Title: Erythrocyte morphology in metabolic syndrome

Summary:

The study of erythrocyte morphology is of great importance in the field of hemorheology, since the deformability of the circulating cells has a fundamental influence on the rheological properties of the blood. Diabetes mellitus, hypertension, dyslipidemia and obesity (mostly central obesity) are the major components of metabolic syndrome. In this review, we focus on the changes in erythrocyte morphology in different components of metabolic syndrome and also discuss the erythrocyte morphology in regards to oxidative stress - a common state of chronic diseases. This paper also addresses the problem of inconsistency in the use of nomenclature and technique to identify the abnormal morphology and we recommend the use of standard terminology by all authors.

Keywords: metabolic syndrome, diabetes mellitus, hypertension, oxidative stress, erythrocyte morphology, hemorheology

The study of erythrocyte morphology is of great importance in the field of hemorheology, since the deformability of the circulating cells has a fundamental influence on the rheological properties of the blood [1]. Erythrocytes may respond by changing their morphology to any form of insult in their membrane or biochemical composition. Biconcave discoid shape is the physiological form of red blood cells. Treatment of physiological biconcave discoid erythrocytes with various agents can transform them into their two extreme opposite form: stomatocytes and echinocytes [2]. Amphipathic compounds that intercalate themselves into the cytoplasmic half of the lipid bilayer (such as cationic amphipaths like phenothiazine tranquilizers) cause expansion of the inner half relative to the outer half causing invagination and thus eventually leading to stomatocyte formation. On the other hand, compounds that are preferentially distributed into the outer half (such as anionic amphipaths like free fatty acids) expand the outer half relative to inner half and induce crenations leading to echinocyte formation [2,3].

Deformability was found to be decreased in stomatocytes and echinocytes when assessed by micropipette technique [4]. In contrast, in another study, spherostomatocytes (transformed by chlorpromazine) showed a decreased deformability whereas spheroechinocytes (transformed by sodium salicylate) showed increased erythrocyte deformability [5]. Increased low shear rate whole blood

viscosity (WBV) has been reported in prediabetes and in diabetes mellitus (DM) with and without cardiovascular complications [6]. Membrane proteins of diabetic erythrocytes such as spectrin, ankyrin, and protein 4.2 are heavily glycosylated than non-diabetic erythrocytes. Oxidative modification of spectrin due to its glycosylation was attributed as a reason for decreased erythrocytes deformability [7]. Erythrocytes in rouleaux can have both discoid and stomatocytes shape. There is the transitions of these two shape in rouleaux [8]. The formation of rouleaux increases the effective volume of erythrocytes which in turn is the fundamental factor in increasing WBV [9]. Altered erythrocytes morphology and reduced deformability leads to increased WBV [10] and hence cardiovascular disease (CVD) complications. Thus, the precise determination of erythrocyte morphology could provide information on the disease process, which is primarily contributing to its deterioration. Change in the morphology from discoid to other forms results in the generation of stiffened erythrocytes with less deformability and this may mechanically traumatise the capillaries leading to microangiopathy [11]. This is suggested by the strong correlation of reduced erythrocyte deformability with retinopathy [12] and nephropathy [13] in DM.

The term 'metabolic syndrome' (MetS), refers to a complex of cardiovascular disease risk factors. We cannot describe the pathophysiology of MetS without referring to blood rheology. There is always the propensity for erythrocytes to be involved in cellular oxidative stress function thereby being a cellular common factor in all components of MetS. However, an extensive review of the literature on the subject of MetS revealed (i) lack of certainty regarding its pathogenesis, (ii) considerable doubt regarding its value as a CVD risk marker, and (iii) much critically important information is missing to warrant its designation as a syndrome [14]. DM, hypertension, dyslipidemia and obesity (mostly central obesity) are the major components of MetS [15]. In this review, we focus on the changes in erythrocyte morphology in different components of MetS like DM, hypertension, obesity and dyslipidemia and also discuss the erythrocyte morphology in regards to oxidative stress - a common state of chronic diseases [16].

