Ultrastructural analysis of platelets during three phases of pregnancy: A qualitative and quantitative investigation

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Objectives: In the past, platelet morphology during normal pregnancy has not been given much attention.
Methods: Electron microscopy analysis of platelets from 60 pregnant individuals (30 early pregnancy (weeks 8–14) participants and 30 late pregnancy (weeks 36–40) participants which were followed up 6–8 weeks post-partum) were compared to platelets from 30 non-pregnant individuals as well as each other to establish whether differences in platelet morphology exist during pregnancy.
Results: Ultrastructural changes pertaining to the external and internal arrangements of platelets were visible. Fixated platelets showed pseudopodia formation and membrane blebbing. Increased and enlarged open canalicular system pores, pseudopodia formation, platelet spreading, and membrane blebbing were visible in vital platelets. Platelets from pregnancy were tightly packed and internal structures were different from the non-pregnant group. The internal granules showed modification in their occurrence within the cell. The α- and lysosomal granule counts were significantly increased during pregnancy while dense granule and mitochondrial numbers were significantly decreased.
Discussion: This may point to a pregnancy-specific modification. The changes in platelet ultrastructure discerned in this study attribute to the hypercoagulable state associated with pregnancy. All ultrastructural alterations associated with pregnancy persist for at least 2 months after birth.

Keywords: Blood platelets, Morphology, Pregnancy, Scanning electron microscopy, Transmission electron microscopy

Introduction

Platelets are small, anucleated blood cells that are discoid in shape. They are derived from megakaryocytes in the bone marrow and have a lifespan of 8–14 days. They move freely through circulation until the integrity of the vascular system is compromised. These cells play an integral role in the maintenance of haemostasis since they form the first line of haemostatic defence by formation of the primary haemostatic plug.[1,2]

Platelets play an important role in primary haemostasis. Platelets adhere to the injury site and are activated. The activated platelets will then secrete their granular constituents to employ additional platelets to form a platelet aggregate known as the primary haemostatic plug.[1]

The coagulation cascade is initiated by thrombin production induced by the activation and aggregation of platelets.[3]

During normal pregnancy platelet aggregation and secretion of ATP is increased compared with the non-pregnant individuals, reaching a prominent peak in the third trimester but returning to normal by 6–12 weeks post-partum.[4,5] Platelet binding to fibrinogen remained unchanged.[5,6]

Fitzgerald *et al.* indicated an elevated biosynthesis of thromboxane during pregnancy. They concluded that this increase was caused by platelet release of thromboxane and that it was consistent with platelet activation associated with pregnancy.[7]

Platelet count remains normal during pregnancy, although benign gestational thrombocytopaenia may present in the third trimester. Platelet activation along with β-thromboglobulin and PF-4 release are also found. Platelet and fibrinogen consumption is increased during delivery. Most haemostatic changes return to normal within 6 weeks after birth; however, the platelet count and protein S levels may be altered for a longer period.[8]

Most research concerning platelets and pregnancy do not focus on normal pregnancy. The majority focus is on preeclampsia,[6,9–12] HELLP syndrome,[13,14]

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and hypertension.[15,16] However, a Pubmed Central search yielded no research focusing on the platelet internal and external structure observed with scanning and transmission electron microscopy during normal pregnancy.

Various granule populations are found within platelets. The α-granules contain proteins involved in coagulation, adhesion, and wound healing. Dense granules mainly contain ADP, calcium, and serotonin. The α- and dense granules release their constituents upon platelet activation while the lysosomal granules secrete acid hydrolyses only upon strong stimuli.[17,18]

Since very little is known about platelet morphology during normal pregnancy, this study aimed to establish platelet internal and external structures during pregnancy.

Materials and methods

Pregnancy participants

Sixty pregnant females participated in this study. Two groups were distinguished: 30 women in the early phase of pregnancy (8–14 weeks) and 30 women in the late phase of pregnancy (36–40 weeks). The second group of late pregnancy participants also participated in the follow-up phase post-partum (6–8 weeks after birth). All participants were between the age of 18 and 35 years. All participant information was handled anonymously. The volunteers were recruited from the Femina Clinic, Pretoria (Ethical clearance number 185/2011). Informed consent was obtained from each participant.

Control participants

Thirty control participants were also employed for this study. The control females were also between the age of 18 and 35 years, and all participant information was handled anonymously (Ethical clearance number 185/2011). Informed consent was obtained from each participant.

All pregnant and non-pregnant participants were non-smokers, did not have a history of thrombotic disease, or used any chronic medication known to disturb coagulation factors and/platelet function. Neither aspirin nor aspirin analogues were used within 48 hours prior to sampling.

Blood collection

A qualified nurse collected 5 ml of blood from each pregnant women participating in the study. Blood was collected in a citrate tube containing 0.5 ml of sodium citrate (3.8% sodium citrate). One citrate tube of blood was collected from the 30 women in the early pregnancy group. One citrate tube of blood was also collected from women in the late pregnancy group on two occasions: between weeks 36 and 40 of birth, as well as their first appointment with the gynaecologist after birth (the post-partum group).

