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Sialic acid and sialidase activity in acute stroke

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Abstract. Stroke is a heterogeneous syndrome caused by multiple disease mechanisms, resulting in a disruption of cerebral blood flow with subsequent tissue damage. It is well known that erythrocytes have a large amount of sialic acid and could represent a model to investigate changes occurring in a pathology like stroke. The aim of this study was to investigate a possible relationship among erythrocyte membrane, plasma and sialic acid content. The possible impact of the sialic acid content and the activity of sialidase on stroke severity was also evaluated.
The study population consisted of 54 patients with a first stroke and of 53 age-and sex matched healthy volunteers.
The total bound sialic acid was substantially decreased in patients. There was a significant correlation between the sialidase activity values and the severity of the neurological deficit defined by the National Institute of Health Stroke Scale.
This study shows that low sialic acid erythrocyte concentrations with contemporary high sialic acid plasma levels and elevated sialidase activity can be considered as markers of ischemic stroke. Further investigations are needed to clarify the possible role of these biochemical changes in producing and sustaining cerebral ischemic damage.

Keywords: Sialic acid, sialidase, erythrocytes, plasma, acute stroke

1. Introduction

Erythrocyte aggregation is one of the main determinants influencing blood circulation at low shear rates by increasing blood viscosity and inducing "sludging" in the capillary bed. Aggregation of red blood cells (RBC) is a reversible process that occurs when the bridging force due to the adsorption of macromolecules onto adjacent cell surface exceeds the disaggregation forces caused by electrostatic repulsion, membrane strain, and mechanical shearing. An increase in erythrocyte membrane aggregation was found to be associated with cardiovascular risk factors such as diabetes, hypertension, and hyperlipoproteinemia, and in clinical situations such as myocardial ischemia, thromboembolic states, and retinal venous occlusion [1].

Sialic acid (SA) refers generically to acetylated derivatives of neuraminic acid that are present in both lipoproteins and glycolipids found in plasma and in cellular membranes [2]. Sialic acids are constituents of acute phase proteins and are highly concentrated in the surface of vascular endothelium [3]. The sialic acid content of cellular membranes may account for up to 50% of the negative charge of the cell surface, suggesting a key role in the maintenance of cellular integrity. The sialic acid content of lipoproteins and erythrocytes also confers surface electronegativity, important for physiological function [1,2]. The role of sialic acid in the pathogenesis of atherosclerosis and as a predictor of cardiovascular events has attracted much attention.

SA is a N-acetylated derivative of neuraminic acid that is an abundant terminal monosaccharide of glycoconjugates. Normal human serum SA is largely bound to glycoproteins or glycolipids, with small amounts of free SA [4]. Negatively charged SA units stabilize glycoprotein conformation in cell surface receptors to increase cell rigidity. This enables signal recogni-

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tion and adhesion to ligands, antibodies, enzymes and microbes [5]. SA residues are antigenic determinant residues in carbohydrate chains of glycolipids and glycoproteins [4], chemical messengers in tissue and body fluids, and may regulate glomeruli basement membrane permeability [2]. Studies have shown an association between serum SA and cardiovascular mortality in the general population [6,7]. However, the mechanisms underlying this are unknown. In healthy arteries, sialic acids contribute to the overall net negative charge of vascular endothelial cells and low density lipoprotein (LDL) surface receptors [2]. It has also been reported that a reduced SA content of LDL has a greater propensity to form aggregates that are readily taken up by smooth muscle cells [8,9], suggesting that a low sialic acid content of LDL may be atherogenic. SA is an acute-phase reactant by itself and moieties are found also at terminal oligosaccharide chains of acute phase proteins [10,11]. Serum SA has been proposed as a marker of an acute-phase response in CVD.

