glucose moieties and the hydroxyl groups of boronate ligands under alkaline conditions. Non-glycated peptides were eluted. Step 2 was a 10–20 min, 100% mobile phase B (100 mM acetic acid) for the elution of glycated peptides under acidic conditions; followed by a 20–30 min, 100% mobile phase A to bring the column back to the initial equilibrium conditions. The glycated fraction was collected for subsequent evaporation and reconstitution in 5% ACN/0.1% FA, for the ensuing desalting and concentration.

2.6. LC–MS/MS detection of acetylated and glycated peptides

The samples from acetylated and glycated data sets were desalted and pre-concentrated using a MacroSpin Column™ C_{18} (Harvard Apparatus), according to the manufacturer's protocol. Peptides were eluted in 400 μL of 50% ACN/0.1% FA and then evaporated to dryness before reconstitution in 5% ACN/0.1% FA, prior to liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) analysis. Peptide digests were analysed using electrospray ionisation in positive ion mode (1.9 kV ionisation voltage), on an Orbitrap hybrid linear ion trap (Thermo Fisher). A reverse-phase separation was carried out using a Waters NanoAquity HPLC system consisting of a pre-column (100 μm inner diameter and 18 mm in length) packed with C_{18} resin, where peptides were initially loaded and trapped at a flow rate of 3 μL/min in water/ACN (95/5 v/v) with 0.1% FA. After loading, peptides were eluted using an ACN gradient developed in an analytical column packed with C_{18} resin, at the flow rate of 200 nL/min with mobile phase A (water, 0.1% FA) and B (ACN, 0.1% FA). The mass spectrometer was operated in data-dependent MS/MS mode with a precursor-ion scan range of 400–2000 m/z and a resolving power of R = 60 000. Each sample, in both data sets, was analysed three times (three technical replicates) using high energy collisional dissociation (HCD) as the activation mode. The five most abundant ions detected by the MS survey scan were selected as precursor ions for fragmentation (normalised collision energy, 35%). This was followed by Orbitrap detection at a resolving power of 7500 and a dynamic exclusion of 120 s to minimise repeated analysis of the same precursor ion. All analyses were carried out using three replicate injections.

2.7. Data analysis

After data-dependent acquisition, peak lists were generated from the raw data using EasyProtConv v1.5 software, and the resulting data files were searched for matches against the UniProtKB/Swiss-Prot database (Release 13 June 2012, 659,907 entries) using the EasyProt v2.3 (build 751) tool [37]. Because this study analysed human plasma, *Homo sapiens* taxonomy was selected in the protein search criteria. The high resolution of the data acquired allowed the scoring model to use an accuracy of 6 ppm on fragments and 10 ppm on precursors. Common amino acid modifications detected were carbamidomethylation of cysteine residues (57.0215 Da) and oxidised methionine (15.9949 Da); these were set as fixed and variable modifications, respectively. Additional variable modifications, for acetylation and glycation, were selected on lysine residues and N-terminal positions. For the analysis of acetylated peptides, the selection of acetylation-induced modification was based on the peptide attachments of the acetyl group (42.0100 Da), while for the selection of glycated peptides, the mass shift was built on the attachment of the entire glucose molecule (162.0523 Da). The number of modifications per peptide was set to a maximum of two in order to reduce the search space of all possible combinations of variable modifications. Endoproteinase Glu-C was selected as the cleavage enzyme, with three potential missed cleavages allowed. The minimum peptide length was eight, with a minimum peptide z-score of four. Three analytical injections per sample each analysed independently, provided replicate data values. The peak list files obtained for each technical replicate were merged and submitted to the database search. For the significance of peptide identification we used a false-discovery rate ≤5% (Supplementary data FDRs_Tables, FDRs_Graphs). Peptides were considered as identified if they were present in all three replicates.

2.8. Peptide quantitation

Label-free quantitation of glycated and acetylated peptides from plasma was done using Nonlinear Dynamics' Progenesis software. This relies on the accurate alignment of the m/z and Rt features for every chromatographic run in each group. A global peak list file, containing all the features detected, was used to query the database, as above, and a pepXML file was exported to Progenesis from the results identified. All the identified peptides were then matched using their relative features. Quantitation was carried out using the normalised abundance for each peak corresponding to features and comparing it to the same peak for all the conditions analysed. The normalisation of peptide abundance was performed automatically by Progenesis. Briefly, the software selects one technical replicate as a reference; after calculating the raw intensities for each peptide on each replicate, it determines the ratio between the replicate being normalised and the reference replicate. Then, a distribution of log (ratios) is calculated and a global scaling factor (the anti-log of the average of the log (ratios)) is determined. The raw intensities for each peptide among different replicates were multiplied by the global scaling factor.

3. Results

3.1. Identification of acetylated and glycated proteins in human plasma

In this study, the influences of aspirin-induced acetylation and protein glycation were assessed using the independent incubation of four separate plasma conditions. An aspirin concentration of 500 μM was selected in order to have homogeneous pharmacodynamic effects, in line with a previous in vitro study using aspirin [38]. A glucose concentration of 30 mM was chosen to mimic the type of chronic extreme glucotoxicity perturbation/imbalance that is often observed in diabetic patients and to thus reduce one of the limitations of this in vitro study. Next, a short

incubation time (30 min) was chosen for aspirin in order to be more consistent with its pharmacokinetic profile [39]. The acetylation and glycation patterns of human plasma were analysed using a tandem mass spectrometry approach. This approach was optimised for the identification of acetylated and glycated peptides and corresponding proteins, as well as for discovering acetyl and glucose attachment sites. These are shown in Supplementary Tables S1 and S2. In total 16, 21, 16 and 26 proteins were found to be acetylated (without a pre-fractionation step) in native plasma, after aspirin incubation, after glucose incubation and after sequential glucose-then-aspirin incubations, respectively. Moreover, 36, 39, 33 and 52 unique acetylated sites were identified using these conditions, respectively (Supplementary data, Fig. S1A). These results suggested an unexpectedly significant increase in acetylation, both at peptide and site levels, after sequential glucose-then-aspirin incubation compared with aspirin incubation alone (Fig. 1A). The glycation pattern of plasma was then characterised after the enrichment of glycated peptides using BAC. The proportion of non-glycated peptides identified in the retained fractions ranged between 17.6% and 26.3% across all conditions, validating the selectivity of the enrichment step. Totals of 26, 29, 60 and 53 glycated proteins were identified, as were 65, 69, 181 and 153 glycated sites, for the four conditions tested, respectively (Supplementary data, Fig. S1B). Again, looking at the mean numbers of proteins, peptides and sites identified among the three replicates, a significant decrease of the glycation content was observable after glucose and 30 min of aspirin incubation in comparison to glucose incubation alone (Fig. 1B).

In order to ascertain which proteins underwent both acetylation and glycation modifications, the data sets for the acetylated and glycated proteins obtained from the sequential incubation of glucose and aspirin were compared. Four specific plasma proteins were found to be both acetylated and glycated (Fig. 2A). These proteins were human serum albumin, immunoglobulin γ – 1, the component C-3 of the complement and apolipoprotein A-2. Among the detected sites, 10 were found to be both acetylated and glycated (Fig. 2B), and seven of them belong to human albumin in Lys and Arg residues located at positions 51, 64, 93, 257, 444, 445 and 545. Most of them had been identified as preferential sites of both aspirin-acetylation and glycation in a previous study [33]. To the best of our knowledge, the present study is the first to have identified three acetylation and glycation sites for the plasma proteins immunoglobulin γ – 1 (Lys 129), complement C-3 (Ser 1) and apolipoprotein A-2 (Lys 23), that are the targets of both aspirin and glucose. Annotated spectra for these sites are furnished in Supplementary data (Annotated_Spectra_PTMs_Sites).

3.2. Assessment of the acetylation and glycation rates of plasma proteins

Qualitative analysis of acetylated and glycated plasma proteins was complemented by quantitative information. The extent of aspirin-acetylation and protein glycation was measured using a label-free approach as described in the Material and methods section. This strategy enabled the quantification of 40 unique acetylated proteins across all the conditions tested (Supplementary Table S3). Most of these proteins were quantified with one unique acetylated peptide. Human albumin proved to be an exception: due to its extremely high concentration in human plasma, nine unique acetylated peptides were quantified.

The quantitative analysis of the glycated data set of human plasma, obtained after enrichment, quantified 110 glycated proteins, the majority of them with more than two unique peptides, over the four conditions studied (Supplementary Table S4). The quantified proteins are representative of the major plasma proteins, such as human albumin (63 glycated peptides), serotransferrin (16 glycated peptides), haptoglobin (4 glycated peptides), apolipoprotein A-1 (4 glycated peptides), vitamin D-binding protein (5 glycated peptides) and fibrinogen (6 glycated peptides), to cite but a few. Quantitation measurements from acetylated and glycated plasma proteins were initially used to perform a Principal Component Analysis (PCA) to highlight the main differences between the four groups and

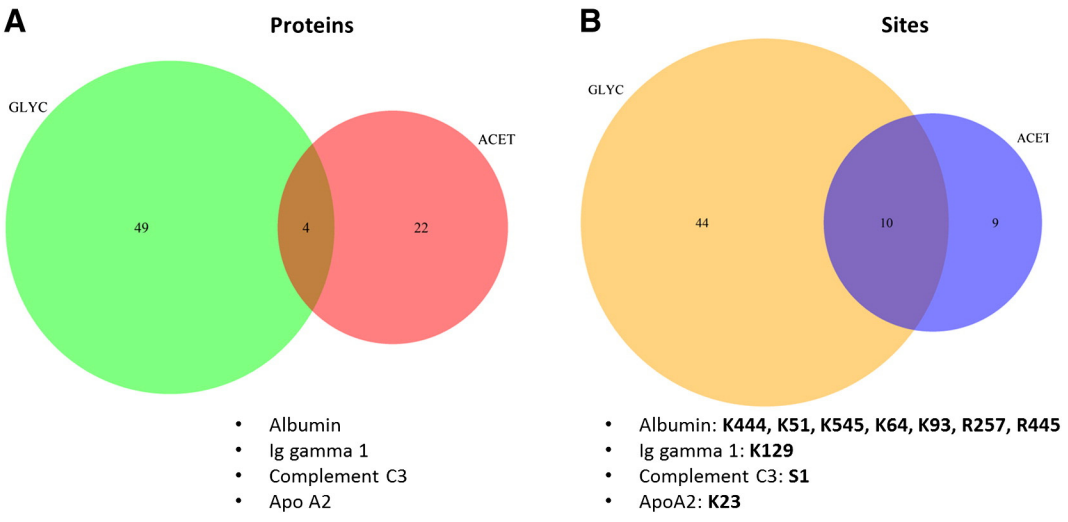

Fig. 2 – Common acetylated and glycated plasma proteins (A) and sites (B) identified after sequential incubation of glucose and aspirin.

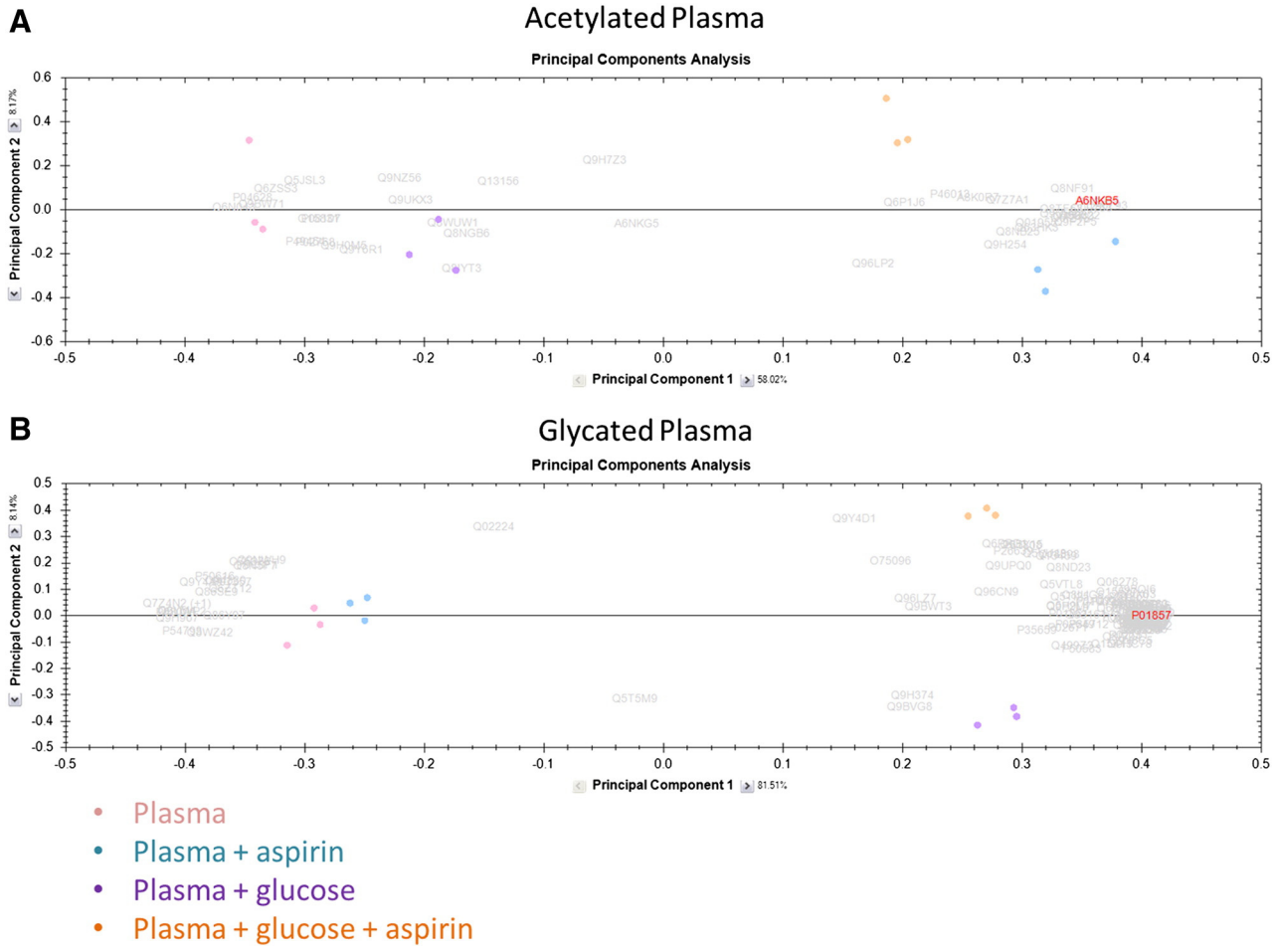

Fig. 3 – Principal component analysis (PCA) of acetylation (A) and glycation (B) data sets represents the separation pattern between native plasma (pink dots), after aspirin incubation (blue dots), after glucose incubation (violet dots) and with glucose followed by aspirin exposition (orange dots).

to represent the inter- and intra-group variability across the replicates. From these results, four clearly separated clusters, each attributable to a sample group, were observed for the acetylation (Fig. 3A) and glycation data sets (Fig. 3B). The minimal degree of intra-group dispersion (around 8.2%) provided the first general confirmation of the impact of aspirin and glucose on human plasma proteins.

3.3. *Quantitation of common acetylated and glycated sites as responses to the effects of glucose and aspirin*

We assessed how acetylation and glycation influenced each other at the binding-site level, focusing on the four proteins found to be the common targets of both PTMs. Interestingly, all ten sites that were observed to be both acetylated and glycated revealed significant variations after the sequential glucose-then-aspirin incubation. In particular, when glucose was incubated before aspirin, the four representative acetylated sites showed significant and unexpected increases in acetylation rates in comparison to aspirin incubation alone (Fig. 4), as also shown for the other six sites (Supplementary data, Fig. S2). On the glycation side, on the other hand, when glucose was incubated before 30 min of aspirin exposure, an interesting and significant decrease in protein glycation was observed, in comparison to glucose incubation alone for the same time period. This is shown for the same four representative sites mentioned above (Fig. 5) as well as for the other six glycated sites (Supplementary Data, Fig. S3). Despite the evident impact of acetylation and glycation on common sites involved in both PTMs, it was of interest to evaluate whether this mutual influence could also be detected in residues that were found to be either acetylated or glycated. Surprisingly, most of the uncommon sites showing a significant variation across conditions, and matching with both common and uncommon plasma proteins, followed the same trend observed at the 10 sites that shared both modifications. This was demonstrated by the quantitation levels of further glycated sites belonging to the same proteins, found to be targets of both acetylation and glycation and also for several other plasma proteins (Supplementary data, Fig. S4), in which a significant decrease of glycation is visible as well. The same was shown for human albumin and other identified acetylated proteins, representing the variation in acetylation levels on uncommon sites (which are not glycated) (Supplementary data, Fig. S5). Again, a significant increase in acetylation was observed after the sequential glucose-then-aspirin incubation, in comparison to exposure to aspirin only. This indicates that the influence of the

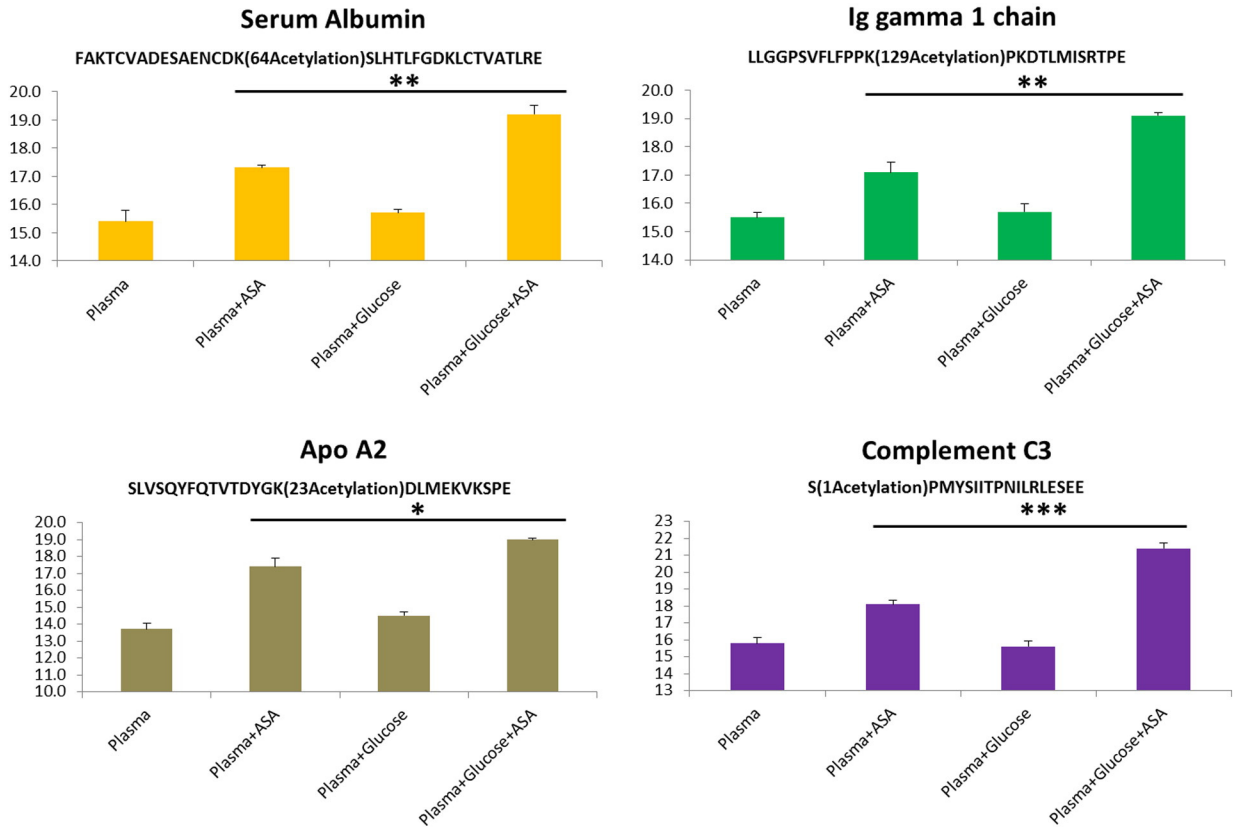

Fig. 4 – Quantification of the peak abundances of 4 acetylated sites from 4 representative common plasma proteins among all the conditions (native, 500 μM aspirin, 30 mM glucose, 30 mM glucose +500 μM aspirin). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.005$.

two reactions spreads to sites usually only modified by one PTM or the other.

In order to confirm the results from LC–MS/MS data, sample conditions were replicated to perform a Western blot analysis. This analysis aimed to determine the level of acetylation in native plasma after 30 min of aspirin incubation, both with and without 24 h of prior exposure to 30 mM glucose. The results showed a clearly visible difference in the acetylation reactions for the four protein bands represented by human albumin, fibrinogen beta chain and the heavy and light chains of Ig G (Fig. 6). For all the revealed bands, the acetylation signal in plasma incubated sequentially with glucose followed by aspirin (Supplementary data, Fig. S6, lane 4) was significantly higher than the signal after aspirin incubation alone (Supplementary data, Fig. S6, lane 2). No signal was detected in native plasma or after glucose incubation alone (Supplementary data, Fig. S6, lanes 1 and 3). These results strongly confirmed the data obtained by LC–MS/MS and, taken together; they reveal a new and interesting aspect of the impacts of aspirin-acetylation and protein glycation.

4. Discussion

4.1. *Qualitative analysis of acetylated and glycated plasma proteins*

In this study we have described an analytical method aimed at understanding the in vitro interactions of aspirin and glucose, through qualitative and quantitative analyses of acetylated and glycated proteins in human plasma. There were several relevant reasons for wishing to identify and measure the extent of aspirin-acetylation and glycation of plasma proteins. One reason is the current lack of basic information, at the molecular level, about the mutual interaction and effects of those PTMs in human bio-fluids. Moreover, few studies have described the protective role of aspirin in preventing protein glycation via the basic concept of competition. This assumption has been confirmed in a few protein models, such as with α-crystallin (showing that acetylation and glycation occur concurrently) [25], but to the best of our knowledge no elucidation of the specific sites modified had as yet been performed. Indeed, in fibrinogen, the characterisation of acetylated and glycated sites showed the aspirin and glucose reactions to be independent [29]. The authors of the present study have previously examined the interaction between aspirin-acetylation and protein glycation on human albumin. Although they were able to observe mutual competition for binding sites, only a few sites were shown to share both PTMs. This demonstrated that the influences of aspirin and glucose could not simply be explained by a direct competitive effect [33]. The present study used a combination of MS/MS mode coupled with HCD activation to identify acetylated and glycated plasma proteins after incubation with a high concentration of glucose (30 mM) followed by exposure to a therapeutic dose aspirin (500 μM) [38]. The high accuracy of the HCD-MS/MS mode in identifying precursor and fragment ions, coupled with Orbitrap detection, enabled the identification of acetylated and glycated peptides in each of the four conditions [40]. Looking at the mean

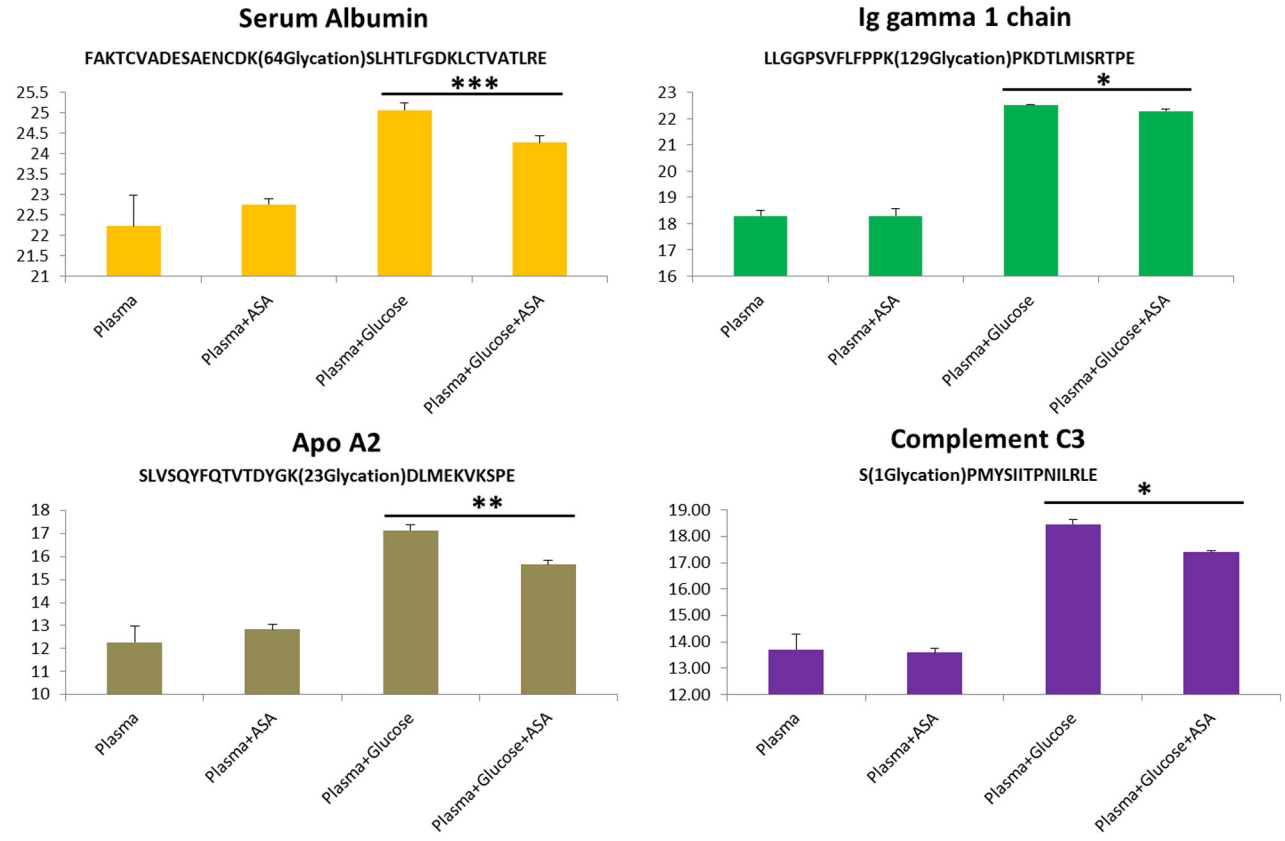

Fig. 5 – Quantification of the peak abundances of 4 glycated sites from 4 representative common plasma proteins among all conditions (native, 500 μM aspirin, 30 mM glucose, 30 mM glucose +500 μM aspirin). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.005.