Blood was collected from the control volunteers by qualified phlebotomists. Five millilitres of blood was collected in a citrate tube containing 0.5 ml of 3.8% sodium citrate during days 1–5 of the menstrual cycle. During this period of the menstrual cycle, defined by menses, the levels of all gonadotropic hormones as well as the ovarian hormones are at their lowest.[19] Since pregnancy is associated with increased oestrogen and progesterone concentrations,[19] control blood samples were taken during menses to eliminate the possible influence of these hormones on platelets.

Sample preparation

Preparation of whole blood for ultrastructural analysis

First, citrated whole blood was used to prepare fixated whole blood samples. Five hundred microlitres of whole blood was placed in 500 μl of a 4% solution of formaldehyde in phosphate buffer overnight. The samples were washed three times 0.075 M phosphate-buffered saline (PBS), fixed in 1% osmium tetraoxide (OsO_4) for 15 minutes, and washed again thrice. Dehydration in ethanol with concentrations of 30, 50, 70, 90, and finally three-fold with 100% ethanol for 5 minutes in each concentration completed the preparation process.

A mixture of ethanol and hexamethyldisilazane (HMDS) in the ratio of 1:1 was added to the samples for 30 minutes, followed by pure HMDS for 1 hour. New HMDS solution was added and the mixture of HMDS and blood was dropped on a glass coverslip and left to dry. The glass coverslips were then mounted and coated with carbon. The coverslips were examined with a Zeiss Ultra plus FEG scanning electron microscope. Photomicrographs revealing the external morphology of the platelets were taken.

Preparation of platelet-rich plasma for ultrastructural analysis

The whole blood sample was centrifuged at 1250 rpm (maximum relative centrifugal force (RCF) = 17.523 g; 1250 g) for 10 minutes. The plasma supernatant was collected from the centrifuged sample and placed in an Eppendorf tube to be centrifuged again for 4 minutes at 1250 rpm (maximum RCF = 17.523 g; 1250 g) to obtain platelet-rich plasma (PRP). The PRP was used to make the platelet smear for scanning electron microscopy (SEM) analysis and transmission electron microcopy (TEM) analysis of platelets' external and internal morphology, respectively.

For SEM analysis, the PRP obtained from centrifugation was used to make a plasma smear on a glass coverslip. No other chemicals were added to this

Table 1 Mean values of different platelet components in non-pregnancy and different phases of pregnancy

	Control ($n = 100$)	Early pregnancy ($n = 100$)	Late pregnancy ($n = 100$)	Post-partum ($n = 100$)
α-Granules	3.24 ± 2.32	11.67 ± 5.21	12.94 ± 6.26	10.39 ± 5.21
Dense granules	1.46 ± 1.34	0.99 ± 1.15	0.37 ± 0.75	0.23 ± 0.53
Lysosomal granules	3.09 ± 2.52	5.67 ± 3.35	8.53 ± 3.37	9.12 ± 3.79
Mitochondria	0.75 ± 0.93	0.12 ± 0.46	0.18 ± 0.56	0.08 ± 0.27

Values given as mean ± SD. One-way ANOVA showed significant differences between the control values and all the three phases of pregnancy for all organelles (α-granules, dense granules, lysosomal granules, and mitochondria) with all P values less than 0.05.

smear. The remaining PRP in the Eppendorf tube was once again centrifuged for 4 minutes at 1250 rpm to obtain a pellet of platelets to be used for TEM preparation.

Preparation of PRP on a glass coverslip to exhibit platelets sample with scanning electron microscope

The plasma smear (consisting of 20 µl of PRP) on the glass coverslip was placed on filter paper dampened with PBS to establish a humid environment and placed at 37°C for 5 minutes. After the 5 minute incubation period, the sample was placed in PBS and washed for 20 minutes. The sample was not placed on a plate shaker, to ensure that all the platelets remained on the coverslip. This washing process ensured the removal of any excess plasma and plasma proteins.

The sample consequently underwent fixation in the solution of 2.5% glutaraldehyde for 30 minutes. Hereafter, triplicate rinsing with phosphate buffer for 5 minutes, secondary fixation with 1% osmium tetraoxide (OsO_4) for 15 minutes, and a second rinsing process as described above were followed. Dehydration in ethanol with concentrations of 30, 50, 70, 90, and finally three-fold with 100% ethanol for 5 minutes in each concentration completed the preparation process.

The sample finally underwent critical point drying, was mounted and coated with carbon, and examined with a Zeiss Ultra plus FEG scanning electron microscope. Photomicrographs revealing the external morphology of the platelets were taken.

Preparation of PRP in epoxy resin to exhibit platelets with transmission electron microscope

The platelet-pellet was prepared as described in the SEM preparation until after the dehydration step. Following dehydration, the sample was infiltrated in a mixture of one part ethanol and one part epoxy resin for 30 minutes and then stored in pure epoxy resin for 4 hours. The sample was then placed in a rubber mould filled with pure epoxy resin and left to set for 3 days overnight in an oven at 60°C. Ultrathin sections of 80–100 nm were cut with a diamond knife using an ultramicrotome. The sections were contrasted with uranyl acetate for 7 minutes followed by contrasting with lead citrate for 5 minutes, after

which samples were allowed to dry for a few minutes before examination with the JEOL transmission electron microscope (JEM 2100F). Photomicrographs revealing the internal structures of the platelets were taken.