Diabetes mellitus and erythrocyte morphology

The erythrocyte membrane has a dynamic shape. Glucose oxidation and protein glycation, caused by diabetes-associated hyperglycaemia, could induce a number of modifications in the mechanical and rheological properties of erythrocytes. To study the effect of DM in erythrocyte morphology, Turchetti

V *et al.* [17] compared erythrocytes morphology of healthy subjects with those of diabetics with and without other complications by light microscopy. In healthy subjects, so called 'bowl-shaped' erythrocytes, which were considered as the most deformable cells, were most abundant (55%) followed by discocytes (44%). Discocytes though physiological cells were considered more rigid cells than 'bowls shaped' erythrocytes. The Authors considered that 'bowl-shaped' erythrocytes are derived from the distortion of discocytes in blood flow and are more deformable than discocytes. Altered cells, mainly 'echinocytes' (cells with 10 to 30 spicules, regularly distributed, characterized by introflexions of the membrane caused by the modification of the surface/volume ratio) and knizocytes (cells with a slightly smaller diameter than discocytes and characterized by two or three concavities in the membrane caused by alterations in their lipidic component), did not exceed a mean value of 1%. In the diabetics without vascular complications, there were no significant differences from the normal subjects. In the vasculopathic patients, a statistically significant increased in discocytes (60%) and decrease in bowl shaped cells were noted when compared to controls. In the diabetics with vasculopathy, there was a significant increase in the discocytes to a mean of 57% and a modest increase in the altered forms (3%). Elevated number of discocytes and diminished number of bowl-shaped cells were shown in vasculopathic patients and diabetic patients with peripheral obliterative arterial disease in another study [18]. Spherocytes were reported in type I diabetics whereas both spherocytes and echinocytes were reported in type II diabetics in the peripheral blood smear [19]. The authors stated that spherocytosis, observed in both types of diabetes, appears to be associated with hyperglycemia whereas echinocytosis in type II diabetic patients was possibly related to the abnormal plasma lipid profile and increased level of lipid peroxides. Similarly, 62% of discocytes were detected in type II diabetics in contrast to 72% in healthy controls by atomic force microscopy. "Crest" erythrocytes (cells with cresentric structure), spherocytes, planocytes (cells with loss of biconcavity), macrocytes, and microcytes were reported as a modified erythrocyte forms [20].

An erythrocyte morphologic index (EMI) as a measure of erythrocyte rigidity given by number of bowl-shaped cells/ discocytes was >1 in normal subjects, whereas it was well below 1 in both vasculopathic and diabetic patients [18]. EMI greater than 1 implies higher number of bowls than discocytes whereas EMI below 1 implies the opposite situation. Therefore, EMI less than 1 has a decreased number of most deformable cells. Vascular complications of DM are widely acknowledged. Abnormal erythrocyte morphology in vasculopathic diabetes reported in these studies suggests abnormal erythrocyte morphology in the etiology of vascular complications of diabetes. Wautier *et al.* [21] reported increased

adhesion of diabetic erythrocytes to endothelium and positively correlated the extent of adhesion with vascular complications. We propose that changes in morphology of erythrocytes in diabetes are mainly responsible for their adhesion to endothelium and endothelial activation leading to vascular complications.

With the increase in glucose concentration, it was shown in another study that, the perimeter of a cell increased and area decreased, along with the development of irregularity in the membrane. This was further confirmed by the significant increase in the perimeter to area ratio and form factor [22]. Further based on another study, diabetic subjects showed higher 'acanthocytes' (surface blebbing cells), distorted forms and 'cup forms' (stomatocytes) in comparison to controls. However, it was shown that after necessary treatment, erythrocyte morphology was restored to normal [23]. Therefore, there is clinical importance in erythrocyte morphologic changes and their consequences in diabetes.

Hypertension and erythrocyte morphology

Sodium ion transport across membrane and calcium binding by the membrane is altered in the erythrocytes of subjects suffering from essential hypertension [24]. It has also been reported that the concentration of free calcium ions is increased in the cytoplasm of cells in essential hypertension [25]. These defects in ion transport and concentration may account for erythrocyte membrane alterations and defective cytoskeleton. Phosphorylation of spectrin is decreased due to increased cytosolic calcium concentrations and hence erythrocytes are less deformable [26]. The ATP pool is affected in patients with essential hypertension [27] and this leads to changes in membranous properties and erythrocyte morphology. Interestingly, Teodori *et al.* [28] showed that the erythrocytes of healthy subjects with familial hypertension were less biconcave whereas erythrocytes of essential hypertension subjects were more biconcave than those of normal healthy controls without familial hypertension. They offered no explanation for the finding. The study of erythrocyte according to Zipursky–Forconi method in the patients affected by essential hypertension showed high number of discocytes and low number of bowls and hence a significant decrease in EMI compared to controls [26,29]. In the method of Zipursky et al [30], the three dimensional shape of glutaraldehyde fixed erythrocytes is assessed by light microscopy. A drop of fixed blood is mixed with glycerol (1:4 ratio) and placed in a microscope slide. 100X immersion lens is used to made the observation. Microscope lens exert a slight pressure on a viscous liquid being observed causing the complete rotation of erythrocytes in viscous medium allowing three dimensional observations. EMI was found to be further decreased in essential hypertensive subjects suffering from