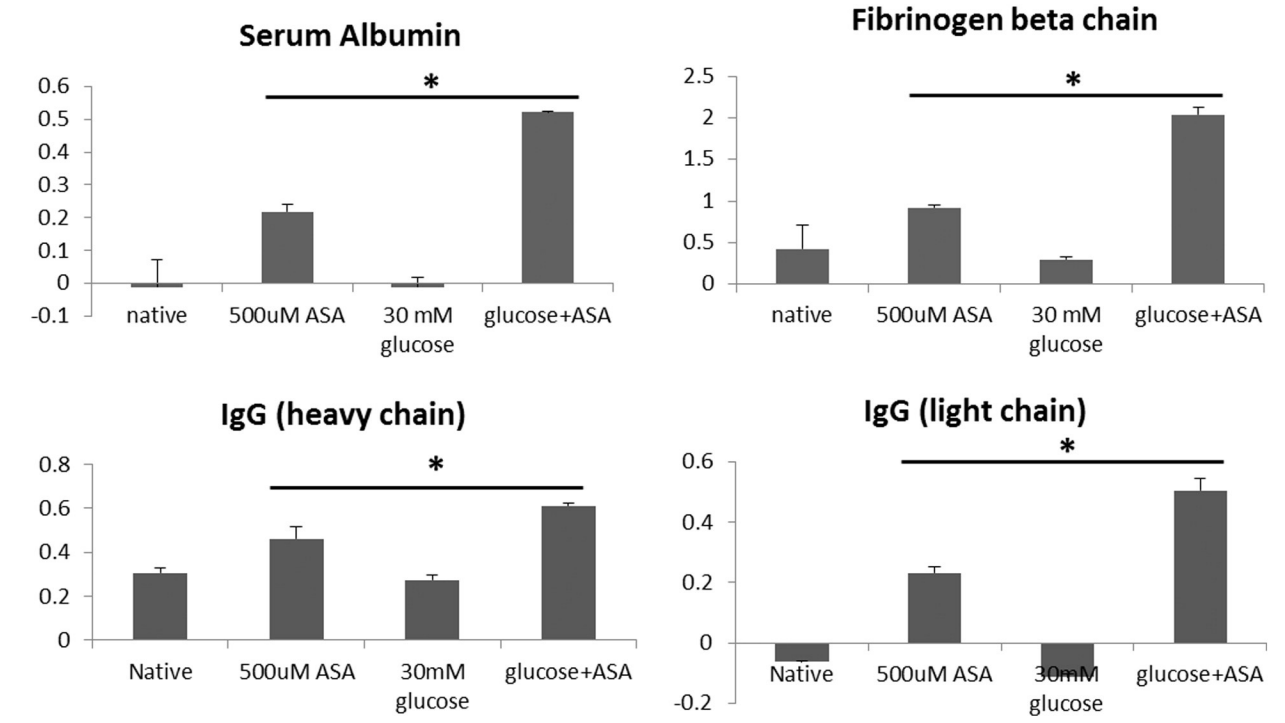

Fig. 6 – Acetylation level measured by Western blot shows a significant increase after 30 mM glucose incubation followed by 500 μM aspirin compared with aspirin incubation alone for the 4 protein bands detected (human albumin, fibrinogen, IgG heavy chain and IgG light chain) (N = 3). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.005.

number of acetylated proteins, peptides and binding sites among replicates (N = 3), an unexpected increase in the acetylated content was observed in plasma after the sequential incubation of glucose and aspirin in comparison to aspirin incubation alone. This increase was significant for acetylated peptides and sites, but not for proteins. Further, a significant decrease (N = 3) in the glycated content at the protein, peptide and site levels was shown after just 30 min of aspirin activity after glucose incubation, in comparison to glucose incubation alone. Both results are of interest because it seems that the presence of glucose enhances the acetylation process, and in parallel, acetylation itself reduces protein glycation highly efficiently. Four specific plasma proteins were shown to be preferentially acetylated and glycated and, among them, 10 sites were characterised as being the targets of both PTMs. The seven acetylated residues found in human albumin had already been observed to be preferential acetylation sites at low aspirin doses [33,41]. The other three, belonging to apolipoprotein A-2, Ig γ – 1 and complement C-3 had never yet been characterised under such conditions. It is worth emphasising that, the small number of plasma proteins undergoing acetylation by aspirin and the presence of single peptides is mainly related to the absence of a pre-fractionation step. Moreover, the reduced chemo-selectivity of such low concentrations of aspirin (500 μM) also explains this small number of acetylated peptides. Antibody-based enrichment could be a further step to implement in future studies. On the other hand, glycation of human albumin has been reported in previous works [33,42], as it has for other plasma proteins found to be glycated in the present study, such as IgG [43,44], Apo A-1 [45–47], complement C-3 [48], haptoglobin and serotransferrin [49], to cite but a few. Most of these studies associated glycation to the alteration of the protein structure.

The total rate of acetylation on native plasma and after glucose incubation alone was estimated around 24.6%, while after aspirin incubation alone it increased to 39.6% and after sequential glucose-then-aspirin incubation it reached a rate of 45.6%. Then, glycation rates of 28.6%, 30.8%, 48% and 44.9% were calculated for native plasma, after aspirin incubation, after glucose incubation and after the sequential incubation of glucose and aspirin, respectively. There was evidently a mutual influence occurring between acetylation and glycation, the degree of which was measured using label-free approach, as described below.

4.2. Quantitation of aspirin and glucose interaction on human plasma

The dual effects of aspirin-acetylation and glycation observed in the qualitative data were confirmed by the quantitation of 10 common sites across the four proteins. Indeed, it was possible to observe a significant increase in the acetylation rate for all 10 residues in the sequential incubation of glucose and aspirin, compared with aspirin incubation alone. It should be noted that other independent acetylated proteins exhibit the same behaviour, although most of them did not show a significant variance between sequential glucose-then-aspirin incubation and aspirin alone.

These observations were reconfirmed using the Western blot test under the same sample conditions. This showed that the rate of acetylation of the three detected plasma proteins (serum albumin, fibrinogen and IgG) increased significantly when glucose and aspirin are incubated sequentially. This may support the interpretation by which protein glycation favours acetylation in a process governed by variation in the

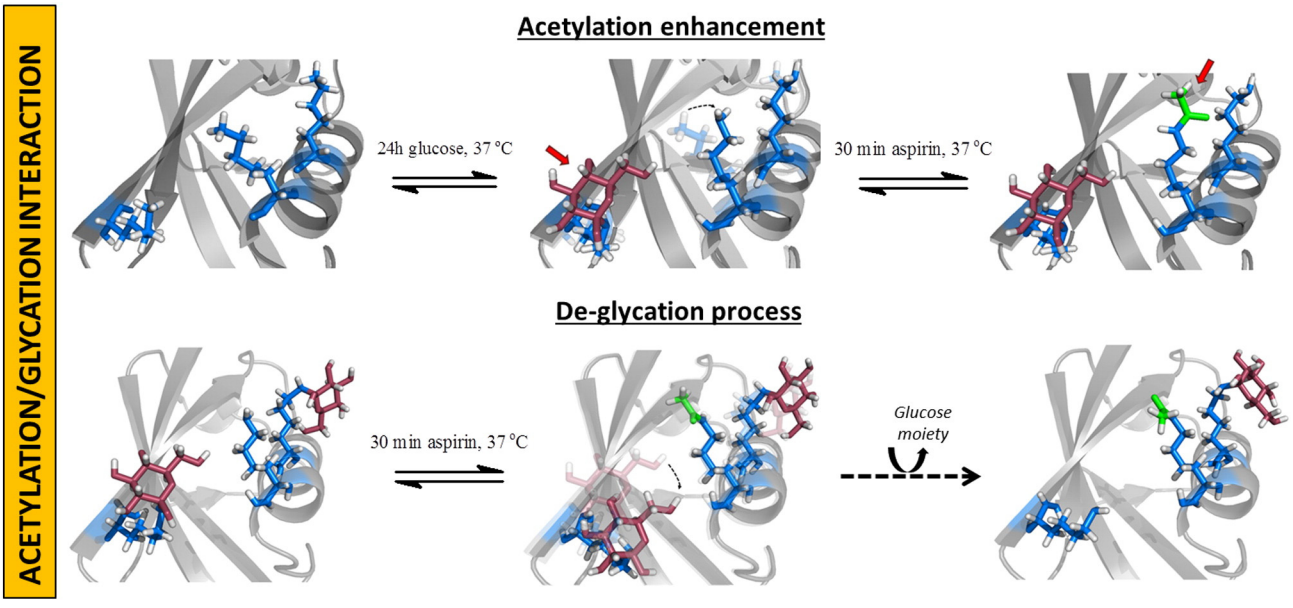

Fig. 7 – The potential mechanism through which aspirin-induced acetylation and protein glycation influence each other at protein level, might be explained by a dynamic equilibrium between these two post-translational modifications, that in one side increase the acetylation process and in the other exert a de-glycation effect. In blue are highlighted lysine residues, in red the glycation motif and in green the acetyl group.

intramolecular chemo-selectivity, potentially due to conformational changes exerted by glycation. This might also explain why independent acetylated sites, which were not also found to be targets of glycation, showed the same increase in the acetylation rate as after glucose incubation alone.

With regard to glycation, for all the 10 commonly modified residues, a significant decrease was observed after sequential glucose incubation followed by 30 min aspirin exposure, in comparison with glucose incubation alone. This highlights, not only the aspirin's protective role in reducing further glycation, but also that this blocking process is efficient even at low aspirin doses and for brief exposure periods, mimicking physiological conditions. The next step was to focus attention on those glycated sites that were not also found to be targets of acetylation, which gave rise to some potential new insights. Indeed, looking at the different glycated residues on the four proteins that share both modifications, it was possible to observe a significant decrease in the glycation rate after aspirin incubation, as was shown before for the common sites. This was also true for other glycated plasma proteins quantified in this study, when comparing the glycation rate of their glycated residues. Taken together, these results give new potential to the behaviours of the dual effects of aspirin-acetylation and protein glycation: on one side, glycated sites favour the acetylation of non-reactive sites due to the kinetic effects supported by chemo-selectivity that make these sites more prone to being potentially acetylated, on the other hand, there is a parallel competitive effect by which acetylation promotes a de-glycation step. De-glycation can only be explained by the conformational changes occurring in the protein. It is important to note that glycation is an equilibrium process involving a condensation reaction that is not 100% efficient. Conformational changes could alter this equilibrium, leading to stable glycated sites being converted into unstable ones which might then undergo acetylation (Fig. 7).

5. Conclusion

The extent of aspirin-induced acetylation and protein glycation in human plasma was evaluated in order to characterise the potential mutual molecular influences of the two PTMs. This study was relevant because human plasma is a source of key proteins with which both aspirin and glucose interact, and it enabled the interpretation of the changes that these PTMs can have on protein structure and function. High resolution tandem mass spectrometry, coupled with a label-free approach, enabled the detection of acetylated and glycated peptides along with their modified residues, and allowed the quantitation of acetylation and glycation rates of acetyl and glucose attachment sites. The application of these techniques allowed the authors to highlight new aspects of the interactions between acetylation and glycation that are the results of potential conformational changes at the protein level. This strategy would be valid as a qualitative and quantitative approach to comparing the acetylation and glycation states of other blood compartments (erythrocytes, platelets or leucocytes) and, finally, of diabetic patients.

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Transparency document

The Transparency document associated with this article can be found, in the online version.

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Chapter 5

ASPIRIN-MEDIATED ACETYLATION OF HAEMOGLOBIN INCREASES IN PRESENCE OF HIGH GLUCOSE CONCENTRATION AND DECREASES PROTEIN GLYCATION

In this section we applied the MS-label free approach presented in the previous chapters to analyse the extent of glycation and aspirin-induced acetylation in red blood cell (RBC) proteins, mainly focusing the attention on haemoglobin. Glycated haemoglobin (HbA_{1C}) represents, to date, an important long-term indicator of glycaemic status of individuals, which correlates with their mean blood glucose level, and is currently used for the diagnosis and follow-up of diabetes. However, in certain situations including alteration of RBC life span, the presence of haemoglobin variants (e.g. HbF) or during therapeutics treatments, HbA_{1C} measurements may be unreliable. Moreover, reports showed that glycation of haemoglobin alters its structure and that this effect can be partially reverted by aspirin. A comprehensive qualitative/quantitative description of the preferential RBC proteins, that were shown to be both affected by glycation and acetylation, revealed that high glucose levels favoured the acetylation on several protein sites. This phenomenon was observed on the major RBC proteins, including different haemoglobin isoforms. The analysis of the primary and tertiary structure regions nearby the modified residue highlighted the presence of acidic and basic amino acids that could favour the glycation/acetylation processes. Importantly, we evidenced that glycation of the N-terminal valine of haemoglobin β chain was significantly reduced by aspirin-acetylation. In this sense, beside its beneficial effects, aspirin might be a factor that leads to the underestimation of HbA_{1C} levels, impairing the robustness of the test for glycaemic control.

For this second part of the analysis of glycated and acetylated proteins in blood, I was in charge of the whole experimental section and data analysis and I wrote the entire paper.

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Aspirin-mediated acetylation of haemoglobin increases in presence of high glucose concentration and decreases protein glycation

Francesco Finamore[a], *Feliciano Priego-Capote*[b], *Severine Nolli*[c], *Pierre Fontana*[c], *Jean-Charles Sanchez*[a,*]

[a] *Translational Biomarker Group (TBG), Department of Human Protein Sciences, University Medical Centre, University of Geneva, 1211 Geneva 4, Switzerland*

[b] *Department of Analytical Chemistry, Annex C-3 Building, Campus of Rabanales, University of Còrdoba, Spain*

[c] *Division of Angiology and Haemostasis, University Hospitals of Geneva and Geneva Platelet Group, Faculty of Medicine, Geneva, Switzerland*

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ABSTRACT

Glycation represents the first stage in the development of diabetic complications. Aspirin was shown to prevent sugars reacting with proteins, but the exact mechanism of this interaction was not well defined. We performed a quantitative analysis to calculate the levels of acetylation and glycation of haemoglobin, among others red blood cell (RBC) proteins, using a label free approach. After glucose incubation, increases in the acetylation levels were seen for several haemoglobin subunits, while a parallel decrease of their glycation levels was observed after aspirin incubation. These results suggest that, a mutual influence between these two modifications, occur at protein level.

1. Introduction

As diabetes progresses, the number of disease-specific complications has a tendency to increase. Among other risk factors, this is mostly due to chronic hyperglycaemia which promotes an uncontrolled, non-enzymatic modification of proteins called glycation. This post-translational modification (PTM) occurs between electrophilic glucose and nucleophilic primary amino groups of proteins (typically the N-terminal and ε amines of lysine) to generate a stable covalently bound intermediate, the Amadori compound. The latter represents the central core for the subsequent irreversible reactions of the glycation process [1]. The reaction's kinetics are enhanced by high and prolonged exposure to glucose, which in turn leads to the chronic health problems commonly observed in diabetes, including neuropathy [2], nephropathy [3], retinopathy [4] and cardiovascular diseases [5,6]. These disorders frequently appear several years after the onset of the illness, therefore, timely glycaemic control is of utmost importance in order to minimize the deleterious effects of glucose [7]. Today, blood glucose is typically monitored using the glycated haemoglobin (HbA1c) test considered, the gold standard for disease definition and the monitoring of anti-diabetes treatment [8]. Nevertheless, increasing evidence has shown that glycated haemoglobin may be affected by different inter-individual

* *Corresponding author at*: Translational Biomarker Group, Department of Human Protein Sciences, University of Geneva, Rue Michel Servet 1, 1211 Geneva 4, Switzerland. Tel.: +41 022 3795486; fax: +41 022 3795505.
E-mail address: Jean-Charles.Sanchez@unige.ch (J.-C. Sanchez).

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factors, leading to a "glycation gap" between the HbA1c and the level of mean plasma glucose [9,10]. This discrepancy can result from any one of a range of physiological variables that leads to an underestimate of the HbA1c levels in diabetic patients. These include any disorder that alters the lifespan of erythrocytes (red blood cells, RBCs) such as renal failure, haemolytic anaemia, iron-deficiency anaemia or a blood transfusion. Moreover, haemoglobin variants and adducts, which result from PTMs of haemoglobin (carbamylated Hb and pre-HbA1c), may alter the outcome of the test, thus generating method-specific interferences [11]. It should be noted that haemoglobin is the most abundant protein in RBCs, and the variation of its glycation levels is mostly due to its continuous interaction with circulating glucose, which also, influences other RBC proteins. As a consequence, hyperglycaemia has deleterious effects on the lifespan of RBCs, altering deformability and oxidative stress, which in turn lead to the hypertension and vascular complications [12–16] typical of diabetes.

Over the last few decades, it has been shown that the severity of these disorders can be suppressed, or at least relieved, by aspirin. Aspirin is a cornerstone treatment for the prevention of diabetic complications such as cardiovascular diseases. Although aspirin mediates its antithrombotic effects by inhibiting the cyclooxygenase (COX 1) enzyme, it not only effects platelet function, but also RBCs. Aspirin decreases the platelet reactivity amplified by RBCs through the down-regulation of the P-selectin and integrin IIb/IIIa, which in turn reduces the pro-thrombotic phenotype of RBCs [17,18]. Moreover, the interaction between platelets and RBCs in blood vessels, during thrombus formation, is strongly influenced by any reduced deformability of RBC membranes. This rheological mechanism can be reduced by aspirin, which decreases the rate of RBC aggregation [19,20]. Furthermore, evidence suggests that aspirin induces RBCs' nitric oxide synthase, which is well known to be helpful in regulating of the vascular tone and immune response [21]. The efficiency of all these processes seems to be reduced in RBCs by an acetyl-hydrolase activity that rapidly decreases the half-life of aspirin in blood [22–24]. Despite the significant number of studies that have attempted to understand the separated roles of non-enzymatic glycation and aspirin acetylation on the biological processes of RBCs, the exact mutual and synchronous interaction between these two modifications has so far been poorly investigated.

In this study, we carried out the first analyses of the simultaneous impact of protein glycation and aspirin acetylation on RBC proteins, with a special emphasis on haemoglobin. An experimental mass spectrometry (MS) approach, previously applied to serum albumin [25] and human plasma [26], was used to obtain qualitative information, by detecting acetylation and glycation sites, as well as quantitative reports determining the levels of these modifications.

2. Materials and methods

2.1. Chemicals

EDTA (10.8 mg) – Vacutainer tubes were from BD Vacutainer®. Dulbecco's phosphate-buffered saline (DPBS; 1X, pH 7.4) was from Invitrogen™. NaCl 0.9% was from Bichsel. Mouse anti-human CD235a, Glycophorin A/RPE antibody was from Dako. Aspirin was from Aspegic Inject®. EDTA-free protease inhibitor cocktail (PIC) tablets were from Roche. Anti-human Nε-acetyl-lysine monoclonal antibody was from Cell Signalling Technologies®. ECL™ detection reagent was from GE Healthcare. Protein assay dye reagent concentrate (liquid) was from Bio-Rad. D-glucose [$^{12}C_6$] (lyophilized powder, ≥99.5%), Naphtol Blue Black (lyophilized powder, dye content *ca* 80%), tris-(2-carboxyethyl) phosphine hydrochloride (TCEP; 0.5 M, pH 7.0), iodoacetamide (IAA, crystalline, ≥99%), endoproteinase Glu-C from *Staphylococcus aureus* V8 (lyophilized powder, 500 U), HPLC-grade water (CHROMASOLV®) and HPLC grade acetonitrile (ACN; CHROMASOLV®, ≥99.9%) were purchased from Sigma–Aldrich®. Bovine serum albumin (BSA, lyophilized powder, ≥96%), triethylammonium hydrogen carbonate buffer (TEAB; 1 M, pH 8.5), ammonium acetate (NH_4Ac, solid, 98.0%), magnesium chloride ($MgCl_2$, solid, ≥99.0%), acetic acid (99.5%), and formic acid (FA; 98.0%) were from Fluka.

2.2. RBC preparation and lysis

RBC fraction was obtained by centrifuging whole blood from three blood bank donors. Briefly, EDTA-Vacutainer tubes containing blood were centrifuged at 900 rpm, at 37 °C, to remove the platelet rich plasma fraction. Then, the remaining part was centrifuged at 3500 rpm for 10 min to separate and remove the plasma from the RBCs. Carefully 1 mL of erythrocytes was aspirated from the bottom of the tube and transferred to a Falcon tube. RBCs were washed four times with 0.9% NaCl and centrifuged at 3690 rpm for 10 min to minimize the presence of contaminants. Before the final washing step, 100 μL of cell suspension was used to determine the concentration RBCs using a Sysmex KX21 N haematology analyser (Sysmex Corporation). A sample concentration of 2.51×10^6 cells/μL was diluted down to 10^4 cells/μL and incubated with an anti-human antibody against CD235a (Glycophorin A) for subsequent fluorescence-activated cell sorting (FACS) analysis. The level of cell purity was assessed using an Accuri C6 fluorescence flow cytometer (BD Accuri™). After sedimentation, the supernatant was removed, and a cell pellet (~1 mL) was lysed by adding eight volumes of deionized water. Cell debris was separated by centrifugation at 13,000 rpm for 30 min at 4 °C, and protease inhibitors were added to the RBC extract at a dilution of 1:7 before aliquoting. The three RBC protein extracts were then pooled together.

2.3. RBCs incubation with aspirin and glucose

Four aliquots of 225 μL of RBC protein extract were diluted in phosphate buffer to reach a final volume of 400 μL, and were subsequently incubated with (1) 500 μM aspirin for 30 min at 37 °C; (2) 30 mM glucose for 24 h at 37 °C; and (3) sequentially, with 30 mM glucose at 37 °C for 24 h followed by 500 μM aspirin for 30 min at 37 °C. As negative control, a further aliquot was incubated using the same time periods, but without glucose and aspirin. Additional five conditions were created in which 100 μL of RBC extract were incubated for 24 h with increasing glucose concentrations (0, 10, 30, 50 and 100 mM) followed by 30 min of aspirin exposure at 37 °C. As a positive control,

20 mM aspirin were incubated in the absence of glucose. After incubation, protein amounts were determined using the Bradford assay, and BSA was used to create a standard calibration curve.

2.4. Western blot analysis of aspirin-mediated acetylation

Samples of 5 μg were separated using electrophoresis on a 15%T/2.67%C polyacrylamide gel, and protein transfer took place for 1 h at 350 mA. Membranes were stained with Amido black to highlight the protein bands [27], and, then, were washed with water to minimize background. A western blot test was performed using an anti-human antibody against N ε-acetyl-lysine (1:5000). ECL™ reagents were used to detect the acetylation signal. The intensity of the immunoreaction (detected by ECL) was normalized over the total protein signal of the corresponding band stained with Amido black. Each sample was assessed using three experimental replicates ($N = 3$).

2.5. Protein purification and Glu-C digestion

After incubation, glucose, aspirin and salts were removed from the samples using Amicon Ultra-0.5 mL, Ultracel® 3 K membrane (Millipore™) devices, and purified proteins were then reconstituted in 500 mM TEAB, pH 8.5. The amount of protein was estimated using the Bradford assay, with BSA to perform the calibration curve. Samples were separated into two groups in order to analyze the acetylated and glycated peptides separately for each experimental set. An amount of 200 μg and 1 mg of protein was dissolved in 400 μL of TEAB for the acetylation and glycation study, respectively. Then, cysteine residues were reduced with 5 mM TCEP (4 μL) by incubation of the reaction mixture for 1 h at 60 °C. Free sulfhydryl groups were alkylated with 20 mM IAA (10 μL) for 30 min at room temperature in dark conditions. Freshly prepared endoproteinase Glu-C (1 mg/mL) was added in a ratio of 1:10 (w/w), and digestion took place for 16 h at 37 °C. For acetylation analysis, digestion mixtures were lyophilized under speed vacuum and reconstituted in 5% ACN/0.1% FA for the following desalting step. For the glycation analysis, peptide digests were evaporated and reconstituted in mobile phase A (200 mM NH_4Ac, 50 mM $MgCl_2$, pH 8.1) to obtain an estimated concentration of 16 mg/mL, for the enrichment of glycated peptides.

