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Carbonic anhydrases: hematologic relevance and a biosensing perspective

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ABSTRACT

Carbonic anhydrases were first identified in red blood cells and have been thus traditionally addressed in a hematological context. However, recently there has been a shift of research interest to therapeutic areas, notably in solid cancers, relegating the impact of carbonic anhydrase function and pathological dysfunction in blood related physiology to secondary importance. This review addresses this paradigm and emphasizes the potential impact of recent studies on blood related carbonic anhydrase isotype expression and modulation in diverse areas such as physiology and pathology, biosensing, their use as biomarkers, and in the development of synthetic blood. A special emphasis is placed on reviewing new dynamic and quantitative studies that allow for the efficient tracking and quantitation of various carbonic anhydrase isozymes within the blood and more generally within the human body, that give new perspectives on the biochemical and physiological role of blood associated carbonic anhydrase in health and pathology.

Keywords: carbonic anhydrase, blood, hematology, biosensors, disease biomarkers, red blood cells, synthetic blood

INTRODUCTION

Carbonic anhydrases (CAs) form a ubiquitous class of metallo-enzymes that catalyze the reversible hydration of carbon dioxide CO_2 leading to the formation of bicarbonates and protons ($CO_2+H_2O \longleftrightarrow HCO_3^- +H_+$) - all important orchestrators of cellular physiology[1]. This reaction is universal in nature, involving the interchange of gaseous and ionic species crucial to a wide range of physiological and biochemical processes. Spanning all areas of the biological realm, from procaryotes to multicellular organisms and humans, CAs are involved in many metabolic functions that involve carboxylation or decarboxylation reactions, thus play a crucial role in key physiological processes such as respiration, inorganic carbon transport, intra and extracellular pH regulation, gluconeogenesis, lipogenesis, tumorigenicity, signal transduction and bio-mineralization[2]. Seven CA polyphyletic classes, indicated as α, β, γ, δ, ζ, η and θ (η proposed in 2014[3] and θ in 2016[4]) were identified in living organisms and current research continuously expands the list of CA isoforms associated with these classes[2].

While it has been suggested that CAs initially evolved to facilitate transcellular carbon dioxide transport rather than its more familiar role in respiratory gas exchange[5], CAs also confer directionality on carbon dioxide transport across membranes, maintaining high levels of the gas in the solution on the upstream side of the membrane. They do this by causing acidification of the downstream boundary layer thus maintaining the concentration gradient to drive diffusion. In addition to facilitating passive diffusion, CAs act in concert with membrane-associated ion transport systems forming functional complexes[6, 7] nicknamed "metabolons" (such as the sodium-hydrogen exchanger and chloride-bicarbonate anion exchanger).

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Biological membranes form an effective barrier to the passive diffusion of bicarbonate and hydrogen ions, whereas carbon dioxide is highly membrane permeable. CO_2, HCO_3^-, CO_3^{2-}, and H^+, are interconnected by equilibrium reactions, and their concentrations are regulated by CAs. However, although each reaction is in principle quite simple, their interlinked complexity makes analytical solutions for even the simplest families of equations (even in the steady state) impossible to achieve[8]. While this is a challenge, it also emphasizes the essential role of CA biosensing, contributing to the aim of this review, to advance basic knowledge on CA modulation in physiology and pathology and exploring new avenues for diagnostic and therapeutic applied research.

Given the exquisite role of blood in O_2 and waste CO_2 transport, of no surprise is that CAs were first identified in red blood cells (RBCs). CAs are among the four major proteins of RBCs, with amounts that range from ~ 7 amol/cell for CA Ⅰ, to 0.8 amol/cell for CA Ⅱ[9], to 900 amol/chain for hemoglobin, Hb alpha-and beta-chains, that confer RBCs the capacity to effectively shuttle O_2 and CO_2.

Blood contains different types of cells, besides RBCs, white blood cells (WBCs), and platelets suspended in plasma. Recent results show intriguing distributions of CAs in all blood compartments. All these highly specialized cells and media are essential in supporting the fulfillment of life supporting complex functions such as: transport (gases, nutrients from the digestive system, removing toxins and waste, move regulatory molecules around the body-hormones, chemokines, cytokines, antibodies, etc.), response to injury by clotting, support for the immune system, and the maintenance of pH and osmotic balance. Recapitulating their diversity and function in artificial blood substitutes is currently an unsolved challenge. Blood is also the premiere target for the clinical analyses and identification of disease biomarkers and, due to the catalytic nature of CAs these enzymes are both putative biomarkers and theranostic tools.

This minireview addresses current knowledge on CA expression and (dys) function in relation to human blood, with specific emphasizes on the potential impact of CA studies on diverse fields such as: physiology and pathology, biosensing and the development of synthetic blood, according to ***Fig. 1***.

CARBONIC ANHYDRASE EXPRESSION

CAs are almost ubiquitously present in human cells. There are 16 CA isoforms in humans all belonging to the α subgroup. Among them, 13 have been shown to be enzymatically active. They show extensive diversity in their subcellular localization and their tissue distribution[2, 10].

