The *PIG-A* gene mutation assay in human biomonitoring and disease

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Abstract

The blood cell phosphatidylinositol glycan class A (*PIG-A*) gene mutation assay has been extensively researched in rodents for *in vivo* mutagenicity testing and is now being investigated in humans. The *PIG-A* gene is involved in glycosyl phosphatidylinositol (GPI)-anchor biosynthesis. A single mutation in this X-linked gene leads to loss of membrane bound GPI-anchors, which can be enumerated using flow cytometry. With many studies published to date measuring this mutation in erythrocytes, there is remarkable consistency across research groups. Moreover, with the low background level of mutant erythrocytes in healthy subjects (2.9 - 5.56 x 10-6 mutants), induction of mutation post genotoxic exposure can be detected. Cigarette smoking, radiotherapy and occupational exposures including lead, have been shown to increase mutant levels. Identification of harmful agents can allow us to recommend new exposure limits, minimising individual risk. Conversely, protective agents such as aspirin and healthy diets could mitigate these effects, reducing baseline somatic mutation levels and such behaviours can be encouraged. This mutational monitoring approach may also provide information on individuals at higher risk of cancer development. Patients with inflammatory bowel disease, oesophageal adenocarcinoma and pancreatic cancer have elevated numbers of *PIG-A* mutant erythrocytes compared to age-matched controls. With further technological progress including protocol standardisation and the development of cryopreservation methods to improve GPI-anchor stability, this assay can be widely employed in rural and low-income countries. Here we review the current literature on *PIG-A* mutation in human subjects and discuss the potential role of this assay in human biomonitoring and disease.

Introduction

Measuring somatic mutations in humans provides an invaluable tool for identifying exogenous mutagenic sources, allowing us to make better informed public health choices to avoid harmful exposures and to introduce regulatory approaches to reduce these adverse exposures. As cancer is driven by DNA mutations, an accumulation of somatic mutations is known to increase an individual's cancer risk (Hanahan & Weinberg, 2011). Hence monitoring individuals for the number and types of somatic DNA mutations will allow the identification of those who may be at increased risk of cancer development which may allow for early intervention and perhaps even cancer prevention. Environmental exposures that can induce mutation include dietary, lifestyle, accidental and occupational exposures. Developing tools that allow us to measure such mutations will aid in the identification of new mutagenic and/or carcinogenic exposures (Loomis, Guha, Hall, & Straif, 2018). Knowledge of the lifestyle factors associated with increased mutation levels could be used to tailor advice to members of the public in relation to avoiding risky behaviours (e.g., smoking) through public health measures. Everyday exposures may conversely reduce our mutational risk, e.g., diets containing anti-mutagenic compounds. Chemo-preventative, or anti-genotoxicity lifestyle factors could be specifically

identified through large-scale human biomonitoring studies (e.g., dietary compounds and medications) using high-throughput approaches needed to unpick the multi-faceted exposures we face daily.

In addition, genetic polymorphisms may modulate an individual's risk of mutation (and hence cancer), either by increasing or decreasing the likelihood of mutational events. For example, if a certain exposure to a genotoxin induces DNA damage which cannot be efficiently repaired due to polymorphisms in genes such as *OGG1* (Jensen et al., 2012) or*XRCC1* (Monteiro, Vilas Boas, Gigliotti, & Salvadori, 2014) (involved in base excision repair), this may increase an individual's sensitivity to DNA damage. In addition, germline mutations in DNA polymerases increases somatic mutational burden in normal tissues (Robinson et al., 2022), and somatic mutation rates are increased in the normal tissues of patients with MUTYH-associated polyposis (MAP) (Robinson et al., 2021). Therefore, a personalised approach to precision exposure monitoring may be most beneficial with the inclusion of these biomarkers of susceptibility.