2.6. Enrichment of glycated peptides using boronate affinity chromatography

Reconstituted peptides were fractionated by boronate affinity chromatography by the interaction between the hydroxyl-groups of glycated peptides, present at low concentrations, and boronic acid, as previously described [28,29]. For this purpose, samples (50 μL) were injected in a Waters 600E HPLC system equipped with a TSK-Gel boronate affinity column from Tosoh Bioscience (7.5 cm length × 7.5 mm inner diameter, i.d., 10 μm particle size). An isocratic chromatographic method based on three steps was used to separate non-glycated from glycated fractions. Step 1 was a 0–10 min, 100% mobile phase A (200 mM ammonium acetate, 50 mM magnesium chloride, pH 8.1) for the retention, under alkaline conditions, of glycated peptides by esterification of the 1,2 cis-diol group of glucose moieties and the hydroxyl groups of boronate ligands with elution of non-glycated peptides. In step 2, 10–20 min, glycated peptides are eluted with 100% mobile phase B (100 mM acetic acid) under acidic conditions; and step 3, 20–30 min with 100% mobile phase A for the equilibration of the column to the initial conditions. The glycated fraction was collected for subsequent evaporation and reconstitution in 5% ACN/0.1% FA. Then, peptides were desalted and pre-concentrated prior to LC–MS/MS analysis. This was carried out with C_{18} MacroSpin Column™ (Harvard Apparatus), and peptides were eluted in 400 μL of 50% ACN/0.1% FA and then evaporated to dryness before reconstitution in 5% ACN/0.1% FA.

2.7. LC–MS/MS detection of acetylated and glycated peptides

Peptide digests were analyzed by electrospray ionization in positive ion mode (1.9 kV ionization voltage), on an Orbitrap hybrid linear ion trap (Thermo Fisher). Nanoflow was performed using a Waters NanoAquity HPLC system consisting of a pre-column (100 μm inner diameter and 18 mm in length) packed with C_{18} resin, where peptides were initially trapped at a flow rate of 3 μL/min in water/ACN (95/5 v/v) with 0.1% FA. Peptide separation was then performed using an ACN gradient developed in an analytical column packed with C_{18} resin, at the flow rate of 200 nL/min with mobile phase A (water, 0.1% FA) and B (ACN, 0.1% FA). A data-dependent tandem mass spectrometry method was used for analysis of acetylated and glycated proteins with a precursor-ion scan range of 400–2000 m/z and a resolving power of 60,000. Fragmentation of the five most abundant ions, detected from the MS1 survey scan, was performed in the octopole collision cell at the rear of the C-trap (normalized collision energy, 35%), followed by Orbitrap detection with a resolving power of 7500 and a dynamic exclusion of 120 s, to minimize repeated analysis of the same precursor ion. The fragment ion isolation window was set to 2.5 m/z units. Precursor ions of charge state 1+ were excluded for the data-dependent acquisition. All analyses were carried out using three replicate injections.

2.8. Data analysis

After data-dependent acquisition, peak lists were generated from the raw data using EasyProtConv v1.5 software, and the resulting data files were searched for matches against the UniProtKB/Swiss-Prot database (Release June 13, 2012, 659,907 entries) using the EasyProt v2.3 (build 751) tool [30]. Because this study analyzed human RBCs, we specified *Homo sapiens* taxonomy for database searching. The acquired high resolution data allowed the scoring model to use an accuracy of 6 ppm for fragment ions and 10 ppm for their precursor ions. Common amino acid modifications detected were carbamidomethylation of cysteine residues (57.0215 Da) and oxidized methionine (15.9949 Da), which were set as fixed and variable modifications, respectively. For the analysis of aspirin and glucose modifications, acetylation of lysine residues or N-terminal positions (42.0100 Da), was selected as variable modification and glycation of lysine and arginine residues

or N-terminal positions (162.0523 Da) was selected as variable modification, as well. The number of modifications per peptide was set to a maximum of 2 in order to reduce the search space of all possible combinations of variable modifications. Endoproteinase Glu-C was selected as the cleavage enzyme, with three potential missed cleavages allowed. The minimum peptide length was 8 amino acid residues, with a minimum peptide z-score of 4. Three analytical injections per sample were each analyzed independently, providing replicate data values. The peak list files obtained for each technical replicate were merged and used to search the database. For the significance of peptide identification, we used a false-discovery rate of 1%. Calculation of the peptide false discovery rate (FDR) is automatically performed by EasyProt v2.3 as $\%FDR = M_R/M_F \times 100$, where M_R and M_F are the number of PSMs (Peptide-Spectrum Matches) from the reverse and the forward database searches respectively.

2.9. *Peptide quantification*

Quantitation of acetylated and glycated peptides from RBC samples was carried out using Nonlinear Dynamics' Progenesis software. Briefly, after the accurate alignment of *m*/*z* and Rt (retention time) features for every chromatographic run in each testing group, a global peak list file, containing all the features detected, was created and used to query the database, and from the identification results, a pepXML file was exported to Progenesis. Each identified peptide was then associated to its relative feature and its raw abundance was normalized multiplying it by a global scaling factor. This scaling factor is defined by the anti-log of the average of the log ratios between the run being normalized and the reference run.

3. Results

3.1. *Identification of acetylated and glycated proteins in RBCs*

This study aimed to evaluate the mutual interaction between protein glycation and aspirin-induced acetylation on RBC cytosolic proteins. After isolating RBCs from whole blood, the purity of the cell population was assessed using FACS through cell-line specific antibodies conjugated with fluorochromes at different emission wavelengths. This analysis revealed that a highly homogeneous RBC distribution had been obtained, with a very low degree of platelet and leucocyte contamination, as shown in Supplemental Fig. 1A and B. Next, after lysis of RBCs and protein extraction through centrifugation, 30 mM glucose incubation was selected to mimic a gluco-toxicity level; 500 μM aspirin concentration was used as this is within the usual therapeutic blood ranges. Aspirin's half-life *in vivo* is approximately 20 min; in order to be more confident with its kinetics in this analysis, an incubation period of 30 min was chosen [31].

A total number of 96 proteins, including acetylated, glycated and unmodified ones, were identified in this study in the cytosolic fraction of RBCs. The name of the proteins with associated their accession number, the sequence coverage and the number of detected peptides with which protein has been identified, are listed in Supplemental Table 1. The MS acetylation pattern of RBCs was characterized using the four test conditions. Table 1 shows the list of acetylated proteins that were identified with information about the peptides detected for each protein with the acetyl attachment sites. A more detailed list of acetylated proteins, their respective acetylated peptides and modification sites, with information about the experimental masses, the *m*/*z* and Rt values and the scan name is furnished in Supplemental Table 2. Among them, haemoglobin subunits α, β and δ show the most of assigned acetylation sites. Other RBC proteins, including peroxiredoxin 2, superoxide dismutase and carbonic anhydrase 1, were found to be significantly acetylated by aspirin. The percentage of acetylated proteins reached in this study is 15.62%. The MS glycation profile of RBC proteins was obtained after their enrichment with BAC. The proportion of non-glycated peptides identified in the retained fraction was below 35% across all the test conditions. As shown in Table 2, most of the glycated sites identified belong to haemoglobin α, β and δ subunits. Other proteins were also distinguishable in the presence of high glucose concentrations such as peroxiredoxin 1 and 2 and carbonic anhydrase 1. A more detailed list of glycated proteins with their glycated peptides and the annotation of their modification sites are provided in Supplemental Table 3, with information about the *m*/*z* and Rt value for each modified peptide and the respective scan name. The percentage of glycation before and after enrichment was estimated at 1.72% and 21.87%, respectively.

To discover which proteins underwent both acetylation and glycation modifications, the data sets were compared. A total of six specific RBC proteins were found to be target of both modifications (α, β and δ subunits of haemoglobin, peroxiredoxin 2, superoxide dismutase and carbonic anhydrase 1. The six proteins had 10 sites which were found to be both acetylated and glycated. Five sites belonged to haemoglobin β in the N-terminal Val and in Lys residues located at positions 9, 18, 59 and 96. All these sites had previously been found to be the targets of glycation [32] and aspirin-induced acetylation [33,34]. The other five acetylated/glycated sites were identified as residues Lys 11 and Lys 16 of haemoglobin subunit α, Lys 95 of haemoglobin subunit δ, Lys 183 of peroxiredoxin 2 and Lys 45 of carbonic anhydrase 1.

To assess the presence of a specific amino acid motif in proximity of these 10 sites, we carried out a motif analysis using the primary structure of the corresponding peptide sequences. The sequences were loaded on WebLogo software [35], specifying 20 amino acids prior to and after the modification site. The frequency of occurrence of each amino acid at a given position is graphically represented by its size. Looking at the logo (Fig. 1A), acidic side chain residues (aspartic acid and glutamic acid) and basic side chain residues (lysine) were identified to be highly representative in close proximity of the modification site. In addition, hydrophobic short chain amino acids like alanine, valine and leucine, were observed to occur most frequently in acetylated/glycated peptides identified in this study. Furthermore, the study of sequence motifs was integrated with a tertiary (3D) structure analysis in order to figure out the importance of specific amino acids located in the neighbouring environment of the potential reactive site. Using PyMOL v1.5 program, we observed that several reactive

Table 1 – Acetylated proteins with information about the detected peptides and the position of the acetylation sites identified in RBCs, after aspirin incubation, after glucose incubation and after glucose and aspirin incubation.

Protein	Peptide	Position	RBCs	RBCs + Aspirin	RBCs + Glucose	RBCs + Glucose + Aspirin
Haemoglobin subunit beta	VHLTPEEKSAVTALWGKVNVDE	1–22	V1, K17	V1, K8	V1	V1, K17
	VHLTPEEKSAVTALWGKVNVDEVGGE	1–26	x	V1, K17	x	K8, K17
	VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFE	1–43	K8, K17	K8, K17	K8, K17	K8, K17
	SFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSE	44–90	x	K59, K61	K59	K59, K61
	LHCDKLHVDPE	92–102	x	K96	x	K96
Haemoglobin subunit delta	NFRLLGNVLVCVLARNFGKEFTPQMQAAYQKVVAGVANALAHKYH	102–146	K120	K120	K120	K120
	LHCDKLHVDPE	91–101	x	K95	x	K95
Peroxiredoxin-2	HGEVCPAGWKPGSDTIKPNVDDSKE	167–191	x	K183, K190	x	K183, K190
	VCPAGWKPGSDTIKPNVDDSKE	170–191	x	K183	x	K183, K190
Haemoglobin subunit alpha	VLSPADKTNVKAAWGKVGAHAGE	1–23	x	K11	x	K11, K16
	VLSPADKTNVKAAWGKVGAHAGEYGAEALE	1–30	x	K16	x	K11, K16
Carbonic anhydrase 1	TKHDTSLKPISVSYNPATAKE	38–58	x	K45	x	K45
Superoxide dismutase [Cu-Zn]	ATKAVCVLKGDGPVQGIINFE	1–21	A1	A1	A1	A1
	ATKAVCVLKGDGPVQGIINFEQKE	1–24	A1	A1	A1	A1
Catalase	ADSRDPASDQMQHWKE	1–16	A1	A1	A1	A1
Glutathione S-transferase omega-1	SGESARSLGKGSAPPGPVPE	2–21	S2	S2	S2	S2
Low molecular weight phosphotyrosine protein phosphatase	AEQATKSVLFVCLGNICRSPIAE	2–24	A2	A2	A2	A2
RNA-binding protein 12	KIDMIRKRLQNFSYDQREMILNPE	513–536	K519	x	x	x
Flavin reductase (NADPH)	AVKKIAIFGATGQTGLTTLAQAVQAGYE	1–28	x	K3	K3	A1, K3
SH3 domain-binding glutamic acid-rich-like protein 3 (SH3BP-1)	SGLRVYSTSVTGSREIKSQQSE	2–23	x	x	S2	S2
AT-rich interactive domain-containing protein 3A	GPGEEHFEDMASDEDMKPKWEEEE	108–131	x	x	x	K124
Alpha-haemoglobin-stabilizing protein	ALLKANKDLISAGLKE	2–17	x	A2	x	x
Peptidyl-prolyl cis-trans isomerase A, N-terminally processed	AMERFGSRNGKTSKKITIADCGQLE	140–164	x	R143	x	R143

Table 2 – Glycated proteins with information about the detected peptides and the position of the glycation sites identified in RBCs, after aspirin incubation, after glucose incubation and after glucose and aspirin incubation.

Protein	Peptide	Position	RBCs	RBCs + Aspirin	RBCs + Glucose	RBCs + Glucose + Aspirin
	VHLTPEEKSAVTALWGKVNVDE	1–22	V1, K8	K8	V1, K8, K17	V1, K8, K17
	VHLTPEEKSAVTALWGKVNVDEVGGE	1–26	K8	K8	K8, K17	K8, K17
	KSAVTALWGKVNVDE	9–23	x	x	x	K9
Haemoglobin subunit beta	VGGEALGRLLVVYPWTQRFFE	24–44	x	R31	R31, R41	R31, R41
	SFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSE	45–91	x	x	K59, K65	x
	LHCDKLHVDPE	92–102	x	x	K96	K96
	FTPPVQAAYQKVVAGVANALAHKYH	123–147	x	x	K133	K133
	LHCDKLHVDPE	91–101	x	x	K95	K95
Haemoglobin subunit delta	VHLTPEEKTAVNALWGKVNVDAVGGE	1–26	x	x	x	K8
	VHLTPEEKTAVNALWGKVNVDAVGGEALGRLLVVYPWTQRFFE	1–43	x	x	K8, K17, R30	K8
	GGLGPLNIPLLADVTRRLSEDYGVLKTDE	93–121	x	x	K118	x
Peroxiredoxin-2	HGEVCPAGWKPGSDTIKPNVDDSKE	167–191	x	x	K176, K183	K176, K183
	VCPAGWKPGSDTIKPNVDDSKE	170–191	x	x	K176	K176
	VLSPADKTNVKAAWGKVGAHAGE	1–23	K7, K11	K7	K7, K11	K7, K11
Haemoglobin subunit alpha	VLSPADKTNVKAAWGKVGAHAGEYGAE	1–27	K7, K11	K7	K11	K11
	VLSPADKTNVKAAWGKVGAHAGEYGAEALE	1–30	x	x	K11, K16	x
Carbonic anhydrase 1	TKHDTSLKPISVSYNPATAKE	38–58	K57	K57	K45, K57	K39, K45, K57
Superoxide dismutase [Cu-Zn]	ATKAVCVLKGDGPVQGIINFE	1–21	x	x	K9	K9
	ATKAVCVLKGDGPVQGIINFEQKE	1–24	x	x	K9	K9
	CFLQHKDDNPNLPRLVRPE	101–119	K106	K106	x	x
	KCCAAADPHECYAKVFDE	359–376	K372	K372	x	x
Serum albumin	LRDEGKASSAKQRLKCASLQKFGE	185–208	K195	K195, R197	x	R197
	RAFKAWAVARLSQRFPKAE	209–227	R218	R218	x	x
	VSKLVTDLTKVHTE	231–244	K233	K233	K233	K233
Peroxiredoxin-1	VCPAGWKPGSDTIKPDVQKSKE	171–192	x	x	K177, K184	K177
Putative IQ and AAA domain-containing protein 1-like	GPDMKEKMKE	289–298	K293, K297	x	x	x
Obscurin-like protein 1	LSREDAPVRWYKDGLE	1013–1028	R1015	x	x	x
WW domain-binding protein 4	KASKEFAAMEAAALKAYQE	60–78	x	K63	x	x
TATA element modulatory factor	KTRSIMAEELVKLTNQNDELEE	1012–1033	x	R1014, K1023	x	x
Protein kinase C and casein kinase substrate in neurons protein 1	KRLVFLKE	240–247	x	R241	x	K246
Nck-associated protein 5	TVNIMVYQEKLSEEERKHKEALE	111–133	x	x	K120, R126	x
Kinesin-like protein KIF20A	TLHVAKFSAIASQLVHAPPMQLGFPSLHSFIKEHSLQVSPSLE	493–535	x	x	K498	x
R3H domain-containing protein 2	KSTKDVSEKEDKDKNKE	115–131	x	x	K118, K128	x
Protein S100-Z	PTQLEMAMDTMIRIFHRYSGKE	1–22	x	x	R13, R17	x
Cyclin-dependent kinase 11B	KRKEKRKHARVKE	70–82	x	x	R71	x
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2	TKTPNLRMSE	16–25	x	x	R22	x
Golgin subfamily B member 1	QAAQQVVREKDARFETQVRLHE	213–234	x	x	x	K222
Ninein (hNinein) (GSK3B-interacting protein)	RIAALKNE	429–436	x	R429	R429	R429

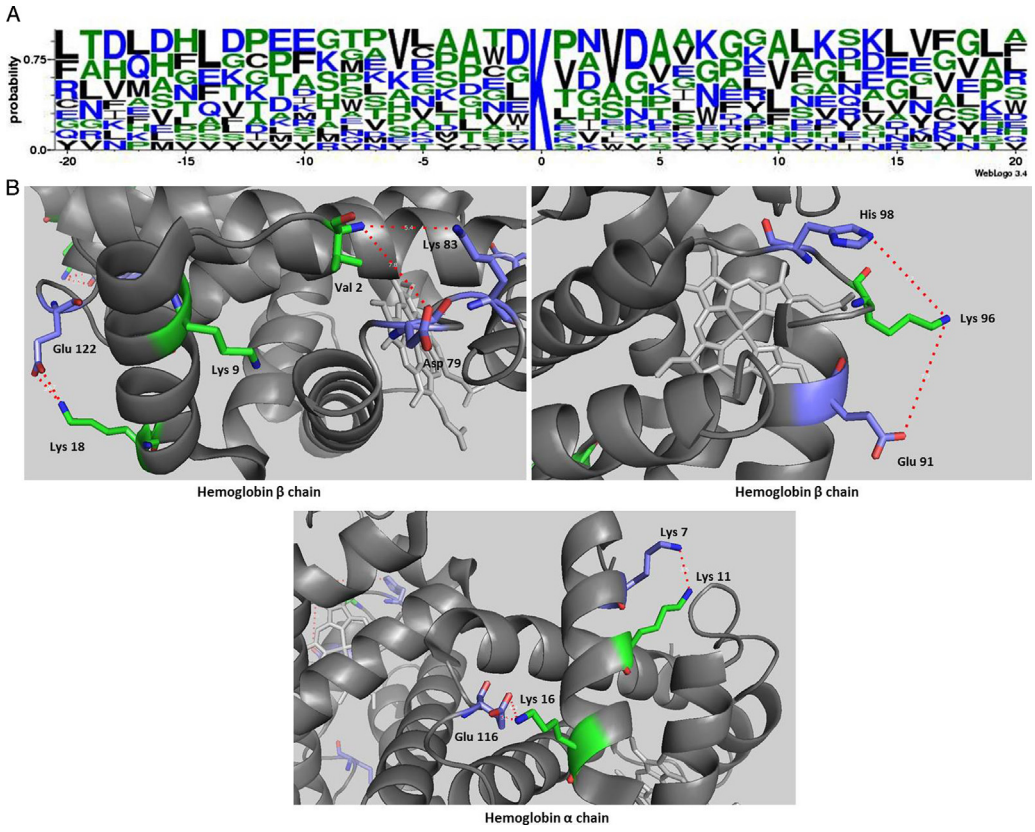

Fig. 1 – Motif logo analysis of acetylated and glycated peptides detected in RBCs after glucose and aspirin incubation. The X-axis represents the neighbouring residues (−20 to +20) around the modified lysine (0 position) in the primary sequence structure; the Y-axis represents the frequency of occurrence of each amino acid present at each location (A). Protein tertiary structure analysis shows the spatial proximity around the modification sites of haemoglobin β and α chains that are detected as both acetylated and glycated. Red dashed lines represent the distances between the modified residues (green) and the neighbouring amino acids (blue) (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sites found in this study to be both acetylated and glycated in haemoglobin α and β chain, were located in the spatial proximity of acidic and basic amino acids (Fig. 1B), confirming, once again, the relevance of this class of residues for lysine modification by aspirin and glucose. These results are in line with those obtained by Zhang et al. [36] on RBCs from diabetic patients, highlighting the specificity of site modification.

3.2. *Assessment of the levels of acetylation and glycation of carbonic anhydrase 1*

The label free approach described in the Experimental Section was used to quantify the extent of aspirin-acetylation and non-enzymatic glycation on RBC proteins. Supplemental Tables 4 and 5 show the lists of acetylated and glycated proteins quantified, with information of the peptides detected for each protein, the site of modification and the relative abundance across all test conditions, respectively.

Most of the proteins quantified are specific to RBCs, such as haemoglobin subunit β (4 acetylated peptides, 6 glycated peptides), haemoglobin subunit α (2 acetylated peptides, 3 glycated peptides), haemoglobin subunit δ (2 acetylated peptides, 2 glycated peptide), peroxiredoxin 2 (2 acetylated peptides, 3 glycated peptides) and carbonic anhydrase 1(1 acetylated peptide, 1 glycated peptide), to cite but a few. Quantification measurements from acetylated and glycated RBC proteins were initially used in a principal component analysis to highlight the main differences between the four groups and to represent the inter- and intra-group variability across the replicates. These results showed that the four sample groups were separate (with a slight degree of overlap between them) for both the acetylation (Supplemental Fig. 2A) and glycation data set (Supplemental Fig. 2B), thus providing a first general proof of the mutual effects of aspirin and glucose on RBC proteins.

