Electron microscopy examination of platelet whole mount preparations to quantitate platelet dense granule numbers: Implications for diagnosing suspected platelet function disorders due to dense granule deficiency

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Abstract

Introduction: Dense granule (DG) deficiency (DGD) is a feature of some platelet function disorders (PFD) with a prevalence similar to von Willebrand disease. Most laboratories assess for DGD using whole mount platelet preparations and electron microscopy (EM). We evaluated our experiences with this test and associations between DGD and bleeding.

Methods: Dense granule EM records for 2006-2017 were examined for patients and simultaneously tested controls, and for an overlapping PFD study cohort to evaluate findings and their relationship to bleeding.

Results: More patient than control samples had reduced DG counts (6.5% vs 0.3%, \( P < .01 \)). DG counts showed no relationship to age or mean platelet volume and had acceptable within-subject variability that was higher for DGD than control participants (28% vs 12%). Repeat tests confirmed DGD in all persons with initial DG counts <4.0/platelet, but not in those with less severe reductions (4.0-4.8 DG/platelet) or normal DG counts (≥4.9 DG/platelet). Aggregometry and adenosine triphosphate release tests, respectively, had only ~52% and 70% sensitivity for DGD. Confirmed DGD by EM was associated with higher bleeding scores and a bleeding disorder.

Conclusion: Whole mount EM is useful for the evaluation of suspected PFD due to DGD and detects abnormalities associated with bleeding.

Keywords
bleeding scores, dense granule deficiency, dense granules, electron microscopy, platelet function disorders

1 | INTRODUCTION

Platelet dense granules (DG) are important for platelet function and store adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, polyphosphate, and calcium for regulated release with platelet activation.\(^1\)\(^2\) Platelet dense granule deficiency (DGD) is associated with mucocutaneous bleeding problems and has a similar prevalence to von Willebrand disease (VWD).\(^3\)\(^4\) The causes of DGD include both nonsyndromic and syndromic platelet function disorders (PFD), including Hermansky-Pudlak syndrome and Wiskott-Aldrich syndrome.\(^5\) Tests for DGD are considered helpful for diagnosing PFD as about one-third of persons with DGD have normal maximal aggregation (MA) responses to agonists by light transmission aggregometry (LTA)\(^6\)\(^6\) and some have normal
findings in lumi-aggregometry assessments of platelet DG ATP release. Furthermore, the International Society on Thrombosis and Haemostasis (ISTH) has recommended that assays for DGD be performed as part of the workup for a PFD.

Many laboratories that evaluate for platelet DGD use the method first described by James White to evaluate the number of electron-dense granules in platelet whole mount preparations by transmission electron microscopy (EM). The calcium and phosphorus content of DG makes these structures electron-dense, allowing DG to be visualized in platelets without fixation or special stains. The whole mount preparations of platelets for DG evaluation are prepared by placing a drop of platelet-rich plasma (PRP) on a Formvar-coated grid, blotting off the excess PRP, followed by a quick wash with distilled water before air drying the grid and examining the sample by EM to determine the average number of electron-dense granules in 30-50 unfixed platelets. Externally quality assurance (EQA) exercises on whole mount EM tests for DGD have shown good agreement between laboratories on which electron-dense structures should be counted as a DG and which samples show normal findings or DGD.

While the finding of DGD by EM has been associated with a bleeding disorder, we decided to address the typical findings and within-subject variability for the assay by examining 12 years of prospectively collected data on diagnostic EM tests for DGD. We postulated that this would generate useful evidence, like our studies of LTA and DG ATP release. To explore relationships between confirmed DGD, other laboratory findings, and bleeding, we also evaluated whole mount EM data for an overlapping cohort that had their bleeding history evaluated as part of a study on PFD.

2. MATERIALS AND METHODS

2.1 Study participants

The study was conducted in accordance with the requirements of the revised Helsinki accord, the Hamilton Integrated Research Ethics Board (HiREB), and the Hamilton Regional Laboratory Medicine Program (HRLMP). All participant identities were anonymized prior to data entry.

Cohort I included all referred patients and simultaneously evaluated controls that were tested by the HRLMP for DGD over a 12-year period from January 1, 2006 to December 4, 2017; HRLMP and HiREB did not require informed consent to assess cohort I findings for quality improvement purposes.

Cohort II participants provided written informed consent for a HiREB-approved study to evaluate the phenotype and causes of PFD associated with DGD and/or impaired MA responses with ≥2 agonists. Cohort II participants included index cases with PFD, and affected and unaffected relatives whose investigations were completed by December 4, 2017.

