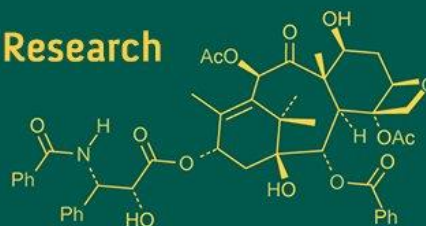
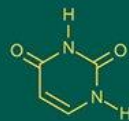
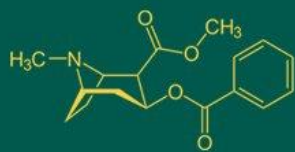


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Biochemistry of an allosteric protein: From normal human hemoglobin to its post-translational glycosylated component as marker of type 2 diabetes mellitus: A focus on several medical implications of this altered protein

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Abstract

Hemoglobin is the erythrocyte protein that transports O₂ from the pulmonary alveoli to the tissues and CO₂ in the reverse direction. The mechanism by which this transport is accomplished is highly intriguing. Hemoglobin is composed of four structured polypeptide chains and four heme groups, one attached to each of them, respectively involved in the coordination of an O₂ molecule. The bond between O₂ and hemoglobin is cooperative, meaning that the binding of an O₂ molecule to a heme group of a hemoglobin polypeptide chain increases the binding affinity between another O₂ molecule and another polypeptide chain of the same hemoglobin. Hemoglobin is subject to post-translational modifications, particularly glycation with glucose or its metabolites, which partially alters its biological properties, making it less able to perform its task. High levels of glycosylated hemoglobin, especially in the form of HbA1c (adduct of hemoglobin with glucose), are a marker of the abnormal blood glucose level in the blood known as diabetes mellitus. Glycosylated hemoglobin and diabetes mellitus are also associated with the onset of several serious disorders, from eye to heart disease.

Keywords: HbA1c, hemoglobin, heme, oxygen, cooperativity, diabetes, glucose

Introduction

Oxygen transport and storage: hemoglobin versus myoglobin

The transition from anaerobic to aerobic life was a fundamental stage in evolution because it allowed higher organisms to utilize a large energy reserve derived from molecular oxygen (O₂) collected through pulmonary respiration. Vertebrate organisms have developed three mechanisms to provide their cells with a continuous and adequate flow of O₂. The first is the respiratory system, the set of organs that facilitates the reciprocal exchange of gases (O₂ and CO₂) between the organism and the external environment (from the nose to the lungs and vice versa). The second is the circulatory system, consisting of the heart, blood vessels, and blood, which transports O₂ directly from the lungs to all cells of the body and expels CO₂ as a byproduct of cellular catabolism. Without the circulatory system, the mobilization of O₂ would be too slow to meet the body's needs. The third is the development of O₂-transporting proteins: hemoglobin ^[1] and myoglobin ^[2], which overcome the limitation imposed by the poor solubility of O₂ in the water that composes the liquid part of the blood (plasma). Hemoglobin, contained in a portion of the cells that make up the corpuscular component of the blood, the red blood cells, serves as an O₂ transporter in the blood, increasing the transport rate fifty-fold compared to what could be achieved by spontaneous diffusion through the blood, in addition to providing assistance in the transport of CO₂ and H⁺ (see below). The binding of hemoglobin to these chemical species is favorably regulated by allosteric interactions, from the ancient Greek words *allos*, "other", and *stereos*, "solid", that is, between spatially distinct sites and transmitted by conformational changes of the subunits that constitute this protein. Hemoglobin, in fact, belongs to the class of allosteric proteins and is a subject to the phenomenon of cooperativity, as described below. In aerobic metabolism, one O₂ molecule is consumed for every CO₂ molecule produced, and hemoglobin participates directly in its transport from the tissues to the lungs by forming a carbamate bond between CO₂ and a terminal α-amino group present in each of its four polypeptide chains.

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The CO₂-transporting capacity of deoxyhemoglobin (*i.e.*, devoid of O₂) is approximately three times greater than that of oxyhemoglobin (*i.e.*, to which O₂ is bound). Myoglobin, found in muscle (skeletal and cardiac), serves instead as an O₂ reserve in the same tissue, facilitates its movement in case of need and is not involved in CO₂ dislocation. The ability of these transporter proteins to bind O₂ is due to the presence of a non-protein unit, the prosthetic group known as the heme group, which consists of an organic part [3] and an Fe²⁺ ion [4] (The Fe atom can naturally be found in the 2+ and 3+ oxidation states; the heme group is able to bind both forms of Fe, but only the presence of Fe²⁺ allows the heme group to bind O₂). The organic part is a protoporphyrin IX, composed of four pyrrole groups joined by methine bridges to form a tetrapyrrole ring to which are linked, according to a rigorous isomerism, four methyl groups, two vinyl groups and two propionic groups (Figure 1).

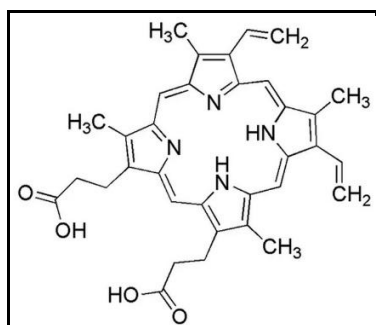


Fig 1: Structure of the precursor of heme: protoporphyrin IX. Biosynthesis of heme is an eight-step process that occurs partly in mitochondria and partly in the cytoplasm. Protoporphyrin IX serves as the substrate for ferrochelatase, the final enzyme in heme biosynthesis, which catalyzes the entry of the Fe²⁺ into the protoporphyrin IX to form heme. Protoporphyrin is ubiquitously present in small amounts as a precursor of heme, particularly in red blood cells becoming part of hemoglobin in the context of O₂/CO₂ transfer.