This behaviour strongly reflects the response of aspirin acetylation and glycation at peptide/site level, as shown for peptide TKHDTSLKPISVSYNPATAKE from carbonic anhydrase 1 (Fig. 2). In particular, a significant increase in its aspirin-mediated acetylation level is evident with prior glucose incubation (Fig. 2A), whereas, a significant decrease of protein glycation is observed in the presence of glucose after aspirin exposure, in comparison to glucose incubation alone (Fig. 2B). The fragment spectra of this peptide (the most representative peptide identified in this study) confirm the presence of acetylation (Fig. 2C) and glycation (Fig. 2D) on

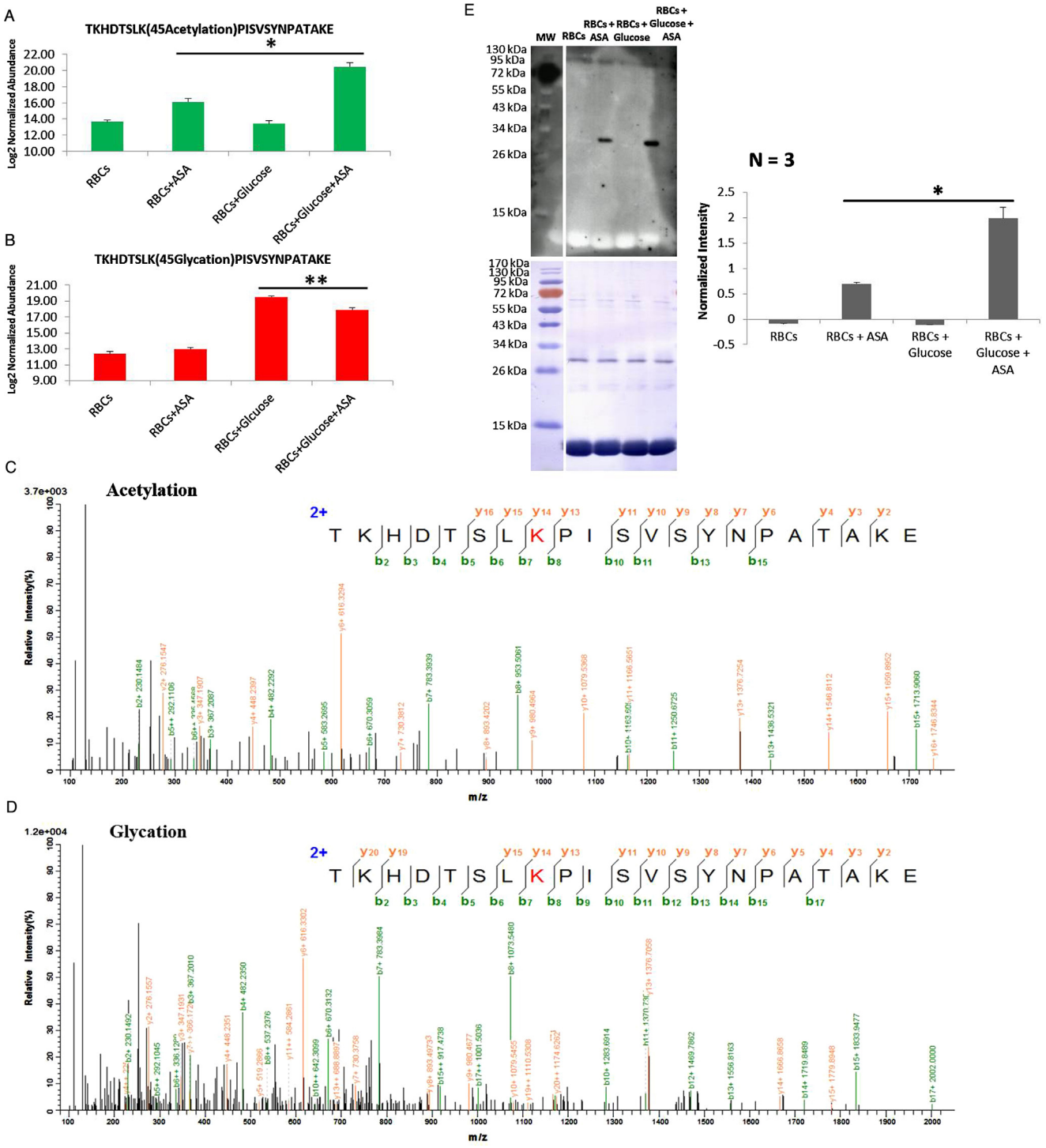

Fig. 2 – Quantification of the peak abundances of carbonic anhydrase peptide TKHDTSLKPISVSYNPATAKE in its acetylated (green bars) (A) and glycated (red bars) state (B). Annotated spectra of the same peptide in its acetylated (top spectrum) (C) and glycated (bottom spectrum) states (D). Acetylation level measured by western blot shows a significant increase after 30 mM glucose incubation followed by 500 μM aspirin, compared to aspirin incubation alone, for the 28 kDa protein band detected (carbonic anhydrase 1) (E). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

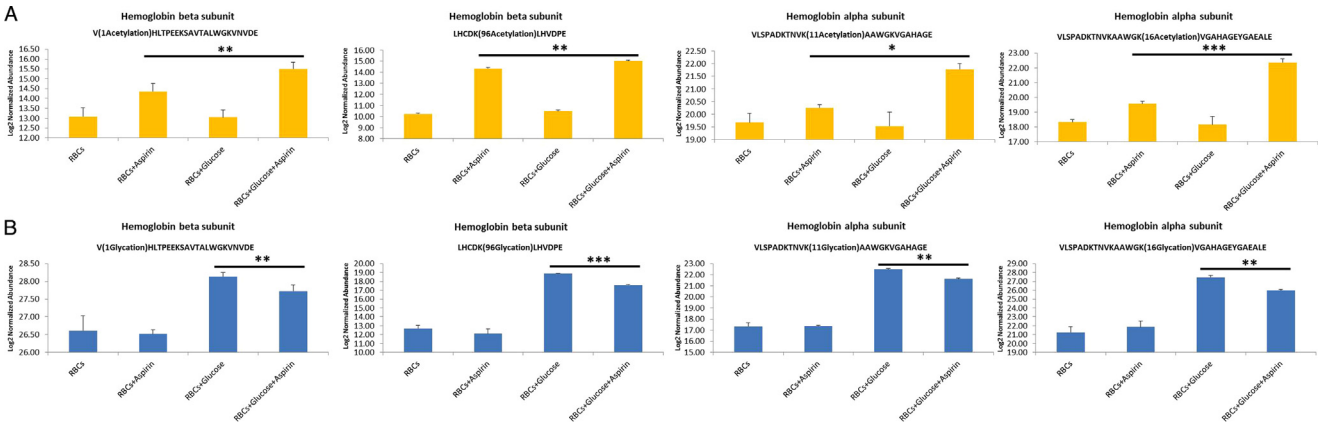

Fig. 3 – Quantification of the peak abundances of 4 acetylated (orange bars) and glycated (blue bars) sites from four representative peptides belonging to haemoglobin β and α subunit, in all the test conditions (native, 500 μM aspirin, 30 mM glucose, 30 mM glucose + 500 μM aspirin). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

lysine 45. These results were clearly confirmed by western blot tests using an anti-acetylation antibody and the same sample conditions ($N = 3$). Indeed, a significant difference in the acetylation reactions for the protein band at 28 kDa, was seen when comparing the different lanes (Fig. 2E). The acetylation signal in RBCs incubated with glucose followed by aspirin (Fig. 2E, lane 4) was significantly higher than the signal after aspirin incubation alone (Fig. 2E, lane 2), and no signal was detected in either native RBCs or after glucose incubation alone (Fig. 2E, lanes 1 and 3). Glu-C in-blot digestion of the band slice followed by LC–MS/MS identification, allows the unambiguous assignment of the carbonic anhydrase 1 (data not shown).

Interestingly, when comparing RBC protein extracts incubated with increasing glucose concentrations followed by 500 μM aspirin exposition, a significant increase in the acetylation levels was evident at 30 mM glucose, in comparison to 10 mM or no glucose (Supplemental Fig. 3, lanes 1–3), but no further significant gain was observed at 50 and 100 mM glucose (Supplemental Fig. 3, lanes 4–5).

3.3. *Quantitation of acetylation and glycation sites on Haemoglobin*

The same trend in the variation of the acetylation and glycation levels that was shown for carbonic anhydrase 1, was also observed for other RBC proteins, including the haemoglobin α and β subunits. The quantitation of the seven Hb sites found to be common targets of both PTMs, revealed a significant variation after the sequential incubation of glucose and aspirin. Fig. 3A shows the level of acetylation of two peptides, from haemoglobin β and α subunits, respectively. Again, a significant increase in aspirin acetylation, after glucose incubation, was evident for Val 1 and Lys 96 from the β subunit and for Lys 11 and Lys 16 from the α subunit, compared to aspirin incubation alone. Furthermore, a parallel decrease of glycation levels was observed after 30 min aspirin exposure in the presence of glucose, compared to glucose incubation alone for the four representative sites (Fig. 3B). Of these, the Val residue at the N-terminal position of the haemoglobin β chain is of interest because it is the target site for measuring the %HbA1c. Supplemental Fig. 4A shows the variation of the peak area of the haemoglobin β subunit N-terminal peptide VHLTPEEKSAVTALWGKVNVDE containing acetylated valine, across all test conditions. The abundance of the acetylated peptide shows a 1.4-fold increase after glucose and aspirin incubation compared to aspirin incubation alone. The corresponding peptide fragmentation spectrum confirms the presence of acetylation on the N-terminal valine residue, as shown in Supplemental Fig. 4B.

In contrast, Supplemental Fig. 5A represents the differences in the peak abundances of the same peptide with the glycated N-terminal valine; its levels show a 1.5-fold decrease when aspirin is incubated in the presence of glucose compared to glucose incubation alone. MS/MS analysis allowed the precise assignment of the glycated residue on the peptide sequence (Supplemental Fig. 5B). Similar behaviour was also observed for the other haemoglobin sites, reinforcing the assumption that high glucose concentrations might favour aspirin acetylation, which in turn protects proteins from further glycation.

4. Discussion

Human blood is usually the first microenvironment in which drugs and metabolites interact with the body's cellular components and for this reason it is the most commonly used bio-fluid for biomarker measurements. HbA1c is a typical example, representing a cornerstone biomarker in the diagnosis and monitoring of diabetes mellitus. Haemoglobin is the most abundant protein in RBCs (~97%), and the level of its glycated state (%HbA1c) should generally correlate with the status of blood glucose for long-term glycaemic control [37]. However, divergences between HbA1c levels and clinical measures of glycaemia (*e.g.*, fructosamine assay), have led to the theory that this "glycation gap" leads to a slight but critical misinterpretation of the real glycaemic state [10,38]. To date, the mechanisms underlying such discordance seem

attributable to a great variety of factors, such as genetic predisposition, age or race and the administration of certain drugs [39–41]. Furthermore, haemoglobin variants, high levels of haemoglobin F and derivatives represent the most common analytical interferences influencing the interpretation of HbA1c results [11,42].

It has previously been shown how aspirin affects the biological functions of RBCs, decreasing their tendency to aggregate and to stimulate the recruitment of platelets and leucocytes [17]. However, the molecular process through which the effects of aspirin and glucose influence each other in RBCs at the protein level, was little known. Using fluorescence and circular dichroism experiments, Bakhti et al. showed that glycation induces conformational changes in the secondary structure of haemoglobin, which are in turn prevented by aspirin [43]. We recently examined the impact of aspirin on human plasma in the presence of a high glucose concentration, theorizing that the observed interplay between these two PTMs may be the result of conformational changes in proteins [26]. The present study used a combination of high resolution tandem mass spectrometry coupled with label-free quantitation in order to measure the extent of acetylation and glycation on RBC proteins after incubation with a high glucose concentration (30 mM) followed by exposure to a therapeutic dose of aspirin (500 μM). The high accuracy offered by the HCD-MS/MS mode for fragment ions, coupled with Orbitrap detection [44], allowed us to identify acetylated and glycated peptides in each test condition. Six specific RBC proteins were shown to be preferentially acetylated and glycated, and on them, 10 sites were characterized as the targets for both PTMs. Most of these are associated to haemoglobin chains, probably due to the high level of this protein in RBC cytosol. By using the WebLogo interface [35], a motif analysis of the primary structures in proximity of these 10 residues revealed a high number of acidic (Asp and Glu) and basic amino acids (Lys), that may favour lysine modification, as previously suggested [33,45,46]. However, information on the sequence primary structure does not reflect the spatial environment of the potential reactive sites, and therefore an evaluation of which amino acids were close to the reactive lysine was carried out on the 3D structure of proteins, using PyMOL v1.5 software. In this way, it was possible to determine that, despite the fact that all the sites were located on the surface of the protein, most of them were also in close proximity to an acidic or basic amino acid (from 2.7 to 7.7 Å) in the tertiary structure of the protein. This suggested the importance of these classes of residues in the promotion of the modifications caused by aspirin and glucose.

Quantitative data demonstrated a significant increase of the degree of acetylation when aspirin was incubated in the presence of glucose, compared to aspirin incubation alone, for the majority of the acetylated sites detected. Among them, the Val 2 residue of the haemoglobin β subunit is of importance because of its role, in its glycated state, in monitoring the HbA1c level for glycaemic control, as previously mentioned.

These observations were also confirmed using a western blot test in which the state of acetylation of carbonic anhydrase increased significantly when glucose and aspirin are incubated sequentially. Moreover, the increase in the degree of acetylation seemed to be dependent on the level of glucose, with a critical threshold at 30 mM. This could support the interpretation by which protein glycation favours acetylation in a process governed by a variation of intramolecular chemo-selectivity, potentially due to conformational changes that alter a dynamic equilibrium between non-reactive and reactive sites towards both PTMs. A similar trend was previously described for different plasma proteins showing the same effect of glucose on aspirin-acetylation [26]. Plasma and RBCs are the most representative components of whole blood, 58% and 42% respectively (with a very low percentage of leukocytes and platelets) and, consequently, their proteins interact more likely with glucose compared to the other two blood compartments. In line with our findings, conformational changes exerted by glycation could in turn make those proteins more prone to be influenced by aspirin. This could explain the match observed in the acetylation response to glucose for both plasma and RBCs data set.

With regard to glycation, a significant decrease in its level was observed after 30 min of exposure to aspirin, compared to glucose incubation alone, for most of the glycated sites. This again highlights the role exerted by aspirin in reducing further glycation. One noteworthy aspect of these results derives from the influence that aspirin exerts on the Val 2 residue of haemoglobin β chain. Indeed, the glycated form of this N-terminal amino acid is commonly used in clinical measurements of HbA1c levels, which correlate with mean blood glucose levels for the diagnosis and follow-up of diabetic complications and treatment. However, the HbA1c test can be biased by the presence of haemoglobin variants (S, C, D, E and F) which can affect the measurement and make it unreliable. Although these variants do not cause haemolytic disease in their heterozygous form, and although their glycation level is the same as that for HbA1c, they can interfere with measurements of HbA1c depending on which test method is used [47]. The presence of different point mutations (HbE, HbD) or the replacement of β chains with γ or δ chains (β-thalassemia), alters the physicochemical properties of haemoglobin (pI and the extent of glycation). This favours the detection and quantitation of haemoglobin variants using methods based on ion exchange chromatography or capillary electrophoresis but not the use of methods based on boronate affinity chromatography and immunoassay [42]. Besides the impact of haemoglobin variants, the present study showed that aspirin reduces the glycation level of Val 2 by up to 30%; thus, *in vitro* diagnostic testing may routinely underestimate %HbA1c. This is relevant and important in the diagnosis and management of diabetes mellitus: aspirin is the most widely used drug to reduce the risk of cardiovascular disease in diabetic patients, but might in fact be a variable that leads to differences between observed and predicted HbA1c levels, thus impairing the robustness of this test relative to glycaemic control. This raises the possibility that alternative tools and new potential biomarkers of glycation would be better placed to assess the diagnosis and performance of diabetic treatments in patients treated with aspirin.

Transparency document

The Transparency document associated with this article can be found in the online version.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.euprot.2015.04.003.

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Chapter 6

POORLY CONTROLLED DIABETES MELLITUS IS ASSOCIATED WITH DECREASED ASPIRIN-MEDIATED ACETYLATION OF PLATELET CYCLOOXYGENASE-1 (COX-1) AT SERINE 529

This chapter can be considered as a translational study in which we investigated the molecular and biological impacts of *in vivo* hyperglycaemia on *in vitro* aspirin-mediated acetylation of platelets from diabetic patients. Aspirin is a cornerstone in the prevention of ischemic events in high risk cardiovascular patients. Its main anti-thrombotic effect relies on the irreversible acetylation of platelet cyclooxygenase 1 (COX-1) at the serine 529 residue. However, evidences have suggested that platelets from diabetic patients are hyper-reactive and less sensitive to the anti-platelet action of aspirin. Moreover, the clinical benefit of aspirin for the prevention of cardiovascular events in diabetic patients has been challenged. Glycation of platelet proteins might be involved in the less-than expected platelet response to aspirin, by interfering with their acetylation level. Nevertheless, to the best of our knowledge, the effect of high glucose on the acetylation state of the COX-1 serine 529 site has never been evidenced. We addressed this issue by using two MS approaches aimed to the evaluation of the impact that diabetes has on aspirin acetylation of platelet proteome and more specifically on COX-1. We identified and quantified more than 1000 acetylated platelet proteins and more than 500 sites were shown to be differentially acetylated ($-1.5 \geq$ fold change $\geq +1.5$) between diabetic patients and control subjects. Seven COX-1 sites were confidently quantified, and among them the acetylation level of serine 529 was shown to be drastically reduced in diabetes compared to controls. Moreover, a decreased inhibition of platelet COX-1 activity by aspirin was also observed with increasing glucose levels. Taking together, these results demonstrated that glucose interferes with the acetylation of COX-1 serine 529 by aspirin, which in turn leads to a decreased inhibition of the enzyme activity and thus contributing to the less-than-expected response of platelets to aspirin, often observed in diabetes. A potential model of this process based on molecular docking simulations was also proposed.

I contributed in performing all the experimental part, the development of the targeted MS method (PRM), data analysis and I wrote the manuscript.

Poorly controlled diabetes mellitus is associated with decreased aspirin-mediated acetylation of platelet cyclooxygenase-1 (COX-1) at serine 529.

Francesco Finamore[1], Jean-Luc Reny[2,3], Sarah Malacarne[4], Pierre Fontana[1,3,5], Jean-Charles Sanchez[1].

[1] Department of Human Protein Sciences, Faculty of Medicine, University of Geneva, Geneva, Switzerland.
[2] Division of Internal Medicine and Rehabilitation, University Hospitals of Geneva, Geneva, Switzerland.
3 Geneva Platelet Group, Faculty of Medicine, University of Geneva, Geneva, Switzerland.
[4] Endocrinology, diabetology and nutrition Unit, Geneva University Hospital, Geneva, Switzerland.
[5] Division of Angiology and Haemostasis, University Hospitals of Geneva, Faculty of Medicine, Geneva, Switzerland.

Corresponding author:
Prof. Jean-Charles Sanchez
Translational Biomarker Group
Department of Human Protein Sciences
University of Geneva
Rue Michel Servet 1
1211 Geneva 4, Switzerland
E-mail: Jean-Charles.Sanchez@unige.ch
TEL: +41 (0) 22 3795486
FAX: +41 (0) 22 3795505

Key Points

- Diabetic patients have a decreased aspirin-induced acetylation level of serine 529 of COX-1 compared to non-diabetic controls.
- The glucose-induced impairment of the acetylation process of serine 529 is translated into decreased inhibition of COX-1 by aspirin.

Abstract

Diabetes is a major risk factor of cardiovascular disease. Although aspirin is considered as a cornerstone in the prevention and treatment of the atherothrombosis-related ischemic events, this antiplatelet drug seems to be less effective in diabetic patients. Glycation of platelet proteins has been suggested to play a pivotal role in aspirin poor responsiveness but a direct effect on the critical residue (serine 529, Ser529) of the catalytic pocket of cyclooxygenase 1 (COX-1) has never been demonstrated. The aim of this study is to elucidate the impact of glycation on aspirin acetylation of the platelet proteome and more specifically on COX-1 by investigating control (HbA1c < 6%) and diabetic (HbA1c ≥ 8%) subjects. Using two mass spectrometry approaches, we found a total of 1937 acetylated proteins, from which 1088 were quantified. We evidenced 565 sites with a differential acetylation level between control and diabetics subjects. Glycation did not impact the overall acetylation level of COX-1, except on the Ser529 residue. Moreover, the functional aspirin-induced inhibition of COX-1 was also decreased in presence of glucose. These results provide new insights on the interplay of glucose and aspirin on the platelet proteome and provide a mechanistic explanation of the phenomenon of aspirin poor-response in diabetic patients.

Introduction

Cardiovascular diseases are the main cause of morbidity and mortality of patients with diabetes mellitus[1,2] and long term antiplatelet therapy[3,4] was long thought to be protective in these patients either in primary or secondary prevention[5-8]. However, platelets from diabetic patients are characterized by an enhanced reactivity phenotype and a decreased biological response to aspirin that is in line with no clear benefit of aspirin in primary prevention[5,9] and a consistent lack of increased bleeding risk related to aspirin[10] in diabetic patients.

The antithrombotic effect of aspirin relies on the inactivation of cyclooxygenase 1 (COX-1) in platelets that hampers the conversion of arachidonic acid to prostaglandin G_2/H_2 and finally to thromboxane (Tx) A_2[11,12]. The exact site on COX-1 where aspirin exerts its function was elucidated about twenty years ago using as protein model the ovine COX-1[13,14], which share 95% of sequence similarity with its human counterpart. According to structural characterizations, COX-1 inhibition by aspirin is mainly due to the irreversible acetylation of the serine residue 529 (Ser529) located, together with Tyr385, at the apex of the peroxidase catalytic site, at the bottom of a long hydrophobic channel. This process occurs through a two-step mechanism involving 1) the electrostatic interaction of the salicylate group of aspirin with the guanidine moiety of Arg120 side chain, located at the entrance of the channel and 2) the position of the aspirin acetyl group in close proximity to the hydroxyl groups of Ser529 through hydrogen bonds and van der Waals interactions with Tyr385[15,16]. The resulting acetylation of Ser529 prevents the access of arachidonic acid to its binding site on the enzyme[17] leading to the inhibition of thromboxane A_2 synthesis. The extent of thromboxane A_2 inhibition was shown to be inversely correlated to the level of COX-1 acetylation, highlighting the importance of the acetylated Ser529 as a specific index of drug action[18].

To date, a large body of evidence supports the relationship between the less-than-expected response to aspirin and diabetes[19] as a result of residual platelet hyper-reactivity[20] due to accelerated platelet turnover[21-24] and increased oxidative stress[25]. Furthermore, COX-1 polymorphisms were also proposed as influencing factors of aspirin effectiveness[26], although the specific effects of these SNPs on COX-1/aspirin interaction remains still unclear[27,28]. Beside the relevance of extra-platelet sources of

thromboxane A_2 such as monocytes/macrophages COX-2[29,30] and increased levels of F_2-isoprostanes[25,31], the residual platelet activity may also result from the impairment of COX-1 aspirin acetylation by hyperglycaemia favouring non-enzymatic glycation. The mutual influence between glycation and acetylation has been previously reported on platelets from diabetic patients[32] suggesting that the poor response to aspirin could be explained by an extensive glycation of platelet proteins that in turn interferes with their acetylation state. Recently, the same group reported a pioneering study in which a number of glycation and acetylation sites were detected on purified ovine COX-1, demonstrating also that very high glucose concentrations affect the ability of aspirin to acetylate the enzyme[33]. However, the precise interplay between glycation and aspirin-induced acetylation on the critical residue (Ser529) of COX-1 remains unknown. Here we report a comprehensive study aimed to elucidate the impact of chronic hyperglycaemia on the aspirin response of total platelet proteins, with a particular emphasis for COX-1, providing new insights on the hampering effect of glucose on the acetylation of Ser529 by aspirin.

Material and methods

Patient selection and blood sampling

Diabetic patients (n=3) were recruited in the diabetic outpatient clinic of the University Hospitals of Geneva. The inclusion criterion was a glycated haemoglobin (HbA1c) ≥ 8%. The main exclusion criterion was aspirin or non-steroidal anti-inflammatory drugs intake in the previous 60 days before blood collection in order to avoid any drug-induced protein acetylation. Age and sex-matched apparently healthy control subjects without any previous medication intake within the last 60 days and with HbA1c < 6% were recruited among the staff of the University Hospitals of Geneva. All study participants gave their written informed consent and the study protocol was approved by the Central Ethics Committee of the University Hospitals of Geneva.

Blood was drawn from an antecubital vein into Vacutainer tubes containing 10.8 mg EDTA (BD Vacutainer®).

Platelet sample preparation

Blood was centrifuged at 150 g for 15 min at 37°C and the platelet-rich plasma (PRP) was used for an additional centrifugation step to remove residual erythrocytes and leukocytes [34]. The homogeneity of platelet population was evaluated by FACS analysis using the Accuri C6 fluorescence flow cytometer (BD Accuri™). Briefly, 10^6 platelets were incubated with 20 µL FITC labelled anti-human CD42b (BD Pharmigen™), 2 µL PC5 labelled anti-human CD45 (IO Test®) and 1 µL RPE labelled anti-human CD 235a for 15 minutes at room temperature and in dark conditions. The level of contamination of erythrocytes and leukocytes from the PRP sample was measured to be around 0.3% for both cell populations. PRP was then centrifuged at 1000 g for 10 minutes in presence of 25 µM PGI_2 and platelet pellets were washed with Dulbecco's phosphate-buffered saline (DPBS; 1X, pH 7.4, Invitrogen™). Platelets were then lysed with a surfactant containing DPBS buffer (Rapigest, Waters, Milford, USA) (pH 7.4) and sonicated to increase the yield of protein extract. Protein concentration was measured by Bradford assay (BioRad).

Glucose and aspirin *in vitro* incubation

Protein extracts from the different platelet fractions were used in two different experimental protocols aimed to characterize the influence of diabetes on aspirin-mediated acetylation. The first one addressed the issue of the effect of chronic hyperglycaemia (in samples from diabetic patients with poor HbA1c ≥ 8%) compared to control subjects (HbA1c < 6%) after *in vitro* exposure of 30 µL of platelet proteins with 500 µM aspirin for 30 minutes at 37ºC. This concentration was chosen as it is still in the range of therapeutic doses of aspirin for cardiovascular protection[35] while the time period for incubation was selected because it covers the half-life of aspirin in plasma. The second experimental protocol addressed the issue of a high glucose concentration exposure of platelet proteins of healthy subjects on the acetylation process induced by aspirin in order to mimic an acute glycaemic environment. For this purpose, 50 µL of platelet protein sample from each control subject were pooled together and used to create four test conditions in which 270 µg of proteins being incubated either with 1) 500 µM of aspirin for 30 minutes at 37ºC, or 2) 30 mM glucose for 24 hours at 37ºC, or 3) 30 mM glucose followed by 500 µM of aspirin. A last 270 µg-sample was incubated at 37ºC during the same

time period without glucose or aspirin (negative control). Samples were then immediately processed for protein digestion.

Protein digestion

After incubation, samples from both studies were ultra-filtrated with Amicon Ultra-0.5 mL, 3K cut-off filters (Millipore™) in order to remove the excess of salts, aspirin and glucose and to exchange the solubilisation buffer with 50 mM ammonium bicarbonate (Sigma-Aldrich®) pH 8.0. Protein amount was estimated again by Bradford assay and 100 µg of proteins were used for subsequent trypsin digestion. Cysteine residues were reduced with 5 mM TCEP for 30 minutes at 60ºC and the resulting free sulfhydryl groups were alkylated with 20 mM iodacetamide for 30 minutes at room temperature in dark conditions. Then, a solution of 200 ng/µL of trypsin was freshly prepared and 10 µL (corresponding to a ratio of 1:50 w/w) were added to each protein sample and incubated for 16 hours at 37ºC. Afterward, 0.5% trifluoroacetic acid was added to each protein digest and incubated for 40 minutes at 37 ºC under shaking in order to induce the acidic cleavage of Rapigest surfactant. After centrifugation at 16000 x g, supernatants were lyophilized and then reconstituted in 5% acetonitrile / 0.1% formic acid to be subsequently desalted and concentrated with C18 MacroSpin Column™ (Harvard Apparatus). Peptides were eluted with 50% acetonitrile / 0.1% formic acid and then evaporated to dryness before the final reconstitution in 5% acetonitrile / 0.1% formic acid for the subsequent mass spectrometry analysis. Each sample was analysed in triplicate under two mass spectrometry conditions.