Statistical analysis of platelet internal components

In the TEM micrographs the internal components, specifically the α-granules, dense granules, lysosomal granules, as well as mitochondria, of a 100 platelets were counted for each of the following groups: controls, early pregnancy, late pregnancy, and post-partum. All values in Table 1 represent mean ± standard deviation (SD). Statistical modelling was performed by employing a computerized software system (IBM SPSS Statistics 21) for statistical analysis of the data. One-way analysis of variance (ANOVA) was used to compare results. A level of significance was set at $P < 0.05$.

Results
Scanning electron microscopy
Scanning electron micrographs of PRP were prepared to investigate the external structure of platelets of the control (non-pregnant) and the pregnant participants.

Figs. 1A and 2A are SEM micrographs of platelets from the non-pregnant participants. They are typical control platelets, showing a spherical structure and no or only one pseudopodium extending from the body of the platelet. A few small pores of the open canalicular systems (OCS) are also shown on the membrane, with no platelet interactions observed.

Fig. 1B shows a platelet from an early pregnancy participant, while Fig. 2B shows a collection of platelets also associated with early pregnancy. The pores of the OCS are much larger in Fig. 1B, when compared with the control platelets. Several pseudopodia extend from the body of all the platelets; and in Fig. 2B, the interaction of these pseudopodia with the adjacent platelets are clearly visible. These platelets are not as compact as the control platelets, but spreading was visible. Blebbing of the membrane is also seen in both the figures. Similar results were found for the late phase pregnancy as well as the post-partum groups. Fig. 1C indicates a platelet from a participant in the late pregnancy phase and Fig. 2C shows the

interaction between several platelets from a late pregnancy participant. Figs. 1D and 2D represent the platelets from participants, 6–8 weeks after birth, showing single platelets and platelet interaction, respectively. These figures show that platelets are activated during different phases of pregnancy.
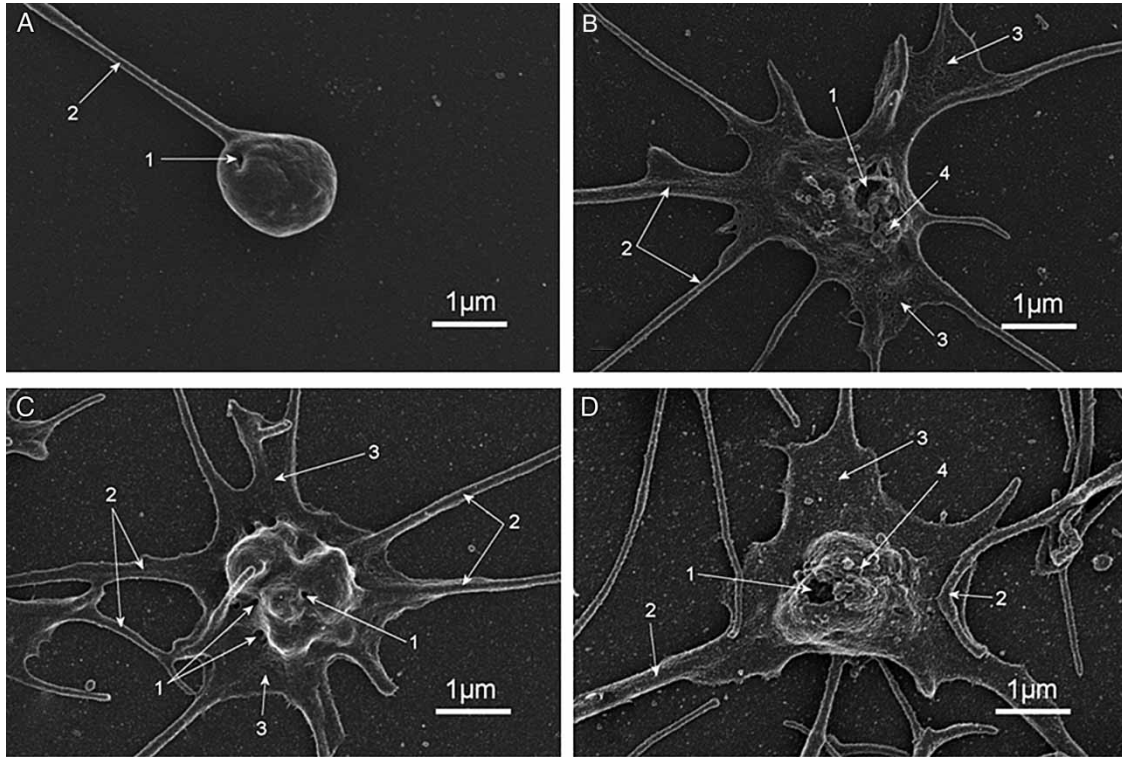