The Fe²⁺ ion is coordinated in the heme plane by the four nitrogen atoms in the center of the protoporphyrin ring, although it undergoes a 0,1 nm out-of-plane shift in the absence of O₂, with which it forms a bond perpendicular to the heme plane on one side and another bond with a particular histidine residue of the hemoglobin polypeptide chain on the other side. Hemoglobin is a protein like myoglobin, but with

different peculiarities. Unlike myoglobin, which consists of a polypeptide chain and a heme group, hemoglobin is made up of four polypeptide chains joined by noncovalent interactions, and each of these polypeptide chains contains a heme group. Hemoglobin therefore has four binding sites for O₂ [5].

Types of normal hemoglobin in humans

Hemoglobin A (Hb-A), the main type of human hemoglobin in adults and children above the age of six months, is composed of two different types of polypeptide chains: two α chains and two β -chains [6]. These chains are coded by separate genes. The structure of Hb-A is therefore $\alpha_2\beta_2$. Substituents in the amino acid chains are very important for the function of hemoglobin as they are involved in the O₂ binding site. In the human fetus, during development, two different types of polypeptide chains exist, alternative to the adult β -chain but with a very similar primary structure. Amino acids are replaced with other amino acids with similar characteristics that preserve the hydrophobic nature and the three-dimensional structure of the protein. The fetal hemoglobin (Hb-F) chains alternative to the β -chain is γ , identifying hemoglobin as $\alpha_2\gamma_2$. In the fetus, Hb-F is preceded by the embryonic hemoglobin (ξ -chain in the place of β -chain), whose production occurs in the yolk sac (from 3rd to 8th week). Its production decreases shortly after Hb-F starts to be produced in the liver and in the spleen (from 6th to 30th week) and finally in the bone marrow, where Hb-A will also be produced by the unborn throughout his life [7]. The sites of red blood cell production are the same as those of various types of hemoglobin. Hb-F has, under physiological conditions, a greater affinity for O₂ than adult Hb-A. This greater affinity of Hb-F allows for higher efficiency in the transport of O₂ from the maternal to the fetal circulation and allows the transfer of O₂ from Hb-A to Hb-F (see below). The α chain is made up of 141 amino acids, while the β , ξ , and γ chains are made up of 146 amino acids, with more substantial differences with respect to their three-dimensional structures. The structure of hemoglobin is essentially spherical, with the chains organized in a tetrahedral structure. The heme groups are in nonpolar niches protected from water near the outer surface. Each α chain is in contact with each β chain, while there is no contact between the two α chains and the two β chains [5].

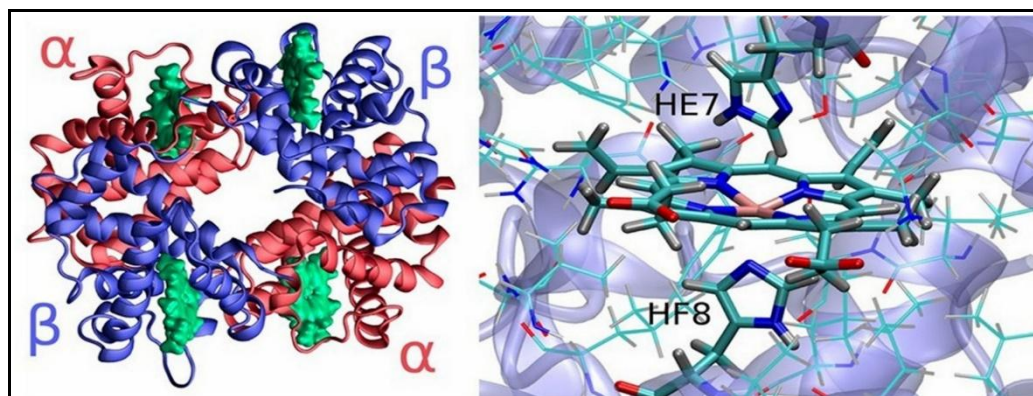


Fig 2: Hemoglobin helical structure (image from [8]). Hemoglobin is a tetrameric protein composed of four globular subunits named α and β . Each subunit is composed of eight α -helices, named A to H, connected by short non-helical regions and holds the prosthetic heme group, coordinated to the protein through histidine F8, called the proximal histidine. The other key residue related to its function is the histidine present in position E7, called distal histidine.

Variation in the oxygen affinity of hemoglobin due to many effectors: Bohr Effect

Varying concentrations of H^+ , CO_2 , and 2,3-diphosphoglycerate (DPG) interfere with the affinity between hemoglobin and O_2 , playing a very important biological role^[9]. There is a close correlation between them, and within hemoglobin, they are bound at spatially distinct sites that communicate with each other through conformational changes in the protein's quaternary structure. In hemoglobin, increasing H^+ and CO_2 concentrations (typically in the capillaries of metabolically active tissues with high O_2 demand, *e.g.* skeletal muscles) reduce the affinity for O_2 , increasing its release into cells requiring it. This phenomenon occurs because of an increase in the pK_a value of hemoglobin amino acid residues with charged side chains (lysine, arginine, histidine, aspartic acid, glutamic acid), including the N-terminal amino groups of the α subunits. High concentrations of H^+ and/or CO_2 in metabolically active tissues then cause the release of O_2 from hemoglobin. This phenomenon, discovered in 1904 by Bohr, Hasselbalch and Krogh, is commonly known as the Bohr effect^[10, 11], while the Haldane effect is the phenomenon which describes the capacity of blood to carry CO_2 from tissues to lungs by the condition of decreased hemoglobin oxygen saturation^[12]. Furthermore, 2,3-diphosphoglycerate (DPG), a product of the glycolysis metabolic pathway, present in trace amounts in all mammalian cells but conversely present in significant concentrations in red blood cells, is of notable physiological importance because it binds the tetramer central cavity (one molecule of DPG per hemoglobin tetramer), and prevents that O_2 from binding to it^[13]. Since the binding of DPG and that of O_2 to hemoglobin are mutually exclusive, it can reduce the affinity of hemoglobin for O_2 , which can then be released into the tissues. Since DPG is negatively charged, it exploits a binding site in hemoglobin composed of specific positively charged amino acid residues present in the β chains, stabilizing the quaternary structure of deoxyhemoglobin by binding the β chains together. Finally, Hb-F binds DPG weaker than Hb-A, which is why hemoglobin F has a higher affinity for O_2 than Hb-A (as discussed previously)^[15].