end-organ damage. The same trend was shown by WBV and fibrinogen level (significantly high in essential hypertension and even higher in essential hypertension with end-organ damage) [29]. Hence, it can be surmised that morphology changes depend on the complexity and severity of underlying pathology.

Postnov *et al.* [31] revealed a threefold increase in monoconcave cup shaped cells in essential hypertension subjects in comparisons to normotensive cases by phase contrast microscopy. When the normotensive erythrocytes were treated with 12-Otetradecanoylphorbol-13-acetate, an activator of proteinkinase c, the concentration of monoconcave cup shaped cells were seven to nine fold higher in comparison to untreated normotensive erythrocytes. It was suggested that appearance of unusual cup shaped monoconcave cells may be due to increased activity of protein kinase C or by the alteration of the membrane skeleton [31,32].

Dyslipidemia and erythrocyte morphology

To demonstrate the effect of cholesterol on erythrocyte morphology, the shape of erythrocytes from a normal population was compared with normocholesterolemic diabetics and hypercholesterolemic diabetics. The range of blood glucose level was same in both normo and hypercholesterolemic groups. The changes in shape parameters of hyperglycemic samples with normo- compared with hyper-cholesterol levels were found to be significant. The 'form factor' [22] showed a significant increase in hyperglycemic subjects with hypercholesterol compared to normo-cholesterol levels indicating more deviation in the erythrocyte shape in diabetic subjects with hypercholesterol than those with normocholesterol compared to normal erythrocytes. Also, perimeter to area ratio and form factor of diabetic subjects was large compared to normal groups [33]. A similar result was shown in another study to demonstrate the effect of cholesterol on erythrocyte morphology [34]. With the increasing concentration of glucose, it was shown that, the perimeter of the cells was increasing and the area was decreasing. Also, these parameters were further deviated from normal when hypercholesterolaemia was present along with hyperglycemia. Glycated and oxidized low density lipoproteins are reported to cause alterations in conformation of normal and diabetic erythrocyte membrane proteins [35]. Increased concentration of cholesterol in plasma implies its increase accumulation in membrane which leads to altered shape and increased stiffness [36].

In the concept of MetS, dyslipidemia refers to high triglycerides and/or low HDL-cholesterol [15]. The former (hyper-triglyceridemia) is cytotoxic to the erythrocytes with a potential for morphological transformation, and consequential decreased deformability, membrane fluidity, and increased osmotic fragility. The erythrocyte morphological changes may include protrusions, irregular appearances or indistinct concaves [37,38].

Obesity and erythrocyte morphology

Obesity is the central and causal component of MetS [39]. Central obesity contributes to decrease red cell deformability [40]. It was shown that greater than three percent of weight reduction was associated with improved blood rheology in obese subjects [40]. Hemorheological profiles of 136 morbid-obese subjects were compared with same number of normo-weight healthy controls. Obese subjects showed higher plasma viscosity and erythrocytes aggregation (3 s^{-1}), and lower erythrocytes deformability. No statistical differences were observed for rheological parameters between obese subjects with and without MetS. Authors concluded that altered rheology is not only related to MetS but with obesity itself [41]. To the best of our knowledge, erythrocyte morphology has not yet been studied in obese subjects without other co-complications to date. The effect of free fatty acids in the generation of free radicals has been demonstrated. Free fatty acids like oleic acid, linoleic acid and arachidonic acid activate the NADPH oxidase system in cultured adipocytes and hence stimulate the generation of reactive oxygen species [42]. Erythrocytes morphologic change may occur due to the toxic effect of free fatty acid and oxidative stress in obese subjects. It was shown by light microscopy that the erythrocytes from healthy subjects changed their shape from biconcave to highly transformed forms like monoconcave forms and 'shrinked cells' after incubation with free fatty acids palmitate (0.2 mM) for 15 minutes. At higher concentration of palmitate, cell swelling and even ghost formation was observed [43].

