Type 2 Diabetes: Platelets and Long-Term Metabolic Control as Estimated from Glycosylated Haemoglobin (HbA1c)

M. Edvardsson[1,2] M. Oweling[1] P. Järemo[1]

[1] Local Healthcare Centre, Finspång, Sweden
[2] Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

Address for correspondence P. Järemo, MD, PhD, Local Healthcare Centre, SE-61236 Finspång, Sweden (e-mail: petter.jaremo@telia.com).

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Abstract

In type 2 diabetes, platelets are likely affected by impaired long-term glycaemic control, but such pathophysiological links are poorly understood. This study thus compares platelet reactivity (i.e. agonist-evoked platelet reactions) *in vitro* with glycosylated haemoglobin (HbA1c), a measure commonly used for monitoring long-term metabolic control of type 2 diabetes. Elders with type 2 diabetes ($n = 35$) were divided according to HbA1c into groups (HbA1c—low and high) consisting of 17 and 18 subjects, respectively. For estimating mitochondria disintegration, a flow cytometer determined mitochondrial transmembrane potentials after whole blood agonist stimulation. The activating agents used were α-thrombin (10 μM) and collagen (0.15 μg/mL). The same apparatus analysed the fibrinogen receptor activity, lysosomal exocytosis (surface lysosomal-associated membrane protein 1), and platelet procoagulant characteristics (membrane-attached annexin V) after stimulation. In type 2 diabetes, after *in vitro* agonist stimulation, platelet mitochondria injury was higher in the HbA1c-high group. The fibrinogen receptor, lysosomal secretion, and the creation of procoagulant platelets proved to be uninfluenced by HbA1c.

Keywords

- annexin V
- $\alpha_{IIb}\beta_3$ activity
- type 2 diabetes
- HbA1c
- lysosomal-associated membrane protein 1
- mitochondria
- platelets
- platelet reactivity

Introduction

Dysregulated type 2 diabetes is a major risk factor for coronary heart disease, and strict glycaemic control is essential for preventing adverse events. Pathophysiological links explaining complications are not understood, but platelets are thought to be important.[1,2] Thus, knowledge about interrelations between diabetic platelets and glycosylated haemoglobin (HbA1c) is scarce. The disease is accompanied by platelet alterations; they enhance during hyperglycaemia; and it is hypothesised that they reverse after HbA1c correction.[3,4] Mean platelet volume, a computer-generated average of platelet size, is increased and associates with glycaemic control.[5] Furthermore, diabetic platelets are characterised by enhanced sensitivity to agonists, exaggerated aggregation, increased surface thrombin generation,[6,7] and an impaired sensitivity to inhibitory agents.[8]

In recent times, a renewed interest in platelet diversity has emerged, and we have comprehensively investigated platelets divided according to density.[9–11] Platelet density covers the span 1.040 to 1.090 kg/L[12,13] and high-density fractions encompass more mitochondria, glycogen, and granules.[12–14] As judged from membrane-exposed activity markers, high- and low-density platelets circulate more activated.[15,16] It is further agreed that platelets exhibit different behaviour in response to agonist stimulation, as some populations activate their fibrinogen receptors while other platelets externalise granules. Finally, when activated, specific sub-fractions exhibit procoagulant properties by exposing phosphatidylserine and releasing vesicles.[17,18]

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Georg Thieme Verlag KG,
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The flow cytometry technique used in this study divides platelets and platelet-derived corpuscles according to size (i.e. normal-sized, small platelets, and vesicles). Little is known about the function of small platelets,[11,17] whereas from a clinical point of view, platelet-derived vesicles have received more attention.[19,20] Most vesicles are created at thrombopoiesis,[21] but they also originate from membrane blebs of activated normal-sized platelets. Vesicles are postulated to be important for several clinical conditions such as diabetes.[22] Thus, dysregulated glucose homeostasis is a potent stimulator of vesicles.[23] Vesicles further augment atherosclerosis[20] and predict adverse cardiac events of patients with type 2 diabetes.[24] Some scientists hypothesise that vesicles in conjunction with enhanced thromboxane A_2 associate with HbA1c.[22,25]