Hemoglobin: Curve of dissociation

The curve representing the O_2 dissociation graph is exemplified on the x-axis by the partial pressure of O_2 (pO_2) values and on the y-axis by the saturation values, as shown in Figure 3^[14]. Saturation is the fraction of O_2 sites occupied: it can vary from 0 (all sites free) to 1 (all sites occupied). It is possible to observe graphically that the two O_2 dissociation curves for hemoglobin and myoglobin differ greatly. In fact, for any given pO_2 , the saturation values are higher for myoglobin than for hemoglobin, meaning that myoglobin has a greater affinity for O_2 than hemoglobin. The p_{50} , which is a useful parameter in defining the position of the O_2

dissociation curve, corresponds to the partial pressure of O_2 at which 50% of the available sites are saturated. For myoglobin, p_{50} corresponds to 1 torr, while for hemoglobin, p_{50} corresponds to 26 torr, demonstrating a lower affinity for O_2 in the latter. This can also be observed graphically; in fact, the O_2 dissociation curve for myoglobin is hyperbolic, while that for hemoglobin is sigmoidal (both curves are completely like those described by enzyme kinetics: the hyperbolic one, according to the Michaelis-Menten model of 1913, and the sigmoidal one, studied in the 1960s based on the cooperativity model). This phenomenon was observed in 1904 by Bohr, Hasselbalch and Krogh who discovered how O_2 molecules bound to hemoglobin in a nonlinear fashion, together with the effect of augmented H^+ and CO_2 concentration on the lessened affinity of O_2 to hemoglobin (called Bohr effect)^[10]. The dissociation curve shows that O_2 release from hemoglobin at low O_2 pressure occurs more strongly than expected due to its sigmoid shape. This phenomenon, studied in detail in subsequent years, is related to the ability of hemoglobin in its tetrameric form ($\alpha_2\beta_2$, for Hb-A), compared to the monomeric form of myoglobin, to acquire new structural and functional properties, suited to its role of acquiring O_2 at the level of the pulmonary alveoli (pO_2 , 100 torr) and then releasing it at the level of tissues, such as muscle, (pO_2 , 30 torr). Unlike the hyperbolic shape of the O_2 dissociation curve in myoglobin, which assumes a simple role of O_2 reserve in the muscles, the sigmoidal shape of the O_2 dissociation curve in hemoglobin demonstrates that the bond between O_2 and hemoglobin is cooperative, in that the binding of an O_2 molecule to a heme molecule facilitates the binding of more O_2 to other heme groups of the same hemoglobin and, *vice versa*, the release of O_2 from a heme group facilitates its release from other heme groups of the same hemoglobin molecule. Consequently, there is true interdependence between the heme groups of a single hemoglobin molecule. The contact regions between the different polypeptide chains can, therefore, amplify and transmit conformational changes from one subunit to another: this represents the phenomenon of allostery. The key to its physiological role concerns the cooperatively regulated differential oxygen affinity between the so-called T (tense, low affinity) and R (relaxed, high affinity) states. Allostery represents one of the ways of regulating the function of very specific oligomeric proteins (human lactate dehydrogenase and *E. coli* transcarbamylase, to name a few), which are therefore defined as allosteric proteins and are subject to the phenomenon of cooperativity. As typically happens for allosteric proteins, hemoglobin modulates its biochemical characteristics by through the binding of other specific chemical species (allosteric effectors): O_2 , CO_2 , H^+ , DPG. Hemoglobin therefore can receive information from the surrounding environment to increase its efficiency^[5, 15].

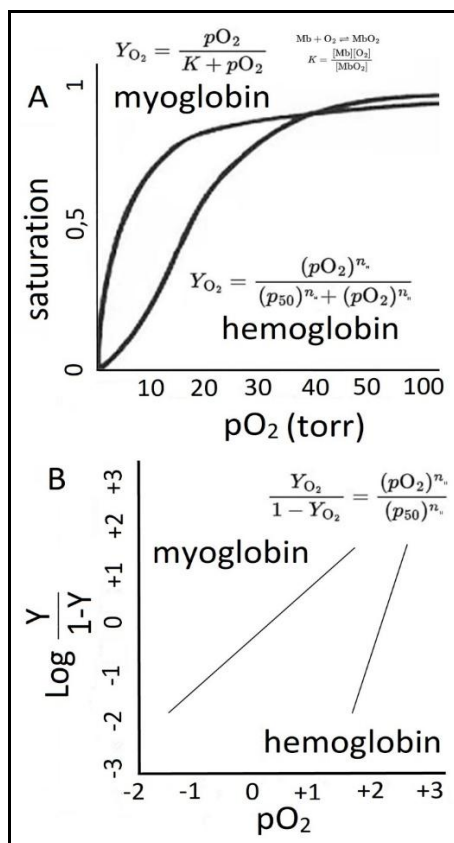


Fig 3: Comparison between the saturation curves of myoglobin and hemoglobin, respectively hyperbolic and sigmoid shaped (A) [5, 15]. By placing the values of Log pO₂ on the x-axis and the values of log Y/(1-Y) on the y-axis, we obtain the Hill diagram (named after its creator, Archibald Vivian Hill, Nobel Prize winner for Medicine in 1922). The different inclinations of the straight line obtained express the angular coefficients (called Hill coefficients, n_H). Myoglobin does not show variations in slope and the n_H is equal to 1 in its entirety. This indicates that the O₂ binding site of a single myoglobin is independent from that of the others monomer molecules (lack of cooperativeness). On the contrary, hemoglobin shows a transition between a weakly bound state and a strongly bound one (since each of the four heme molecules in the tetramer of hemoglobin does not bind O₂ in a single step), thus reflecting the changes in the slope of the curve, which is essentially linear within the range of saturation values between 0,1-0,9 and resulting in the n_H values of 2,8-3,0. This indicates that the binding between O₂ and hemoglobin is of the cooperative type (B) [5, 15].