Figure 1 SEM analysis of external platelet morphology. External morphology of single platelets show the following: (1) indicates the OCS; (2) indicates pseudopodia; (3) shows platelet spreading; and (4) shows membrane blebbing. Bar represents 1 μm (original magnification ×40 000).
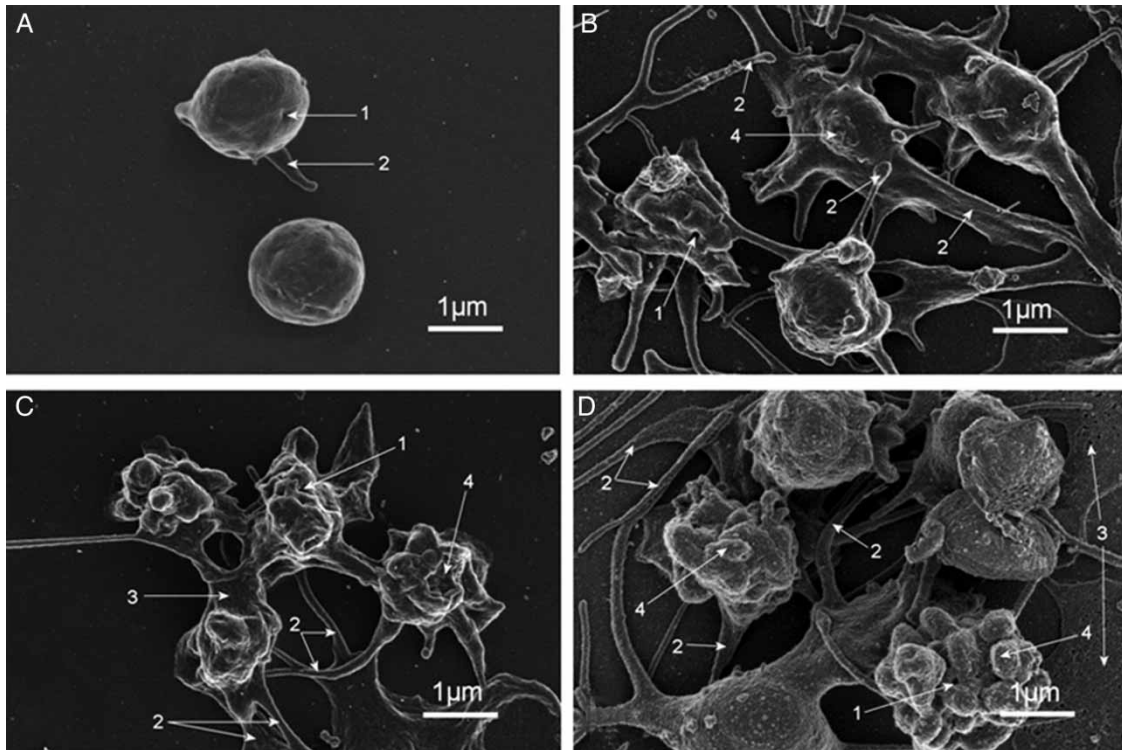

Figure 2 SEM analysis of external platelet interaction. External morphology of platelet aggregates show the following: (1) indicates the OCS; (2) indicates pseudopodia; (3) shows platelet spreading; and (4) shows membrane blebbing. Bar represents 1 μm (original magnification ×40 000).
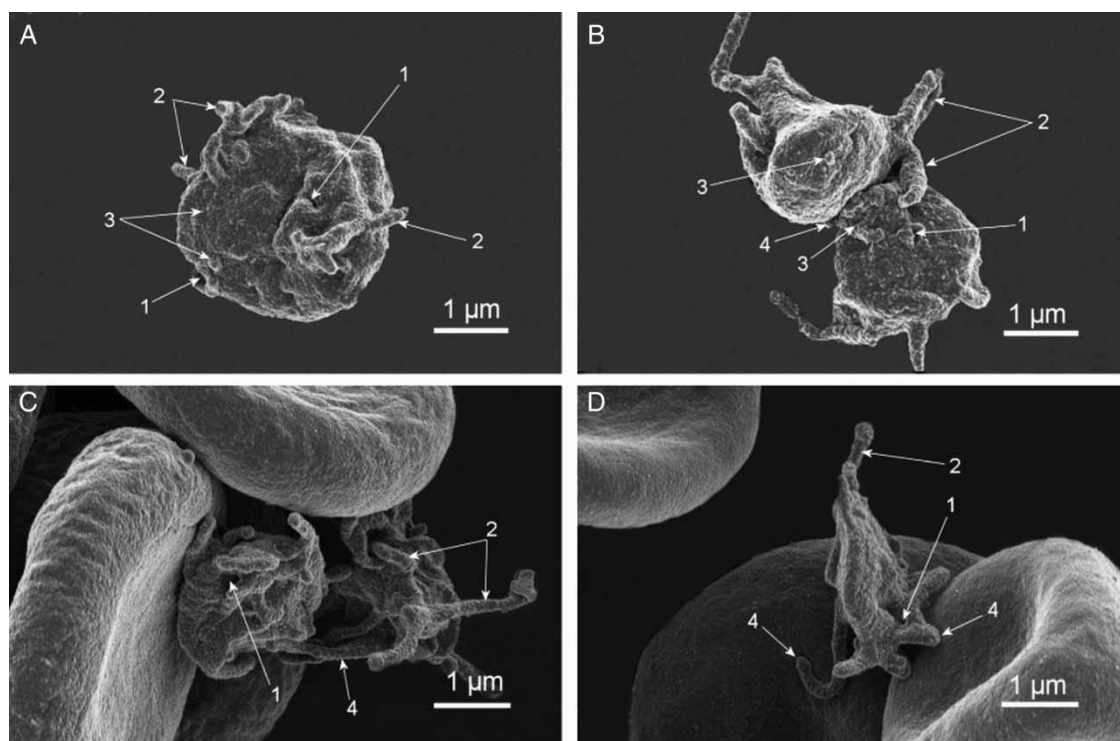
Figure 3 SEM analysis of fixated whole blood. External morphology of platelets in whole blood show the following: (1) indicates the OCS; (2) indicates pseudopodia; (3) shows platelet spreading; and (4) shows membrane blebbing. Bar represents 1 μm (original magnification ×40 000).