Oxidative stress and erythrocyte morphology

Alterations of erythrocytes morphology in MetS could be explained on the grounds of prooxidant-antioxidant imbalance because oxidative stress is a common state in metabolic chronic diseases [16]. Erythrocytes play a significant role in scavenging free radicals [44-46]. It was proposed that in carrying out their role of free radical scavenging, erythrocytes become damaged by oxidation, which consumes endogenous reducing substances [47]. This damage then leads to shape changes and increased rigidity by alteration in the erythrocytes lipid bilayer and oxidation of labile groups in the proteins of the

cytoskeleton. To demonstrate the effect of oxidant on erythrocyte morphology, normal erythrocytes were incubated with varying concentrations of H_20_2. 2.5% of the cell population were echinocytes following treatment with 135 μM H_20_2As erythrocytes were treated with increasing concentrations of H_20_2, they became progressively more echinocytic when viewed from scanning electron microscopy, their membrane rigidity increased, cell surface alterations occurred and altered cells were recognized by monocytes [48]. *In-vitro* adherence and/or phagocytosis of erythrocytes with monocytes were considered as the sign for cell membrane alterations. Phagocytosis of altered erythrocytes *in-vivo* generates reactive oxygen species [49] further complicating the scenario.The authors [48] stated that the mechanism of echinocyte formation may be related to condensation of the inner monolayer lipids as a result of spectrin-hemoglobin complex formation because carbon monoxide treatment which inhibited the formation of this spectrin-hemoglobin complex also inhibited cell shape transformation. Erythrocytes from healthy donors treated with tert-butyl hydroperoxide were transformed to echinocytes. At a higher concentration of oxidant, spheroechinocytes, echinocytes III (spherical cellswith the large number of spicules evenly distributedover the surface), stomatocytes and ghost cells were also observed [50]. These studies suggested that erythrocytes morphology is affected by oxidant in a dose dependent manner.

Certain phenomena such as oxidation of sulphydryl groups on the membrane of cytoskeleton proteins, oxidation of membrane fatty acid residues or oxidation of the hemoglobin molecule could alter the membrane properties and shape of the cell [51]. Watanabe *et al.* [52], showed that erythrocytes obtained from healthy donors incubated in hypoxanthine/xanthine oxidase system and hydrogen peroxide had decreased membrane fluidity. Also, significant morphologic changes were reported when erythrocytes were incubated with H_2O_2. 'Spiculated form' (cells with spicules as seen in the figure of manuscript) and spheroechinocytes were seen after 5 and 30 min of incubation respectively. >96-100% of methemoglobin content was reported in erythrocytes incubated with 2.5 mM peroxynitrite (reactive nitrogen species) for 10 minutes in comparison to 4-5% methemoglobin content in erythrocytes untreated with peroxynitrite. Peroxynitrite treated cells demonstrated changes in the mechanical and physical properties of its membranes when studied by atomic force microscopy [53]. Crenated forms (cells with protrusions) of erythrocytes were observed in a dose dependent manner when blood samples from healthy donors were incubated with peroxynitrite [54]. The authors claimed that crenation of erythrocytes appears to be caused by both water and ion imbalance between the cells and surrounding medium and cytoskeletal structure changes.

Discussion: the relationships between erythrocyte morphology and metabolic syndrome

In a study that determined erythrocyte membrane parameters in components of metabolic syndrome, it was found that all membrane parameters differed significantly in metabolic syndrome compared to controls. In particular, it was concluded that erythrocyte membrane structural changes are associated with features of metabolic syndrome [55]. A complete haemorheological profile was studied in MetS subjects by Vaya A et al [56]. The Authors reported higher corrected WBV, increased erythrocytes aggregation and reduced erythrocyte deformability in subjects with MetS compared to controls without MetS who might have one or two components of MetS. Inflammatory markers such as CRP, fibrinogen, neutrophils and total leukocytes were significantly higher in MetS subjects compared to controls. Among five components of MetS, waist circumference significantly predicted corrected high shear rate WBV in MetS. Higher level of inflammatory markers in MetS subjects and strong association of waist circumference with WBV emphasizes on the role of central obesity and adipokines in haemorheological alterations.