Glycated minor components of adult hemoglobin

The diversity of proteins is considerably enhanced by physiological post-translational modification, and a wide variety of proteins owe many of their functional properties *in vivo* to covalent attachment to different chemical species, including carbohydrates (mainly glucose). These specific modifications are generally under precise enzymatic control by glycosyltransferase enzymes (*N*-glycosylation with asparagine linkage, *O*-glycosylation with serine or threonine linkage, *C*-glycosylation with tryptophan linkage). Certain proteins can spontaneously undergo *in vivo* to non-enzymatic glycosylation (or rather glycation, because the concepts of glycation and glycosylation, still interchangeably used in the scientific literature, are not actually the same). Glycation rate depends on the presence of unnaturally high concentrations of the blood glucose or its metabolites for a long time, generally due to severe alterations in insulin availability (diabetes mellitus disease) [16].

Among the various proteins, hemoglobin can spontaneously undergo these non-enzymatic changes, which end up negatively altering its biochemical properties, mainly with carbohydrates or with other natural (glutathione [17]) or unnatural (glycidol [18], acrylamide [18]) molecules, whose

adducts with the last two have been used as biomarkers for the exposure to these chemicals, that are mutagenic and carcinogenic to humans.

Glycated hemoglobin is generated by spontaneous, post-translational, irreversibly, and non-enzymatic reaction between red blood cell glucose or several types of glucose metabolites (including those containing one or two phosphate moieties) and N-terminal valine residue of β -chain of hemoglobin A to form an N-1 deoxy fructosyl by ketoamine linkage. Particularly, the chemical mechanism is based on the formation of Schiff base then shifting into rearrangement by means of the Maillard reaction providing the final Amadori product, as shown in Figure 4 [19]. Glycation does not depend solely on the concentration of different glycated metabolites within the red blood cells, but also the rate with which they react with hemoglobin must be taken into consideration. Adducts with fructose 1,6-diphosphate (HbA1a1), glucose 6-phosphate (HbA1a2), pyruvate (HbA1b), and glucose (HbA1c), all intermediates of glycolysis, increase the O₂ affinity compared to native hemoglobin HbA0 (n_H values of the glycated hemoglobins are decreased compared to HbA0 and negatively affect the cooperativity).

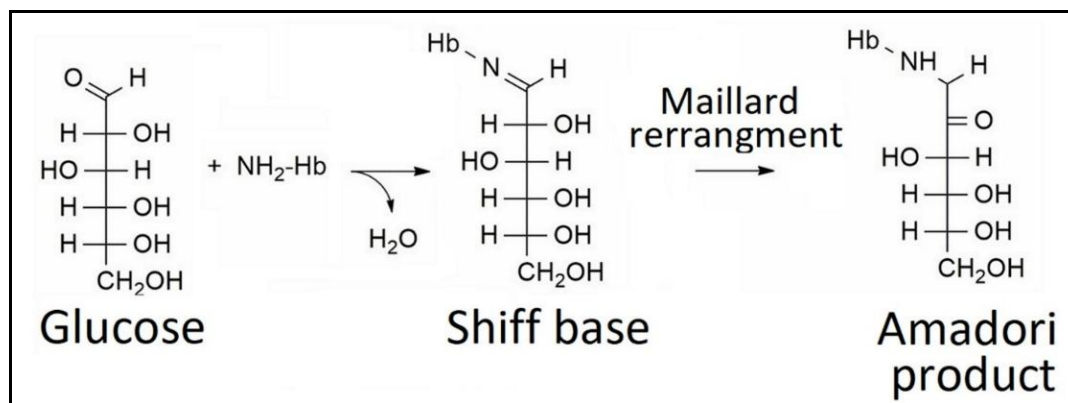


Fig 4: Chemical mechanism in the glycation of human adult hemoglobin (Hb-A) β -chain. The example in the picture shows the reaction between hemoglobin and glucose, resulting in the hemoglobin-glucose linkage by the formation of amine bond (Schiff base) with elimination of water, and finally the rearrangement of Maillard with Amadori product formation (ketoimine linkage) ^[20].

The glycated minor components of human hemoglobin are reported in Table 1. As shown in the same table, glycated derivatives, particularly phosphorylated ones, show the lowering of n_H , affecting the cooperativity of glycated hemoglobin.

Table 1: glycated minor components of human hemoglobin, their Hill coefficient (n_H) at pH 7 ^[21] and their percentage in normal and in diabetic red blood cells ^[22].