Platelets show metabolic flexibility by being capable of switching between aerobic glycolysis[26] and an active energy metabolism, mediated by mitochondria, the hub of cellular oxidative homeostasis.[17,27] Mitochondria constitute important regulators of platelet function,[27] but when platelets activate, they do not influence adhesion and thrombi growth.[23,28] Information about energy utilisation of diabetic platelets is sparse, but the condition involves dysfunctional mitochondria.[3] It is further theorised that mitochondria of diabetic platelets enhance ATP production, elevate respiration, and reduce their transmembrane potentials as an adaptation to durable high exposure to energy substrates (i.e. a poor metabolic control).[29–31] With this background in mind, the present experimental protocol is designed to examine relations between mitochondria integrity after *in vitro* agonist stimulation and enduring glycaemic control (HbA1c) of type 2 diabetes.

Patients and Methods

Subjects and Blood Sampling

After authorisation by the local ethics committee (Regionala Etikprövningsnämnden i Linköping, Medicinska Fakultetens Kansli, Linköpings Universitet, SE-581 83 Linköping [registration number 2018/54-31]), patients with type 2 diabetes ($n = 35$) were enrolled. Demographic and anthropometric details are given in ►Table 1. The participants signed informed consent, and exclusion criteria were not applied. Participants had regular appointments with their family physicians and were enrolled when laboratory staff was available. For flow cytometry platelet reactivity studies, whole blood (9 mL) was drawn from the antecubital vein into sodium citrate (3.2%) test tubes. Agonists were α-thrombin (10 µM) and collagen (0.15 µg/mL). For platelet density separations (see later), an additional citrate anticoagulated whole blood specimen (9 mL) was transferred to a blocking mixture composed of equivalent quantities of the following stock solutions:

A. 1 mg/L prostaglandin E1 dissolved in 95% (w/v) ethanol.
B. 0.13 M Na2 EDTA and 0.15 M Na2 citrate (pH 7.4 at 25 °C).
C. 2.7 mM theophylline dissolved in 150 mM TRIS chloride buffer (pH 7.4 at 25 °C).

Table 1 Clinical and demographic characteristics of patients with type 2 diabetes ($n = 35$)

	HbA1c Low (mmol/L) ($n = 17$)	HbA1c High (mmol/L) ($n = 18$)
Male/female (*n*)	8/9	7/11
Age (years)	70 ± 12 (SD)	73 ± 10 (SD)
Body weight (kg)	80 ± 13 (SD)	85 ± 12 (SD)
Duration of diabetes (years)	9 ± 4 (SD)	13 ± 7 (SD)
Previous coronary disease (*n*)	3	1
Previous cerebral disease (*n*)	3	1
Insulin (%)	8[a]	67[a]
Metformin (%)	85[a]	53[a]
Other oral antidiabetics (%)	21[a]	50[a]
β-Blockers (%)	36	33
Diuretics (%)	36	47
Ca^{2+}-blockers (%)	33	36
Aspirin (%)	20[b]	38[b]
Clopidogrel (%)	8	0
ACE inhibitors (%)	38	44
A2 inhibitors	28[b]	13[b]
Statins (%)	92[b]	67[b]
Haemoglobin A1c (mmol/L)	49 ± 6 (SD)	67 ± 10 (SD)
Creatinine (µmol/L)	77 ± 26 (SD)	85 ± 27 (SD)

Abbreviations: ACE, angiotensin-converting enzyme; SD, standard deviation.
Note: The Student *t*-test (unpaired) and chi-square test were used where applicable.
[a] $p < 0.01$.
[b] $p < 0.05$.

Subsequently, the mixture was used for the separation of platelets according to density.[9,10] EDTA-anticoagulated whole blood (3 mL) was also collected for routine analysis (►Table 1). The flow cytometry procedures were started approximately 120 minutes after venipuncture. The unpaired Student *t*-test and the chi-square tests were used as statistics where appropriate.