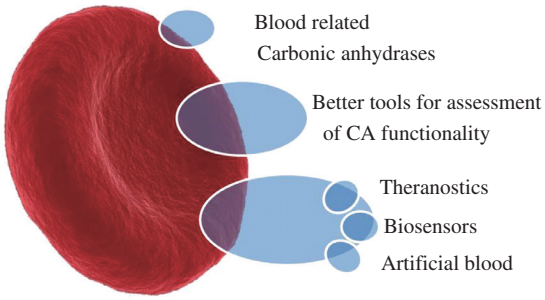

Fig. 1 **Carbonic anhydrases of hematologic relevance from a wider, including (biosensing) perspective**

In terms of subcellular localization, there are isozymes which act in the cytosol-isozymes Ⅰ, Ⅱ, Ⅲ, Ⅶ and Ⅷ which are connected with cell differentiation, others are membrane-bound, Ⅳ, Ⅸ, Ⅻ, ⅩⅣ and ⅩⅤ (with Ⅸ and Ⅻ associated with tumors), and those expressed in mitochondria-isozyme Ⅴ, or salivary/mammary gland the secreted isozyme Ⅵ (e.g., the extracellular enzyme present in breast milk)[2].

Cells/organs where CA expression is most abundant include red blood cells, brain, lung, gut, liver, kidney, and testis; here, various CA isoforms contribute to CO_2 hydration (***Fig. 2***), breathing, synaptic transmission, cerebrospinal fluid secretion, production of ocular fluid, bone resorption, secretion of gastric acid, urine acidification or sperm maturation[2, 10].

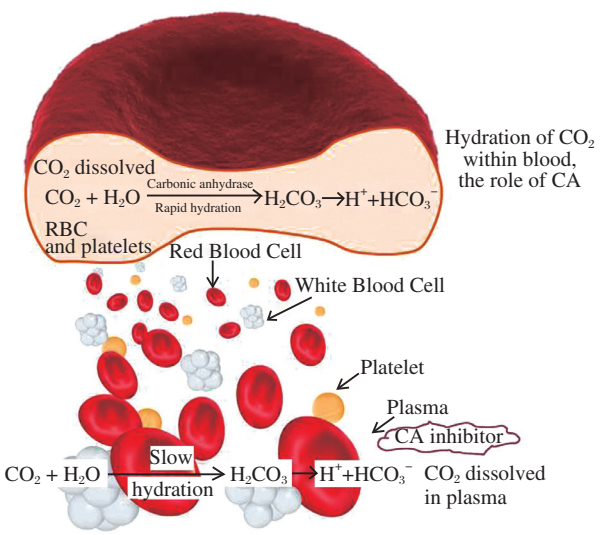

Fig. 2 **Carbonic anhydrase role in CO_2 hydration in blood compartments**

Excellent overviews of the exciting history of CA discovery can be found elsewhere[2, 10]. It is worth mentioning here however, that early experiments in the late 1920 s performed with hemolyzed blood demonstrating a higher than expected rate of carbon dioxide release from the blood, provided the first known

indicators of CA activity. It was only in the 1960 s, that the putative enzymatic catalyst of this reaction, containing one zinc ion per molecule and having a molecular weight of approximately 30 kDa, was isolated and purified. Two CA isoforms were detected in human erythrocytes by a procedure involving ethanol-chloroform extraction. These isoforms were called hCA Ⅰ and hCA Ⅱ, which were purified with a high degree of homogeneity by ion exchange chromatography or zone electrophoresis on cellulose columns.

Carbonic anhydrase Ⅰ (CA Ⅰ)

CA Ⅰ with relatively low specific activity occurs in the red blood cells of all vertebrate groups, with the exception of the cat family and a few other species; it is absent from the red blood cells of smaller species of cat, lions, jaguars, tigers, leopards, oxen, chickens, and frogs. It is somewhat less prone to sulfonamide inhibition and is highly susceptible to anions. It is an early specific marker of normal human erythroid differentiation[5].

Carbonic anhydrase Ⅱ (CA Ⅱ)

CA Ⅱ is a cytoplasmic enzyme with a very high affinity for sulphonamides and high catalytic activity. It is one of the fastest working enzymes in the human body at 10^6 cycles of enzyme per second[11], approaching the diffusion limit and is essential in the transport of CO_2 from tissues to the lungs (in the form of bicarbonate) through blood. CO_2 released as a part of respiration by tissues is not very soluble in blood and thus, in order to be transported, is converted to HCO_3^- by HCA Ⅱ. CA Ⅱ is present in numerous tissues besides erythrocytes: eyes, gastrointestinal tract, bone osteoclasts, kidney, lung testis, and brain and it constitutes a possible drug target in certain diseases (glaucoma, oedema, epilepsy, and altitude sickness). However, it has been mostly studied in red blood cells, where it has been extracted for the purpose of *in vitro* studies.

The platelet CA Ⅱ was described for the first time in the ´80 s. Taking into account the limited number of studies directly analyzing CA properties in platelets, its direct role in platelet physiology and pathology still remains poorly understood[11]. The modulation of platelet CA Ⅱ action as a therapeutic approach holds promise as a novel strategy to reduce the impact of cardiovascular diseases.