Hemoglobin component	Carbohydrate metabolite	n_H	Hemoglobin in normal subject	Hemoglobin in diabetic subject
HbA0	No adduct	2,6	96%	80-90%
HbA1a1	Fructose 1,6 diphosphate	1,8	0,2%	0,2%
HbA1a2	Glucose 6 phosphate	1,7	0,2%	0,2%
HbA1b	Pyruvate	1,7	0,5%	0,7%
HbA1c	Glucose	2,3	3,3%	7,5%

HbA1c, among the other minor and less common glycated components of hemoglobin, is the most abundant fraction normally present in the red blood cells. It increases two- to three-fold during type 2 diabetes mellitus while the others glycated components remains almost at a normal level during this chronic metabolic disease, which is characterized by persistent increased blood glucose. HbA1c has an altered cooperativity due to structural changes because of glucose attachment to hemoglobin, which increases its O_2 affinity (HbA1c has approximately ten-fold higher O_2 affinity than HbA0), causing the theoretical leftward shift of the dissociation curve graph and a decrease of its delivery to tissues. This alteration is a direct result of the glycation process itself, which modifies the hemoglobin structure and affects its function. By its additional negative charge, HbA1c interferes with the linkage of DPG in the central cavity of the tetramer of hemoglobin, theoretically reducing its ability to transfer O_2 to the tissues through the Bohr Effect. However, the O_2 affinity in blood from diabetic patients does not differ from that in healthy subjects. DPG, which lowers the O_2 affinity of hemoglobin by stabilizing deoxyhemoglobin, keeping the overall O_2 delivery to tissues normal and compensates for the harmful effect of HbA1c. In diabetes, in fact, red blood cells increase DPG levels, which makes hemoglobin release O_2 more easily, effectively balancing the dissociation curve.

By providing an integrated measurement of blood glucose, HbA1c is useful in assessing the degree of diabetic control. HbA1c is a marker of the average blood glucose level over the past 8-12 weeks and has less variability, is unaffected by acute factors such as stress and physical exercise and better represents relative long-term glycemic control. For this reason, it is used with excellent results as a rapid, standardized and less affected by analytical variables, diagnostic test of hyperglycemia and diabetes (6,5%, least

HbA1c value in diabetic subjects). HbA1c is formed by direct reaction of hemoglobin with glucose and therefore reflects the average plasma glucose over the previous eight to twelve weeks, period in which it is slowly, irreversibly and continuously formed, also indicates its production during the 120-days lifespan of red blood cells. Because the measuring of plasma glucose allows merely to monitor the immediate condition, it is often less reliable compared to the several-weeks retrospective survey. These properties have made HbA1c the preferred parameter for assessing glycemic control in persons with full-blown diabetes or in screening test for persons at high risk of this metabolic disease. Although studied little, an additional biomarker for diabetes alternative of HbA1c can be HbA1d3 (adduct composed by glutathione and the β chain of hemoglobin) ^[17], forms normally *in vivo*, and that may provide a useful marker of oxidative stress in diabetes and potentially in other pathological situations related to oxidative stress.

HbA1c and its role in diabetes mellitus complications

Diabetes mellitus, a chronic metabolic disease characterized by persistent hyperglycemia, is a group of disorders caused by lack of insulin secretion and/or impaired insulin action (insulin resistance). Insulin, which is an anabolic hormone produced by the pancreatic cells of Langerhans (so-called islet of Langerhans), plays an important role in the metabolism of carbohydrates, lipids, and proteins. The metabolic abnormalities associated with diabetes mainly affect adipose tissue, skeletal muscles and several organs often in a very serious way, even causing death. The severity of symptoms can vary depending on the duration and type of diabetes. They are: type 1 diabetes (an autoimmune disease, in which the body attacks and inactivates the cells of Langerhans, that therefore cannot produce insulin), type 2 diabetes (insulin resistance disease in which the body does

not use correctly the insulin or does not produce enough of it), and hybrid forms of diabetes, such as gestational diabetes^[23]. Type 2 diabetes accounts for the majority (90-95%) of diabetes disease and is typical of elderly people but is also significantly increasing in adolescents and young adults due to obesity and physical inactivity, which often characterizes their bad lifestyle. It is chronic, progressive, medically incurable and poorly controlled by pharmacotherapy^[24].

Both the incidence and prevalence of every types of diabetes have increased worldwide over the past three decades by 103% and it is unstoppably growing^[25]. Present in a third of diabetic patients, the main collateral diseases promoted by diabetes include: autonomic and peripheral neuropathies, respiratory system diseases, kidney, hepatic, and gastrointestinal diseases, retinopathy, cardiovascular failure, osteoporosis, pancreatic cancer, stroke, Charcot's acute neuroarthropathy, Alzheimer's disease, bacterial infections, periodontitis, and COVID-19 infection.

Autonomic and Peripheral neuropathies

Neuropathies are common and often severe microvascular complications of diabetes, significantly impacting patient quality of life and contributing to morbidity and mortality, affecting various nerves, both autonomic (*e.g.* involving heart rate, digestion, bladder and sexual function) and peripheral (relating hands, feet, legs and arms). Major clinical manifestations of diabetic autonomic neuropathy, present in 54% of type 1 and 73% of type 2 diabetic patients, include resting tachycardia, exercise intolerance, orthostatic hypotension, constipation, gastroparesis, erectile dysfunction, sudomotor dysfunction, impaired neurovascular function, fluctuation between blood hyperglycemia and hypoglycemia (brittle diabetes), and hypoglycemic-associated autonomic failure. Most cases of diabetic peripheral neuropathy, present in 29% of type 1 and 42% of type 2 diabetic patients, include burning, numbness, tingling, limbs paresthesia and dysesthesia often resulting in difficulty with ambulation and other basic activities of daily living. This disease is typically characterized by severe limbs ulcerations, which can result in their amputation. The development or progression of diabetic autonomic/peripheral neuropathy is associated with the 3-year mean high HbA1c level, suggesting the importance of monitoring the long-term mean HbA1c level and keeping them as much as possible within acceptable values, but also the variability in the HbA1c level is associated with a higher risk of microvascular complications in the diabetic patients, recognizing it as an important predictor of serious or really lethal diabetes complications^[26].