LC-MS/MS analysis, identification and label-free quantitation of the platelet acetylome

Data-dependent acquisition (DDA) was performed on a Q Exactive Plus (ThermoFisher, San Jose, CA) mass spectrometer equipped with a Thermo EASY-nLC coupled with an EASY-Spray source operating at 1.8 kV in positive ion mode. Peptides were trapped at on a 2 cm x 75 µm i.d., PepMap C_{18} precolumn packed with 3 µm particles and 100 Å pore size. Then, separation was achieved in a 50 cm x 75 µm i.d., PepMap C_{18} column packed with 2 µm, 100 Å particles and heated at 50°C. Liquid chromatography was performed using a 120 minutes gradient at a flow rate of 250 nL/min using as

mobile phase A 0.1% (v/v) formic acid (Fluka) in HPLC-grade water (CHROMASOLV®) and as mobile phase B 0.1% (v/v) formic acid in HPLC-grade acetonitrile (CHROMASOLV®). The gradient program was as follows: 5% B at 5 min; slow ramping to 28% B in 105 min followed by a higher slope increase up to 40% B in 15 min; rapid ramping to 95% B over 10 min and washing column for 15 min. The column was re-equilibrated to 5% B for 24 min after each run. All peptides were analysed with a data-dependent tandem mass spectrometry method that relies on a MS survey scan from which a maximum of 15 precursor ions were selected with an isolation window of 1.6 m/z for subsequent fragmentation (normalized collision energy 27%). MS survey scans were acquired at a resolving power of 70,000 in profile mode, whereas MS/MS data were acquired at a resolving power of 17,500 in centroid mode. A dynamic exclusion of 30 sec was enabled and precursor ions of charge state 1+ were excluded from data-dependent selection. To identify peptides, raw data were first converted to .mgf file using msConvert tool[36] and the Mascot algorithm (Version 2.5, MatrixScience) was used to query all the MS/MS spectra against the UniProtKB/Swiss-Prot database (Release March 2015; 547,964 sequences) in which we preferentially select the human organism (20.203 sequences) as taxonomy. For database search we selected trypsin as endoprotease with a maximum of four missed tryptic cleavages; a 10 and 6 ppm error tolerance were used for precursor and fragment ion masses, respectively; carbamidomethylation of cysteine residues was selected as fixed modification while methionine oxidation and acetylation at lysine, serine, threonine and N-terminal residues were selected as variable modifications. False discovery rate (FDR) was derived by searching the same MS/MS data against a decoy database composed of reversed protein sequences. The estimated number of false positive peptide identifications was then used to adjust and filter the true positive matches according to an FDR $\leq$ 1%. Label-free quantitation was performed using Nonlinear Dynamics' Progenesis QI software, as described elsewhere[37]. Briefly, after run alignment and features extraction, m/z and retention time (Rt) pairs were associated to their relative peptide sequence and peak abundances were extracted by integrating the area under the peak curve. Raw abundances were then normalized to a global scaling factor derived from the log space distribution of the ratios between each run and a reference run.

Detection and quantitation of platelet COX-1 acetylation by a targeted approach

Parallel reaction monitoring (PRM) analysis was carried out using the same chromatographic system and mass spectrometer as for the DDA approach with some setting modifications. Liquid chromatography was performed with a 60 minutes gradient where mobile phase B composition ramped from 5% up to 35%. Six-teen target masses (doubly and triply charged ions) corresponding to 8 COX-1 peptides in their acetylated and non-acetylated state (Supplemental Table 1), were previously chosen based on stability, sequence (modified and unmodified) and strong transition signals. This inclusion list triggered targeted scans at a resolving power of 17,500 with an isolation width of 3 Th around the m/z of interest, an AGC target of 2 x 10^5, a maximum injection time of 100 ms and a normalized collision energy of 27% in a higher-energy c-trap dissociation (HCD) cell. According to these parameters, an entire cycle time of ≈ 2 sec was required to acquire all transitions, with an average of 12 points per peak (30-40 sec elution width). Data were analysed using the targeted MS/MS feature implemented in Skyline v3.5 software[38]. For each peptide, transition peak areas for the most intense fragments with the modification site were normalized by the sum of the transition peak areas for all the peptides across the runs.

COX-1 activity measurements

The activity of platelet COX-1 in presence of glucose and aspirin was monitored using a COX Activity Assay Kit (Cayman Chemical) that measures the peroxidase reaction of COX-1. First, platelet protein extracts were incubated with 500 μM aspirin for 30 minutes, 30 mM glucose for 24 hours and with a sequential incubation with 30 mM glucose for 1 day followed by 500 μM aspirin for 30 minutes. Platelet protein sample with neither glucose nor aspirin exposure was used as reference. For the end-point study, platelet proteins were incubated with an increasing glucose concentration (0, 10, 30, 50, 100 mM) for 24 hours and subsequently exposed with 500 μM aspirin for 30 minutes. The same conditions without adding aspirin were used as reference. In order to exclude the presence of other peroxidase activities that could generate false positive outcomes, an aliquot of sample was incubated with an inhibitor of COX-1 (SC-560) provided by the kit. The activity of COX-1 was determined by an absorbance reading taken at 590 nm. Samples were analysed in triplicate.

Functional networks and Gene ontology (GO) Term analysis

Functional networks based on protein-protein interaction were created with the STRING[39] database (version 1.0.4) using the Cytoscape[40] plug-in MCODE[41] and applying a confidence score cut-off of 0.6 to extract the highly interconnected regions of the network (clusters). Gene Ontology (GO) analysis was performed using the Cytoscape plug-in ClueGO[42] (updated 09.02.2016). GO parameters used for functional grouping pathways were: a p-value $\leq$ 0.01 integrated with a Bonferroni step down correction, a GO tree interval between 4 and 8, a minimum number of genes per cluster of 5 with a 5% of genes, a kappa score of 0.4 and an initial group size of 3 terms with a percentage of overlapping terms per group of 50%.

Model structure prediction and molecular docking analysis

The prediction of the three-dimensional (3D) structure of human COX-1 was performed using Phyre2 (v 2.0) homology prediction tool[43]. Briefly, primary sequence of human COX-1 is queried in PSI-BLAST to detect sequence homologues and position specific scoring matrix (PSSM) is calculated from the multiple alignment. Then, secondary structure elements are estimated using the neural-network prediction tool Psi-pred[44]. A hidden Markov model (HMM) is generated from the query sequence and matched against a library of HMMs from proteins of known structure. The 3D model of human COX-1 is then constructed depending on this alignment that in turn depends on the similarity of residue probability in each position and of the secondary structure. A confidence higher than 90% and an identity (accuracy of the model) higher than 40% establish a true structural homology between the protein and the template. To predict a potential binding of glucose on the COX-1 catalytic pocket, a molecular docking simulation was performed with AutoDock Vina[45], using the homology model of human COX-1 as receptor and the linear form of glucose as ligand. A grid box size of 12 x 12 x 12 with a grid spacing of 1 Å was centred at the entrance of the pocket. A total of 20 poses were calculated and a “score” (binding energy) was determined for each ligand conformation via a scoring function which uses the AMBER force field to infer the free energy associated to the complex.

Statistical analysis

Statistically significant differences were estimated using the analysis of variance (ANOVA) when comparing the two groups of different control and patient samples, nested for biological and technical replicate levels and to analyse the consistency of changes in the acetylation level of the 4 *in vitro* platelets condition tests. The data were expressed as mean ± SD and a value of $p \leq 0.05$ was considered to be statistically significant.

Results

Impact of chronic hyperglycaemia on aspirin-induced platelet proteome acetylation

The effect of diabetes on aspirin-mediated acetylation was assessed on platelet protein extracts from healthy controls (HbA1c < 6%) and diabetic patients (HbA1c > 8%) exposed *in vitro* with aspirin for 30 minutes. Samples from each subject were analysed separately in order to take into account the biological variability in the same group. A total of 1937 acetylated proteins were identified among the two groups (Supplemental Table 2) and 1088 were used for label-free quantitation after filtering features (Rt, m/z pairs) for their abundance ($\geq 10^4$), their match with MS/MS spectra (≥ 1), their reproducibility across replicates (CV ≤ 30%) and their significant variation between groups ($p \leq 0.05$, Supplemental Table 3). From the 3292 acetylated residues quantified in this study, 3.5 % and 11.6 % showed a 2- and 1.5 fold significant decrease, respectively in their acetylation level in diabetic patients compared to controls while 2 % and 5.6 % evidenced a 2- and 1.5 fold significant increase of their acetylation state in diabetic patients and in controls, respectively (Figure 1). All proteins containing the significantly different acetylation sites with a fold change higher and lower than ±1.3 were used to generate a functional protein-protein interaction network (Supplemental Figure 1 A) and finally to perform a GO term enrichment. The most significant GO terms for each functionally grouped network were “blood coagulation”, “haemostasis” and “platelet activation/degranulation” ($p \leq 1.1E^{-10}$) and represented biological processes with the highest number of genes, as evidenced by the color-coded heat map (Figure 2) and by the highest scoring sub-network extracted from the global one (Supplemental Figure 1 B). Among the different proteins involved in those functional pathways, we

found several classes of integrin receptors, structural proteins involved in cell shape change, coagulation factors, platelet agonists and enzymes that trigger platelet activation, including COX-1, from which 5 different sites (Lys168, Lys221, Lys252, Lys565 and Lys572) were quantified. The acetylation level of most of these residues showed no significant variation between control and diabetic group with the only exception for Lys168 and Lys572 residues ($p \leq 0.05$) while no information on the critical site of Ser529 was obtained with this method.

Impact of chronic hyperglycaemia on aspirin-induced COX-1 acetylation

Since the residue of Ser529 was not previously detected with the data-dependent approach, a targeted mass spectrometry method (PRM) was used in order to increase the specificity and sensitivity of the analysis. A total of 6 out of 8 acetylated peptides (and their non-acetylated counterpart) were detected and quantified with a coefficient of variation (CV) of 22.3% and 26.4% for control and diabetic groups, respectively, with a correlation of $r^2 = 0.99$ between replicates was achieved (Supplemental Table 4). The acetylation level of 2 out of 6 sites (Lys167 and Lys168) showed a slight variation between the two groups with a fold-change less than 1.3, suggesting that diabetes does not influence the aspirin-acetylation on these residues (Supplemental Figure 2). In contrast, a significant decrease of acetylation associated to a fold-change of 2.7 was observed for Ser529 ($p < 0.001$) in diabetic patients compared to controls (Figure 3 A), as evidenced by the extracted ion chromatograms (XICs) in each group (Figure 3 B). Although the level of acetylation of this specific residue was already determined in a recent study[18], here we have reported for the first time a direct impact of diabetes on this preferential COX-1 target of aspirin. Moreover, the acetylation of new COX-1 residues by aspirin was here shown.

Impact of *in vitro* acute hyperglycaemia on aspirin-induced COX-1 acetylation

In order to validate the results derived from the comparison of diabetic patients and controls on the acetylation level of COX-1, we performed an *in vitro* study on a pool of platelet protein extracts from the cohort of healthy volunteers. Incubation with glucose was performed to mimic an acute hyperglycaemic event, followed by the subsequent exposure with aspirin. The PRM method allowed

the detection and quantitation of all the 8 peptides/sites in their acetylated and non-acetylated state, corresponding to the 16 selected target m/z values, with a correlation coefficient (r^2) around 0.996 (Supplemental Table 5). The abundance of all the peptides (acetylated and non-acetylated) used in this study remains quite similar across the different conditions, stating that the variation between conditions is related to a real biological effect with a low level of experimental variability. We found that high glucose levels (30mM) had no significant impact on the aspirin acetylation of the majority of the sites reported in this study (Supplemental Figure 3). Of particular interest among the acetylated sites is the Ser529 residue which acetylation state was quantified using the 7 most abundant fragment ions containing the modification (Figure 4 A), showing a significant decrease of aspirin-induced acetylation after glucose exposure ($p < 0.05$, fold change > 2) (Figure 4 B-C). These results further support the idea that glucose influences the aspirin-mediated acetylation of Ser529 of COX-1.

Impact of glucose on aspirin-induced inhibition of COX-1 activity

In order to figure out whether the reduced level of acetylation of Ser529 by high glucose was associated to an impairment of aspirin inhibition of COX-1 activity, we evaluated the conversion of arachidonic acid into PGH_2 on a pool of platelet proteins from healthy controls in different conditions. As expected, aspirin alone decreased the activity of COX-1 by 90%, while glucose alone showed no evident effect (Figure 5). However, a significant decrease in the inhibition of COX-1 activity was observed when aspirin was incubated after glucose (Figure 5). In a second experiment, we assessed the dose response relationship between different glucose concentrations and the inhibition of the activity of COX-1 by aspirin. COX-1 activity increased despite aspirin exposure along with increasing glucose concentrations, while high glucose concentrations by itself did not affect the enzyme function (Figure 6). Taken together these results suggest that high glucose levels decreased aspirin-induced acetylation on Ser529 in a dose-dependent manner, which in turn leads to an uncomplete inhibition of COX-1 activity, contributing to the residual platelet hyper-reactivity observed in diabetes.

Homology 3D model of human COX-1 and docking analysis

Due to the lack of experimental 3D structures of human COX-1, we used the Phyre2 (v 2.0) homology prediction tool to generate a homology model of the tertiary structure of human COX-1. The model was built up using the known structure PDB: c2oyuP as template that yields a sequence coverage of 92% with the highest confidence value of 100%, and an identity of 94% after alignment. The generated structure was then visualized with PyMOL (The PyMOL Molecular Graphic System, Version 1.7 Schrödinger, LLC) in order to figure out the spatial distribution of the preferential sites that were found to be acetylated by aspirin. We observed that all the acetylated residues found in our study lie on the solvent accessible surface of the protein (Supplemental Figure 4 A-C), whereas only serine 529 is placed in a buried region of the enzyme surrounded by a 7 Å width hydrophobic channel (Supplemental Figure 4 D), corresponding to the peroxidase and cyclooxygenase domain of COX-1. Additional acetylated residues were not detected in the catalytic site of the enzyme. Then, to gain insight into the understanding of how glucose can influence the acetylation of Ser529, a docking analysis was performed. Two major clusters of glucose binding poses were located nearby Arg120 and Tyr354 residues surrounding the entrance of the active site groove, with free energy values that range from -3.4 and -4.3 kcal/mol. The glucose conformations with the lowest binding energies were chosen from both clusters and represented in Figure 7. As shown, glucose can adopt two similar but reversed poses at the exterior of the pocket, where it interacts with Arg120 residue alone through hydrogen bonds between the carbonyl oxygen of the sugar and the η amines of the guanidine group, together with those formed between the hydroxyl group of carbon atoms 5 or 6 of the sugar and the ε amine of the guanidine group (Figure 7 A). A reverse configuration of glucose may generate H – bonds between the hydroxyl group of carbon 6 and the η amines of the guanidine group, together with H – bonds between hydroxyl group of carbons 2, 3 or the carbonyl oxygen and the ε amine of the guanidine group (Figure 7 B). Alternatively, glucose could be stabilized into the pocket at the interface between Arg120 and Tyr354 residues by two potential sets of H – bonds: 1) between the hydroxyl group of carbons 4 or 5 and the η amines of the guanidine group of Arg120 residue and 2) between the hydroxyl group of carbon 3 and that one of the aromatic moiety of Tyr354 residue (Figure 7 C). On

the other side, carbonyl oxygen and the hydroxyl group of carbon 3 of glucose can interact by H – bonds with the η amines of the guanidine group of Arg120 and with the hydroxyl group of Tyr354, respectively (Figure 7 D). According to our theoretical model, the positioning of glucose in close proximity to the cavity that surrounds the COX-1 catalytic domain and the subsequent generation of polar contacts with Arg120, might interfere with the interaction of aspirin with this fundamental residue, preventing in turn the access of aspirin into the pocket and its acetylating effect on Ser529.

Discussion

The correlation between residual platelet activation and a suboptimal responsiveness to antiplatelet-agents is a phenotype frequently observed in diabetes. Potential explanations rely on an increase of newly produced platelets from bone marrow megakaryocytes with a new replenished cargo of proteins involved in platelet activation pathway[24,46], an enhance of oxidative stress through a reduced synthesis of NO and the increase of lipid hydro-peroxides and F_2-isoprostanes levels[25] and an uncomplete inhibition of COX-1. The latter is a matter of concern since several suggestions were proposed to explain the less than expected response of COX-1 to aspirin. Among them, additional copies of active COX-1 from immature platelets may shift the balance in the distribution of inactive enzyme towards its functional state making insufficient the effect of aspirin. Non-enzymatic glycation may have a role as well, but to date the mechanism by which high glucose might contribute to the reduced sensitivity of COX-1 to aspirin is far from a clear comprehension. Glycation on purified ovine COX-1 was recently shown to interfere with its acetylation state and on the other hand, acetylated COX-1 evidenced less susceptibility to glycation compared to the unmodified enzyme[33], suggesting that a mutual competition between these two non-enzymatic modifications might occur. In the same study, it was reported that the activity of COX-1 is strongly impaired by glycating agents (i.e. glucose and derivatives) and further inhibition was observed after aspirin incubation. As stated by the same authors, these results seem to be not in good agreement with the large body of evidences that show the association between diabetes and the higher levels of platelet thromboxane A_2 measured by its stable metabolite, the 11-dehydro-thromboxane B_2, excreted with the urines[47]. Moreover, although a number of sites that undergo to glycation and aspirin acetylation were clearly identified in the aforementioned

report, the direct impact of glucose on the acetylation state of the catalytic residue of Ser529, as well as of other potential sites of human COX-1, remains to be still demonstrated. In this study, we aimed to determine the role of glucose on the aspirin response of human platelet COX-1 performing two *in vitro* approaches close to physiological conditions. First, the characterization of the acetylation levels of platelet proteins was performed using an *in vivo* diabetes model after *in vitro* incubation with 500 μM aspirin. This concentration was chosen because it is in the range of the typical *in vivo* therapeutic levels (0.1 – 2 mM) [35] and time exposure (30 min) closely reflects the half-life of aspirin in plasma. From the thousands of acetylated sites quantified we filtered out for those that showed a highly differential variation in their acetylation level. We observed that the level of most of these acetylated sites showed a decrease in diabetes compared to controls, likely reflecting the hampering effect of glucose or glycation on aspirin-mediated acetylation. Some of them, however, evidenced higher acetylation levels in diabetes than controls, a fact that may be explained by possible conformational changes in the protein structure induced by glycation that may in turn favour the acetylation reaction by aspirin. Despite a number of proteins containing those differential acetylated sites span on several functional clusters related to metabolic processes, a large enrichment of genes was found to be involved in blood coagulation, platelet activation and degranulation. The acetylation of different integrin receptors, coagulation factors, mediator of platelet aggregation (e.g. vWF, Rap1, Talin) and degranulation (e.g. VAMP) and enzymes like PI3K and COX-1 was shown to be lower in the group of diabetic patients compared to that of healthy controls. The exact role of aspirin on the biological function of most of these proteins is not known despite a correlation between diabetes, platelet hyper-reactivity and low responsiveness to aspirin was already evidenced. In this study we were able to elucidate the preferential sites of aspirin-induced acetylation on a significant fraction of the platelet proteome and to quantify the impact of diabetes on the effect of aspirin on several platelet proteins, mainly focusing our attention on COX-1. Both data-dependent and targeted (PRM) approaches were sensitive enough to detect with high confidence some preferential acetylation sites, including Lys167, Lys168, Lys221, Lys252, Lys472, Lys572 and Ser529. The latter is of high interest since its reduced acetylation level may reflect the relation between hyperglycaemia and highly reactive platelets observed in diabetic patients compared to controls, while no drastic changes were evidenced for the

other sites. To the best of our knowledge, this is the first report that shows the impact of manifested diabetes on the effect of aspirin on platelets COX-1 and in particular on the acetylated state of its fundamental residue (Ser529) involved in the complete enzyme inhibition upon the irreversible acetylation by aspirin. Then, the decreased acetylation level of Ser529 by aspirin was further validated using a pool of platelet protein extracts from healthy volunteers with a % HbA1c < 6 incubated with 30 mM glucose followed by 500 µM aspirin exposure. The choice to use such high glucose levels is justified by the fact that early glycation is a process that naturally occur in a time period that ranges from days to weeks at physiological (or pathophysiological) glucose concentrations (6 – 11 mM), pH and temperatures. To compensate for the short incubation time used in this predictive analysis (24 hours), a concentration of 30 mM glucose represented a good compromise because it is neither so low to render inadequate the glycation process nor too high to greatly exceed what happens *in vivo*. Eight preferential acetylated sites of COX-1 were identified and quantified from human platelet protein samples (with and without glucose and aspirin) using a PRM approach. The acetylation level of most of these sites was shown to be not significantly affected by glucose, indicating that if some competing glycation occurs this would be a kinetically unfavoured process (high K_m constant) than aspirin-induced acetylation. In contrast, the acetylation level of Ser529 showed a large significant decrease (4-fold decrease) in presence of glucose, providing the first direct proof of the hampering effect of glucose on the acetylation of this crucial target of aspirin. This assumption was strongly supported by the observation that inhibition of overall enzyme activity by aspirin is significantly reduced in presence of high glucose levels compared to aspirin alone. In addition, no changes in COX-1 function were evidenced when platelet proteins were incubated with glucose alone compared to the control condition, meaning that its activity tends to be retained at such glucose concentrations. Our findings differ from those obtained by Kassassir et al. showing a reduced activity of COX-1 after *in vitro* glycation with glucose[33]. One potential explanation for this difference may be related to the large variation in the experimental set up used in these two studies including the nature of the sample (ovine vs. human), the complexity of the sample (purified protein vs. complex sample) and the incubation conditions (extremely high glucose levels [300mM] vs. more physiological ones). Instead, these results are perfectly compatible with the high platelet reactive phenotype associated with elevated

baseline levels of thromboxane A_2 and poor responsiveness to aspirin frequently observed in diabetes. The analysis of the 3D model of human COX-1 highlighted the prevalence of the acetylated residues on the solvent exposed surface of the protein, favouring the modification by glucose and/or aspirin in a process mainly governed by the reaction with faster binding kinetic. This could explain the reason why most of the acetylated sites found in this study showed no significant variation in their acetylation state even in presence of high glucose levels or in diabetes. Only two of those sites (Lys168 and Lys572) evidenced a decrease in their acetylation degree, potentially amenable to their close proximity with basic residues (Lys167 and Lys565, respectively) that, upon acetylation, increase the pK_a of their neighbours, making them less prone to be modified. However, it is worth to state that the fold change of variation of these sites was much lower than the threshold of 1.5 fixed to have a robust difference between conditions. Unlike the different sites found to be acetylated, Ser529 is located at the bottom of a narrow internal hydrophobic channel but nevertheless, its acetylation state was found to be drastically affected by *in vitro* high glucose concentrations and chronic hyperglycaemia. Our results implicate that under an excessive hyperglycaemia stimulus and in diabetes as well, a high glucose level is able to interfere with the acetylation of Ser529, making the enzyme less responsiveness to the normal effect of aspirin. A likely explanation of this effect came from molecular docking analysis showing that glucose can pose at the entrance of the pocket and interact with the Arg120 and Tyr354 residues through hydrogen bonds formation. The former is responsible of the first interaction of COX-1 with aspirin and the occupancy of glucose may prevent this process, contributing to the reduced acetylation of Ser529. Nevertheless, non-enzymatic glycation may exert a pivotal role, as well, through the modification of amino acid side chains that form the solvent exposed surfaces of the internal pocket, hindering the access of aspirin. Another mechanism might rely on allosteric conformational changes induced by glycation of residues spatially distant from the catalytic sites of COX-1. Additional structural studies are underway in our lab to figure out all these assumptions. In conclusion, our findings show for the first time the impact of diabetes on aspirin-induced acetylation of human platelet proteins, including COX-1. The acetylation state of the critical catalytic residue of Ser529 was shown to be strongly affected by chronic hyperglycaemia. These results were validated as well after *in vitro* exposure with high glucose levels and therapeutic aspirin concentrations. The

reduced acetylation of this crucial site is associated with a lowering of the inhibition of COX-1 activity by aspirin, providing new insights on the potential harmful role of glucose on the less sensitivity to aspirin in diabetes. We propose that glucose may dock at the entrance of the functional pocket of the enzyme interfering with aspirin interaction but nevertheless we cannot exclude a mechanism involving glycation by a direct effect on the catalytic site of the enzyme or through allosteric conformational changes. Taking together these results provide us a new prospective that improves our understanding on the pathological role of glucose on platelet hyper-reactivity in diabetes.

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Authorship

Contribution: F.F. carried out the experimental section of the study, including sample preparation, mass spectrometry experiments, COX-1 activity assay, performed data analysis and wrote the first draft of the manuscript; J.L.R. provided fundamental insights for data analysis and helped with the project design and with the manuscript revision; S.M. recruited the patients, contributed to the data interpretation and to the manuscript revision; J.C.S. and P.F. were responsible of the overall direction and supervision of the present study and contributed to the data interpretation and to the manuscript revision.

Figure Legends

Figure 1

Volcano plot showing the differentially acetylated platelet protein sites in healthy subjects compared to diabetic patients. In orange and in red are those sites with an acetylation level higher than 1.5 and 2, respectively (diabetes reduces their acetylation). In green and blue are those sites with an acetylation level lower than -1.5 and -2, respectively (diabetes increase their acetylation).