Light transmission aggregometry and DG ATP release findings for many cohort I participants were previously reported but as participants were anonymized with different codes than used for the present study, LTA and DG ATP release data for DGD participants (both cohorts) were retrieved from original medical records.

DG counts for samples used for North American Specialized Coagulation Laboratory Association (NASCOLA) EQA exercises on quantitating DG numbers by EM (offered twice annually, between 2003 and 2012) were exclusively used to evaluate within-subject variability. To assess within-subject variability in DG counts for persons with DGD, older EM records (from January 1999 to December 2005) were searched for additional data on cohort I patients with 1 test showing reduced DG numbers/platelet.

2.2 Evaluation of bleeding scores

The ISTH bleeding assessment tool (ISTH-BAT) was used to quantify bleeding symptoms for cohort II participants, in comparison with general population controls (n = 60; 40 females, 20 males; ages: median = 51 years, range 15-64) whose ISTH-BAT scores were previously reported.

2.3 Evaluation of platelet dense granule numbers

Whole mount preparations of platelets were typically prepared within 4 hours of blood collection using PRP prepared by the Special Coagulation Laboratory, HRLMP, from blood anticoagulated with 0.105 mol/L sodium citrate. No method changes were introduced during the 12-year period of data evaluation. Each count was derived from a fresh sample, blinded to previous determinations to avoid bias. The number of platelets evaluated was based on Dr. James White’s method and his recommendation to count DG in about 30 platelets, when we consulted him about setting up the method in our laboratory, as described previously. A healthy control sample was drawn and tested on each day that patient tests were performed. Some controls provided a sample on many different occasions.

Samples were first examined at a low magnification (~100×) to assess the distribution of platelet DG before proceeding to count representative regions at a higher magnification (~4000×). Representative grid openings were examined systematically, from one side to the next, before moving onto the next fields. DG number was not counted in platelets that overlapped, showed bubble artifacts or shape change. The structures counted as DG were typical of the electron-dense structures that the majority of NASCOLA laboratories classified as DG, which included round DG and DG with tails or a “purse-like” shapes but not faint, tiny, or chain-like granules. Ten random samples were used to compare DG counts based on the examination of 30 vs 100 platelets.

Reports for samples with 3.8-4.8 DG/platelet had included a recommendation to consider repeat testing. Decisions on repeat testing were left to the ordering hematologist’s discretion.

2.4 Data collection

Data collected for cohorts I and II included gender, age (inconsistently recorded for cohort I controls), date of testing, and the DG count for each unique sample. For cohort II, data were also collected on: whether the participant had a bleeding disorder (based on the
hematologist's opinion, obtained by chart review, as described15; ISTH-BAT scores7,16,17; the most recent HRLMP platelet count, mean platelet volume (MPV), DG ATP release findings (determined by lumiggregometry as described,7 using an agonist panel that included: 1 U/mL thrombin, 5.0 μg/mL Horm collagen, 6 μmol/L epinephrine, 1 μmol/L thromboxane analogue U46619, and 1.6 mmol/L arachidonic acid [AA]) and MA responses (evaluated by LTA, as described,14-16 with the agonists: 1.25 and 5.0 μg/mL Horm collagen, 2.5 and 5.0 μmol/L ADP, 6 μmol/L epinephrine, 1 μmol/L thromboxane analogue U46619, 1.6 mmol/L AA, and 0.5, and 1.25 mg/mL ristocetin).

2.5 | Statistical analyses

After assessing for differences in data for male and female controls, the lower limit of the reference interval (RI) for DG counts (which was last updated in October 2008 using 124 values from 33 unique adult controls) was estimated using stepwise logistic regression to determine the lower limit of the 95% confidence interval (CI) limits.18 Briefly, data for all unique cohort I general population controls (60 males, 66 females) were analyzed by the method of Taylor et al,19 which allows the inclusion of repeated measures on subjects as it takes into account within-subject variability and adjusts weightings for repeated measures so that each participant’s data contribute equally to the RI.