A common conjecture is that CAs are absent from blood plasma. Furthermore, the localization pattern of CA in blood is further complicated by the presence of a CA inhibitor in the plasma of some species, which ensures the absence of any soluble CA activity in plasma. This plasma inhibitor completely neutralizes erythrocytic CA released from any hemolyzed red blood cells throughout the blood vessels, thus reducing the dehydration/hydration reaction in the plasma to an uncatalyzed velocity[8, 12, 13].

Moreover, CA activity available to plasma appears to be confined to precise localizations within the circulation that are equipped with a capillary CA, thus in vascular regions with membrane-bound CA, e.g., muscles, heart, lungs, and others, a marked catalysis of the dehydration/hydration reaction can take place even in the presence of a plasma inhibitor. Intriguingly, the effect of the presence of CA in the plasma (as studied in rainbow trout) shows that CA attenuated post-exercise increases of P_{CO_2} and decreases of arterial pH is achieved by increasing CO_2 excretion during exercise. Moreover, it appears that plasma available CA plays a key role in enhancing the body´s ability to extract oxygen from their blood[14].

CARBONIC ANHYDRASE MODULATION

At the single-cell level, carbon dioxide diffuses more rapidly in buffer solutions and across artificial membranes than would be expected from its diffusion coefficients, and this facilitated diffusion is abolished by the inhibition of CA activity[8, 13]. Numerous compounds have been demonstrated to modify carbonic anhydrase activity: from physiological substances, drugs currently used in clinical practice and other chemicals tested *ex vivo* in laboratories. CAs have become an interesting enzyme for clinicians when inhibiting drugs were first established as a promising group of diuretics.

There are a wide variety of substances tested in laboratory conditions for their CA inhibitory properties[15], with current research either incrementally addressing the specificity and inhibitory potential of existing compounds[15] or generating novel classes of inhibitors. Selenols[16] were recently demonstrated to inhibit four human isoforms, CA Ⅰ, Ⅱ, Ⅶ and tumor-associated CA Ⅸ. To date, X-ray crystallographic, physical and computational studies have all provided insights into the binding mode of this conceptually new class of CAs.

Interestingly, acetazolamide remains a useful drug for cases of intracranial hypertension and reducing the risk of mountain sickness at high altitudes. CA inhibition is one of the multiple mechanisms underlying the effects of anticonvulsant compounds (such as Topiramate and Zonisamide). Furthermore, not only does this effect seem to be important for the main (antiepileptic) activity of these drugs, but another side effect might also be weight loss. Therefore, it potentially

provides a basis for designing new anti-obesity drugs[2, 15].

Sustained research is also devoted to CA activators[17] and interestingly, besides synthetic activators, there are potential CA Ⅱ activators physiologically present in the human organism. These include biogenic amines (histamine, catecholamines, and serotonin) and amino acids (phenylalanine and histidine)[11].

Moreover, environmental and dietary factors were reported to influence CA activity, including heavy metals, organic chemical pollutants, exercise, altitude, and low dietary Zn. Interestingly, the detection of these compounds is typically laborious and still challenging for *in vivo*, for environmental or food applications. The detection avenues offered by novel CA biosensors are of high relevance and are relatively less known across disciplines.

Heavy metals

CA activity shows a marked sensitivity to heavy metal exposure. Recent studies[18, 19] demonstrated the inhibition of the cytosolic CA Ⅰ and CA Ⅱ by lead, cobalt and mercury. Lead was a non-competitive inhibitor for CA Ⅰ and competitive for CA Ⅱ, cobalt was competitive for CA Ⅰ and non-competitive for CA Ⅱ, and mercury was uncompetitive for both CA Ⅰ and CA Ⅱ. Lead was found the best inhibitor for both CA Ⅰ and CA Ⅱ.

Organic chemical pollutants

Regarding the sensitivity of CA to organic chemical pollutants, most available data were obtained from pesticides, which represent one of the most worrying classes of chemical contaminants in terms of toxicological risk for humans and wildlife. In the last two decades, interest in the toxicity of pesticides has been extended, not only including the direct effects on man but also the far more subtle effects that pesticides exert on natural biota.

Common herbicides and fungicides used in agriculture (imazethapyr, 2,4-D dimethylamine salt, glyphosate isopropylamine salt and propamocarb HCl) were assayed as inhibitors of human erythrocyte CAs (CA Ⅰ, CA Ⅱ isozymes) with imazethapyr being the most effective (IC_{50}: 9.3×10^{-5} mol/L)[20]. The sensitivity of CA to organic chemical pollutants other than pesticides was also demonstrated by Lionetto *et al*.[20], who found CA Ⅱ extracted from bovine erythrocytes to be highly sensitive, not only to the carbamate pesticide carbaryl, but also to the polychlorinated biphenyl (PCB) arochlor, showing an inhibition of 34.4% at a concentration of 10 ng/L. This study also emphasizes the importance of the study of CA as a pollution biomarker for both human and environmental health monitoring.

Exercise

Ever since Kowalchuk *et al*.[21] reported a lower but similar power output after acute infusion of acetazolamide compared with an uninhibited condition during 30 s of maximal-intensity exercise, the effect of exercise has been constantly demonstrated[22]. This effect was not associated with any difference in plasma acid-base status before or immediately after the exercise bout, suggesting that CA inhibition may directly affect exercise response.