Platelet Density Fractionation

As platelet density fluctuates between 1.040 and 1.090 kg/L,[12,13] linear polyvinylpyrrolidone-coated silica (Percoll) gradients covering that span were employed for separating platelets according to density.[9] To circumvent *in vitro* activation in the laboratory, the gradients enclosed EDTA, theophylline, and prostaglandin E_1. After centrifugation, the gradients were split into subpopulations ($n = 16$)[9] with high-density corpuscles situated in low digit subpopulations and vice versa.

Table 2 The flow cytometry technique (i.e. the determinations, antibodies, probes, final concentrations and manufacturers)

Platelet identification Detection of the platelet receptor α_{IIb}	**Antibody**[a] CD41; PE 0.69 µg/mL
Lysosomal exocytosis Analysis of membrane-bound LAMP-1	**Antibody**[a] LAMP – 1 (CD107a, clone: H4A39); PE 0.5 µg/mL
Fibrinogen $\alpha_{IIb}\beta_3$ receptor activity Analysis of membrane-bound PAC-1	**Probe**[b] PAC-1; FITC 0.56 µg/mL
Determination of surface-attached annexin V Membrane-exposed phosphatidylserine	**Probe**[b] Annexin V-V450; PE 2.67 ng/mL
Mitochondria membrane potential Determination of DiIC1(5) retention	**Probe**[c] 1,1′,3,3,3′,3′-hexamethyl-indodicarbo-cyanine iodide 30 nM

Abbreviations: DiIC1(5), 1,1′,3,3,3′,3′-hexamethyl-indodicarbo-cyanine (relates inversely with mitochondria damage); FITC, fluorescein isothiocyanate; LAMP-1, lysosomal associated membrane protein; PE, phycoerythrin.
[a]Beckman Coulter (Brea, California, United States).
[b]Becton, Dickinson and Company (Franklin Lakes, New Jersey, United States).
[c]Molecular Probes (Eugene, Oregon, United States).

Flow Cytometry

The Gallios Flow Cytometer (Beckman Coulter, Brea, California, United States) equipped with three lasers (405, 488, 638 nm) was used. The device has a multi-colour design. The following parameters were determined.

Mitochondrial transmembrane potentials = retention of 1,1′,3,3,3′,3′-hexamethyl-indodicarbo-cyanine iodide (DiIC1(5)).
Membrane phosphatidylserine = surface-bound annexin V.
$\alpha_{IIb}\beta_3$ (Fibrinogen) receptor activity = PAC-1.
Lysosomal discharge = surface-attached LAMP-1.
Corpuscle size = normal-sized platelets, small platelets, and vesicles.

A published flow cytometry protocol was used without major changes[11,17] and ►**Table 2** lists probes and antibodies in detail. Platelets were identified by GPIIb receptor fluorescence and forward scatter (size). The gating of the apparatus has been explained extensively elsewhere.[17] Samples were added to probes and antibodies (►**Table 2**) as published previously.[11,17] The percentage of positive corpuscles was used as an experimental parameter, and we did not analyse mean fluorescence intensities. After 10 minutes, the samples were diluted with HEPES-Ca^{2+} which terminates the reactions. Flow cytometry particle acquisition finished either after 2 minutes or after counting more than 5,000 particles. Thus, the quantity of evaluated corpuscles fluctuated corresponding to subfraction counts.