Coefficients of variation (CV)20 were used to assess the average DG counts/platelet for all participants (cohorts I and II combined) that had ≥3 separate samples assessed (each on different days). Linear regression was used to assess relationships between (i) the average number of DG/platelet and MPV; (ii) CVs for mean DG counts for participants with ≥3 sample assessments; and (iii) relationships between the average number of DG/platelet and age for cohort I participants <18 years old on their first test because a recent study suggested DG numbers are lower in young children.21 Chi-square analysis was used to assess the proportions of (i) cohort I patient vs control samples with DG numbers below the RI (excluding NASCOLA EQA samples); and (ii) cohort II participants with PFD, with or without DGD, that had reduced MA with ≥2 agonists by LTA. Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess whether data were normally distributed. Data that were normally distributed were analyzed by the Student’s t test. The Mann-Whitney U test was used to analyze non-normal data. Kruskal-Wallis and Mann-Whitney U tests were used to evaluate the relationships between confirmed DGD and ISTH-BAT scores. Odds ratios (OR) and 95% CI were used to assess relationships between confirmed DGD by whole mount EM and the diagnosis of a bleeding disorder (based on the hematologist’s opinion of the bleeding history). P-values less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Cohort I

Between January 1, 2006 and December 4, 2017, the HRLMP evaluated platelet whole mount preparations from 1115 unique patients (age in years: median 37, range 0.25-89; females: 77%) and 126 unique controls (ages, if recorded: 18-64; females: 52%). Patients in cohort I included 24/29 persons with PFD in cohort II. No patients in either cohort had Hermansky-Pudlak syndrome.

Some participants in cohort I had been tested on multiple occasions (number of unique samples/participant, as medians [ranges]: patients: 1 [1-6]; controls: 1 [1-63]) (total number of samples: patients: 1195; controls: 615) and the DG counts for individuals with the largest number of determinations indicated that the data had a normal distribution. Accordingly, means were used for comparisons when there were multiple determinations/subject (Figure 1).

Dense granule counts were similar for pediatric (<18 years old) and adult patients (respective means [ranges]: 6.6 [2.7-11.6] vs 6.7 [0.8-15.5] (P = .28) (Figure 1). Among participants <18 years of age, there was no relationship between age and DG counts (R^2 = .005). Among patients, there were no significant differences in mean DG counts for females vs males (6.7 vs 6.7, P = .32); however, among the controls, the mean DG counts were slightly higher for females than males (6.9 vs 6.5, P = .03). Accordingly, the RI for DG counts was determined separately for males (RI: 4.9-8.2) and females (RI: 4.9-8.8). The lower RI limit of 4.9 DG/platelet was the same for both males and females, and it was identical to the 2008 estimate. Although the mean DG counts for patients and controls in cohort I were also identical (respective values: 6.7 vs 6.7, P = .84), proportionately more patient samples had reduced DG counts (% <4.9 DG/platelet for patients vs controls: 6.5% vs 0.3%, P < .01). A cohort I participant with a PFD, thrombocytopenia, and an elevated MPV (MPV: 12.1, upper

![FIGURE 1](image)
limit of RI: 10.4 fL) had the highest DG counts (2 determinations, averages: 14.5 and 15.8 DG/platelet) of all study participants.

Dense granule counts, determined by the examination of 30 or 100 platelets for 10 random samples, were comparable (respective: means [ranges]: 5.2 [1.8-7.6] vs 4.9 [1.7-6.8]; standard deviations: 1.6 vs 1.3; \(P = .60\)).

3.2 | Cohort II

In cohort II, there were 26 females and 16 males, whose ages ranged from 7 to 80 years (median 47 years). In this cohort, 29/42 had a PFD, whereas all 13 unaffected relatives had normal DG counts and LTA findings. Among cohort II participants, platelet DG numbers showed no relationship to MPV (\(R^2 = .06\), \(P = .15\)). In the subgroup with PFD, ~34% (10/29) had confirmed DGD, based on 2 or more tests.

3.3 | Within-subject variability for dense granule counts

Figure 2 shows data for participants in both cohorts (n = 120) that had DG counts determined for multiple unique samples, each drawn on a different occasion, including samples drawn for NASCOLA EQA exercises. Almost all controls with repeat tests had DG counts ≥ the lower RI limit on all tests (124/126, 98%), and 16 had ≥10 determinations because they volunteered to donate a sample on many different occasions (Figure 2A). Among patients with multiple tests, the median time between first and second tests was 63 days (range: 7-2882 days). Many patients that were retested had confirmed normal findings on each test (41%, 26/64), whereas others had confirmed DGD on each test (30%, 19/64) or discordant results (30%, 19/64) (Figure 2B). Among patients with discordant results, the majority (17/19, 89%) had a mild reduction in DG numbers (ie, 4.0-4.8 DG/platelet) on the first test only (Figure 2B).