Whole blood was also used to investigate platelet external morphology using SEM analysis. Fig. 3A–D are representative micrographs of platelets from the fixated whole blood samples of the pregnant participants. The platelets exhibited several pseudopodia, membrane blebbing, and some OCS pores. No platelet spreading was visible, but the platelets did interact with each other (Fig. 3B and C) and erythrocytes (Fig. 3D). These results indicate that platelets are activated during pregnancy, and not by the method used to prepare PRP for SEM analysis.

Transmission electron microscopy
TEM was employed to investigate in the internal structures of the platelets. Fig. 4A shows the internal structure of a platelet from a non-pregnant participant. This is the typical composition of internal compounds from a healthy individual. The α-granules, dense granules, and lysosomal granules can be distinguished. Glycogen and mitochondria are present. The OCS and dense tubular systems (DTS) are seen. A few pseudopodia are also visible.

Fig. 4B shows the platelet internal structure of an early pregnancy participant. No mitochondria or glycogen can be distinguished. The OCS and DTS are also not visible. More α-granules can be seen with only one dense body. Lysosomal granules are present. The platelets are also closely packed together. Similar results were found for the late phase and the post-partum groups. Fig. 4C and D shows platelets

from the late phase pregnancy and the post-partum participants, respectively. These results suggest that the incidences of platelet organelles are altered during pregnancy.

Statistical analysis
Fig. 5 show the differences in mean values of each platelet component. Fig. 5A represent differences in the α-granule counts, Fig. 5B shows differences in the dense granule counts, Fig. 5C shows the lysosomal granule counts, and Fig. 5D indicates differences in the number of mitochondria present within the platelets.

A one-way ANOVA revealed significant differences between the control values and all three phases of pregnancy for all organelles (α-granule, dense granules, lysosomal granules, and mitochondria) with all P values less than 0.05.