It has recently been reported that sialic acid levels rise in myocardial infarction [12,13] and in different inflammatory disorders [5]. It is well known that the activation of the coagulation system is closely associated with the development thrombotic episodes in the evolution of acute ischemic stroke. The presence of blood factors that reflect enhanced thrombogenic activity, would not only be associated with thrombotic process but also with atherogenesis and inflammatory process. Sialidase, is a very common enzyme that hydrolyzed terminal sialic acid residues on polysaccharide chains, most often exposing a galactose residue. This enzyme is present in the erythrocytes, as opposed to other cell types. It is well known that erythrocytes have a large amount of sialic acid and could represent a model to investigate the changes occurs in a pathology like stroke [14].

The aim of this study was to investigate a possible relationship among erythrocyte membrane, plasma and sialic acid content. The possible impact of the sialic acid content and the activity of sialidase on stroke severity was also evaluated. Moreover the study was performed to asses changes in sialidase activity in patients with recent onset stroke in red blood cells.

2. Material and methods

Patients ($n = 114$) admitted to the Stroke Unit of the Department of Neuroscience of Polytechnic University of Marche, between May 2005 and November 2006, were initially recruited in the study.

Admission criterion was the presence of a clinical syndrome suggestive of a large artery involvement [15]. All recruited subjects gave informed consent prior to the drawing of peripheral venous blood; the study was performed in accordance with Declaration of Helsinki as revised in 2001 and the study was approved by the Institutional Review Board of the University.

The diagnosis of stroke was based on a focal neurological deficit that lasted for a least 24 hours [16]. The diagnosis of ischemic stroke was then confirmed through CT scan within 12 hours from stroke onset.

The exclusion criteria were as follows: hemorrhagic lesion in computed tomography (CT scan) (24 patients); transient ischemic attack or TIA (9 patients whit neurological deficit lasting $<$ 24 hours); a history of chronic liver disease (5 patients); chronic renal disease (6 patients), neoplastic disease (4 patients), cardioembolic stroke (12 patients).

Thus, the study population consisted of 54 patients with large-artery stroke (16 women, 38 men, mean age: 70.9 ± 13.7 and 68.9 ± 16.3 respectively) which did not undergo previous cerebrovascular diseases. The control group consisted of 53 age-and sex matched healthy volunteers (20 women, 33 men, mean age: 71.2 ± 16.8 and 69.3 ± 17.4 respectively), which presented a negative anamnesis for past ischemic stroke or TIA. Further, all these subjects were submitted to a careful clinical evaluation, electrocardiogram, haematological screeningand ultrasonographic evaluation of neck arterial vessels to exclude the presence of neoplastic, inflammatory and atherosclerotic conditions.

In patients, the severity of the neurological deficit on admission was assessed using the National Institute of Health stroke scale (NIHSS) [17]. The venous samples were performed at entry before the administration of any medication; different measurements were made only on samples with confirmed diagnosis of ischemic stroke. The venous samples were performed at entry within 12 h from symptoms onset and prior of any drug administration.

In each sample, sialic acid levels both in plasma and in erythrocyte were determined. Moreover sialidase activity in erythrocyte were determined. Plasma was obtained through a centrifuged for 15 min at 200 $\times$ g. Plasma samples were immediately stored at $-80\,^{\circ}\mathrm{C}$ after withdrawal.

2.1. Erythrocyte membrane preparation

Heparinized blood samples (10 mL) collected after overnight fasting were centrifuged (4,500 $\times$ g)

to remove plasma. RBCs were washed twice with NaC1 0.9% isotonic solution, lysed hypotonically in 5 mmol/L ice-cold phosphate buffer solution (pH 8), and processed in a Kontron (Milano, Italy) centrifuge at 20,000 $\times$ g. The resulting membranes were washed with phosphate buffer of decreasing molarity to completely remove the haemoglobin. The membrane yield was similar in all groups studied ($\sim$2 μg membrane proteins) [18].

2.2. Determination of SA content

SA content of RBC membranes was determined by the periodate thiobarbituric acid method of Denny et al. [19].

Briefly, membranes (1 mg membrane proteins/mL) were first hydrolyzed in 0.05-mol/L I-I2SO 4 in a final volume of 0.1 mL for 1 hour at 80 °C to release SA [20].