Among participants who had their platelet DG counts assessed ≥3 times, the within-subject CV for DG counts/platelet showed an inverse relationship to the subject’s mean DG count (\(R^2 = .65\), \(P < .01\)) with non-DGD participants having a lower within-subject CV than DGD subjects (respective means [ranges] for within-subject CV for normal vs DGD participants: 12% [5%-24%] vs 28% [18%-46%]) (Figure 3). Nonetheless, DGD was evident on each sample for all participants with <4 DG/platelet.
3.4 | Aggregation and dense granule secretion findings for dense granule-deficient participants

The data for the most recent platelet function tests for the 19 participants with confirmed DGD are summarized in Figure 4, except for LTA findings with 0.5 mg/mL ristocetin as it was normal for all participants. While none of the unaffected relatives in cohort II (n = 13) had abnormal LTA findings (not shown), 52% of the DGD participants (10/19) had reduced MA with ≥2 agonists (Figure 4A). Comparisons of aggregation data for cohort II participants that had a PFD, with or without DGD (2 studied with limited numbers of agonists due to their young age), indicated that proportionately less with DGD had impaired MA with 2.5 μmol/L ADP (0/19 vs 6/18; P < .01); 5 μmol/L ADP (0/19 vs 5/18; P = .01); 1.6 mmol/L AA (7/18 vs 14/18; P = .02); and 1 μmol/L thromboxane analogue U46619 (9/18 vs 17/18; P < .01).

Figure 4B summarizes the lumi-aggregometry data for DG ATP release, which was available for 52% (10/19) of participants with confirmed DGD (median [range] of average DG counts/platelet: 2.2 [0.7-4.7]). While most individuals with DGD had reduced ATP release with at least 2 agonists (70%, 7/10), only half showed reduced release with all agonists (5/10) and others (30%, 3/10) had nondiagnostic findings, including an individual with only 1.4 DG/platelet (this participant’s normal ATP release was verified on additional samples). We estimated that the sensitivities of LTA and DG ATP release, for detecting platelet function abnormalities due to DGD, are ~52% and 70%, respectively (based on the detection of impaired responses to ≥2 agonists).

3.5 | Relationship between DGD and bleeding

Nonparametric analysis of cohort II data indicated that persons with confirmed DGD had higher ISTH-BAT bleeding scores (median: 9.5, range: 4-17) than unaffected relatives (median: 1, range: 0-8; P < .01) and general population controls (median: 0, range: 0-6, P < .01) (Figure 5). However, ISTH-BAT scores were not significantly different for persons with a PFD with or without DGD (respective medians [ranges] = 8 [0-17] vs 9.5 [4-17], P = .20). The finding of confirmed DGD by EM was associated with a bleeding disorder (OR = 97, 95% CI = 5.4-1740, P < .01).

4 | DISCUSSION

The main goal of this study was to evaluate our experiences with using platelet whole mount EM to diagnose DGD over a 12-year period, including the findings for an overlapping cohort whose bleeding symptoms, DG counts, platelet function, and MPV were assessed for a study on PFD. We evaluated typical findings, including the lower limit of the assay RI by gender; age differences in findings;
Values indicate significant differences (n = 60). Unaffected relatives (n = 13), and general population controls (n = 10), PFD without DGD (n = 19, indicated as Not DGD), with all agonists. Given our major findings, we recommend that specific testing for DGD, by a validated method (such as whole mount EM) that has an acceptable, within-subject variability, be considered when testing for a suspected PFD, even if LTA and ATP release findings are nondiagnostic or inconsistent.

In our study, we formally evaluated the variability of the whole mount EM test for DGD as this had not been previously evaluated and tests with high CV (such as DG ATP release) perform poorly for diagnostic applications. Based on comparative data for 10 samples, we observed very similar means, ranges, and standard deviations for the average DG count/platelet whether 30 or 100 platelets were evaluated. We found that an EM assessment for DGD using whole mount preparations had an acceptable within-subject CV (12% for samples with normal DG counts). Although the within-subject CV was higher (28%) for samples with significantly reduced DG counts, DGD was confirmed in all samples from persons with an initial count that was <4.0 DG/platelet. The observation that many mild reductions in DG numbers (i.e., 4.0-4.8 DG/platelet) were not confirmed when another sample was collected and tested, illustrates that it is important to verify reduced DG counts with another sample. Some of the mild reductions in DG counts that we observed could reflect false positives and also the biology of some PFD. For example, familial PFD due to RUNX1 haploinsufficiency reduces the DG content of platelets; however, only ~50% of the affected family members have DG counts below the RI. Our observations on the test reproducibility and within-subject CV are important as whole mount EM is the most commonly used method for diagnosing platelet DGD in North America. We acknowledge that our study reports an expert center’s experience as 2 expert EM technologists performed the test during the period of data collection, using PRP prepared by our Special Coagulation Laboratory. While many centers that use EM to evaluate platelet DG cannot perform energy dispersive spectrometry, our site had the technical capability during the initial assay validation which was helpful to verify that the platelet structures counted as DG did indeed contain calcium and phosphorus. Nonetheless, EQA exercises have shown excellent agreement between laboratories on what electron-dense structures in platelets should be counted as a DG.