α-granules
A one-way ANOVA showed a significant difference in the amount of α-granules during pregnancy compared with the control group $F(3, 396) = 76.239$, $P < 0.05$. *Post hoc* comparisons using Tukey's honestly significant difference (HSD) test showed an increase in α-granule count. This increase seen in pregnancy was almost four-fold to that of the control group. There was also a significant difference between the α-granule count of the late pregnancy group and the post-partum group ($P < 0.05$), but no significant difference between the
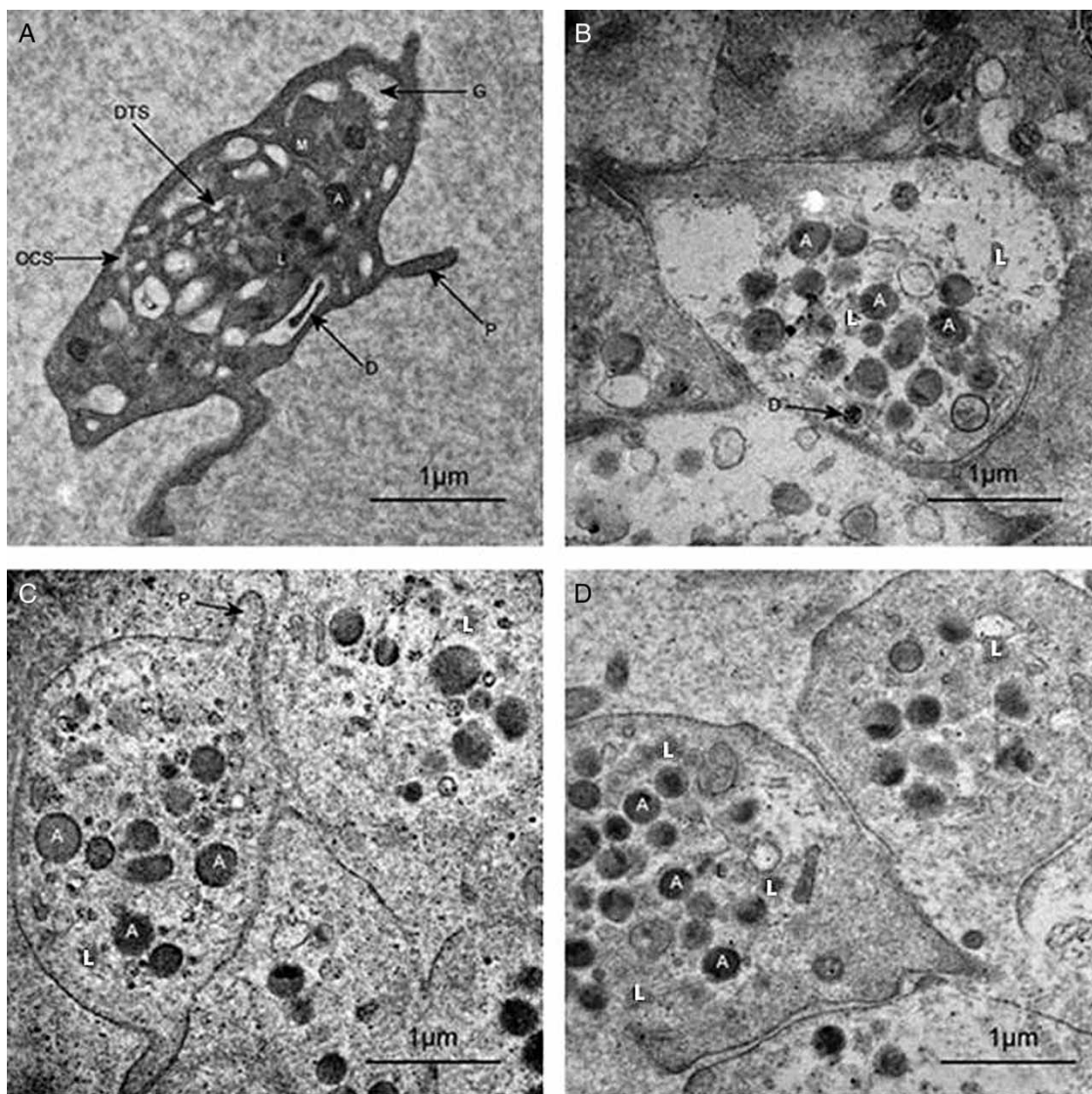
Figure 4 TEM analysis of internal platelet morphology. Internal morphology of platelets show the following: (A) α-granules; (G) glycogen; (D) dense granules; (DTS) dense tubular system; (L) lysosomal granules; (OCS) open canalicular system; (M) mitochondria. Bar represents 1 μm (original magnification ×40 000).

early pregnancy values and either the late pregnancy or the post-partum values.

Dense granules

One-way ANOVA indicated a significant difference in dense granule numbers during pregnancy when compared with the control group $F(3, 396) = 32.958$, $P < 0.05$. *Post hoc* comparison using Tukey's HSD test indicated a significant decrease in dense granule count for all the pregnancy groups. This decrease seen in pregnancy ranged from nearly two- to six-fold when compared with the non-pregnant group. There was also a significant difference between the dense granule count of the early pregnancy compared with both the late pregnancy and the post-partum groups (individually and combined) with a P value less than 0.05, but no significant difference between late pregnancy and post-partum.

Lysosomal granules

A one-way ANOVA revealed significant differences in lysosomal counts from all the pregnancy groups compared with the control group $F(3, 396) = 71.674$, $P < 0.05$. *Post hoc* comparisons using Tukey's HSD test showed a significant increase in the number of lysosomal granules for all the pregnancy groups. This increase seen in pregnancy was nearly three-fold to that of the non-pregnant, control participants. There was also a significant difference between the lysosomal granule numbers of the early pregnancy compared with both the late pregnancy and the post-partum groups (individually and combined) with $P < 0.05$. However, no significant difference between late pregnancy and post-partum were found.