Both standards and samples were incubated with 0.25 mL periodate solution (0.025 mol/L periodic acid in 0.25 mol/L HCI) at 37°C for 30 minutes. After reduction of excess periodate with 0.25 mL 0.32 mol/L sodium thiosulfate, the reaction was completed by addition of 1.25 mL 0.1-mol/L thiobarbituric acid. The samples were heated at 100°C for 15 minutes and cooled to room temperature. The product was extracted with acidic butanol and colorimetrically assayed with a spectrophotometer at 549 nm.

Protein content was determined by Bradford method to normalize the sialic acid content using crystalline BSA as the standard [21].

The total plasma sialic acid (TSA) level was measured by a colorimetric assay for a commercial enzymatic kit (Sialic acid Farbtest, Boehringer Mannheim, Germany).

2.3. Enzyme assays

The assays of resealed membrane sialidases obtained from control and patients erythrocytes were routinely determined by fluorimetric methods [22]. The assay mixtures, containing in a final volume of 0.1 mL, 50 mM citric acid-sodium phosphate buffer (at established optimal pH), 0.15 M NaCl, 0.6 mM MU-NeuAc (optimum concentration), 10 to 60 μg protein of enzyme preparation, and 0.6 mg albumin, were incubated for up to 30 minutes at 37°C. The blank mixtures consisted of the incubation mixtures lacking the enzyme preparation. Enzyme activities were expressed as units (U), ie, the amount of enzyme that liberates 1 μM of product per minute at 37°C under optimal conditions.

2.4. Statistical analysis

Statistical analysis was performed using the SAS statistical package (Statistical Analysis System Institute, Cary, NC). All experiments were carried out in duplicate and were repeated three times. Data were compared using unpaired Student's t-test. Correlations were performed by using Pearson's coefficient. All values were reported as mean $\pm$ SD. Significance was established at the level of $p < 0.05$.

3. Results

The content of sialic acid in red blood cells was significantly decreased in patients with respect to controls (34.82 $\pm$ 1.95 μgAS/mgprot vs 45.92 $\pm$ 2.87 μgAS/mgprot, $p < 0.001$) (Fig. 1A). Moreover content of sialic acid in plasma was significantly increased in patients compared to controls (82.33 $\pm$ 6.77 μgAS/mgprot vs 70.75 $\pm$ 8.92 μgAS/mgprot, $p < 0.001$) (Fig. 1A). Sialidase enzyme activity in red blood cells of patients was significantly increased in respect to controls (15.03 $\pm$ 1.92 mU/mL vs 6.00 $\pm$ 0.65 mU/mL, $p < 0.001$) (Fig.1B). As a consequence, the total bound sialic acid was substantially decreased in patients. Moreover there was a significant positive correlation between the sialidase activity values and the severity of the neurological deficit defined with the NIHSS score ($p < 0.001$ r $= 0.834$) (Fig. 2). It has been found a significant positive correlation between sialic acid plasma level and NIHSS ($p < 0.001$ r $=$ 0.832) (Fig. 3). It has been highlighted no differences in gender in sialic acid levels and sialidase activity.

4. Discussion

Sialic acid plays a central role in the functioning of biological systems, being commonly positioned at the terminal positions of complex carbohydrates. Free SA is poorly present in organisms – SA occurs mainly at terminal positions of glycoprotein and glycolipid oligosaccharide side-chains [2].

Since cell surfaces and membrane components play a prominent role in neoplastic behaviour, neoplasms often have an increased concentration of sialic acid on the tumor cell surface, and sialoglycolipids are secreted by some of these cells, increasing their concentrations in blood [23]. On the other hand, since as integral parts of the cell membrane, the gangliosides or sialic