Figure 2

Heat map of the most significant ($p < 0.01$) biological processes obtained from the GO analysis of the differentially acetylated proteins. The number of genes for each GO term are color-coded depending on the degree of gene enrichment in each functional network.

Figure 3

Acetylation level of the COX-1 peptide CHPNSIFGESMIEIGAPFS(Ac)LK detected by PRM in platelets from healthy subject and diabetic patients. Bar plots are shown as the sum of the most abundant fragment ions that contains the acetylated Ser529 (A). Extracted ion chromatograms (XICs) of the fragment ions used for quantification in diabetic and control groups (B).

Figure 4

Representative fragment ion-MS2 spectrum of the COX-1 peptide CHPNSIFGESMIEIGAPFS(Ac)LK detected by PRM (A) in platelets *in vitro* incubated with glucose and aspirin. The most abundant fragment ions containing the acetyl-Ser529 were used for quantification. The bar plot shows the acetylation level among the different *in vitro* conditions, as a sum of the different detected fragments (B). The comparison between the XICs clearly shows the difference in the acetylation state of this peptides across the different *in vitro* conditions (C).

Figure 5

The level of COX-1 peroxidase activity was assessed from platelet protein extracts (see text) and expressed as U/mL. Bar plot shows the change of the enzyme activity in presence of 500 μM aspirin, 30 mM glucose and after a sequential incubation with glucose followed by aspirin. A significant decrease of aspirin-inhibition with prior glucose incubation is clearly evident compared to aspirin exposure alone.

Figure 6

COX-1 peroxidase activity was evaluated in presence of a growing glucose concentration (0, 10, 30, 50, 100 mM) followed by 500 μM aspirin incubation. A progressive significant ($p < 0.01$) decrease of COX-1 inhibition by aspirin is shown when increasing glucose concentration.

Figure 7

The most favoured poses of glucose at the entrance of the catalytic pocket of COX-1 has been derived from molecular docking simulations. Glucose can stably interact with Arg120 alone through the formation of 5 likely polar contacts (A), or through non-covalent H-bonds with Arg120 and Tyr354 (B).

Supplemental figure 1

Functional network of protein-protein interaction generated using the STRING database. Nodes represent the differentially acetylated platelet proteins and the colours indicate different acetylation levels, in red the acetylation level is higher in controls than in diabetes, in green the acetylation is higher in diabetes than in controls (A). The highest scoring sub-network extracted from the global one shows the interconnection of proteins involved in platelet activation and degranulation (B).

Supplemental figure 2

Acetylation level of other 5 COX-1 sites detected by PRM in platelets from healthy subjects and diabetic patients (A). XICs for each acetylated peptide in control and diabetic groups (B).

Supplemental figure 3

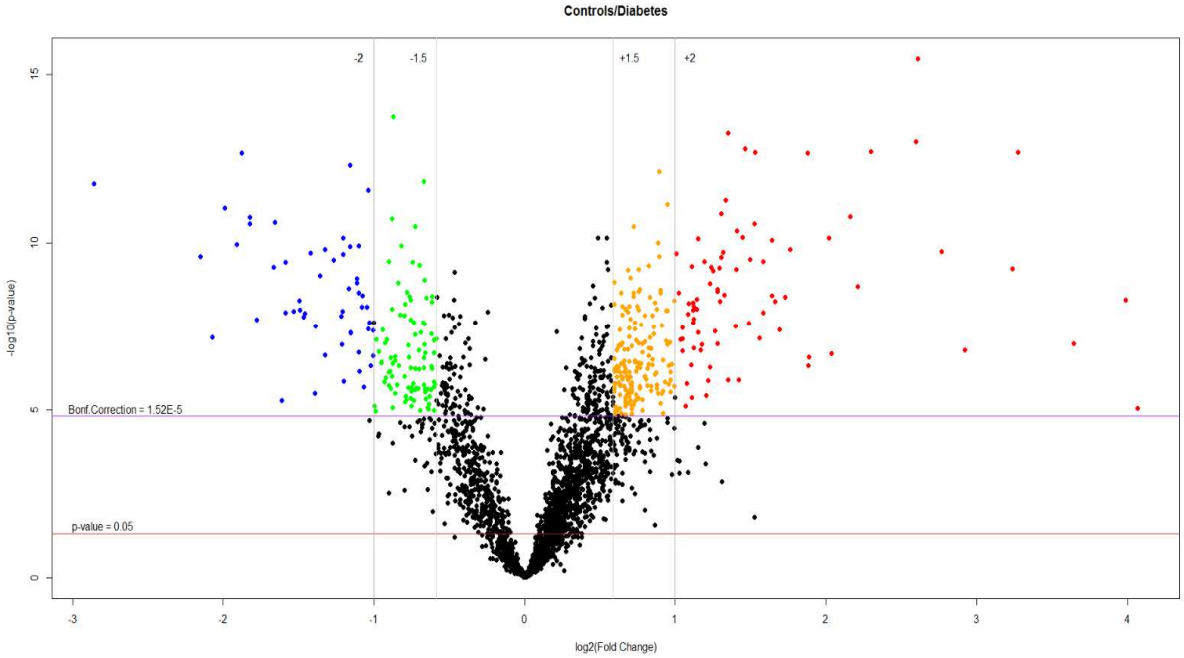
Acetylation level of 9 COX-1 sites detected by PRM in platelets *in vitro* incubated with glucose and aspirin (A). XICs for each acetylated peptide among the different conditions (B).

Supplemental figure 4

3D structure of human COX-1 obtained by homology modelling. Most of the detected acetylated sites (green residues) are located at the solvent-exposed surface of the protein (A-B-C), while Ser529 is placed at the bottom of an internal hydrophobic pocket (yellow residues) (D).

Figures

Figure 1



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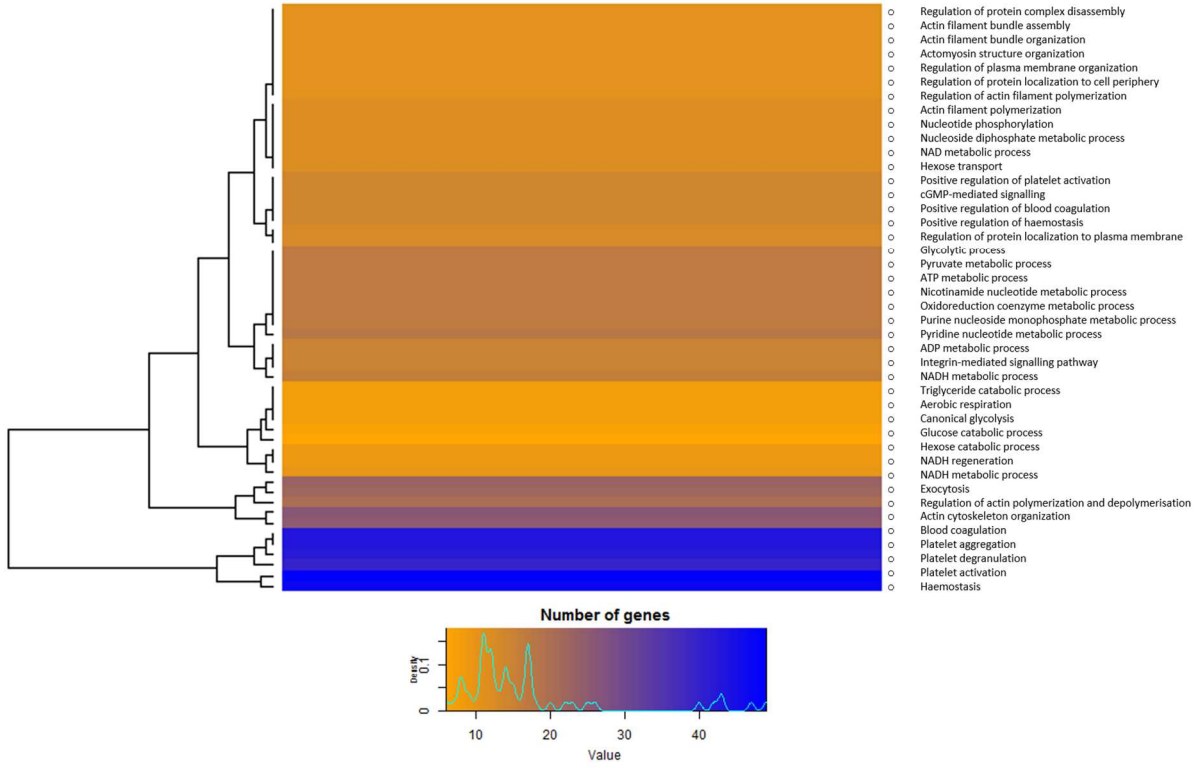

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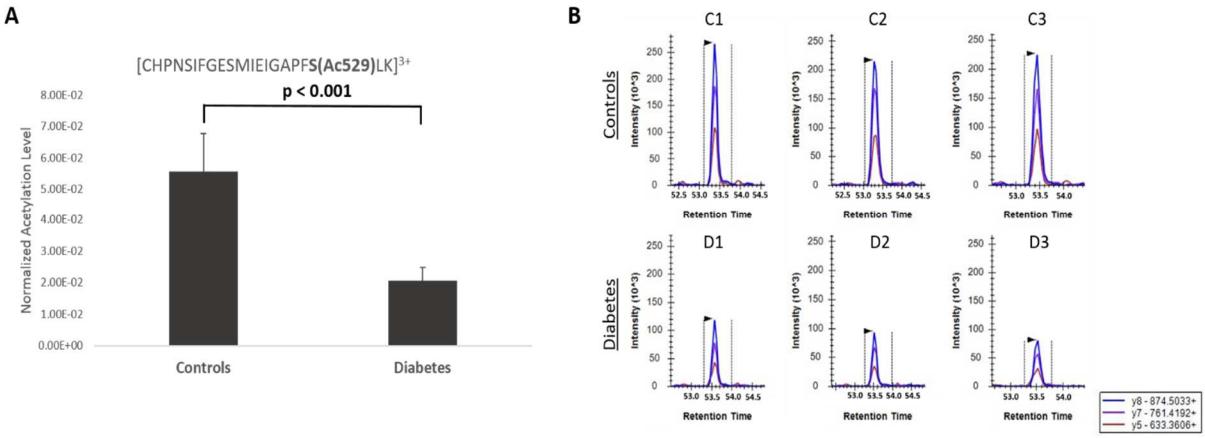

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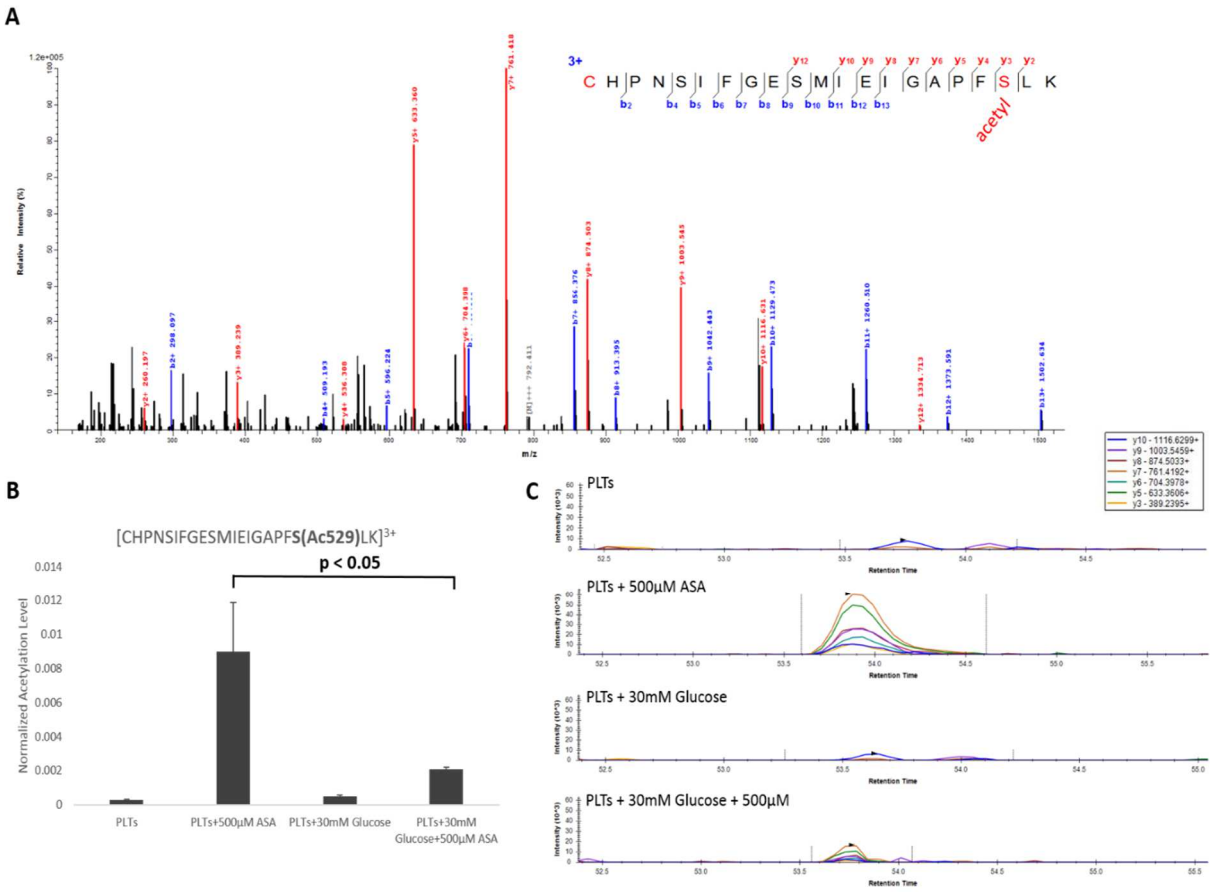

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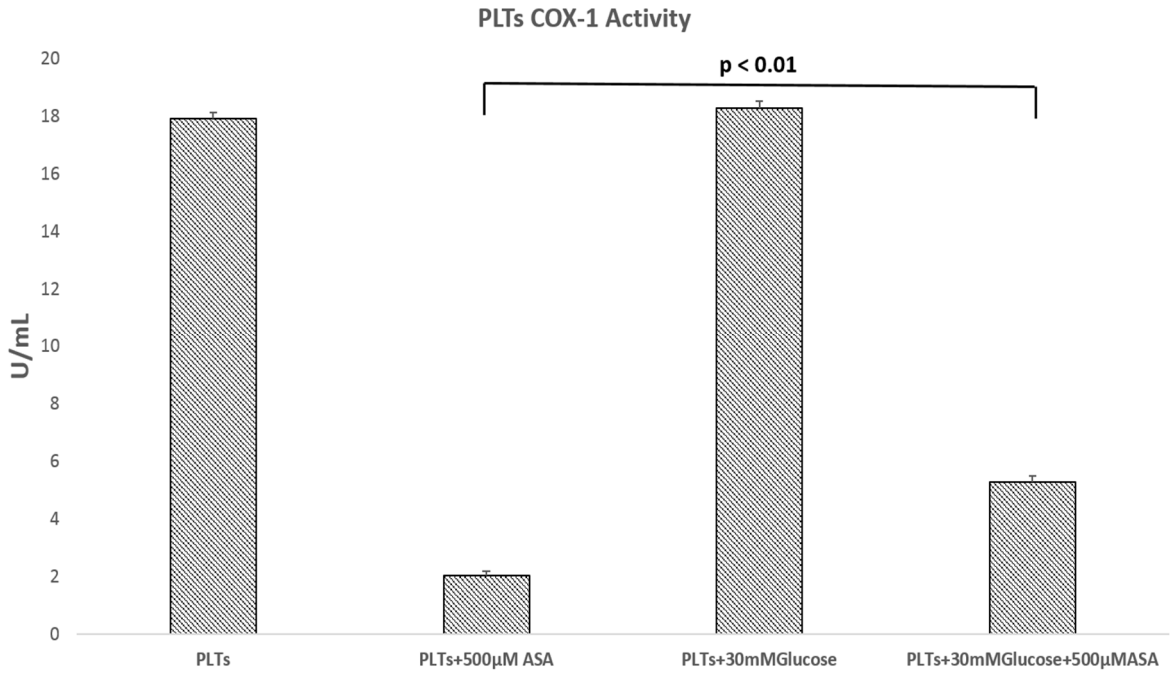

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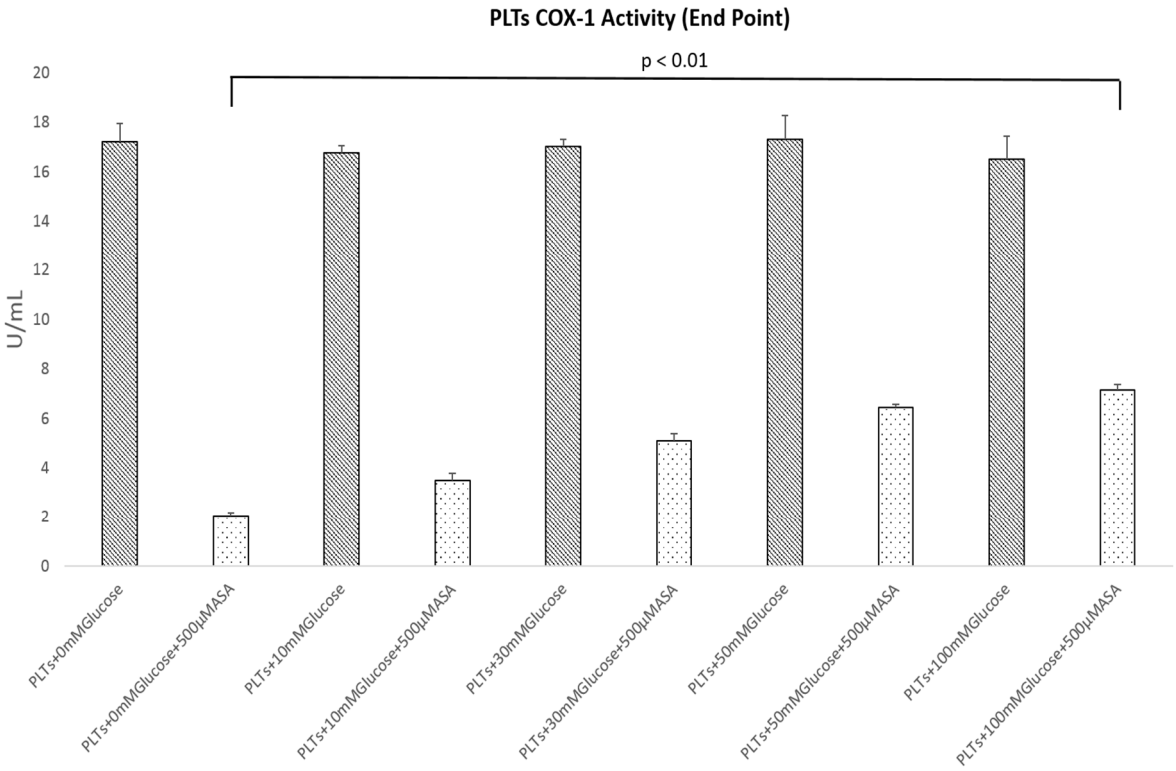

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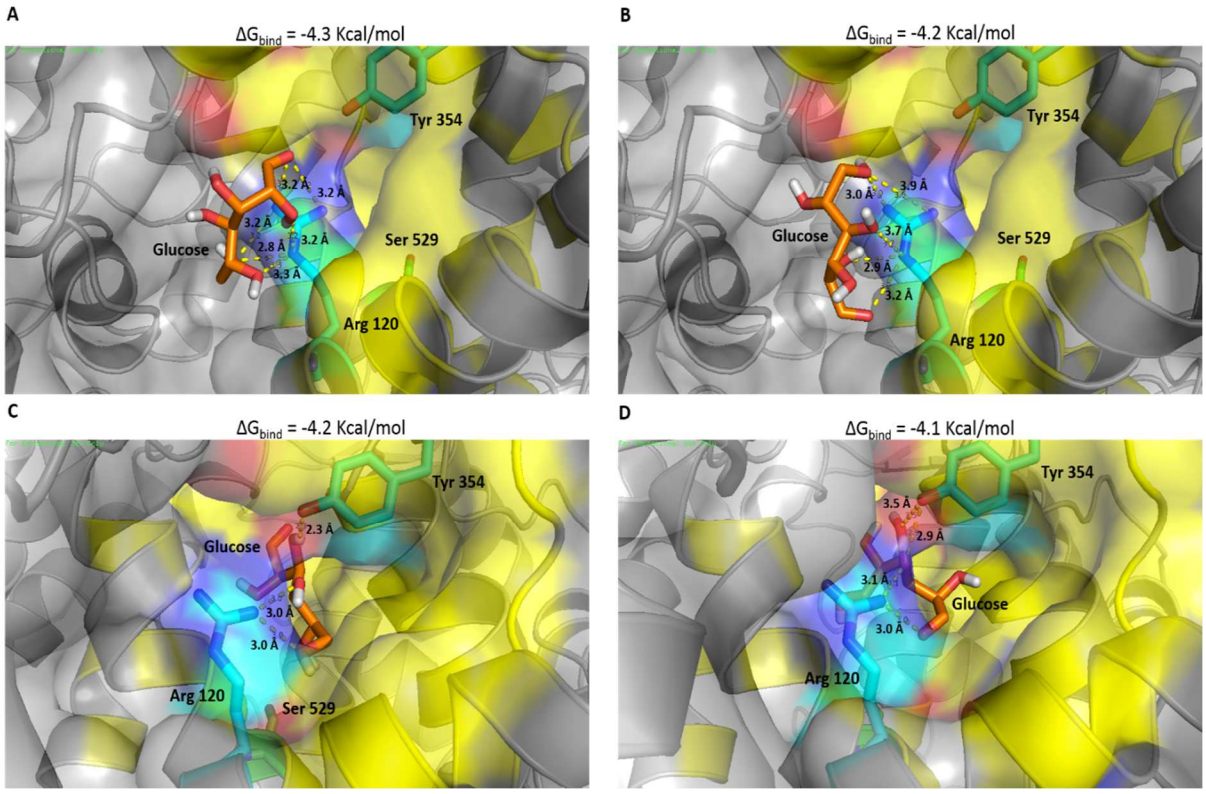

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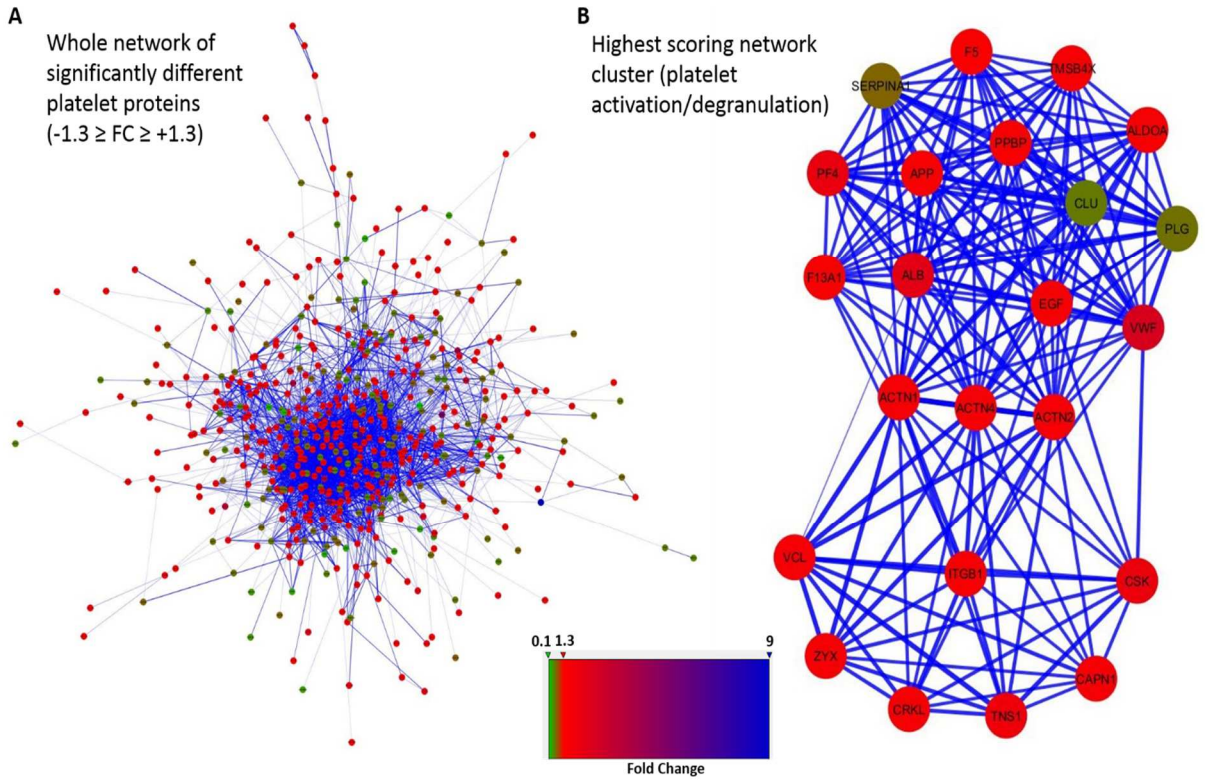