Implication for athletic performance

New research[14] indicates that plasma-accessible carbonic anhydrase (paCA) - an enzyme anchored to the walls of salmons´ blood vessels - helps reduce cardiac work rates during exercise by up to 27% when expending a comparable amount of energy. The researchers also found the paCA enzyme kicked in when the fish were exposed to low water oxygen levels - hypoxia - and helped the salmon recover faster from exercise. Interestingly, elevated temperature was demonstrated to recruit paCA in a similar way to hypoxia, rising the intriguing perspective that CA could develop evolutionarily for better acclimatization and better preparedness to deal with elevated temperatures associated with climate changes. Altitude - typically associated with chronic hypoxia, induces dramatic changes in erythrocyte proteins: the erythrocyte lactate-H^+ co-transporter increased by 230%–405% in lowlanders spending time at altitude and was 324 % higher in natives. The erythrocyte inorganic anion exchanger (Cl^-–HCO_3^- exchanger AE1) was increased by 149%–228%[23, 24].

Moreover, a recent study[22] focused on CA activity in erythrocytes from three groups of athletes experiencing interval and continuous training for 6 weeks, in cold weather and high altitude (>1,600 m). This showed a 50% increase in the blood CA activity at the second week after initiation of training in both the interval and continuous running groups, whereas the control group did not experience any variation in enzyme activity levels. In the trained individuals a mild decrease in body mass, BMI and an increased V_{O2max} were also observed. The CA activity returned at the basal values after 4–6 weeks after the training started suggesting that a metabolic compensation occurred without the need for enhanced enzyme activity[22].

Low dietary Zn

Low dietary Zn results in lower serum and erythrocyte zinc concentrations, zinc retention, and total

CA and CA isoform activities in RBCs, as well as lower peak oxygen uptake, carbon dioxide output, and respiratory exchange ratio, and greater ventilatory equivalents for metabolic responses during exercise[25]. Similar functional responses are also observed during prolonged, submaximal exercise. One could conclude that low dietary zinc is associated with significant reductions in zinc status, including RBC CA activities, and impaired metabolic responses during exercise.

Moreover, it is claimed that natural products, resveratrol, catechin, curcumin, and silymarin are good inhibitors of CA Ⅰ and CA Ⅱ, however the field of dietary supplements and herbal extracts requires a higher degree of standardization.

Autoantibodies

Mounting evidence demonstrates that anti-carbonic anhydrase autoantibodies could be involved in modulation of the effectiveness of CA: measurable levels of autoantibodies against CA are present in rheumatoid disorders (such as systemic lupus erythematosus, polymyositis, systemic sclerosis or Sjögren´s syndrome and rheumatoid arthritis - where they bind to CA Ⅲ in synovial membranes) and acute myeloid leukaemia among others. Moreover, autoantibodies against CA Ⅱ were proved to play an important role in the pathogenesis of retinopathy and interestingly, they might be produced due to cross-reactivity with the CA of pathogens (such as those of *Helicobacter pylori*[11]). The binding of these antibodies also provides a quantitative way to assess the distribution of CA across different cell types: epidermal cells, hair follicles, sweat glands, and renal tubular cells.

A special emphasis on CA Ⅱ activators in platelet physiology has been recently revealed[11]. According to this review, potent CA agonists (with effects on platelet aggregation) are adrenaline and serotonin. Adrenaline can sensitize platelets to thrombin by induction of CA Ⅱ activity, is able to initiate platelet aggregation in a concentration dependent fashion and has an overall proaggregatory effect. Serotonin is also an activator of CA; it enhances adrenaline, adenosine diphosphate (ADP) and collagen induced aggregation, and since serotonin plasma levels are increased in stress conditions, it provides a clinically relevant association between plasma CA and coronary artery disease and predisposition to cardiac events. Selective serotonin reuptake inhibitors are also potential activators of CA Ⅱ; although their effect is more nuanced and their presentation outside the scope of the review, it is worth emphasizing this dual modulation potential as an indication of the complex interplay between activation and inhibition and the need for precise and dynamic detection assays. This idea is further suggested to be based on the role of histamine on platelet CA modulation. While in principle a poor CA Ⅱ activator, histamine present in human platelets increases platelet sensitivity to aggregation agonists like ADP, thrombin, collagen, arachidonic acid and adrenaline.

CARBONIC ANHYDRASE INVOLVEMENT IN PATHOPHYSIOLOGY

Numerous diseases result from inappropriate function (deficiency, inhibition, overexpression) of CAs. In terms of deficiency - it is generally considered that the erythrocyte CA Ⅰ deficiency has no clinically relevant effects[26]. CA Ⅱ deficiency can cause osteoporosis, renal tubular acidosis, and brain calcification[27-30]. CA Ⅲ deficiency (mostly in skeletal muscles) seems to play an import role in pathogenesis of myasthenia gravis[11]. CAVA deficiency may present as early-onset liver failure with hyperammonemia, hyperlactatemia, and ketonuria[11]. CAs activity in erythrocytes (CA Ⅰ and CA Ⅱ) has recently been observed to be associated with various pathological conditions especially in diabetes mellitus, hypertension and lipid disorders, and the occurrence of high sensitivity to sulfonamide inhibitors opens up new therapeutic avenues. The field of CA inhibitors and activators and their impact on different treatments and diseases is authoritatively reviewed elsewhere[15, 17].