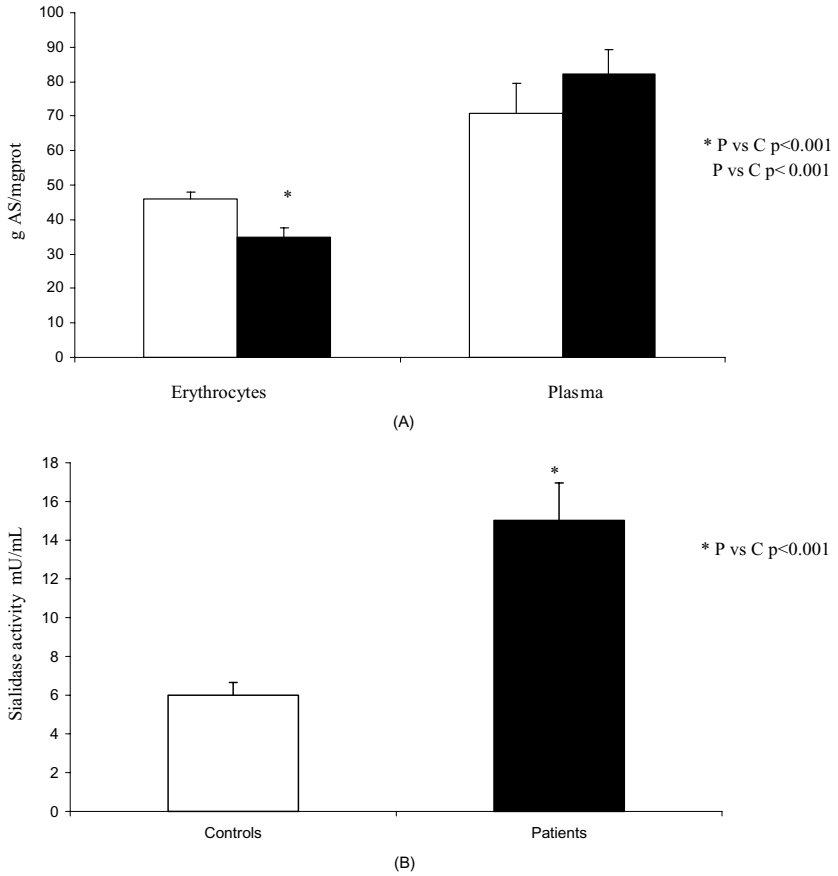

Fig. 1. (A) Sialic acid levels in controls (C, white column) and in patients (P, black column) with acute stroke both in red blood cells and in plasma. Means $\pm$ standard deviations are shown $p < 0.001$. (B) Sialidase activity in controls (C, white column) and in patients (P, black column) with acute stroke in red blood cells. Means $\pm$ standard deviations are shown $p < 0.001$.

acid may play a controlling role in the process of cell-to-cell recognition and in the feedback growth inhibition, and hence the carbohydrate moiety may influence growth and cell-to-cell interaction and thus may be of importance in the development of this disease [23].

Increased sialic acid concentrations have been reported during inflammatory processes [2] in serum and in plasma. In addition, in previous study, the sialic acid level it has been shown to be correlated with the presence of carotid atherosclerosis, independently of major cardiovascular disease risk factor [24].

There is considerable evidence that serum sialic acid rises during acute phase response such as after a myocardial infarction and it is higher in people with established cardiovascular disease [25]. In previous studies, there is mounting evidence that serum sialic acid is a marker of both atherosclerosis and progression of atherosclerosis [26]. This means that serum sialic acid is raised in people who have sub-clinical disease and/or more rapid progression of atherosclerosis and therefore explains any association with future events in people who are currently clinically free of disease. The increase in total serum sialic acid reflects increased sialylation of glycoproteins or glycolipids due to increased sialyltransferase activity, and/or increased secretion of sialic acid from cell membranes due to elevated sialidase activity. The activity of these enzymes is increased in atherosclerosis [27].

The previously reported data, raise the hypothesis that investigating SA functions could help to gain insights into molecular nature of many physiological and pathophysiological pathways; in fact, the mechanisms underlying the elevated SA concentrations in plasma and the diminished values in erythrocyte, in different diseases, are still not clear.