Mitochondria

A one-way ANOVA revealed significant differences in the number of mitochondria from all the pregnancy
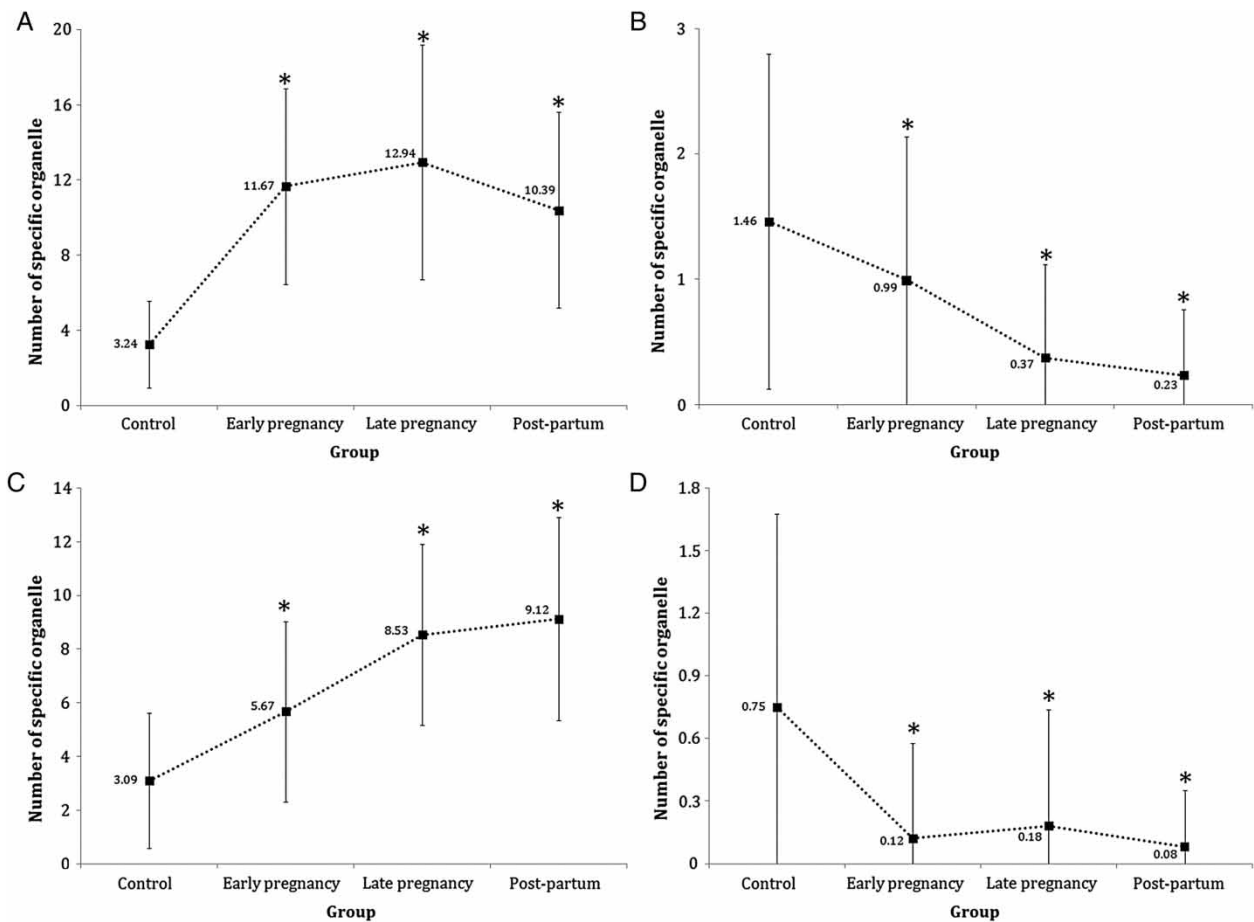
Figure 5 Differences in mean values of each platelet component counted in platelets from non-pregnant individuals and different phases of pregnancy. (A) The α-granule content; (B) the dense granule content; (C) the lysosomal granule content; (D) the mitochondrial content.

groups compared with the control group $F(3, 396) = 27.285$, $P < 0.05$. *Post hoc* comparisons using Tukey's HSD test showed a significant decrease in the mitochondria count for all the pregnancy groups. This decrease seen in pregnancy ranged from four- to nine-fold when compared with the non-pregnancy group. No significant difference was found between the different phases of pregnancy.

Taken together, these results suggest that pregnancy influence platelet components including all populations of granules (α-granules, dense granules, and lysosomal granules) and also mitochondria. Early pregnancy differs significantly from late pregnancy and post-partum (combined and separately) for the dense granules and lysosomal granules but not for α-granules and mitochondria. Late pregnancy differs from post-partum only for α-granules but not for dense granules, lysosomal granules, or mitochondria. Significant increases can be seen in Fig. 5A and C while Fig. 5B and D show significant decreases.

Discussion
The fixated whole blood samples seen in revealed some interesting features of circulating platelets during pregnancy. Platelets were activated, since pseudopodia were prominent and interactions, especially platelet–platelet interaction, could be distinguished. The platelets, however, did not show any platelet spreading and the OCS was small. This would be the characteristic of platelets that have not released their granular content. In the PRP smear, the platelets did exhibit platelet spreading and large open canalicular pores characteristic of platelet granule release. This shows that platelets are activated while in circulation and will start to spread and release their granules once it comes in contact with a surface like the glass coverslip.

Platelet external structure
Platelet pseudopodia formation is the initial morphological change associated with adhesion and aggregation.[20] These processes are suggested to aid adherence to other platelets and the forming fibrin strands.[21] The increased pseudopodia formation seen during pregnancy, and the visible interaction of the processes with other platelets, therefore support platelet adhesion and aggregation.

Platelet spreading facilitates the process of covering the damaged area. Subsequent granule release will recruit additional platelets and trigger the formation

of the fibrin network to form the stable, secondary haemostatic plug.[22]

Membrane blebbing is one of the traits of apoptosis.[23] Elevated thrombin induces platelet activation resulting in blebbing of the membrane similar to apoptosis.[24] The membrane blebbing seen during pregnancy can therefore be the result of elevated thrombin concentrations. And since no thrombin was added to the platelet samples, it can be deduced that the elevated thrombin concentrations were present in the plasma.