CA Ⅰ overexpression has been associated with tumor differentiation and negatively with vascular invasion, while overexpression of CA Ⅱ is associated with tumor differentiation and a better prognosis for pancreatic cancer patients[5]. CA isoenzymes´ involvement in various types of anemia is complex: CA Ⅰ concentration is significantly lower in patients of autoimmune hemolytic anemia, CA Ⅱ provides the CA activity necessary for maintaining ion transport in erythrocytes-interestingly, CA activity and CA Ⅱ concentration in anemia patients were significantly higher than those of the control subjects; CA Ⅲ may work as an agent against oxidative damage in iron deficiency and beta-thalassemia anemia (CA Ⅲ concentration was lower in iron deficiency anemia, but higher in beta-thalassemia anemia). CA Ⅱ is found in the majority of acute leukaemias[5].

As fully demonstrated for CA Ⅸ, which has become an essential biomarker and therapeutic target for cancer treatment[31-33], CA isoforms have gained an important place as disease biomarkers. Based on the primary sequence identity (above 50%) and the number of conserved residues between catalytically active CA isoforms (above 130), we have evaluated the reported involvement of: CA Ⅰ, CA Ⅱ, CA Ⅲ,

CA Ⅶ, CAX Ⅲ as disease biomarkers. Since at the border line, with primary sequence identity >43%, CAⅤ (A, B) were also included.

Carbonic anhydrase Ⅰ as a disease biomarker

According to Zamanova *et al*., the amount of free CA Ⅰ protein in blood is normally very low in normal subjects, but can significantly increase in certain pathological conditions and displays a high cancer/normal condition selectivity; thus CA Ⅰ can be regarded as a suitable biomarker for breast cancer, non-small cell lung cancer (NSCLC), prostate cancer. Accordingly, there are methods for the early diagnosis of breast cancer, by relying on selective detection and the quantification of a multiple biomarker set (included two apolipoproteins, fibronectin, neural cell adhesion molecule L1-like protein and CA Ⅰ).

CA Ⅰ might serve as a novel biomarker for early detection in sera of NSCLC; CA Ⅰ can serve as a potential biomarker for the detection of prostate cancer. While plasma CA Ⅰ levels in prostate cancer patients were significantly higher than those in healthy controls, the combination of prostate-specific antigen (PSA) and CA Ⅰ assays may enhance the accuracy of early diagnosis of prostate cancer in patients with gray-zone (inconclusive) PSA level.

Outside the cancer field, measuring CA Ⅰ/leucine-rich alpha-2-glycoprotein in blood samples was patented as a method for diagnosing sepsis. A positive diagnosis of sepsis is made if the concentration of CA Ⅰ/leucine-rich alpha-2-glycoprotein is significantly higher than in healthy persons or non-septic kidney injury patients.

Also, CA Ⅰ has been identified among the most promising negative biomarkers for parasite infections (i.e. schistosomiasis) in human samples. Where interestingly, a progressive reduction of serum CA Ⅰ levels over a 12-week infection period was recorded. Similarly, CA Ⅰ may be an indicator to differentiate autoimmune hemolytic anemia from other types of anemia (CA Ⅰ concentration is significantly lower in patients of autoimmune hemolytic anemia).

Carbonic anhydrase Ⅱ as a disease biomarker

CA Ⅱ is the most active CA isozyme, having a CO_2 hydration turnover rate approaching the diffusion limit and has the widest distribution in the body, being expressed in the cytosol of cells from virtually every tissue or organ. Accordingly, the impact of this CA isozyme in the human body is best exemplified by CA Ⅱ deficiency syndrome, a human autosomal recessive disorder characterized by osteopetrosis, renal tubular acidosis. and cerebral calcification. Subjects suffering from this disorder are characterized by developmental delay, short stature, cognitive defects, and bone fragility.

Notable, besides its presence in erythrocytes, CA Ⅱ is found in large amounts in platelets and neutrophils. Thus, it can be used in assays to detect hemolysis within a blood sample. When hemolysis occurs, both CA Ⅱ and CA Ⅰ are released in the blood stream. The assay exploits the catalytic properties of CA towards the CO_2 hydration reaction. However, the circulation time of the free proteins is short (about 2 h) due to their removal by reticulo-endothelial system after binding by a transferrin-like protein or the plasma CA inhibitor.

It has been reported that the assay can be utilized to assess RBC hemolysis in patients on drugs that would predispose them to hemolysis, as well as patients suffering from other conditions such as hemoglobinopathies, disseminated intravascular coagulation, malaria, pulmonary hypertension, and anemia. The assay might be also useful for assessing RBC status/fragility prior to blood transfusion, to complement impedance/dielectric assays[34, 35] and CA release following lysis of a wide range of cells and tissues.