Results

It is evident from ►**Table 1** that the study groups differed significantly with respect to current medications in that $HbA1c_{high}$ individuals more often had insulins ($p < 0.01$) and 'other' oral antidiabetic drugs ($p < 0.01$). In contrast, they less frequently used metformin ($p < 0.01$). It is also apparent that the $HbA1c_{high}$ group had more aspirin prescriptions ($p < 0.05$) but used less statins and A2 receptor inhibitors (both; $p < 0.05$). ►**Table 3** reveals that mitochondria injury of normal-sized platelets after stimulation (α-thrombin [10 µM; $p < 0.01$] and collagen [0.15 µg/mL; $p < 0.05$]) as judged from DiIC1(5) was higher in the $HbA1c_{high}$ group. Furthermore, basal surface PAC-1 *in vitro* (i.e. platelet activity) and HbA1c related inversely ($p < 0.05$). However, agonist-evoked PAC-1, lysosomal release (LAMP-1), and procoagulant platelet creation (annexin V) failed to associate with HbA1c (►**Table 3**). ►**Fig. 1** reveals that HbA1c did not affect mitochondria integrity of density-separated normal-sized platelets. In contrast, ►**Figs. 2** and **3** demonstrate that denser and lighter circulating small-sized platelets and vesicles of $HbA1c_{high}$ subjects displayed more injured mitochondria (for both corpuscles: fraction nos. 1–6, density span 1.090–1.071 kg/L [$p < 0.05$], fraction no. 7, density span 1.068 kg/L [small platelets, $p < 0.05$] and fraction nos. 13–15, density span 1.049–1.043 kg/L; small platelets [$p < 0.05$]; vesicles [$p < 0.01$]) and fraction no. 16 [density span 1.040 kg/L; small platelets [$p < 0.01$] and vesicles [$p < 0.05$]). ►**Fig. 4** summarises basal surface PAC-1 for each normal-sized density subpopulation ($n = 16$)—the proportions (%) of platelets expressing surface PAC-1 (mean ± SD), i.e. activated fibrinogen receptors. The $HbA1c_{high}$ group displayed lower surface PAC-1 of circulating dense platelets (fractions nos. 1–4, density span 1.090–1.078 kg/L; $p < 0.01$). The population nos. 8, 14 (density span 1.065 and 1.046 kg/L, respectively) performed similarly ($p < 0.05$).

Discussion

The main finding of this study investigating relationships between type 2 diabetic platelets and long-term glycaemic control can be summarised as follows: (1) agonist-evoked platelet mitochondria injury was increased in the $HbA1c_{high}$

Table 3 Associations of basal activity (without *in vitro* stimulation) of normal-sized platelets together with whole blood reactivity after agonist stimulation and long-term metabolic control of individuals with type 2 diabetes ($n = 35$) as judged from HbA1c

Normal-sized platelets	HbA1c Low (mmol/L)	HbA1c High (mmol/L)	*p*-Value
Proportions of DiIC1(5)-positive normal-sized platelets (%)			
No agonist	98 ± 1	98 ± 1	NS
α-Thrombin (10 μM)	98 ± 2	97 ± 2	<0.01
Collagen (0.15 μg/mL)	95 ± 9	88 ± 8	<0.05
Proportions of PAC-1-positive normal-sized platelets (%)			
No agonist	4 ± 3	2 ± 1	<0.05
α-Thrombin (10 μM)	52 ± 37	38 ± 26	NS
Collagen (0.15 μg/mL)	55 ± 30	52 ± 26	NS
Proportions of LAMP-1-positive normal-sized platelets (%)			
No agonist	5 ± 5	2 ± 3	NS
α-Thrombin (10 μM)	56 ± 26	54 ± 12	NS
Collagen (0.15 μg/mL)	56 ± 39	59 ± 18	NS
Proportions of annexin V positive normal-sized platelets (%)			
No agonist	2 ± 1	2 ± 2	NS
α-Thrombin (10 μM)	2 ± 1	3 ± 3	NS
Collagen (0.15 μg/mL)	4 ± 5	5 ± 4	NS

Abbreviations: DiIC1(5), mitochondrial transmembrane potentials, i.e. retention of 1,1′,3,3,3′,3′-hexamethyl-indodicarbo-cyanine iodide; HbA1-c_{high}, subjects ($n = 18$), mean erythrocyte HbA1c level 67 ± 10 (SD) (mmol/L); HbA1c_{low}, subjects ($n = 17$), mean erythrocyte HbA1c level 49 ± 6 (SD) (mmol/L); HbA1c, haemoglobin A1c (mmol/L); LAMP-1, lysosomal-associated membrane protein 1; PAC-1, fibrinogen receptor ($\alpha_{IIb}\beta_3$) activity; SD, standard deviation.

Notes: α-Thrombin (10 μM) and collagen (0.15 μg/mL) were employed as agonists. Mitochondria transmembrane potentials (DiIC1(5)) as measures of organelle integrity were determined, with 'lower' DiIC1(5) suggestive of 'more' disintegrated mitochondria. Surface PAC-1, LAMP-1, and annexin V assessed fibrinogen receptor ($\alpha_{IIb}\beta_3$) activity, lysosomal release, and procoagulant platelet production, respectively.