It is possible that pre-analytical and analytical differences, and differences in the number of control samples used to determine RI, contribute to differences in RI between laboratories for whole mount EM DG counts. For example, a recent study that reported a lower limit cutoff (based on estimating the 95% CI) of 1.96 DG/platelet used single measures for 40 control samples from participants with a median age of 10 (range: 2 months to 20 years), whereas we used data for all 615 samples from 126 unique adult controls and the method described by Taylor et al. that allows inclusion of all data when some subjects have multiple determinations and takes within-subject variability into account in the RI determination. Our estimate is closer to the RI reported by several other groups, which were 4-6 and 4-8 DG/platelet. We found no differences in DG numbers/platelet between pediatric and adult cohort I samples, nor did we find any significant correlation between age and DG numbers for the participants <18 years of age. This suggests that the lower limit of the RI validated for adult samples

**FIGURE 5** Bleeding scores for cohort II participants. ISTH-BAT scores are compared for participants with PFD and confirmed DGD (n = 10), PFD without DGD (n = 19, indicated as Not DGD), unaffected relatives (n = 13), and general population controls (n = 60). P values indicate significant differences.
(ie, 4.9 DG/platelet for both males and females) is suitable for samples from children. Nonetheless, we would recommend confirming reduced DG numbers on all individuals with abnormal counts and retesting, at an older age, all very young children whose findings suggest DGD.

For diagnostic tests, it is important to evaluate the test performance in a prospective cohort of individuals that might reasonably be tested for the condition that the test is designed to assess. While there is no generally accepted “gold standard” for diagnosing DGD, our current study illustrates that whole mount EM provides estimates of platelet DG counts with limited variability, good reproducibility, and important clinical associations. One limitation of whole mount EM (which is fairly simple to perform) is that it requires an expensive piece of equipment: a transmission electron microscope. Recently, super-resolution light microscopy (SRM) assessment of CD63-stained platelets was proposed as an alternative way to detect DGD, based on observations for known DGD individuals vs control subjects.23,24 A prospective study comparing SRM to whole mount EM for diagnosing PFD due to DGD, among patients referred for diagnostic PFD testing, would be the ideal way to establish whether SRM is noninferior or superior to whole mount EM for diagnosing DGD. However, this would be impractical as it would require multicenter recruitment to achieve sufficient power given how infrequently new cases of DGD are diagnosed, even at tertiary referral centers, like our own.

With the growing emphasis on evidence-based decisions in health care, it is important to establish whether diagnostic assays detect clinically important problems. A key observation of our study is that persons with DGD by whole mount EM have a high likelihood of having a bleeding disorder (OR = 97, 95% CI = 5.4-1740). Furthermore, the test detects an abnormality associated with increased bleeding, reflected by higher ISTH-BAT scores than unaffected relatives and general population controls. It was interesting that the bleeding scores of our DGD participants were not different from those that had a PFD without DGD.

Based on our study findings, we recommend the following when evaluating for platelet DGD by whole mount EM:

- Centers that have the capability to assess DG numbers by whole mount EM should perform the test as part of an evaluation for PFD, even if other tests exclude defects in platelet aggregation and DG ATP release.
- If a person has normal DG numbers/platelet, further testing for DGD is not required as repeat determinations are likely to confirm normal numbers of DG/platelet.
- If a person has reduced numbers of DG/platelet, based on a validated RI, the test should be repeated on another sample as many mild reductions in DG numbers are not confirmed on repeat testing and DGD should be confirmed to verify the PFD diagnosis.
- If a very young child appears to have DGD, based on the adult RI, repeat testing should also be performed at an older age to verify the findings.

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REFERENCES