Respiratory system diseases

The diabetic population is at elevated risk of diseases involving in the respiratory system, including asthma, chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, pulmonary hypertension, and lung cancer. Pulmonary function parameters have significant changes in subjects with uncontrolled diabetes, compared to healthy individuals. Many studies demonstrate the direct correlation between diabetes and HbA1c levels with the reduced lung function, and furthermore these factors are a reliable predictor of inadequate lung function. Thus, high HbA1c level impairs lung functions, that is a very important target organ for diabetes, independently of other risk factors, such

as cigarette smoking. Emerging confirmations suggest that diabetes may affect the pathogenesis, development, and progression of several lung diseases and their prognosis and clinical outcome. The pro-inflammatory properties of hyperglycemia have been shown to have an important role in affecting pulmonary vasculature, airways, and lung parenchyma. Type 2 diabetes significantly influences lung cancer biology and prognosis, and the increased risk is associated with elevated HbA1c level above 8%. Integrating glycemic control with cancer therapy offers promising avenues for improving outcomes^[27].

Kidney diseases

Approximately one third of individuals with diabetes worldwide have impaired kidney function, defined by decreased glomerular filtration rate. This dysfunction is the main cause of severe kidney diseases, accounting for more than 40% of all renal failure cases and increasing the risk of its fatal outcome. Increased HbA1c is related to the development of kidney disease in diabetes, and its containment is at the center of the clinical management of hyperglycemia. The association between HbA1c and the incidence of chronic kidney disease is strong, and this suggests that it is a significant modifiable risk factor in chronic kidney disease among patients with diabetes. These results indicate that screening of long-term HbA1c, as a main marker of glycemia, is an important instrument to prevent the development or progression of chronic kidney disease among diabetic individuals. Furthermore, diabetes progression is strongly associated with an increased risk of kidney cancer, especially carcinoma, and is linked to higher rate of recurrence and a greater number of distant metastases, contributing to a reduction in survival. HbA1c values are significantly different depending on the size of the tumor: a recent study found that an HbA1c level of 5,9% corresponded to tumors with dimensions of 1-5 cm, while an HbA1c level of 4,8% corresponded to tumors with dimensions of >10 cm, suggesting that HbA1c may provide a useful marker for early detection of silent or small kidney cancers before they manifest clinically^[28].

Hepatic diseases

The pathophysiology of diabetes is fairly/extensively characterized by abnormal lipid profile. Moreover, non-alcoholic fatty liver disease has been shown to markedly increased in diabetes. It is considered the hepatic expression of the metabolic dysfunctions induced by insulin resistance and the overall mortality is significantly higher than the background population. HbA1c is a predictor of lipid metabolism and hepatic steatosis in these patients and provides evidence that subjects at high level exhibit heightened cardiovascular risk due to an unfavorable lipid profile, liver steatosis, increased fat mass and alanine transaminase levels. Furthermore, increased levels of HbA1c may contribute directly to the progression of non-alcoholic fatty liver disease or indirectly through the promotion of nitric oxide, hypoxia and receptor for advanced glycation end products. Diabetic patients have a significantly higher risk of hepatic carcinoma and poor control of HbA1c/glucose is a significantly unfavorable factors for survival. Furthermore, high level of HbA1c may also be a risk factor for morbidities after hepatectomy^[29].

Gastrointestinal diseases

Diabetes, in terms of a high HbA1c level, is associated with an increase in gastrointestinal abnormalities, particularly in the upper part, including erosive esophagitis. It can also be implicated in the onset of peptic ulcer disease, complications from acute diverticula, and colonic neoplasms, the latter ranging from benign polyps that can become precancerous to malignant tumors, often starting as adenomatous polyps that progress to invasive adenocarcinoma through DNA mutations, often with symptoms like changes in bowel habits, blood in stool, and abdominal pain. Acute diverticulitis in diabetic patients is associated with a higher rate of diverticular bleeding, but lower rates in abscess, obstruction, colectomy, and mortality or diverticulitis-related severe complications. Furthermore, elevated HbA1c level is associated with adverse outcomes in diabetic subjects with gastrointestinal cancer and is correlated with a longer hospitalization. Early detection of elevated HbA1c levels, in combination with a rigorous antidiabetic treatment, may improve the prognosis of gastrointestinal cancer patients with diabetes [30].

Retinopathy

Diabetic retinopathy is a microvascular complication of diabetes and the most common cause of blindness among individuals of every age. HbA1c was found to be a good indicator of 120-days control for glycemic level in diabetes retinopathy patients and could be correlated with the severity of this disease. The early identification and management of risk factors associated with diabetes retinopathy severity such as glycemic control and diabetes duration is very important. Many studies showed a significant association between HbA1c level and severity of this degenerative disease. Prolonged hyperglycemia leads to microvascular damage but maintaining HbA1c levels below 7% reduces the risk of diabetic retinopathy progression. Accordingly, to mitigate the onset of retinopathy and at least to reduce its progression, it is necessary to intensify glycemic management strategies of control, in which HbA1c plays a key role [31].

Coronary heart disease

Coronary heart disease is a macrovascular complication in diabetic patients and is closely linked to fluctuations in blood glucose levels. It is manifested by atherosclerosis and spasms of coronary arteries, resulting in narrowing and occlusion of the cardiovascular lumen and inadequate or interrupted blood supply to the myocardium. Patients with both diabetes and cardiovascular diseases tend to have diffuse, occlusive lesions throughout the coronary arteries, with a high risk of bleeding, ulceration, and calcification (the latter as an active process triggered by pro-inflammatory signals and itself a risk factor for cardiovascular morbidity and mortality). HbA1c is directly associated with the severity of cardiovascular disease and is a risk factor for adverse cardiovascular events in diabetes patients: each 1% elevation in HbA1c level is linked to an approximate 13% increase in the risk of cardiovascular events. Controlling HbA1c level <7,0% generates significant benefits of attenuating the advancement of coronary artery calcification, thereby reducing the incidence of cardiovascular diseases [32].