Increase flux of glucose through polyol pathway, activation of protein Kinase C, and formation of advanced glycation end products in diabetes all lead to excess production of superoxide ion in mitochondria and hence generation of oxidative stress [57]. Consequently, many different oxidative stress markers accumulate in blood and tissues of diabetic patients. Lipid peroxidation products were shown to be higher in diabetics compared to healthy subjects [58]. Similarly, oxidized proteins and markers of oxidative DNA damage were reported to be higher in diabetics [59] whereas antioxidant level are reported to be low [60,61]. Oxidative damage of the erythrocyte is a contributor to its membrane/morphological changes and one of the consequences is decreased deformability. Lipid peroxidation products like malondialdehyde can cause polymerization of membrane components leading to increased membrane rigidity [62,63]. Twenty type II female diabetic subjects without any other complications were treated with the antioxidizing drug N-acetylcysteine (NAC), 1200 mg for 20 days. Blood was collected from the subjects before NAC treatment and after 20 days of NAC treatment for the analysis of erythrocytes morphology. Seven female healthy volunteers were chosen as controls. Diabetic subjects showed higher acanthocytes, distorted forms and cup forms in comparison to controls. However, it was shown that after NAC treatment for 20 days, erythrocyte morphology was restored to normal [23].

Further, there is the cytotoxic potential of dyslipidemia to induce erythrocyte membrane transformation with consequential decrease in deformability, increased rigidity, increased viscosity and hypertension. Yet, another confounding factor is the propensity for obesity to induce erythrocyte oxidative damage through free fatty acids' stimulation of the NADPH system [42], which in turn exacerbates reactive oxygen production. Thus, there is the erythrocyte serving as a cellular common factor in all components of MetS. Straface E et al [64] showed increased number of acanthocytes in subjects with MetS with respect to that of samples from healthy donors. Further, the authors demonstrated that, though the expression of glycophorin A and CD 47 were similar in erythrocytes from subjects with and without MetS, these were lower in male subjects than in female subjects with MetS. Similarly, externalization of phosphatidyl serine was higher in erythrocytes of MetS subjects in comparison to healthy donors. Glycophorin A, CD 47 and phosphatidyl serine are the markers of erythrocyte injury and senescence and their alterations in MetS indicate that erythrocytes could be used as a bioindicators of MetS. Therefore, with regards to (i) lack of certainty regarding its pathogenesis, (ii) considerable doubt regarding its value as a CVD risk marker, and (iii) much critically important information is missing to warrant its designation as a syndrome; we propose the erythrocyte morphological changes and its consequences as critical cellular basis in pathogenesis. We also propose that the cell membrane changes as a critical physiologic basis and erythrocyte oxidative damage as a critical biochemical basis. The concurrence of any two or more of the four (DM, dyslipidemia, hypertension and obesity) components of MetS could have additive effect in enhancing erythrocyte oxidative stress and morphological changes and feed forward to vasculopathy through increase in blood viscosity. Hence, establishment of MetS may no doubt be an invaluable risk marker for CVD. To our knowledge, the quantitative level of as an indicator of severity of diabetes has yet to be established. Nevertheless, it is apparent that morphometric monitoring of patients with alterations of erythrocyte morphology can indicate whether a treatment regimen is significantly counteracting the abnormal changes[23], and aid in the diagnosis of hypertension [28].

Techniques and parameters used in studying erythrocyte morphology

Variations in the dimensions of erythrocytes are documented in different pathologies like anaemia and thalassemia but this has not been used in the field of metabolic diseases like CVD, DM or MetS. Though several papers have addressed the importance of studying erythrocyte morphology in metabolic diseases, the lack of a standard common technique used by researchers has resulted in conflicting