The OCS and α-granules are closely related. They support platelet function in two ways: For α-granules to accomplish their physiological tasks, their constituents need to be liberated from their intracellular storage areas. The α-granule membrane fusion to the surface-connected membranes of the OCS or the plasma membrane will trigger granule secretion.[25] Therefore, the OCS first links the α-granules to the external medium. Secondly, the OCS channels and pores, along with α-granules, provide additional membrane surface area to the platelet upon stimulation. The platelet surface area can increase up to four-fold in size and result in platelet spreading.[26] Since α-granules facilitate the aggregation of platelets, the increased incidence and enlargements of the pores seen in the PRP samples may support increased secretion of α-granules constituents, resulting in the close association of platelets. The smaller pores in the fixated samples may thus indicate that the α-granule content is not continually released in circulation, but only upon initiation of platelet spreading by an external stimulus which will ultimately result in the release of granular content.

Platelet internal structure

Various blood coagulation mediators are secreted by platelets. The α-granules contain large polypeptides that play a role in primary as well as secondary haemostasis. Fibrinogen and von Willebrand factor along with adhesive proteins are secreted by α-granules. These mediate primary haemostasis by supporting both the interactions between platelets as well as platelet interaction with the endothelium.[26]

Platelet α-granules also contribute to secondary haemostasis since they secrete numerous coagulation factors, including factor V, XI, and XIII upon activation. Prothrombin, the inactive precursor of thrombin, is also contained within the α-granules. Platelet α-granules also play a role in the inhibition of fibrinolysis by secreting plasminogen activator inhibitor-1 and a2-antiplasmin.[26]

The α-granules furthermore support haemostatic balance. Antithrombin and C1-inhibitor are both involved in controlling clot formation and are stored in the α-granules. Plasmin along with its inactive precursor (plasminogen) is stored in the platelet α-granules. The α-granules therefore regulate coagulation by playing a significant role as both pro-coagulant and anticoagulant.[27]

The increased number of α-granules seen during all the three phases of pregnancy may point to a pregnancy-specific modification. Since haemostatic factors are altered from the commencement of pregnancy to preserve the pregnancy and prepare the female body for delivery, changes within the platelets may also take place. More α-granules are produced to increase the production of their constituents involved in coagulation and wound healing. It may be that the α-granules are responsible for secreting a constant stream of haemostatic factors, thus promoting coagulation throughout pregnancy. The increase in α-granules thus contributes to the pro-thrombotic state associated with pregnancy. The α-granule count increases during pregnancy, but starts to decrease 6–8 weeks post-partum. Normal α-granule counts are, however, not achieved within 8 weeks after birth. This shows that the pro-thrombotic state of pregnancy persists till at least 2 months after birth. It also shows that this pregnancy-specific modification may return to normal levels or levels close to normal counts and not persist throughout the female's life.

Serotonin, calcium, pyrophosphate, and a non-metabolic adenine nucleotide pool consisting of ATP and ADP are contained within the dense granules.[28,29] ADP and collagen trigger the secretion of dense granules contents.[30] These constituents play a role in the recruitment of additional platelets during platelet activation.[17,18,31]

The decreased number of dense granules may also be a pregnancy-specific modification. Along with the steady stream of α-granule constituents maintaining the pro-thrombotic state of pregnancy, the decreased dense granule numbers may act as a countermeasure for the increase in coagulation. Since dense granule constituents promote platelet recruitment and ultimately platelet aggregation, the decreased numbers may prevent an 'over-aggregation' of platelets in circulation. Early pregnancy dense granule count is higher than late pregnancy and post-partum. It may be that this modification progresses with gestation. However, it does not return to normal within 2 months after delivery.

Lysosomes are present in all types of cells. They enclose acidic hydrolase, responsible for the degradation of proteins.[32] The increase in lysosomal granule count may also be a pregnancy-specific countermeasure like the dense granules. The acidic hydrolase within the lysosomal granules are only liberated during strong stimulus. Increased lysosomal granule numbers will increase the amount of acidic hydrolase which in turn will increase the degradation of fibrin clots. Once again the coagulation system is controlled to maintain a distinctive haemostatic balance that will prepare the body for delivery but not destroy it in the process.

Mitochondria supply the necessary energy needed for platelet reactivity.[31] The decrease in mitochondrial numbers seen throughout pregnancy may result from the pro-thrombotic state of pregnancy. Joseph *et al.*[33] attributed decreased mitochondrial count to the possible release or consumption of mitochondria in the activated platelets compared with the resting platelets. Platelet activation is apparent during all the three phases of pregnancy. Mitochondrial release and/or consumption may therefore explain the decreased counts.

Conclusion

In this study, ultrastructural changes pertaining to the external as well as the internal arrangements of platelets are shown. These are the first morphological study pertaining to pregnancy and its effect on platelets, and that these changes persist at least 2 months after birth. Therefore, we conclude that the hypercoagulable state associated with pregnancy may be accredited to the platelet ultrastructural changes which have been identified in this study.

Disclaimer statements

Contributors ACS contributed to the concept and design of this study as well as analysis, interpretation and writing of this article. EP contributed to the analysis and interpretation of the data along with revising of the intellectual content.

Conflicts of interest None.

Ethics approval Ethical approval was obtained from the University of Pretoria, Human Ethical Clearance Committee.

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