Osteoporosis

Osteoporosis, which affects both men and women, insidiously deteriorates the mineral microstructure of bone

(reduction in bone mineral density, especially the calcium ion), particularly at trabecular sites, such as vertebrae, ribs and hips, culminating in bone fractures, pain and disability, which can lead to significant morbidity and mortality. Osteoporosis is normally associated with senescence and estrogen deficiency during menopause. Furthermore, diabetes contributes to aggravates this condition: diabetic patients, characterized by high glucose and HbA1c level in the blood, exhibited an increased risk of bone fracture. Hyperglycemia and high HbA1c level in diabetic subjects promote osteoclast mediated bone resorption (and conversely suppresses osteoblast mediated bone formation and increases fat accumulation in the marrow cavity) which deteriorates bone strength and increases susceptibility to fracture. The level of osteocalcin, that is a bone-specific protein produced by osteoblasts and involved in the regulation of bone energetic metabolism, is inversely associated with HbA1c level, supporting the theory that a poor glycemic control can affect osteoblast function [33].

Pancreatic cancer

Hyperglycemia detected by elevated HbA1c level is associated with an increased risk of pancreatic ductal adenocarcinoma, that is related to very poor survival (one- and three-year corresponding to 22,5% and 6,6%, respectively). Elevated HbA1c level can be a cause and/or an early symptom of pancreatic cancer, especially in cases of new-onset diabetes. The risk of pancreatic cancer, compared to subjects without diabetes and that have HbA1c value in the normal range, is approximately 15-times greater in the first year following a diabetes diagnosis and decreased approximately to four-fold in the following year, to then settle to three-fold for the remainder of the follow up period (typically more than ten years, if cancer does not appear). In addition to HbA1c, high circulating levels of insulin or C-peptide (a component of proinsulin used as a marker of endogenous insulin production) in diabetic patients might increase pancreatic cancer risk. Potential mediators of diabetes, such as the peptide hormone adrenomedullin, are locally produced by the pancreatic tumor and inhibit the action of Langerhans cells [34].

Stroke

Both ischemic and hemorrhagic strokes are conditions that result in high mortality rates and induce a severe disability in case of survival, due to prolonged interruption of blood flow to the brain. Stroke severity and patient age are the main predictors of stroke outcome in its acute phase. Additional important predictors include functional status prior to stroke, presence of comorbid medical conditions, etiologies and the vascular districts affected. Diabetes is a well-established independent but modifiable risk factor for ischemic stroke but remains unclear for the hemorrhagic one. The severity of both types of strokes correlates with the glycemic/HbA1c status of the patient. In fact, the mortality is higher, or the outcome is poorer, in patients with high levels of both these parameters. In a recent study, the risk of ischemic stroke was higher for either type 1 or type 2 diabetes. For hemorrhagic stroke, individuals with type 1

diabetes had significantly higher risk starting at HbA1c > 6.9%, while in those with type 2 diabetes, a modest risk increase was observed only at HbA1c >8.7^[35].

Charcot neuroarthropathy

Charcot neuroarthropathy is a rare, chronic, weakening, devastating and destructive disease of bone structure presenting with peripheral and/or autonomic neuropathy that typically affects elderly diabetic people, most of whom had been suffering from diabetes for more than ten years. Charcot neuroarthropathy can be painful or painless, characterized by painful or be painless, joint destruction in limbs that have lost sensory innervation, and its late diagnosis and treatment (by drugs or surgical management) can have serious consequences, including deformity, foot ulcers, joint destruction, amputation, and death. A significant risk factor for Charcot neuroarthropathy development includes diabetes, whose presence is represented by high levels of glucose and HbA1c in the blood, whose persistent exposure contributes to vascular changes and neuropathy that underline Charcot neuroarthropathy. Poor glycemic/HbA1c control is a prominent feature among Charcot neuroarthropathy patients, with an average HbA1c level above 9%. Diabetes thus plays a cardinal role in the pathophysiology of this disease, which also includes sensory neuropathy, repetitive trauma, inflammation, and microvascular changes^[36].

Alzheimer's disease

Poor glycemic control, insulin resistance and high level of HbA1c (typically present in type 2 diabetes) are associated with mild cognitive impairment up to established Alzheimer's disease and related dementia disorder. The exact connection between Alzheimer's disease and type 2 diabetes is still in discussion. However, poorly controlled blood glucose may increase the risk of developing this neurodegenerative disorder. This relationship is so strong that Alzheimer's disease is often called "type 3 diabetes". Cognitive deficits in persons with diabetes mainly affect the areas of psychomotor efficiency, attention, learning and memory, mental flexibility and speed, and executive function. In Alzheimer's disease, cerebrovascular alterations, insulin action/resistance, altered amyloid metabolism, and recurrent hypoglycemic/hyperglycemic episodes seem to play a major role in this neurodegenerative disorder. Most recent studies have focused on the role of insulin action and insulin resistance as possible links between diabetes and Alzheimer's disease, in which insulin signaling could disturb the brain functions. Maintaining the recommended glycemic levels may prevent diabetes-related general complications and slow the progression of cognitive decline. Furthermore, the association between dementia and greater glycemic and HbA1c variability appears to be physiopathologically related. The American Diabetes Association recommends the HbA1c level of 7-7.5% for intact cognitive/functional status adults with diabetes, while the HbA1c level of up to 8% is acceptable for mild-to-moderate cognitive impairment status adults, but recommends avoiding reliance on HbA1c for moderate-to-severe cognitive impairment status adults, because this population has a higher risk of conditions that affect its red-blood cell turnover rate, and thus HbA1c level, that is dependent on the length of time which red blood cells circulate in the blood^[37].