evidence (Table 1/Table 2). Erythrocyte morphology was studied, for instance, by a light microscope with a 100X immersion lens and the three-dimensional evaluation was carried out by varying the focus in a study of Turchetti V *et al.* [17]. On the other hand, scanning electron microscopy [48] and atomic force microscopy [54] has been used by other researchers. Use of different nomenclature system/terminologies/parameters for the identification of changes in erythrocyte morphology is another cause for not reaching a definite conclusion. Turchetti V [17,18,29] has used the term 'bowl shaped' for most deformable cells and discocytes, though physiological, were considered less deformable than bowls. Most other studies [20,23] have considered discoid shape as the gold standard for morphologic comparisons without taking 'bowl shape' into account. 'Spiculed cells' with surface projections are called echinocytes. These spicule cells are also interchangeably called crenated cells or acanthocytes by different authors [65]. Watanabe *et al.* [52] have used the term 'spiculated form', Starodubtseva MN *et al.* [20] have used the term 'crenated form' and Straface E *et al.* [23] have used the term 'acanthocytes' (surface blebbing cells as shown in the figure of manuscript) in their article and all these cells most probably referred to echinocytes. 'Area', 'perimeter' and 'form factor' has also been considered to discuss erythrocytes morphologic changes [22,34]. In the same way, different sample preparation techniques and components also affect the erythrocytes morphology. For example, erythrocytes can be transformed into echinocytes when located between glass surfaces. This 'glass effect' depends on the material composition of the glass surface, the distance between the two adjacent surfaces, haematocrit and temperature [66]. In addition, a low concentration of serum albumin [67,68] and glutaraldehyde (used as a fixative in erythrocytes electron microscopy) [66] can restore the normal discocytes shape of the erythrocytes.

We have used the terminology 'biconcave disc' for the most deformable physiological erythrocyte (fig 1a); 'stomatocytes' for the cup shaped monoconcave cells (fig1c); 'acanthocytes' for cells with surface blebbing (crenated cells) (fig 1d); 'leptocytes' for flat cells (fig 1b) and 'burr cell' for cells with surface changes (one or more surface bumps or spicules) (fig 1e) when viewed by scanning electron microscope [69] (fig 1). The nomenclature system described by Bessis M [70] has been considered the gold standard in erythrocytes morphology studies. Normal disc shaped cells were referred to as 'discocyte'. When erythrocytes are washed in isotonic saline and viewed in glass slide under coverslip, discocytes are transformed to sphere cells covered with crenations or spicules. These cells were typically referred to 'echinocytes'. 'Acanthocytes'- though superficially resembling echinocytes were considered to be different cells with fewer spicules in their surface which are irregularly arranged and bent back at their

tips. The cup shaped cells were referred to as ‘stomatocytes’. Spherical cells were referred to as ‘spherocytes’ and so on. We strongly recommend the use of these terminologies given by Bessis for the purpose of consistency.

Expert commentary

Altered hemorheology as well as oxidative stress has been found in various components and states associated with MetS. Frequently, the biochemical basis of altered erythrocyte rheology has been explained on the ground of glucose toxicity [71], glycation of membrane [22], elevated ketone bodies [72] and oxidative stress [73]. Changes in erythrocyte morphology might be the prior event that leads to altered hemorheology and other complications. Since altered erythrocyte morphology is associated with MetS, it could be a useful bioindicator in the assessment of metabolic syndrome, its severity and progression but techniques and parameters to assess the morphologic changes should be standardized.

Five-year view

During the next five years, in addition to established biomarkers, erythrocytes morphologic study will be used to predict cardiovascular diseases and complications. The proposed definition of MetS may be updated keeping in mind the fact that MetS has a hemodynamic basis. Finally, parameters used and techniques should be standardized that will enable the comparison of results between laboratories.

Key issues

- **The study of erythrocyte morphology is of great importance in the field of haemorheology, since the deformability of the circulating cells has a fundamental influence on the rheological properties of the blood**
- **The term ‘metabolic syndrome’, refers to a complex of cardiovascular disease risk factors. The underlying pathophysiology is presumably inseparable from blood as there is always the propensity for erythrocytes to be involved in cellular oxidative stress function thereby being a cellular common factor in all components of MetS.**
- **Erythrocyte morphological changes and its consequences may be critical cellular basis in pathogenesis of metabolic syndrome.**

- **We propose that the cell membrane changes as a critical physiologic basis and erythrocyte oxidative damage as a critical biochemical basis of metabolic syndrome.**
- **The concurrence of any two or more the four (DM, dyslipidaemia, hypertension and obesity) components could have additive effect in enhancing erythrocyte oxidative stress and morphological changes and feedforward to vasculopathy through increase in blood viscosity.**
- **Though several papers have addressed the importance of studying erythrocyte morphology in metabolic diseases, different technique and terminology used by several researchers has resulted in conflicting evidence.**