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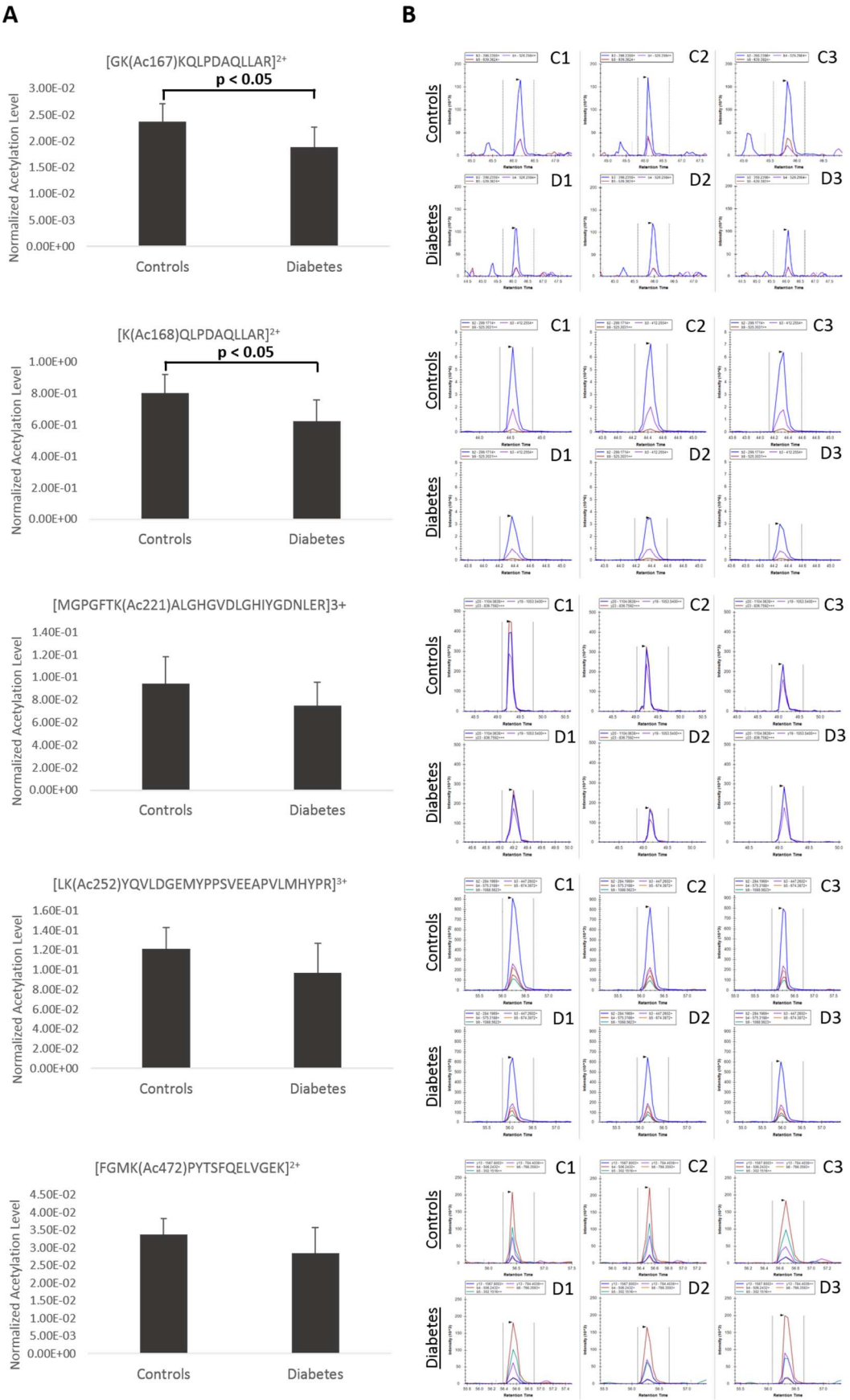

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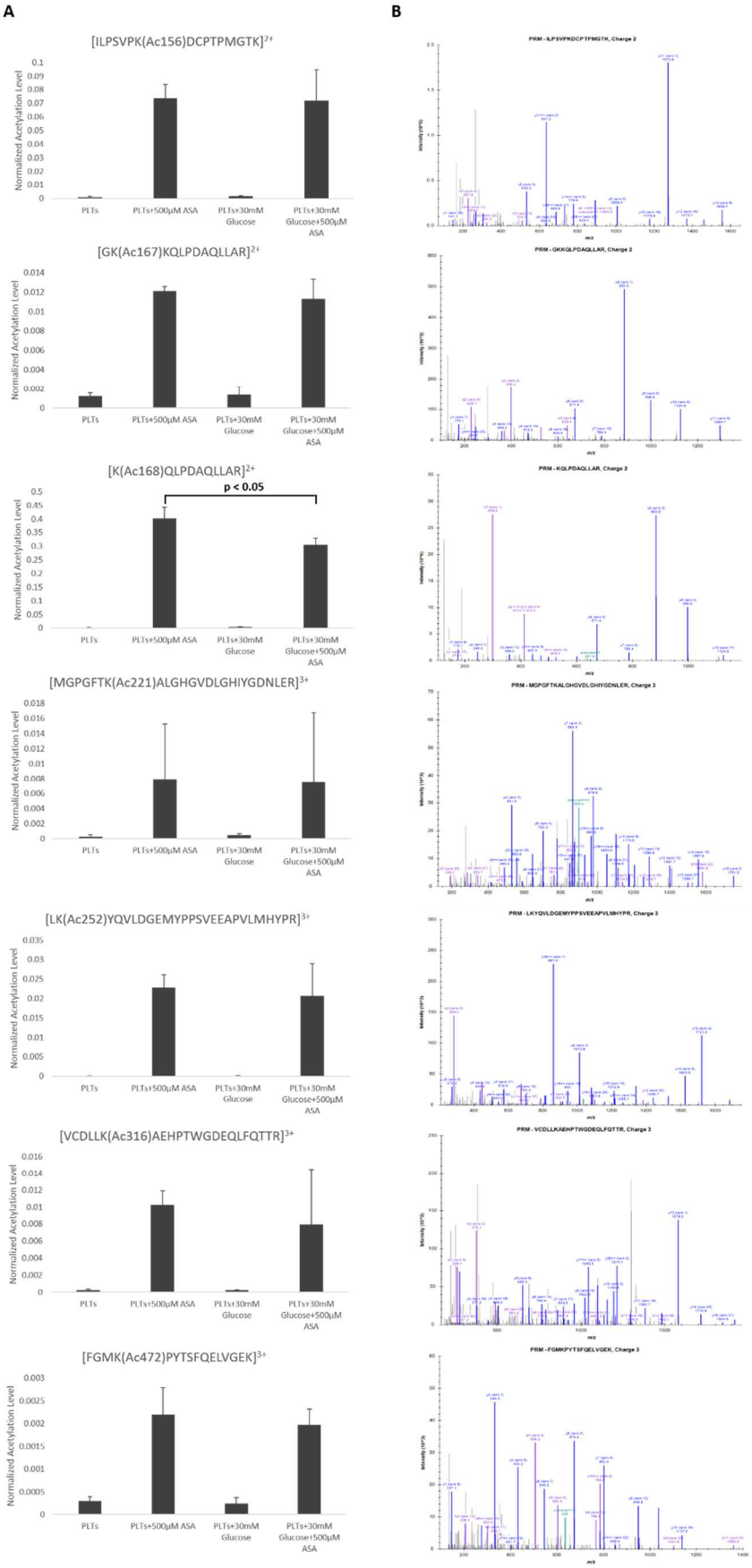

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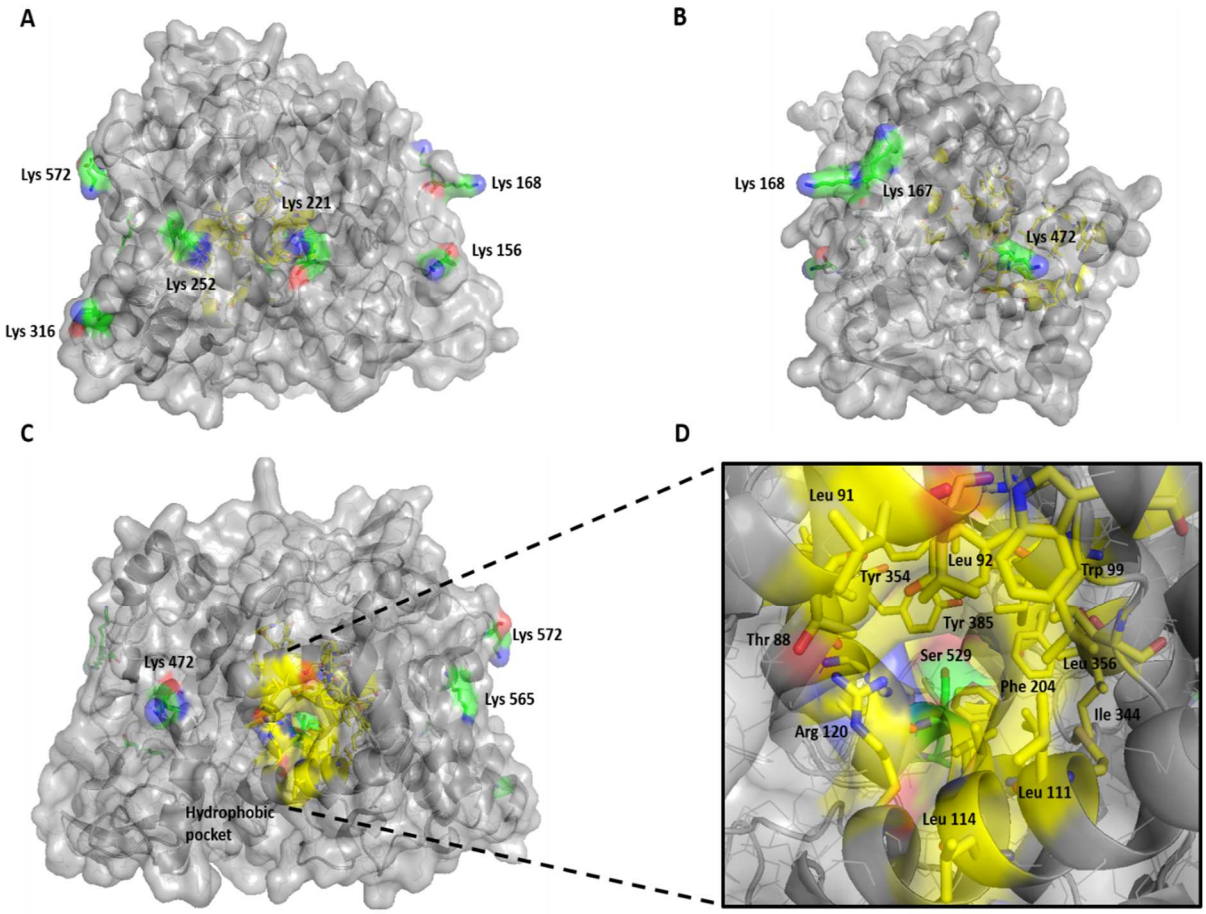

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Chapter 7

DISCUSSION, PERSPECTIVES AND CONCLUSIONS

The presented thesis project aimed at describing the biological relevance of non-enzymatic glycation as a consequence of unbalanced glucose levels, mainly focusing the attention to the protective role of aspirin-induced acetylation in preventing the propagation of this detrimental process in hyperglycaemic conditions and in patients with diabetes mellitus.

After a first description of a comprehensive method for large-scale characterization of glycated proteins in different complex samples (chapter 2), we extended the entire study project to the analysis of the mutual impact between protein glycation and aspirin-mediated acetylation *in vitro* and *in vivo*. Human serum albumin (HSA) was used as a model protein to assess the feasibility of a mass spectrometry (MS) label-free approach for the identification and quantification of glycated and acetylated sites (chapter 3). Then, the same experimental strategy was applied for the analysis of *in vitro* induced glycation and acetylation of proteins from the main blood compartments, including plasma (chapter 4) and erythrocytes (red blood cells, RBCs) (chapter 5). Finally, the specific effect of aspirin-induced acetylation on platelet proteins from diabetic patients was reported, with a special focus on COX-1 protein and its functional consequences (chapter 6).

This concluding chapter will encase the thread that runs through the overall results presented in this thesis manuscript, and provide potential hints for future developments on the molecular mechanisms underlying the pathophysiology of diabetes.

1. Predictive method to unravel the dynamism of human glycated proteome

Chronic hyperglycaemia is now considered as a significant promoting factor of non-enzymatic glycation in a large number of protein tissues, being one of the potential leading cause of glucose toxicity in diabetes. The effects on biological function strongly depends on the dynamic nature of protein glycation, which in turn is related to the uncontrolled fluctuations of blood glucose levels. Monitoring glycaemic state through the measurement of glycated haemoglobin (HbA_{1C}) represents the primary strategy for diagnosis and long-term follow-up of diabetes. Based on the assumption that any protein could be potentially glycated, the discovery of novel protein biomarkers other than HbA_{1C}, is paramount in identifying tissues particularly affected by the glycation process, even before functional

impairment. Moreover, the overall profile of glycated proteins represents a more exhaustive indicator of the glycaemic state of a given patient. We therefore characterized the extent of glycation in biologically different samples using a qualitative and quantitative analytical strategy based on differential modification of proteins with isotopically labelled glucose. The main advantage of this approach relies on the chemo-selectivity of the glycation process that is extremely useful both to confirm the presence of a glycated peptide and to measure its relative abundance. The presence, at MS1 level, of a doublet signal (spaced + 6 Da per glycation site) for each glycated peptide, enhances the confidence of detection and subsequent identification. The high accuracy provided by Orbitrap acquisition is thus fundamental to achieve this goal, in order to avoid any overlapping peak that can eliminates the information on glycation detection provided by the labelling step. Furthermore, peptides labelled with isotopic $^{13}C_6$-glucose create an internal normalization system for the measurement of the native level of glycation from samples in different glycaemic states. A combinatorial MS approach based on HCD-high resolution MS2 and CID-neutral-loss triggered MS3 modes was proposed here for qualitative analysis of glycated proteins from plasma, red blood cells and cerebrospinal fluid. The strength of HCD-MS2 mode relies on its faster and highly energetic fragmentation than CID, resulting in a predominance of informative y-ions that, coupled with the high accuracy and sensitivity of fragment ion detection, make of it a good alternative to ETD to increase the identification rate of glycated peptides. This is particularly true if Glu-C is used as protease for the protein digestion step, since this enzyme produces longer peptides (up to 40 residues) compared to those generated by trypsin (up to 15 residues) due to the low incidence of glutamate residues in proteins. These large highly charged peptides (3+ and 4+) are well suited for HCD fragmentation more than CID (2+), allowing the increase of precision in sequence assignment. Another important benefit from HCD-MS2 is based on the detection of diagnostic immonium ions in the low-mass-range of the MS2 spectrum, from glycated lysine and arginine residues. These ions are, to all intents, specific markers that can be used to assess the level of native glycation of target samples, which in turn reflects their glycaemic state. The utility of these ion species can be evidenced by the comparison of the MS2 extracted ion chromatogram profiles of glycated lysine immoniun ion (192.102 Da) from normoglycaemic (5% HbA_{1C}) and severe chronic hyperglycaemic (> 11% HbA_{1C}) individuals. Since each glycated immonium ion roughly corresponds

to a glycated peptide, the appearance of multiple signals reflects the increased background glycation typical of the hyperglycaemia state. CID neutral loss MS3 is another way to estimate the level of native glycation due to the selection selectivity of those glycated peptides that undergo to neutral loss upon the first activation step. Further fragmentation lead to the generation of MS3 spectra specific for each glycated peptide, from which a total ion MS3 chromatogram can be extracted to compare different glycaemic states. A clear difference in the global MS3 chromatographic profile is evident when comparing normoglycaemic with hyperglycaemic states, and particularly when looking at the increasing intensity of the peptide signal containing the glycated valine of haemoglobin β chain at higher glycaemic states. It is worth to note that, since the fulcrum of glycation analysis relies on the specificity of glycated peptides detection, neutral-loss MS3 approach is effectively more suited than multi-stage activation method, in which the high sensitivity is compensated with a low specificity[1]. The glycaemic state can be inferred by measuring the background glycation in relative terms through incubation of target sample with $^{13}C_6$-glucose. In vitro labelling with $^{13}C_6$-glucose offers a normalized view that can be used to estimate the extent of native glycation at each potential attachment site between different glycaemic states. In addition, this approach has proven to be useful for the elucidation of new glycation targets in samples of different nature like plasma[2], RBCs[3] and cerebrospinal fluid[4], highlighting the wide tissue distribution and dynamism of this PTM and the importance to characterize, and specially prevent, its toxic systemic effects. The mere characterization of the glycated protein profiles from different biological samples, however, can provide only limited and indirect information on the potential effect of aspirin in preventing glycation. A deeper analysis of the acetylation pattern of proteins target of aspirin is thus mandatory, in order to improve our knowledge on the mazy mechanism underlying the mutual interaction between aspirin-induced acetylation and protein glycation.

2. The mutual competition between glycation and aspirin-mediated acetylation on human serum albumin (HSA)

The protective role of aspirin over protein glycation has long been evaluated on a small number of reference proteins such as lens crystallins[5], haemoglobin[6], collagen[7] and fibrinogen[8]. Most of these studies have shown that aspirin can affect the extent of protein glycation, reducing all the structural

alterations associated with this process. In addition, glycation of platelet protein was shown to interfere with their acetylation level[9], confirming once again the strong biological impact between these two PTMs. Although a large burden of evidences have suggested that the interaction between aspirin-induced acetylation and glycation occurs through a competitive process, the exact interplay between these two non-enzymatic PTMs is far from being elucidated. The main objective of this project intended to perform a comprehensive *in vitro* study in which HSA was used as model protein to identify the preferential acetylated and glycated protein sites and to quantify them by a label-free approach as an alternative to the stable isotope labelling. In order to keep the overall MS strategy as homogeneous as possible for the analysis of both acetylated and glycated peptides, we excluded the CID neutral loss MS3 mode and used only the HCD-MS2 mode for fragment ion acquisition because of two main reasons: 1) we observed that the rate of identification of glycated sites in HCD-MS2 is greater (88%) than CID neutral loss MS3 (36%), with a 67% overlap between the two modes, showing that HCD-MS2 mode alone is able to cover the entire glycated profile of HSA; 2) it is well known that acetylation is highly stable upon CID, thus neutral loss MS3 will be useless for this specific modification, while HCD-MS2 is more informative in terms of sequence coverage. The first point of the analysis was to characterize the acetylation profile of HSA in presence of a growing aspirin concentration. Native acetylation of HSA was ascribed to only one site (2.8%) while a rapid increase to 16.7% and 27.8% of acetylated residues were found at very low and middle-low aspirin concentration, respectively. These residues are supposed to be highly reactive towards aspirin, even at low doses, because of their location at the solvent-accessible protein surface or due to the low pK_a of their side chains. Examples of such kind residues are Lys 519 and Lys 199, which were previously reported to be acetylated by aspirin, though not at such low doses[10,11]. At middle-high and very high aspirin concentrations the level of acetylated sites increased to 50% and 88.9%, respectively, albeit this is less likely to occur *in vivo* since the severe toxic effects of aspirin above 3.6 mM[12]. A relevant point of this study was to measure the extent at which aspirin and glucose influence each other on HSA. For this purpose, we used an aspirin concentration 20 times higher than the physiological plasma levels and an incubation period 50 times longer than the half-life of aspirin in plasma in order to speed-up the acetylation process and to obtain a quasi-saturated state of the protein; in such way, any variation of the acetylation level would be

confidently attributed to the direct effect of glucose. We observed that in presence of 10 mM glucose, the total acetylation level of HSA drastically decreased compared to aspirin incubation alone, as evidenced by Western blot and by label-free quantification at site level. Similarly, the quantification of glycated HSA sites in presence of aspirin showed a significant decrease in their extent compared to glucose incubation alone, demonstrating the protective role of aspirin over glycation. The influence of aspirin on glycation is also associated as well to the variation in the glycation content of HSA in its native state (45.5%), which increased after glucose incubation (81.8%) and decreased in presence of aspirin (63.6%). Taken together these results confirm the hypothesis of a mutual competitive effect between aspirin-acetylation and protein glycation in which in one hand glycation reduces the reaction of aspirin with proteins and in the other hand aspirin prevents further protein glycation. Moreover, this approach allowed us to identify novel preferential glycation sites on HSA, besides those already reported in previous studies[13-15], and found that the large majority of them were strongly affected by aspirin. Finally, we cannot exclude the impact that glycation and acetylation could have on HSA structure, and consequently on its biological function. Indeed, most of the acetylated and glycated sites found in this work lie on solvent-exposed pockets of the protein involved in the binding with drugs and metabolites, while others sites are involved in its anti-oxidant activity[16,17]. In this pilot study, the mutual impact between aspirin's effects and glycation was assessed on a single reference protein which was shown to have a strong biological importance, however more consistent information can be drawn from samples which complexity reflects the biological variability associated to *in vivo* systems.

3. Impact of glycation and aspirin-induced acetylation on human blood proteins

Human blood represents the first microenvironment where the interaction between drugs, metabolites, proteins and cells takes place and, indeed, it can be considered as the "mirror" of the general status of the organism. The dynamic nature of blood makes it an ideal sample of study for biomarker discovery, proteomic analysis and PTMs characterization. Obviously, this is a no-trivial task when considering the complexity of blood composition and the wide dynamic range that characterize the proteomes from its different compartments. Several efforts have been made to characterize the extent of glycation in blood in order to determine the precise role that this PTM has on proteins belonging to specific haematic

components and its relationship with the severity of diabetes. As repeatedly shown, aspirin plays a key role in the inhibition of glycation, as evidenced by its protecting effect on two major blood proteins, HSA and haemoglobin[18], while on the other hand, its acetylating action seems to be hampered in glycated platelet proteins[9]. A mutual competition between aspirin-acetylation and glycation has been suggested, however the exact mechanism by which these two PTMs influence each other has never been shown on blood proteomes. The objective of this project aimed to perform a large-scale analysis based on the characterization and quantification of aspirin-induced acetylation and non-enzymatic glycation on the main blood compartments, and specifically on plasma, RBCs, WBCs and platelets. In this study, the protein extracts from the different blood fractions were *in vitro* incubated with 30 mM glucose followed by 500 μM aspirin exposure. The choice to use such high glucose concentration is justified by the low kinetic reaction (days to weeks) of the glycation process under physiological conditions (5-11 mM glucose) and to compensate to the short incubation time (24 h) employed to induce extensive protein glycation. On the other hand, a concentration of 500 μM of aspirin was selected because is within the range of the typical *in vivo* therapeutic levels (0.1-2 mM)[19] and 30 min time exposure encompass the half-life of aspirin in plasma. The qualitative and quantitative characterization of glycated and acetylated sites on plasma proteins confirmed the protective effect of aspirin to reduce glycation in most of detected glycated proteins and, furthermore revealed that glycation, in some way, may favour acetylation by aspirin on specific plasma protein sites. HSA was shown to be the most affected protein by this process, since its high abundance in plasma, followed by immunoglobulins, complement C3, apolipoprotein A2 and fibrinogen. Interestingly, all these proteins were previously shown to be highly glycated in plasma from diabetic patients[20,21], highlighting the correlation between glycation and the alteration of the protein structure[22-24]. In this sense, the hypothesis that glycation may induce allosteric conformational changes that expose previously buried sites for acetylation cannot be excluded. Likewise, acetylation may alter the secondary structure of a certain glycated protein region, unbalancing the dynamic equilibrium between stable and unstable glycated sites and consequently leading to the loss of the energetically un-favoured glucose adduct. If experimentally confirmed, this

potential mechanism may be a valid alternative to the well accepted theory based on the direct competition between aspirin and glucose.

A similar trend was also evidenced in RBC proteins including different haemoglobin subunits (α, β and δ), carbonic anhydrase 1, peroxiredoxin isoforms and superoxide dismutase to cite some of them. The glycation level of the quasi-totality of RBC protein sites showed a significant decrease in presence of aspirin, while the acetylation state of several of them manifested a clear increase in presence of glucose and aspirin compared to aspirin incubation alone, as shown for carbonic anhydrase 1 and haemoglobin α and β subunits. In addition to the mechanism mentioned above, we found that both acetylation and glycation are favoured by the presence of an acidic (aspartic acid and glutamic acid) and/or a basic (lysine) amino acid in the spatial proximity of the modification site either in the primary or in the tertiary structure of the protein, confirming the importance of these residues for the modification by aspirin and glucose[25]. Another interesting implication of this study derive from the effect that aspirin exerts on the glycation state of the N-terminal valine of haemoglobin β chain.