It has been also suggested that anti-CA Ⅱ antibodies have the potential to be utilized for imaging vulnerable plaques in atherosclerosis[36]. CA Ⅱ was identified as the target for single-chain antibodies that target the vascular endothelial layer and sub-endothelial cell surface proteins that stained an area rich in macrophage- and smooth muscle cell-derived foam cells under the endothelium and a deeper area rich in necrotic cells adjacent to the internal elastic lamina in advanced lesions. Of note, anti-carbonic anhydrase Ⅱ (anti-CA Ⅱ) antibodies have been reported to be related in some cases to renal manifestations of primary Sjögren´s syndrome. While of apparently no direct relevance to blood, this is an interesting facet of Sjögren´s syndrome, an autoimmune disorder that often accompanies other immune system disorders, such as rheumatoid arthritis and lupus (lupus being with direct relevance for autoimmune hemolytic anemia), that is characterized by decreased tear and saliva secretions due to immune attack of the mucous membranes and moisture - secreting glands of eyes and mouth. Whereas with genetic implication it appears that a triggering mechanism - such as infection with a particular virus or strain of bacteria-is also necessary. Corroborated with the fact that serum antibodies to both CA Ⅰ and Ⅱ were reported for Sjögren´s syndrome[37], further analysis for correlation with other autoimmune disorders (e.g. autoimmune hemolytic anemia) could be worth pursuing. A whole range of

patents related to CA (including CA Ⅱ) assays for other diseases is reviewed elsewhere[15, 36, 38, 39].

Carbonic anhydrase Ⅲ as a disease biomarker

CA Ⅲ is a low activity isozyme, with a K about 100 smaller than that of CA Ⅱ. This is due to particularities in its active site, which also make this isozyme much more resistant to inhibition with sulfonamides as compared with CA Ⅱ and other (fast) isozymes. It can be found in large amounts in skeletal muscles (up to 8% of the soluble protein) in adipocytes, and, in low amounts in the uterus, red blood cells, prostate, lung, kidney, colon, and testis. This particular biodistribution of CA Ⅲ isozyme, its high abundance in skeletal muscles and its absence from myocardial muscle suggests CA Ⅲ as a serum marker for skeletal muscle damage and also for acute myocardial infarction (AMI).

The presence of both myoglobin and CA Ⅲ in blood (low myoglobin/CA Ⅲ ratio) indicates skeletal muscle damage, while the presence of only myoglobin (or high myoglobin/CA Ⅲ ratio) indicates potential AMI. During early onset of key symptoms of myocardial infarction, myoglobin/CA Ⅲ ratio is a sensitive parameter for the detection of early AMI. The distinctive nature of CA Ⅲ relative to other CAs is the presence of two reactive sulfhydryl groups, which can form a disulfide linkage. Through these S-containing groups, CA Ⅲ can contribute to resistance to oxidative stress, from either natural sources or induced via the consumption of alcohol or various drugs, etc. Interestingly, a down-regulation of CA Ⅲ due to ethanol-induced biochemical stress was reported in liver cells[36].

CA Ⅲ was reported as a target protein recognized by autoantibodies, with obvious modulation of its activity. Interestingly, CA Ⅲ, as the target protein recognized by autoantibodies in rheumatoid arthritis sera showed the highest specificity when compared to non-autoimmune diseases: CA Ⅲ is essentially absent from the synovial membrane of non-rheumatoid arthritis patients.

The use of CA Ⅲ autoantibodies quantification has been expanded to the diagnosis of vasculitis - clinical syndromes characterized by blood vessel wall inflammation that lead to tissue or end organ damage. Corroborating these results to CA Ⅲ for use as a biomarker, although never attempted, the evaluation of CA Ⅲ expression in assessing of the quality of organs to be transplanted in conjunction with electrical assays could be interesting[40, 41].

Carbonic anhydrases Ⅴ A and Ⅴ B as disease biomarkers

CAs ⅤA and ⅤB are mitochondrial isozymes with medium-high activity important for gluconeogenesis and ureagenesis. CA ⅤA is found mainly in the liver, while CA ⅤB is found in skeletal and heart muscles, kidneys, the pancreas, GI tract, brain, and spinal cord. The important role played by these isozymes in the body is evidenced in the CAⅤ-deficiency syndrome. This deficiency is an inherited disorder characterized by episodes during which the balance of certain substances in the body is disrupted (known as metabolic crisis) and brain function is abnormal (known as acute encephalopathy). These potentially life-threatening episodes can cause poor eating, vomiting, weight loss, tiredness (lethargy), rapid breathing (tachypnea), seizures, or coma. During an episode, people with CA ⅤA deficiency have excess ammonia in the blood (hyperammonemia), problems with their acid-base balance in the blood (metabolic acidosis and respiratory alkalosis), low glucose in the blood (hypoglycemia), and reduced production of bicarbonate in the liver. These imbalances lead to the signs and symptoms that occur during the episodes through the neonatal period and early childhood.

These episodes may be triggered by fasting for longer than usual periods between meals or when energy demands are increased, such as during illness. The risk of metabolic crisis and acute encephalopathy is thought to decrease after childhood. Because of the small number of people with CA ⅤA deficiency who have come to medical attention, the effects of this disorder in adults are not well understood. However, the diagnostic molecular analysis of CA ⅤA in newborns and other persons displaying this biochemical imbalance has recently been considered valuable[36]. Moreover, CA Ⅴ A deficiency was proposed to be added to the list of treatable inborn defects of the metabolism, potentially causing intellectual disability[36].