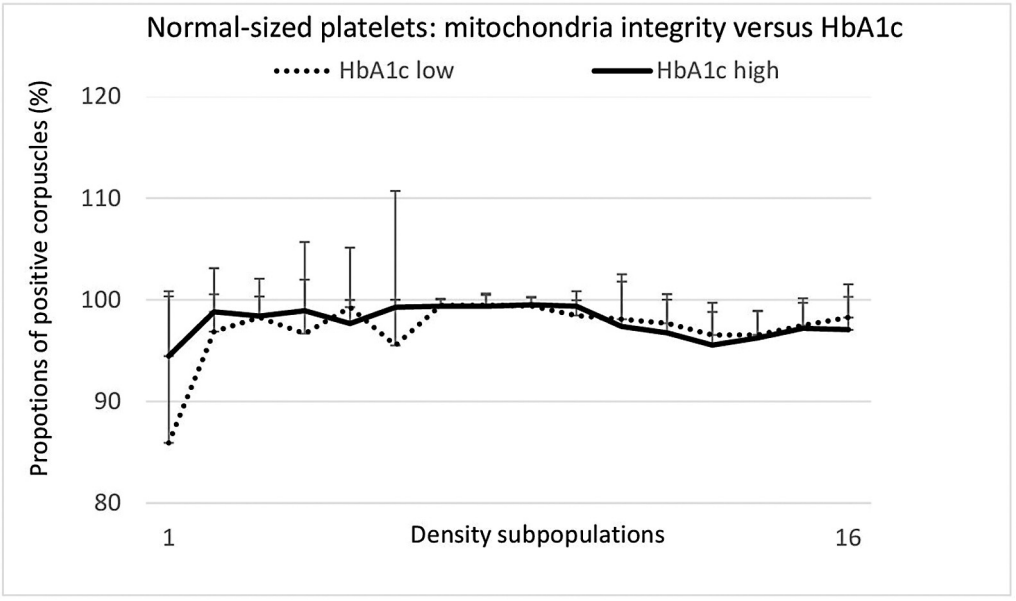
group; (2) after agonist-induction *in vitro*, the $\alpha_{IIb}\beta_3$ receptor (PAC-1), lysosomal release (surface LAMP-1), or procoagulant platelet counts (annexin V) were not affected by HbA1c; (3) mitochondria damage of both lighter and denser circulating small platelets and vesicles, unstimulated *in vitro*, was higher in the HbA1c_{high} cohort.

As compared to previous work investigating resting type 2 diabetic platelets with respect to energy utilisation,[29] this work broadens the observations in showing that elevated HbA1c enhances mitochondria injury when platelets activate as well (►**Table 3**). It could be part of other activation pathways not investigated by us, such as an augmented thromboxane A_2 synthesis.[22,27] Further research is necessary to elucidate if the current mode for platelets to activate (i.e. augmented mitochondria injury without increased fibrinogen receptor activation, lysosomal release, and procoagulant platelet generation) impacts adverse events in conjunction with dysregulated diabetes.

Mitochondria integrity in circulating small platelets and vesicles displayed a substantial heterogeneity in that elevated HbA1c (HbA1c_{high}) affected only some sub-populations (►**Figs. 3**, **4**). For both corpuscles, mitochondria injury of high- (fraction nos. 1–7, density span 1.090–1.069 kg/L) and low-density (fraction nos. 13–16, density span 1.051–1.040 kg/L) subpopulations were lower in the HbA1c_{high} group. In contrast, mitochondria damage of intermediate dense, small platelets, and vesicles (fraction nos. 8–12, density span 1.069–1.051 kg/L) failed to associate with HbA1c.