The present study confirms, on stroke, the results obtained in previous research regarding sialic acid level in other pathologies. Moreover, this study extends previ-

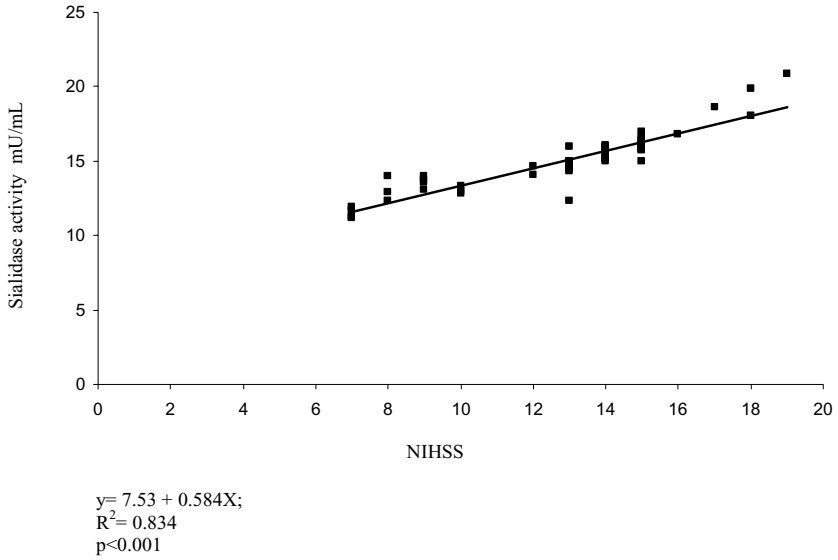

Fig. 2. Correlation between erythrocytes sialidase activity and the severity of the neurological deficit on admission using the National Institute of Health stroke scale (NIHSS).

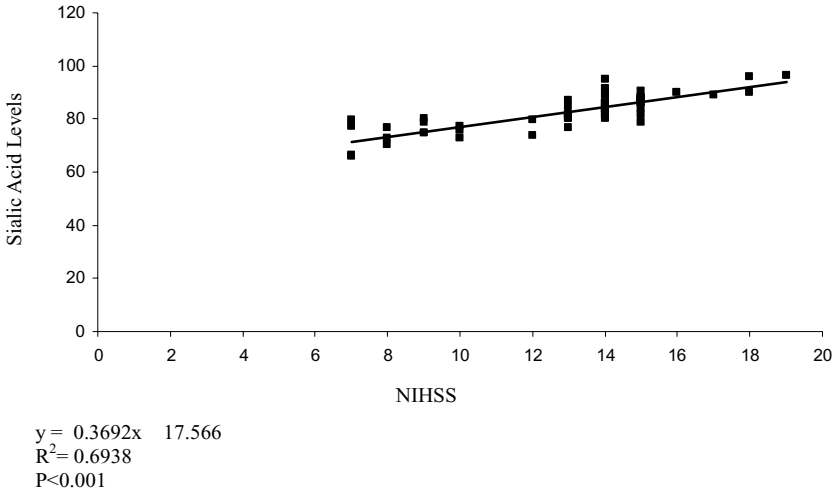

Fig. 3. Correlation between plasmatic sialic acid level and the severity of the neurological deficit on admission using the National Institute of Health stroke scale (NIHSS).

ous investigations showing a positive relation between erythrocytes sialidase activity and the severity of the neurological deficit after stroke.

In patients with stroke, the marked observed increase of the sialidase, the only form of enzyme responsible for the release of sialic acid from endogenous sialoderivatives, present either at the erythrocyte membrane or in the plasma [22,23], is likely responsible for the lower sialic acid content (40%) found in erythrocyte membranes of these subjects. At the same time, the sialidase is responsible of the significant increase of SA in plasma of the same subjects. Thus, the observed enhanced sialidase activity leads to the detachment of sialic groups positioned at the non-reducing end of complex carbohydrates in erythrocyte membranes, and to the consequent increase of free SA levels in plasma. In this way, negative charge of the cell surface results decreased, leading to a lost of cellular integrity. The decrease of sialic acid content of erythrocytes causes also a lost of physiological function, as our group have been demonstrated in previous study [28], that can be related to the pathophysiology and the severity of stroke.