Also related to metabolism, CA ⅤA and ⅤB constitute major targets for anti-obesity therapy. The identification that mitochondrial CAs are implicated *in de novo* lipogenesis has allowed for the consideration of selective inhibitors of such enzymes to be useful for the development of new antiobesity drugs. This includes treatment with two primary sulfonamide drugs, topiramate and zonisamide with potent CA inhibitory activity, as well as their combination with other agents. Moreover, via transcriptional profiling of peripheral blood mononuclear cells, CA ⅤB gene and CA ⅤB were identified as potential biomarkers for the diagnosis of pancreatic cancer (for which the main PC approved biomarker, carbohydrate antigen 19–9, lacks selectivity and specificity)[36].

Carbonic anhydrase Ⅶ as a disease biomarker

CA Ⅶ is a fast isozyme found in large amounts in

central nervous system (CNS), but also encountered in colon, liver, skeletal muscles, duodenum, and stomach. In CNS, CA Ⅶ is found in neurons of hippocampus (but not in glial cells), together with CA Ⅱ. The co-expression of these two cytosolic isoforms enhances bicarbonate-driven GABAergic excitation during intense GABAA-receptor activation. CA Ⅶ is an important modulator of long-term synaptic plasticity, with direct implications for memory and learning processes. Moreover, CA Ⅶ is proposed to be a key molecule in age-dependent neuronal pH regulation, while CA Ⅱ and CA Ⅴ are linked to ageing and neuronal degeneration (including Alzheimer disease)[42–47].

Like with other CA isoforms, CA Ⅶ was recently suggested as a genetic marker for detecting colorectal cancer within a larger group of biomarkers (there is an increased expression of CA Ⅶ in colorectal cancer cells *vs*. normal counterparts) and CA Ⅶ levels were shown to be useful as a predictor of malignancy propensity within sampled tissues (Zamanova *et al*. 2019). However, no clear evidence was identified for blood related diseases.

Carbonic anhydrase XⅢ as a disease biomarker

CA XⅢ is recognized as among the recently characterized isozymes of CA in humans (Zamanova *et al*. 2019 see references within). It is a compact monomeric globular protein, with low-moderate catalytic activity (actually the second least active form among other cytosolic isozymes of CA, after CA Ⅲ. CA XⅢ expression has been reported in the cytosol of cells of different body organs including colon, small intestine, testis, uterine cervix, certain endometrial glands and thymus (Zamanova *et al*. 2019 and references within). Owing to its abundance in reproductive system organs, CAXⅢ is known to play a crucial role in regulating HCO_3^- ion concentration and pH homeostasis in the cervical and endometrial mucus, by maintaining the mobility of spermatozoids and ensuring normal fertilization process.

While no clinical significance has been reported for the level of expression of CAXⅢ in humans, intriguingly, a down-regulation of the level of expression of CAXⅢ (as well as of CA Ⅰ and CA Ⅱ) has been reported in patients diagnosed with colorectal cancer (Zamanova *et al*. 2019), with the lowest signal being detected in carcinoma samples. No clear evidence however has been identified for blood related diseases.

Carbonic anhydrases as biosensors

CAs have been studied for over 90 years and have ever since been at the forefront of scientific discovery; from basic enzymology, to the application of structural biology and *in silico* approaches to study protein dynamics, discovery and clinical medicine[10].

The intrinsic expression and dynamics of CA isozymes at the level of blood compartments of different tissues/organs, is used to early detect, confirm and assess the progress or stage of different diseases and even predict patient prognosis. The field has matured in recent years, shifting from the predominant use of CA Ⅸ and CA Ⅻ in cancer diagnostics (including staging and prognosis assessment) towards the use of a wider list of CA isozymes as disease biomarkers. In the medical field, CA isozymes have been successfully included in sets of reliable biomarkers and in designs of specific chips dedicated to particular diseases. Essentially all CA isozymes associated with blood cells either directly, i.e. CAⅠ and CA Ⅱ (or based on the ones having a high degree of homology with them i.e. CAⅢ, CA Ⅴ, CA Ⅶ, CAX Ⅲ) or indirectly have been proved useful as biomarkers. Either alone or in combination with other proteins, CAs are used for the detection, staging, and prognosis of a huge repertoire of human dysfunctions and diseases, ranging from slight imbalances and dysfunctions to autoimmune diseases and cancer.

With the development of new, improved modulators (activators and inhibitors), as well as antibodies that can be targeted against different areas of the core catalytic region of CA isozymes or can be designed to recognize structural regions unique only to one isozyme (e.g. the proteoglycan region of CA Ⅸ), the biosensing area has gained primary tools for the detection and quantitation of CA isozymes in different human and animal samples, ranging from blood, urine and saliva, to biopsy specimens and different tissue samples.

Surface plasmon resonance assays[12, 48–59], impedimetric techniques[60, 61], or free-solution interaction assays[54] were added to more standard techniques such as immunostaining, ELISA, Western blotting, to enable quantitation of CA isozymes in normal and diseased tissues and assessment of their enzymatic activity/inhibition, and propel CAs as actual theranostics tools (belonging to the field of medicine which combines specific targeted therapy based on specific targeted diagnostic tests).