Bacterial infections

Despite there being no consensus on the impact of diabetes on bacterial infections, such as lung, urinary tract, skin/mucous membranes, kidney and bone, the improvement of glucose/HbA1c control may reduce infection risks. HbA1c levels or fluctuations of them significantly increase the risk of infections, presenting in primary care or hospitalizations, in diabetic subjects. Infection rates increase together with HbA1c level, particularly among those with HbA1c >11%. Poor blood glucose/HbA1c control weakens the immune system, impairing the ability to fight off pathogens and showing a notable rise in serious bacterial infections, also exacerbated by antibiotic resistance. Good glycemic control is crucial for reducing infection risk, enhanced by the high HbA1c level and eventually by its variability. Age ≥65 years diabetic patients are not at significantly increased risk of hospitalization for most infections, but high HbA1c levels of 8-9% are associated with the increased critical issues of infection for the skin, soft tissue, and bone^[38].

Periodontitis

Periodontitis represents a clinical condition globally prevalent, characterized by both infectious and immune-mediated inflammatory components, making it one of the most predominant oral disease conditions worldwide. Periodontitis is characterized by the formation of periodontal pockets, detachment of gingival tissue, alveolar bone loss below the soft tissue, tooth mobility, and ultimately its loss. This pathology begins from a complex interaction between the host and the invading Gram-negative bacterium (*Aggregatibacter actinomycetem-comitans*, *Porphyromonas gingivalis*, *Prevotella intermedia* and *Tannerella forsythia*). Diabetes is a modifiable factor responsible for the onset/progression of periodontitis, which shows a relationship with elevated blood glucose and HbA1c levels. The severe clinical progression of periodontitis and the incidence of tooth loss are closely related to increased blood glucose/HbA1c levels, in fact diabetic patients with high glucose (> 160 mg/dL) or HbA1c (>7%) levels have up to a three-fold higher risk of tooth loss. HbA1c measurement may also represent a valuable double diagnostic tool: predicting diabetes risk in periodontitis-affected individuals and assessing periodontal disease progression in diabetic patients^[39].

Viral infection by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)

Due to its recent origin (2019), still a little is still known about the β -coronavirus SARS-CoV-2 resulted in a global pandemic called COVID-19. The primary human receptor for it is the angiotensin-converting enzyme-2 (ACE2) receptor, that is highly expressed in the upper and lower respiratory epithelium and in other tissues, including those of kidneys and intestines. Although SARS-CoV-2 infections in humans primarily take place in the respiratory tracts, there are strong evidence that infection also occurs in the liver, intestinal tract, brain, and pancreas. Among the numerous comorbidities that accentuate its virality and especially increase the mortality in elderly people (hypertension, ischemic heart disease, myocardial infarction outcomes, pulmonary comorbidities, neoplasms), diabetes plays a significant role. Diabetes is associated with a greater risk of hospitalization (intensive care and invasive ventilation) and mortality from COVID-19. A higher risk of severe outcomes is related proportionally with the increase in the HbA1c level, therefore considered a

clear predictor of COVID-19 severity. HbA1c is also associated with higher risk of “Long COVID”, characterized by many symptoms attributable to myalgic encephalomyelitis/chronic fatigue syndrome for one-six months following infection [40].

Glycated hemoglobin imagined in an artistic scene

According to the writer's imagination, the painting made by the prominent renaissance artist Lorenzo Lotto shows several Catholic saints that can fantastically represent hemoglobin both in its fully active form suitable in the transporting of O₂ through the bloodstream, and in its glycated adducts typical in diabetes mellitus disease, which are less active in carrying out their physiological function (Figure 5).

True to the narrative handed down to us by the so-called

Legenda Aurea by the Dominican friar *Jacopo da Varagine* [41], Saint Christopher (ideally representing hemoglobin) is depicted when, close to reaching the shore of the river (representing the bloodstream), discovers the exact nature of the baby (really Jesus, representing a molecule of O₂), that he has pledged to carry from shore to shore on his shoulder, whose weight has now become unbearable even for his very vigorous giant body. He no longer seems to suffer the overwhelming weight of the world weighing on his shoulders alone, while a sudden gust of wind stirs the red cloak that until a moment before had enveloped his mighty chest, in stark contrast to the placid calm of the waters behind, suggesting the mystic origin of that whirlwind. At his side, Saint Rocco (on the left) and Saint Sebastian (on the right), respectively sick and injured, evoked those minor glycated forms of hemoglobin less active in their role of O₂ transporter, characteristic of diabetes mellitus patients and that induce many diseases difficult to cure.

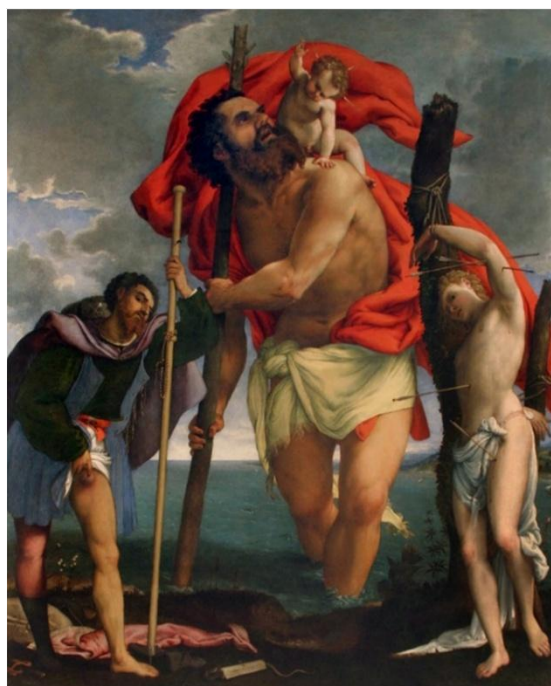


Fig 5: Lorenzo Lotto: “Saint Christopher between Saints Rocco and Sebastian” (1532-33); Loreto (Italy), Museum in the Sanctuary of Santa Casa di Loreto.

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