At this point it is important to remember that the sialidase is present in a "cryptic" form [29,30]. Therefore, it may be suggested that its activity could be unmasked when major changes of the membrane enzymes and proteins occur, especially as a consequence of specific physiologic events involving certain domains of the erythrocyte membrane such as vesiculation processes [31,32].

In the acute phase of ischemic stroke, an activation both coagulation and inflammation systems occurs. In a longer period following the acute event, an increased thrombogenic activity can be detected. The presence of a correlation between NIHSS score and sialidase reflects the importance of this enzyme activity as a potential contributor to cerebral ischemic damage. A possible explanation of these findings is that the erythrocyte sialic acid concentration may reflect the existence or the activity of a thrombotic process, and this may warrant further investigation.

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References

[1] N. Moretti, R.A. Rabini, L. Nanetti, G. Grechi, M.C. Curzi, N. Cester, L.A. Tranquilli and L Mazzanti, Sialic Acid Content in Erythrocyte Membranes From Pregnant Women Affected by Gestational, *Diabetes Metabolism* **5** (2002), 605–608.

[2] R. Schauer, Sialic acids and their role as biological masks, *Trends Biochem Sci* **10** (1985), 357–360.

[3] G.V. Born and W. Palinski, Unusually high concentrations of sialic acids on the surface of vascular endothelia, *Br J Exp Pathol* **66** (1985), 543–549.

[4] M.A. Crook, Sialic acid and cardiovascular disease, *Med Biochem* **1** (1999), 123–130

[5] P. Sillanaukee, M. Ponnio and I.P. Jaaskelainen, Occurrence of sialic acids in healthy humans and different disorders, *Eur J Clin Invest* **29** (1999), 413–425.

[6] G. Lindberg, L. Rastam, B. Gullberg and G.A. Eklund, Serum sialic acid concentration predicts both coronary heart disease and stroke mortality: multivariate analysis including 54 385 men and women during 20.5 year follow up, *Int J Epidemiol* **21** (1992), 253–257.

[7] G. Lindberg, G.A. Eklund, B. Gullberg and L. Rastam, Serum sialic acid concentration and cardiovascular mortality, *BMJ* **302** (1991), 143–146.

[8] V.V. Tertov, A.N. Orekhov, O.N. Martsenyuk, N.V Perova and V.N. Smirnov, Low-density lipoproteins isolated from the blood of patients with coronary heart disease induce the accumulation of lipids in human aortic cells, *Exp Mol Pathol* **50** (1989), 337–347.

[9] V.V. Tertov, I.A. Sobenin and A.N. Orekhov, Characterisation of desialylated low-density lipoprotein which cause intracellular lipid accumulation, *Int J Tissue Reac* **14** (1992), 155–162.

[10] M.A. Crook, P. Tutt and J.C. Pickup, Elevated serum sialic acid concentration in NIDDM and its relationship to blood pressure and retinopathy, *Diabetes Care* **16** (1993), 57–60.

[11] M.A. Crook, P. Tutt, H. Simpson and J.C. Pickup, Serum sialic acid and acute phase proteins in type 1 and type 2 diabetes mellitus, *Clin Chim Acta* **219** (1993), 131–138.

[12] E. Reganon, V. Martınez-Sales, V. Vila, A. Vaya, M. Martinez and M.A. Palencia, Relationship between fibrinogen protein and fibrinogen function in post-myocardial infarction patients, *Thromb Res* **104** (2001), 413–419.

[13] E. Reganon, V.Martınez-Sales, V. Vil, A. Vaya and J. Aznar, Inflammation, fibrinogen and thrombin generation in patients with previous myocardial infarction, *Haematologica* **87** (2002), 740–745.

[14] C.J. Murray and A.D. Lopez, Mortality by cause for eight regions of th word: Global burden of disease study, *Lancet* **349** (1997), 1269–1276.

[15] A. Davaloas, E. Cendra, J. Teruel, M. Martinez and D. Genis, Deterioratine ischemic stroke: risk factor and prognosis, *Neurology* **40** (1990), 1865–1869.