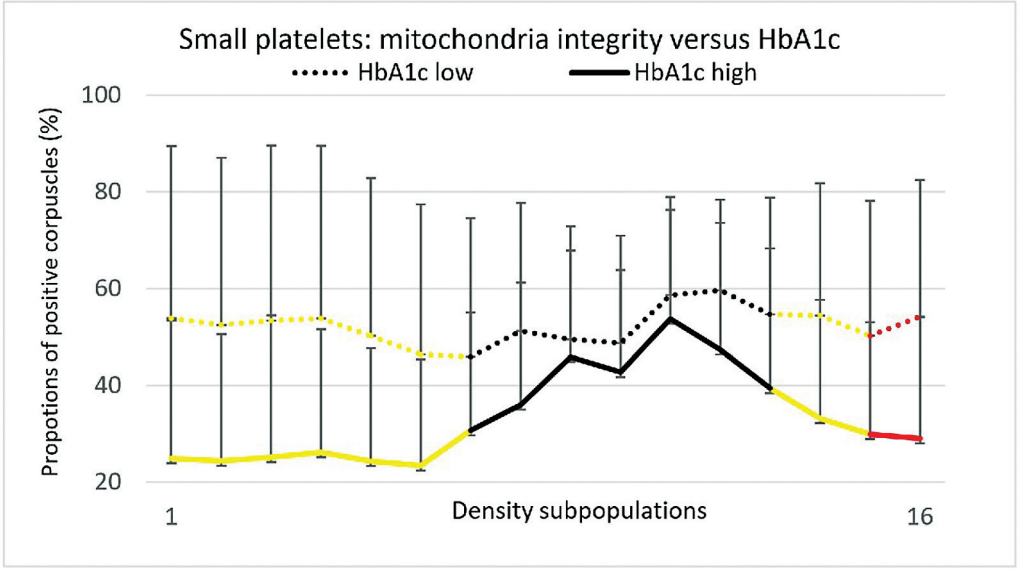
In this study, mitochondria injury (DiIC1(5)) of normal-sized platelets did not associate with the glycaemic control as judged from erythrocyte HbA1c (►**Fig. 1**). It disagrees with earlier work.[29] It could be that mean fluorescence intensity determinations yield more favourable results. It is open to speculation whether the present technique for estimating mitochondria damage is not sensitive enough for detecting minor differences within normal-sized platelet density sub-populations. Furthermore, some small platelet and vesicle sub-populations of the HbA1c_{high} group displayed more disintegrated mitochondria (►**Figs. 2**, **3**). Previous work did not separate platelets according to size.[26,28] In our setting, when evaluating normal-sized platelets, small platelets and vesicles were removed. This offers a tenable explanation for discrepancies in findings.

It is an unproven, but widespread, understanding that dysregulated type 2 diabetes adversely affects the reactivity of circulating platelets. This study fails to verify such clear-cut conclusions. It is, however, evident from ►**Table 3** that $\alpha_{IIb}\beta_3$ receptor activity (PAC-1) of circulating whole blood platelets, unprovoked *in vitro*, decreases with a deranged glycaemic metabolism. Basal PAC-1 of some normal-sized subpopulations (fraction nos. 1–4: density span 1.090–1.077 kg/L and fraction nos. 8, 14: densities 1.065 kg/L and 1.046 kg/L,



HbA1c: haemoglobin $A1_c$ (mmol/L)
HbA1c $_{low}$: (subjects (n=17); mean HbA1c level 49±6(SD) (mmol/L)
HbA1c $_{high}$: (subjects (n=18); mean HbA1c level 67±10(SD) (mmol/L)

Fig. 1 Associations between platelet mitochondria integrity as estimated from DiIC1(5) of normal-sized platelets and glycaemic control (erythrocyte HbA1c) of patients with type 2 diabetes ($n = 35$). 'Lower' DiIC1(5) indicates 'more' disintegrated mitochondria. For each participant, normal-sized platelets were split into density sub-fractions ($n = 16$) and divided into groups ($n = 2$) depending on $HbA1c_{(high\ and\ low)}$. The dotted line shows the $HbA1c_{low}$ group, and the vertical marks display standard deviations. No significant differences between experimental groups were detected.