Carbonic anhydrase applications for synthetic blood products

Owing to their high catalytic rate and the relatively simple procedure of expression and purification, relatively stable and extensive biophysical studies dedicated to CAs (in particular CA Ⅱ) have made CA

an exciting candidate for incorporation into various biomedical applications such as artificial RBCs, artificial lungs and in biosensors and CO_2 sequestration systems, among others. This overarching innovative merge of basic knowledge and nanotechnology is possibly best described in the title of Chang *et al´*s review[62]: CAs as enabling tools for potent artificial red blood cells, therapeutics, artificial cells, nanomedicine and beyond.

Indeed, bio-inspired nanoscale and microscale engineering of synthetic (or semi-synthetic) blood cells using a variety of biomaterials-based systems[63] has generated significant academic and clinical research interest. Continuous research is aimed at developing technologies that can simulate the structure and/or property of blood cells as well as plasma, to allow for the development of "blood substitutes" and theranostics. As authoritatively reviewed developments have led in the last years to polyhemoglobin-catalase-superoxide dismutase-carbonic anhydrase constructs that harness the concept of micro-nanoscale encapsulation of blood relevant compounds within particulate vehicles to gain protection from plasma-induced effects, increased circulation time and sustained delivery to cells, tissues, and organs[63]. These fulfill all three major functions of red blood cells in acting as an O_2 and CO_2 carrier with enhanced antioxidant properties: (1) oxygen transport; (2) carbon dioxide transport; and (3) antioxidant functions. Although there are so far no FDA approved blood substitutes, the field is expected to gain momentum leading to results in breakthrough products, especially through the integration of new biosensing approaches, synthesis[16, 64–69] and nanotechnology[63, 70].

Moreover, in conjunction with other compounds, this paves the way for novel therapeutics. For several decades, researchers have used erythrocytes for drug delivery of a wide variety of therapeutics in order to improve their pharmacokinetics, biodistribution, controlled release, pharmacodynamics, and immunogenicity. Approaches include the coupling of drugs onto the red blood cell surface[71, 72] as well as the encapsulation of drugs within erythrocytes. One such approach led to self-monitoring artificial RBC with autocatalytic (including sufficient oxygen supply) for enhanced photodynamic therapy (PDT). Biomimetic artificial red blood cells were produced by loading complexes of oxygen-carrier (hemoglobin) and photosensitizer (indocyanine green). Such a nanosystem provides a coupling structure with a stable self-oxygen supply and acts as both a fluorescent and photoacoustic imaging probe, dynamically monitoring the nanoparticle biodistribution for the treatment of PDT. The artificial red cells capable of self-monitoring with boosted photodynamic efficacy could serve as versatile theranostic platforms[71, 72].

Carbonic anhydrase biosensing for pollutants

Metals play a key role in the bioactivity of this metalloenzyme, not only as both cofactors of CA, but also as inhibitors of CA activity and modulators of CA expression[19, 20, 73]. Accordingly, CAs have gained a prominent role in the development of biosensors (including genetically encoded fluorescent ones) for the assessment of metals in cells and in blood.

In vitro and *in vivo* studies carried out to date reveal dose-response sensitivity of CA activity to a number of pollutants, heavy metals and xenobiotics, suggesting the possible use of CA activity alterations as a general pollutant biomarker. However, the high species- and tissue-specificity of the observed responses suggest the need for a thorough knowledge of pollutant induced CA alterations in the specific bioindicator species utilized.

As recently revealed[74], following pollution exposure there is a significant induction of CAs in the hepatopancreas of a bioindicator organism (*Mytilus galloprovincialis)*; it thus demonstrated its suitability to integrate CA assessment into multimarker approaches for the detection and characterization of the stress status induced by pollution exposure in bioindicator organisms, further expanding the range of biosensing avenues based on CA.

Interestingly, more and more emphasis has been placed on gaining access to CAs specific dynamics (as opposed to end point analyses) and to their prognostic and diagnostic relevance is gained from only recently advanced multiparametric analyses[39, 73–78]. The concept of dynamic monitoring and cell reactivity modulation has been recently demonstrated[79] using optogenetic stimulation. Importantly, the light protein channel (rhodopsin) belongs to *Chlamydomonas reinhardtii*, an algae where the CA isoforms of intracellular location, expression, and physiological roles have been thoroughly investigated[80]. In conjunction with the demonstrated[81–83] capacity to modulate and assay the intra/extracellular pH of cells with high spatial resolution using reflected light microscopy, this opens up interesting avenues for multiparametric studies, not possible up to now. The evaluation of CA using scanning photo-electrochemical microscopy (SPECM) although has not been demonstrated until now, is expected to be achieved for both purified versions of the enzyme and "intact" cells, based on biocompatible redox polymers used for the immobilization and electrical wiring of enzymes[84].

Looking ahead

It is hoped that new dynamic and quantitative studies will allow for the efficient tracking and quantitation of various CA isozymes within the blood and more generally within the human body and increase their use as disease biomarkers, while also contributing to a better understanding of the biochemical and physiological role of blood associated CA isozymes in normal physiology and pathology.

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