[16] K. Aho, P. Harmsen and S. Hatano, Cerebrovascular disease in the community: results of a WHO collaborative study, *Bull WHO* **58** (1980), 113–130

[17] R.J. Wityle, M.S. Pessin, R.F. Kaplan and L.R. Caplan, Serial assessment of acute stroke using the NIH stroke scale, *Stroke* **25** (1994), 362–365.

[18] L. Mazzanti, R.A. Rabini, E. Salvolini, M. Tesei, D. Martarelli, B. Venerando and G. Curatola, Sialic acid, diabetes, and aging: a study on the erythrocyte membrane, *Metabolism* **46** (1997), 59–61.

[19] P.C. Denny, P.A. Denny and S.E. Allerton, Determination of sialic acidbusing 2-thiobarbituric acid in the absence of hazardous sodium arsenite, *Clin Chim Acta* **131** (1983), 333–336.

[20] L. Warren, The thiobarbituric acid assay of sialic acids, *J Biol Chem* **234** (1959), 1971–1975.

[21] M.M. Bradford, A rapid and sensitive method for the quantitation of migrogram quantities of protein utilizing the principle of protein-dye binding, *Ann Biochem* **72** (1976), 248–251.

[22] S. Marchesini, B. Venerando, A. Fiorilli and A. Preti, Use of 2-(4 methyl-umbelliferyl)-D-N-acetylneuraminic acid for the determination of the sialidase activity in different tissues, *Perspect Inherited Metab Dis* **4** (1981), 191–203.

[23] T. Idota and H. Kawakami, Inhibitory effects of milk gangliosides on the adhesion of Escherichia coli to human intestinal carcinoma cells, *Biosci Biotechnol Biochem* **59** (1995), 69–72.

[24] K.P Gopaul and M.A. Crook, Sialic acid: a novel marker of cardiovascular disease? *Clin Biochem* **39** (2006), 667–681.

[25] M. Haq, S. Haq, P. Tutt and M. Crook, Serum total sialic acid and lipid associated sialic acid in normal subjects and those having a myocardial infarction and their relations to acute phase proteins, *Ann Clin Biochem* **30** (1993), 383–386.

[26] G. Rastam, A.R. Lindberg, G.L. Folsom, P. Burke, P. Nilsson-Ehle and A.M. Lundblad, Association between serum sialic acid concentration and carotid atherosclerosis measured by B-mode ultrasound, *Int J Epidemiol* **25** (1996), 953–958.

[27] M.W. Knuiman, G.F. Watts and M.L. Divitini, Is sialic acid an independent risk factor for cardiovascular disease? A 17-year follow-up study in Busselton, Western Australia, *Ann Epidemiol* **14** (2004), 627–632.

[28] R.A. Rabini, A. Vignini, D. Martarelli, L. Nanetti, E. Salvolini, M.R. Rizzo, E. Ragno, G. Paolisso, C. Franceschi and L. Mazzanti, Evidence for reduction of pro-atherosclerotic properties in platelets from healthy centenarians, *Exp Gerontol* **38** (2003), 367–371.

[29] B. Venerando, A. Fiorilli, G. Croci and G. Tettamanti, Presence in human erythrocyte membranes of a novel form of sialidase acting optimally at neutral pH, *Blood* **90** (1997), 2047–2056.

[30] L. Mazzanti, R.A. Rabini, E. Petruzzi, R. Staffolani, E. Salvolini, A. Vignini, M. Braconi and C. Franceschi, Erythrocyte plasma membranes obtained from centenarians show different functional properties, *Am Geriatr Soc*, **48** (2000), 350–351.

[31] B. Venerando, A. Fiorilli, G. Croci, C. Trincali, G. Goi, L. Mazzanti, G. Curatola, G. Segalini, L. Massaccesi, A. Lombardo and G. Tettamanti, Acidic and neutral sialidase in the erythrocyte membrane of type 2 diabetic patients, *Blood* **99** (2002), 1064–1070.

[32] R.H. Sills, J.H. Tamburlin, N.J. Barrios, P.L. Yeagle and C.A. Glomski, Physiologic formation of intracellular vesicles in mature erythrocytes, *Am J Hematol* **28** (1988), 219–226.