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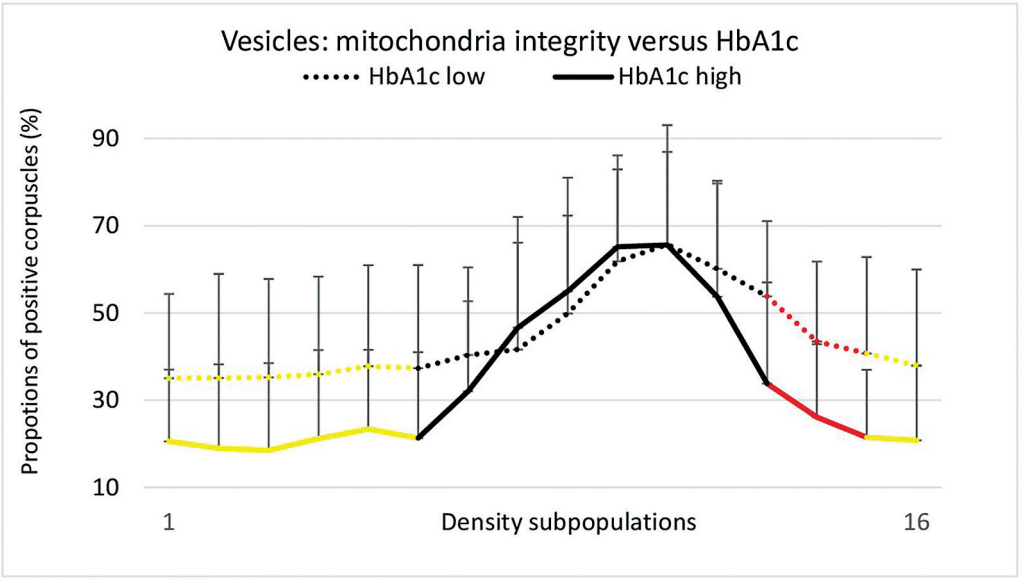
HbA1c $_{high}$: (subjects (n=18); mean HbA1c level 67±10(SD) (mmol/L)

Fig. 2 Associations between mitochondria transmembrane potentials (DiIC1(5)) of density-separated small platelet subpopulations ($n = 16$) and long-lasting glycaemic control of type 2 diabetes as judged from HbA1c. A 'lower' DiIC1(5) denotes 'more' disintegrated mitochondria. Subjects ($n = 35$) were divided, according to HbA1c, into groups ($n = 2$) comprising 17 and 18 ($HbA1c_{high\ and\ low}$) individuals. The dotted line denotes the $HbA1c_{low}$ cohort. For each small-sized density subpopulation ($n = 16$), the groups ($HbA1c_{low\ and\ high}$) were compared with respect to mitochondria integrity (DiIC1(5)). The vertical lines indicate standard deviations and colours imply significance (black: not significant; red: $p < 0.01$; yellow: $p < 0.05$).



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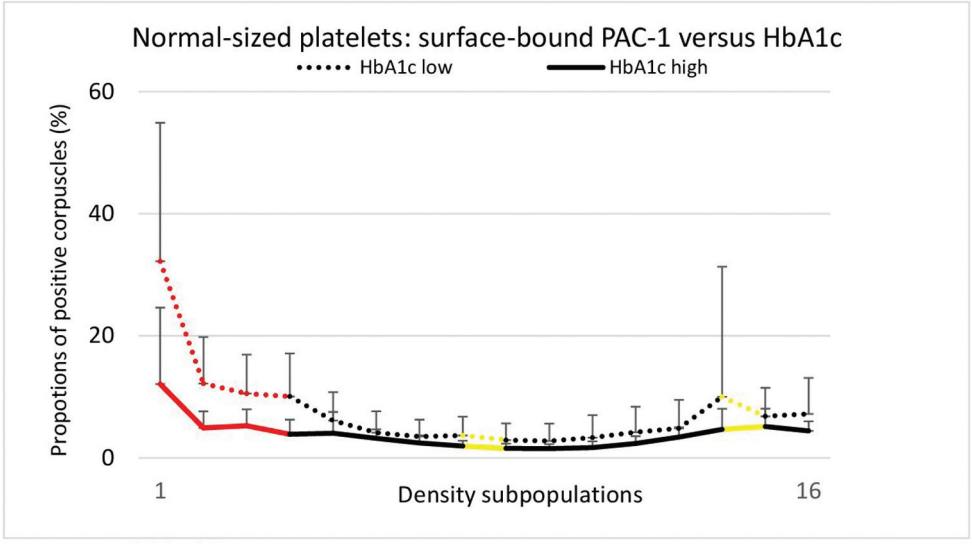
PAC-1: fibrinogen receptor ($\alpha_{IIb}\beta_3$) activity
SD: standard deviation