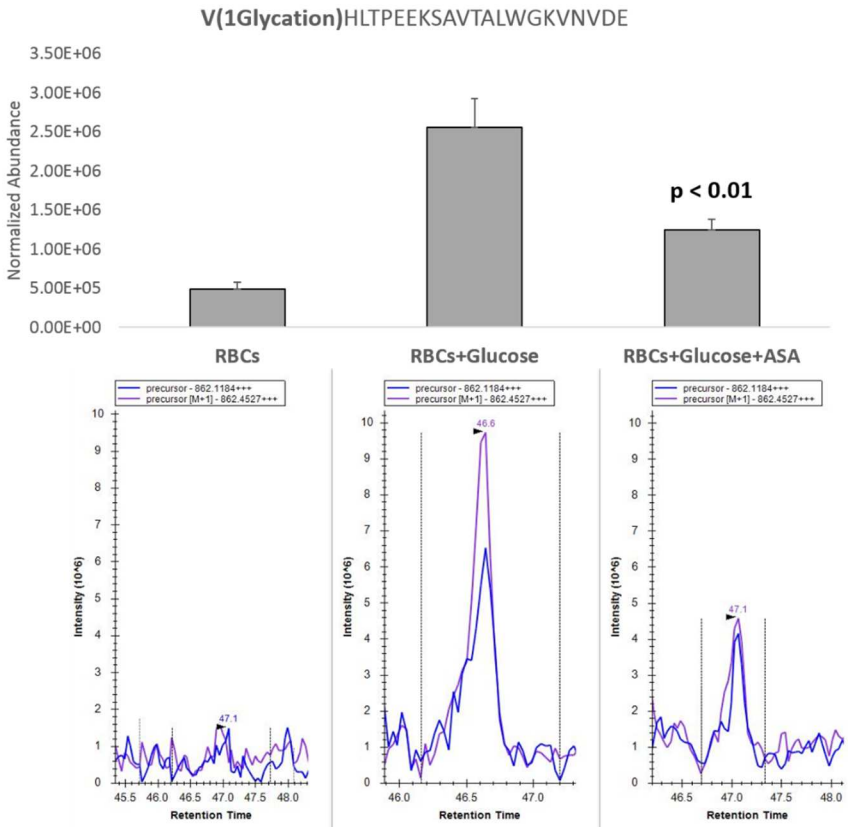

Figure 8. Impact of aspirin on the glycation state of N-terminal Val of haemoglobin β chain. At the top, the bar plot shows the significant decrease of the glycation level of this residue. At the bottom, the XICs of the same glycated peptide are shown.

The clinical measurement of the glycated form of this amino acid currently represents the gold-standard for the determination of HbA_{1C} levels, which correlates with the mean plasma glucose levels for the diagnosis and follow-up of diabetes[26]. However, discordances between HbA_{1C} levels and other measurements of glycaemia, like the fructosamine assay, pinpoint the existence of a "glycation gap" that lead to the misinterpretation of the effective glycaemic state[27]. This divergence seems to be related to various factors such as the presence of haemoglobin variants that can bias the HbA_{1C} measurement[28], and drug interaction[29]. The latter could be the particular case observed in this study, since we have shown that aspirin decreases the glycation level of N-terminal valine by 33% (Figure 8), likely contributing to the underestimation of HbA_{1C} levels for those subjects taking aspirin for primary and secondary prevention of cardiovascular complications in diabetes.

Furthermore, the mutual impact between aspirin and glucose was assessed on WBC and platelet proteins. To date, the relationship between diabetic vascular complications, glycation and WBCs has been mostly ascribed to the enhanced inflammatory response induced by the excessive activation of RAGE receptors after the binding with circulating AGE-modified proteins[30]. Aspirin was shown to alleviate this phenotype through the transient acetylation of COX-2, turning the activity of this enzyme from the production of pro-thrombotic and chemiotactic cytokines to the generation of anti-inflammatory lipoxins[31-33]. Nevertheless, the direct effect of glycation and aspirin-acetylation on WBCs proteins has never been demonstrated. Contrary, *in vivo* and *in vitro* studies supported the evidence that glycation on various platelet proteins make them less susceptible to aspirin, and the resultant acetylation becomes greatly reduced[9,34]. Despite the relevance of these works, few information on the specific platelet proteins, targets of glycation and acetylation, as well as the identification of the preferential glycated and acetylated sites and their quantification have been shown so far. In this study, a total of 58 proteins corresponding to 76 sites in WBCs and more than 100 proteins corresponding to 153 sites in platelets, were found to be both acetylated and glycated. For both WBCs and for platelets, aspirin-mediated acetylation reduces the level of glycation for most of the quantified glycated sites, while for others no significant aspirin effect was evidenced. Similarly to what we observed in plasma and RBCs, glycation seemed to favour acetylation by aspirin for several acetylated sites, while for others it reduces or has no impact on their acetylation state. Modification by aspirin and glucose was found to occur at

the same residue in 52 % of WBCs proteins and 73 % of platelets proteins. Gene ontology (GO) analysis of the glycated/acetylated proteins evidenced a significant enrichment ($p < 0.001$) of functional terms associated to inflammation and platelet activation/aggregation for WBCs and platelets, respectively (Figure 9).

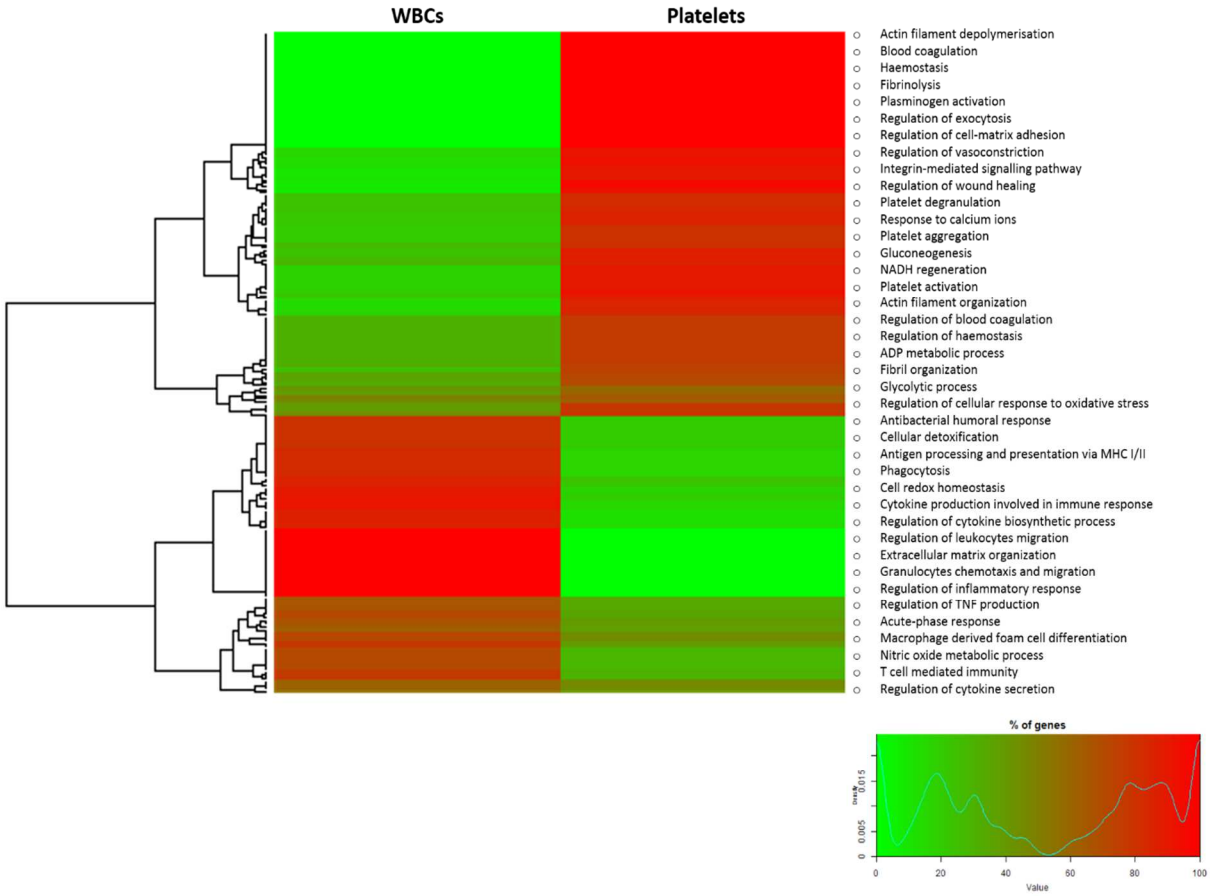

Figure 9. Heat map representing the GO terms of platelet and WBC proteins found to be both glycated and acetylated. Colour coding corresponds to the percentage of proteins enriched for each functional pathway.

Taken together these results highlight for the first time a direct influence of glycation and aspirin-mediated acetylation on WBC and platelet proteins; the fact that most of those proteins are involved in inflammatory and pro-thrombotic pathways, may indicate new potential mechanisms associated to the development of diabetic vascular complications, which can be modulated by non-enzymatic glycation and aspirin-mediated acetylation.

4. Diabetes reduces the aspirin-mediated acetylation of COX-1 serine 529 residue and affects the acetylation level of other platelet proteins.

Among the different consequences of hyperglycaemia, excessive platelet activation was shown to play a key role for the development of diabetic cardiovascular complications[35]. Myocardial infraction and ischemic stroke represent in fact the main causes of morbidity and mortality in diabetes due to their strong association with atherosclerosis and with an increased inflammatory response[36]. Despite the spread out of new generation anti-platelets agents[37], aspirin still remains the most widely used drug for long-term anti-platelet therapy in patient with diabetes[38]. However, platelets from diabetic patients seem to be less sensitive to aspirin and the mechanism underlying this low responsiveness could be ascribed to an accelerated platelet turnover[39], an increased oxidative stress[40] or to an uncomplete inhibition of COX-1. Glycation could be a leading cause for the less-than expected inhibition of COX-1 by aspirin, as recently suggested by the evidence that glycation on specific COX-1 sites interferes with their acetylation levels in a competitive way[41]. However, it is well known that the negative modulation of COX-1 activity by aspirin relies on the irreversible acetylation of the catalytic serine 529 residue and, to the best of our knowledge, no information on the direct effect of glucose on the acetylation state of this crucial site has been reported yet. In this final part of this project, we investigated the impact of *in vivo* diabetes on the *in vitro* aspirin-induced acetylation of the overall platelet proteins, with a particular emphasis for COX-1.

It is worth to highlight that the analytical strategy used in this study was slightly different from that one performed in the previous works: we reintroduced the use of trypsin as proteolytic enzyme instead of Glu-C, because in this special case tryptic peptides yield the greater protein sequence coverage; moreover, MS acquisition was carried out exploiting the much higher resolving power, accuracy, sensitivity and scan speed of a new generation instrument, that make it ideal as well, for targeted analysis.

We found that the majority of differentially acetylated sites belong to proteins associated to blood coagulation and platelet activation functional pathways. Among them, some integrin receptors, coagulation factors, and enzymes like PI3K were already reported to play a key role in platelet

dysfunction in diabetes[42,43] and may contribute to the platelet hyper-reactive phenotype frequently observed in diabetic patients, with and without aspirin treatment. Beside the thousands of platelet protein sites found to be target of aspirin-mediated acetylation, we identified 7 preferential acetylated residues on COX-1, including serine 529. Using a targeted MS approach named parallel reaction monitoring (PRM), the acetylation level of this particular site was shown to be reduced in diabetic patients compared to healthy controls, while no significant changes were found for the other sites. These findings indicate that chronic hyperglycaemia hampers, to some extent, the acetylation of serine 529 by aspirin, leading to a reduced inhibition of COX-1 activity and consequently to increased levels of TXA_2, in line with previous reports[44,45]. These findings suggest that increasing glucose concentrations was associated with a proportional decrease of aspirin-induced inhibition of COX-1 activity and contribute to platelet hyper-reactivity in diabetic patients taking aspirin. Although our results identify this phenomenon with diabetes and with high glucose levels, a fundamental question still remains: how does glucose hampers the aspirin-induced acetylation of COX-1 serine 529, making this protein less responsive to aspirin? Molecular docking simulations provided us a likely interpretation on the dynamic of this process, showing that glucose can pose at the entrance of the pocket at the bottom of which serine 529 is located, and interact with the arginine 120 and tyrosine 354 residues through polar contacts. Since the first step of COX-1 acetylation relies on the non-covalent interaction between aspirin and the arginine 120, the occupancy of glucose may hinder this binding, contributing to the reduced acetylation of serine 529. Apart from this potential model, other mechanisms might underlie the reduced effect of aspirin on COX-1 in chronic hyperglycaemia conditions, opening new perspectives for the improvement of our understanding on the pathological role of glucose on platelet hyper-reactivity in diabetes.

In conclusion, the complicated interplay between the effect of glucose and aspirin-mediated acetylation has been extensively characterized *in vitro* and *in vivo* on different types of biological conditions, starting from single model proteins, moving to more complex samples and ending with a clinical study. Throughout this translational project, we encountered several experimental “crossroads” that have

spawned new possibilities for facing the biological problem under investigation and improving the quality of the analysis.

5. Perspectives

The determination of the dynamic profile of the glycated proteome in human plasma, red blood cells and cerebrospinal fluid provided us qualitative and quantitative information in relative terms (chapter 2). The approach based on the differential labelling with $^{13}C_6$-glucose coupled to HCD-MS2 and CID-neutral loss MS3 methods, allowed characterizing the preferential glycation sites as a function of glycaemia level. Although this method gave interesting results, a complementary MS strategy based on ETD fragmentation could be useful to further increase the confidence of glycated peptide analysis. It is worth to note that ETD is outperformed by HCD for peptide identification (especially for charge states lower or equal to z = 4+)[46], however its non-ergodic nature makes of ETD the method of choice when dealing with labile PTMs, like glycation. Compelling evidences supported the effectiveness of this activation mode to improve the identification rate of glycated proteins in plasma and RBCs from diabetic patients[25,47]. Moreover, the preference of ETD fragmentation for highly charged peptides ($z \geq 3+$), as those produced after Glu-C digestion, could represent a valid alternative to CID in the second dissociation step of the neutral loss MS3 mode, in order increase the confidence of glycated peptide identification.

Beside the relevance to elucidate the role of glucose in protein modification, an important objective of the study was to characterize the mutual influence of aspirin-induced acetylation and protein glycation. Stable isotope labelling with "heavy" glucose provided the particular advantages to localize the presence of native glycated peptides, by the mass spacing of +6 Da in the MS1 spectrum, and to quantify their glycation level. However, this approach allows only the indirect measurement of the effect of aspirin on native glycation, with no qualitative/quantitative information on the potential acetylation sites target of aspirin. An interesting solution to overcome this issue would be the differential labelling of samples with the heavy isotopic form of aspirin, namely $^{13}C_2$-$^{2}H_3$-aspirin that, due to its chemo-selectivity, can attach the preferential acetylation sites on proteins. The distinction between the *in vitro* and the endogenous acetylation would be detected by the characteristic doublet signal separated by a

mass shift of +5 Da in the MS1 survey scan. Similarly to what occurs with heavy glucose labelling, heavy acetylated peptides can be considered as an internal standard for relative quantification of the acetylation levels of biological samples under different conditions. The integrative dual labelling with glucose and aspirin might thus represent a promising approach for the follow-up of aspirin treatment in diabetes.

The excellent advances in MS analyzers capable of high resolution, scan speed and thus sensitivity, make them perfect tools for PTMs analysis. However, due to the low abundance of modified proteins, an enrichment step during sample preparation is often mandatory to maximize the yield of PTM-peptides identification. This is particularly true for glycation since low reaction kinetic of this process makes it one of the most challenging PTM to be detected in complex samples. Contrarily, aspirin is a highly efficient acetylating agent capable to acetylate its target in a very short time frame (minutes compared to days of the glycation process), making the amount of acetylated peptides higher than that of glycated ones in samples not subjected to enrichment (e.g. 43% of acetylated plasma proteins v.s. 4% of glycated plasma proteins). It is worth to remind that the extent of cellular acetylation catalysed by acetyltransferases is much lower (1% - 2%) than the non-enzymatic reaction mediated by low-dose aspirin, thus an enrichment method would be necessary especially for this class of samples. Although we have been successful in the identification of several acetylated proteins in different no-enriched samples, fractionation of acetylated peptides would certainly improve the yield of the analysis. Antibody-based affinity purification could be an attractive approach to reduce the sample dynamic range and to ensure the identification of a larger percentage of acetylated proteins[48]. However, the small acetyl group has a low antigenicity, and thus it will be challenging to produce a high-affinity antibody. Despite this limitation, immune-affinity purification remains the most used enrichment technique for the analysis of protein acetylation[49-51], and thus could represent a valid tool for further *in vivo* studies.

The analysis of the synergic influence between acetylation and glycation, carried out in the large part of this project, employed an experimental design based on the *in vitro* incubation with aspirin and glucose of protein extracts of the main blood compartments, with aspirin and glucose. Although we tried to mimic as best as we could *in vivo* conditions, a validation of our results in clinical samples would be needed. The analytical methods presented in this project could be used to determine the extent

of chronic hyperglycaemia-induced glycation on blood proteins from diabetic patients, and to measure the level at which it changes before and after aspirin administration. Furthermore, a glycation fingerprint based on the presence of a specific diagnostic ion pattern at MS1 and MS2 level, can be derived from each blood component under certain conditions. These profiles can be used as standard for the correlation of the glycated state of each individual with their glycaemic level. In addition, the acetylation profile of specific protein markers could be used as indicator of the effectiveness of aspirin treatment in preventing glycation of different key proteins related to diabetes complications. An example is illustrated by the study of platelet COX-1 which acetylation level at serine 529 residue was shown to be reduced in presence of *in vitro* high glucose and in patients with diabetes, after *in vitro* incubation with aspirin. As stated in chapter 6, this specific site is of fundamental importance since it represents the primary target of aspirin for COX-1 inhibition, which acetylation state correlates with the decreased platelet production of TXA_2. The MS targeted method developed in our laboratory allowed the detection and quantification of the peptide containing this acetylated site by a label-free approach. A preferred option, needed to extend this methodology to clinical applications, would be the use of absolute quantification (AQUA) using the synthetic heavy peptide that contains the serine 529 site in its acetylated state, as internal standard. In this sense, platelet protein extracts from patients taking aspirin can be digested and spiked with the synthetized acetylated peptide in order to provide the absolute level of acetylation on this residue. The automatization of this analytical workflow could lead to significant improvements on the follow-up of aspirin treatment in patients suffering of cardiovascular diseases and in diabetes.

The final part of this study mainly relies on the understanding of the molecular mechanisms by which glucose hampers the acetylation of COX-1 serine 529 residue by aspirin. A model generated by molecular docking predictions evidenced that glucose can affect the first step of the interaction between aspirin and COX-1 (chapter 6), however other potential dynamics can be involved in this process. One of them could be ascribed to the direct glycation of those amino acid side chains that surround the catalytic pocket of COX-1, hindering the access of aspirin. The elucidation of the preferential glycation sites in COX-1 was already performed in our laboratory, using high *in vitro* glucose concentrations as preliminary test, without evidencing any glycated residue in the internal active sites of the enzyme.

Despite glycation was not found in this hydrophobic channel, several glycated sites were detected to be widely distributed on the solvent-exposed surface of the protein, raising a new fascinating hypothesis of its role in COX-1. As evidenced by Bakhti et al., glycation can alter the conformation of the haemoglobin structure[6]; if this is also valid for other proteins, it could be true for COX-1 as well. Glycation of residues spatially distant from the catalytic site of COX-1, might induce allosteric conformational changes that can modify the tertiary rearrangement of different protein regions, including the active site. A MS-based approach for probing structure and dynamics of proteins, named hydrogen deuterium exchange-MS (HDX-MS), represents the ideal tool to track these potential conformational changes[52] (Figure 10).

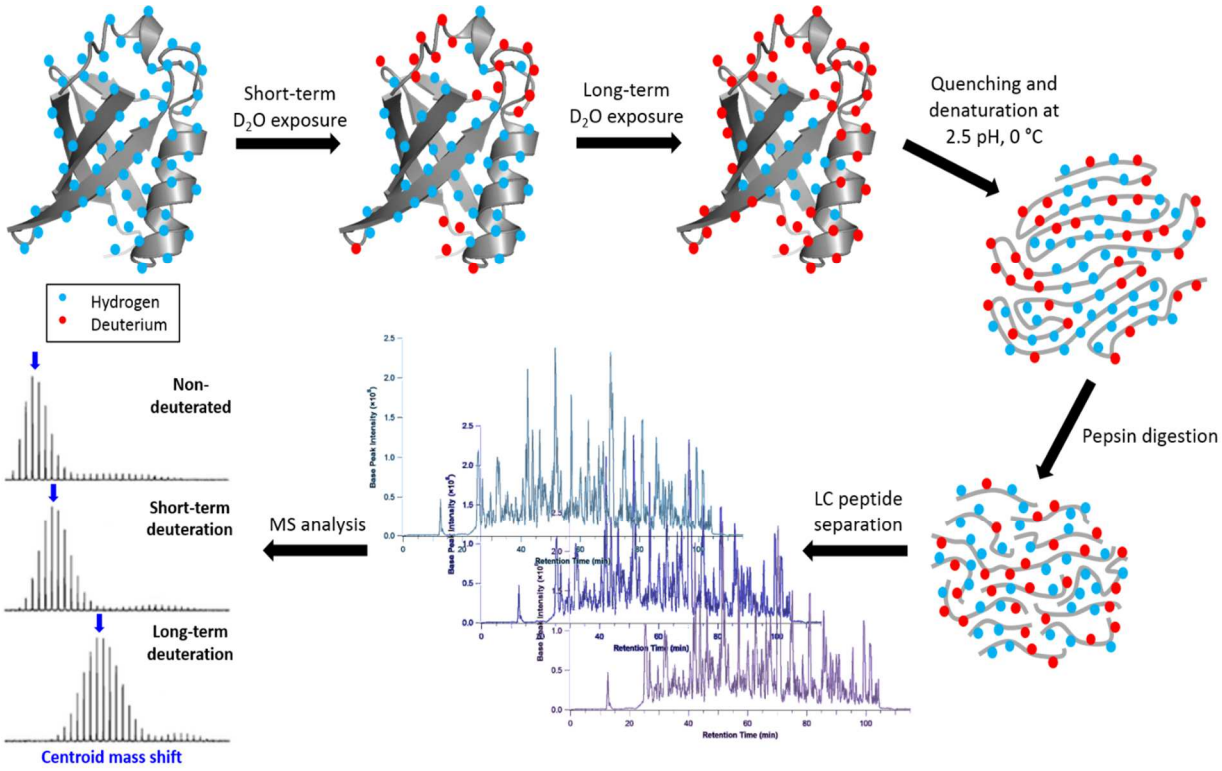

Figure 10. Hydrogen deuterium exchange mass spectrometry (HDX-MS) workflow.

This technique is based on the exchange of amide hydrogens in protein backbone with solvent deuterons, at rates that are mostly dependent on their involvement in secondary structure elements and their solvent accessibility. Amide hydrogens located on the surface of the protein and those that are not involved in the secondary structure exchange more rapidly than those buried, or H-bonded or making part of the interface between two interacting proteins. Under identical condition of pH and temperature,

the rates of exchange can be compared between samples upon varying stimuli, whereas a greater exchange levels indicate less protection and therefore a more exposed region. In this sense, the measured rates are primary dependent on conformational changes and can be sensitively and accurately detected and quantified by MS. This would provide information on protein structure, subunit interaction, membranes and small-molecule binding site, as well as differential conformations induced by mutations and the presence of PTMs[53,54]. In our study, structural dynamics of human COX-1 could be assessed in presence of aspirin and glucose in order to pinpoint the potential protein regions that undergo to major changes upon acetylation and glycation. The combination of HDX-MS, high resolution MS data and predictive approaches based on molecular dynamic simulations will allow for a much more detailed view on the molecular mechanism by which glucose interferes with the normal effect of aspirin on COX-1 in diabetes.

6. Conclusion

Chronic hyperglycaemia is the main risk factor for the development of diabetic complications. Non-enzymatic glycation plays a pivotal role in this scenario through the alteration of protein structure/function and the enhancement of inflammatory and pro-thrombotic pathways. Aspirin was shown to partially prevent this detrimental process through its acetylating effect, although the exact understanding of the interplay between protein glycation and aspirin-induced acetylation is far from a clear comprehension. The MS-based proteomics approaches used in the present work provided fundamental key results to improve our knowledge on the role of these two PTMs in diabetes and in its aspirin treatment. This has been performed at three experimental levels: 1) the analysis of glycation and its dynamics on cellular extracts and body fluids; 2) the characterization of the mutual influence between *in vitro* glycation and aspirin-mediated acetylation on blood proteins; and 3) the impact of chronic hyperglycaemia on the aspirin function, in platelet proteins from diabetic patients.

The qualitative and quantitative approaches developed for the analysis of glycated proteins allowed the determination of a comprehensive glycation profile for each biological sample, with information on the preferential glycation amino acid sites as a function of the level of glucose. This method was then slightly modified for the analysis of glycated and acetylated proteins in plasma proteins, RBCs, WBCs

and platelets. Finally, the level of aspirin-mediated acetylation was extensively measured in a large number of platelet proteins from diabetic patients, focusing the attention on the response of COX-1 to aspirin. Our results indicated that chronic hyperglycaemia strongly affects COX-1 acetylation, leading to a reduced aspirin-induced inhibition of the enzyme activity, partially explaining the poor biological effectiveness of aspirin in the diabetic population.

This work might be considered as the first step towards a better understanding of the molecular mechanisms underlying the mutual interplay between protein glycation and aspirin-mediated acetylation and its impact on protein function. Furthermore, it lays the foundations for future clinical applications aimed to the profiling of glycated proteins in different tissues and bio-fluids and to determine the effects of daily-dose aspirin treatment on the evolution of the detrimental cardiovascular complications associated to diabetes.

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