Table 1. Techniques and assessment parameters used to study erythrocytes morphology – diabetes & hypertension subjects

Authors	Subjects	Techniques used	Results
Turchetti V *et al.* (1997)	Diabetics with vasculopathy	Zipursky–Forconi method	Decreased bowls; increased discocytes. Other altered forms: echinocytes and knizocytes
Turchetti V *et al.* (1998)	Vasculopathic and Diabetics with POAD		
Babu N *et al.* (2004)	Diabetics	Transmission video microscopic system. Shape analysis after image processing	Perimeter of the erythrocytes increased; area decreased
Cimbaljević B *et al.* (2007)		MGG* stain of peripheral blood smear	Spherocytes in type I and spherocytes and echinocytes in type II
Starodubtseva M *et al.* (2008)		Atomic force microscopy	62% discocytes , "Crest" erythrocytes, spherocytes, planocytes, macrocytes, and

			microcytes
Hanjing F at al (1998)		Scanning electron microscopy	stomatocytes and ridgecytes
Straface E *et al.* (2002)			acanthocytes, distorted forms and cup forms
Teodori L *et al.* (2007)	Essential hypertension	optical microscopy and image processing software	more biconcave erythrocytes than healthy controls
	Familial hypertension		less biconcave erythrocytes than healthy controls
Turchetti V *et al.* (1999)	Essential hypertension	Zipursky–Forconi method	Decreased bowls; increased discocytes
Cicco G *et al.* (1999)			
Tripsa MF *et al.* (1990)		Zeiss inverted light microscope	echinocytes/total cells ratios increased
Postnov Y *et al.* (1988)		Phase contrast optics microscopy. Cells counted in counting chamber.	monoconcave cup shaped erythrocytes

*May-Grunwald Giemsa Stain

Table 2. Techniques and assessment parameters used to study erythrocytes morphology – other subjects

Authors	**Subjects**	**Techniques used**	**Results**
Zavodnik B *et al.* (1997)	normal erythrocytes treated with Free fatty acids Palmitate	MGG stain of peripheral blood smear	monoconcave forms, shrinked cells and ghosts cells
Babu N (2009)	hypercholesterolemic	Transmission video	Perimeter of the

	diabetics	microscopic system. Shape analysis after image processing	erythrocytes increased and area decreased
Snyder LM *et al.* (1985)	normal erythrocytes treated with H_2O_2	Scanning electron microscopy	Echinocytes
Zavodnik LB *et al.*(1998)	normal erythrocytes treated with tert-butyl hydroperoxide	MGG stain of peripheral blood smear with phase contrast microscopy	Echinocytes, spheroechinocytes, echinocytes III, stomatocytes and ghost cells
Watanabe H *et al.* (1990)	Normal erythrocytes treated with H_2O_2	Scanning electron microscopy	Spiculated form and spheroechinocytes
Starodubtseva MN *et al.* (2008)	Normal erythrocytes treated with peroxynitrite	Atomic force microscopy	Crenated form

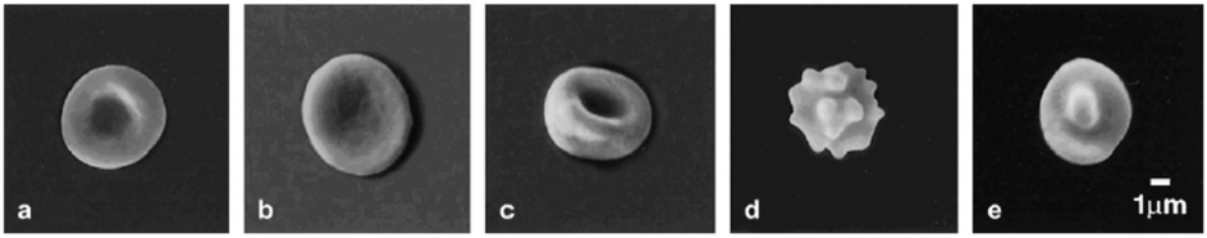

Figure 1. Erythrocyte morphological variation identified from a scanning electron microscope. (A) Biconcave disc, **(B)** leptocytes, **(C)** stomatocytes **(D)** acanthocytes, **(E)** burr cell.

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