Fig. 3 Reviews mitochondria integrity (DiIC1(5)) of density-separated vesicle subpopulations and displays associations with the long-term metabolic control of type 2 diabetes (erythrocyte HbA1c). In this setting, a 'higher' DiIC1(5) indicates 'more' retained mitochondria. Participants ($n = 35$) were split depending on HbA1c into groups ($n = 2$) consisting of 17 (HbA1c $_{low}$) and 18 (HbA1c $_{high}$) subjects, respectively. The dotted line summarises the HbA1c $_{low}$ group (mean ± SD; vertical lines) with the colours indicating significance between HbA1c $_{low\ and\ high}$ (black: not significant; red: $p < 0.01$; yellow: $p < 0.05$).



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PAC-1: fibrinogen receptor ($\alpha_{IIb}\beta_3$) activity
SD: standard deviation

Fig. 4 Relationships between proportions (%) of density-separated normal-sized platelets, demonstrating activated $\alpha_{IIb}\beta_3$ receptors (PAC-1) and long-term metabolic control of type 2 diabetes as ascertained by HbA1c. For each subject ($n = 35$), normal-sized platelets were split according to density into sub-populations ($n = 16$) and the sub-fractions were divided into groups depending on the metabolic control (HbA1c $_{low}$ [$n = 17$], HbA1c $_{high}$ [$n = 18$]). For the two groups, the figure gives the percentages of PAC-1 expressing circulating normal-sized platelets (mean ± SD [vertical bars]) not stimulated *in vitro* (black: not significant; red: $p < 0.01$); yellow: $p < 0.05$).

respectively) demonstrates close inverse relationships with HbA1c (►Fig. 4). It makes us theorise that $\alpha_{IIb}\beta_3$ receptors of well-defined normal-sized density subpopulations react depending on the glycaemic control (►Fig. 4). Platelet lysosomal release and surface annexin V failed to display such phenomena (data not shown).

Limitations

To enhance statistical power, we increased the type 2 diabetes group with a subsequent exclusion of healthy controls. As expected, $HbA1c_{high}$ individuals had more insulin prescriptions (►Table 1). The patients further represent a mixed group with respect to metformin (►Table 1), a remedy that may affect platelet mitochondria.[30,32] In addition, $HbA1c_{high}$ subjects more often had aspirin and, in this setting, it has the potential to diminish differences between study groups. Venipuncture was performed on a single occasion only, making it impossible to secure individual reproducibility of platelet activity measures by repeated sampling.

Conclusion

In type 2 diabetes, mitochondria injury after in vitro platelet activation was increased in the $HbA1c_{high}$ group. No such associations were found for whole blood agonist-evoked aggregation, lysosomal discharge, and procoagulant reactions.

Plain Language Summary

What did we know?

- Little is known about the influence of long-lasting hyperglycaemia upon platelets in type 2 diabetes.

What did we discover?

- Mitochondria injury after agonist stimulation associates with impaired metabolic control. Other platelet activation pathways failed to show such relationships.

What is the impact?

- In type 2 diabetes, the communicated route for platelets to activate could affect thrombotic events associated with increased HbA1c.

Raw Data

Data are available from the corresponding author upon reasonable request.

Authors' Contribution

M.E.: Conceived the research, performed sample and data collection, and did the practical flow cytometry work.
M.O.: Formulated and partly financed the investigation and supervised its findings.
P.J.: Devised and partially financed the study, carried out data analysis, and wrote the first draft of the manuscript. Subsequently, all authors revised the manuscript critically for intellectual content.

Conflict of Interest

The authors declare that they have no conflict of interest.

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