There are a handful of cytogenetic techniques that can detect DNA abnormalities in human cells. Currently available genotoxicity tests suitable for measuring DNA damage and mutation include the lymphocyte cytokinesis-block micronucleus test (CBMN) (Fenech et al., 2011), the lymphocyte COMET assay (Fenech et al., 2011) and the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) test (Townsend, Robison, & O'Neill, 2018) as well as approaches measuring sister chromatid exchanges (Sunada, Haskins, & Kato, 2019) and chromosomal abnormalities (Sunada et al., 2019). Recently, increased accessibility to DNA sequencing technologies able to overcome difficulties associated with background mutation rates, have identified DNA mutational signatures (Alexandrov et al., 2020; Alexandrov et al., 2013), particularly those that correlate with certain human exposures including Aflatoxin B1 and ultraviolet irradiation (Phillips, 2018). Whilst whole genome/exome sequencing allows us to measure mutations across the whole genome, and not just those produced in reporter genes such as *hprt* or phosphatidylinositol glycan class A*(PIG-A)* , the technology is still relatively expensive and not therefore suitable for wide-scale roll out in population-based biomonitoring studies. Moreover, not all exposures have known mutational profiles or signatures at this stage and therefore it is not informative to measure *de novo* mutational signatures if we do not know the cause.

The *PIG-A* assay measures *de novo* somatic mutations in the*PIG-A* gene. The test measures the steady state accumulation of mutations in blood cells, to provide a snapshot of mutant cell levels at any given point in time relative to the lifespan of erythrocytes (approximately 115-120 days) (Franco, 2012; Thiagarajan, Parker, & Prchal, 2021). The *PIG-A* gene is located at Xp22.2 in humans. The protein product of the gene is responsible for the production of a catalytic subunit of N-acetylglucosamine transferase, an enzyme involved in one of the early steps of glycosyl phosphatidylinositol (GPI)-anchor biosynthesis (Takeda et al., 1993). Although there are over 20 genes involved in GPI-anchor production, only one mutation in the *PIG-A*gene is required to inactivate the gene and result in a GPI deficient phenotype (Miyata et al., 1993). As shown in Figure 1, *PIG-A*wild-type cells present many GPI-anchors and their corresponding GPI-anchored proteins (GPI-AP) on the cell surface which can subsequently be detected using fluorescently labelled antibodies targeting the GPI-AP of choice through flow cytometry. *PIG-A*mutant cells lack these GPI anchors and crucially lack the GPI-APs and therefore GPI-AP targeted antibodies are unable to bind to the extracellular surface.

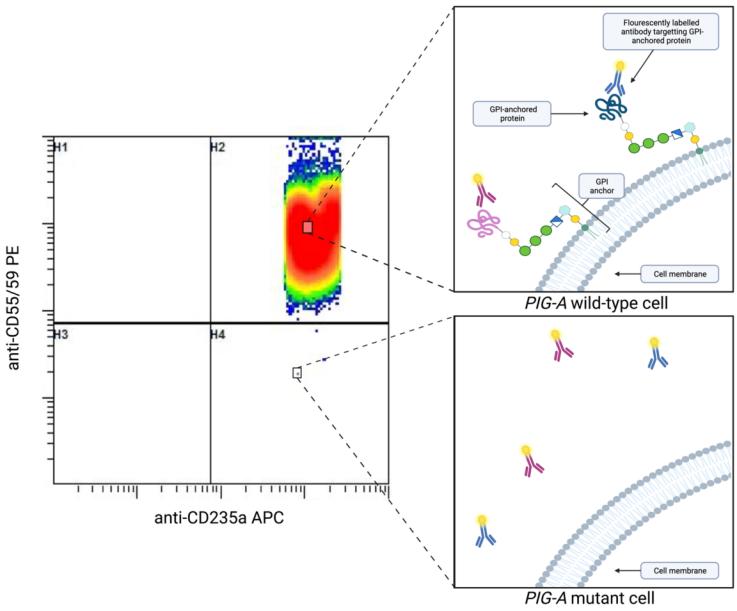

Figure 1. Flow cytometric analysis of red blood cell phosphatidylinositol glycan class A (*PIG-A*) gene status. Anti-CD235A antibodies can be used to isolate erythrocytes in a whole blood sample and anti-CD55/CD59 antibodies are used to detect the presence of the glycosylphosphatidylinositol (GPI)-linked proteins CD55 and CD59. *PIG-A* wild-type cells present functional (GPI) anchors and corresponding GPI-linked proteins at the extracellular surface (top panel) whilst *PIG-A* mutant cells do not fluoresce as they lack GPI-anchors and corresponding anchored proteins (made using biorender.com).

Blood cell *PIG-A* mutations were first described in patients with paroxysmal nocturnal haemoglobinuria (PNH). A *de novoPIG-A* mutation in haematopoietic stem cells results in clonal expansion of the mutant and subsequent GPI (and GPI-AP) deficiency (measured using antibodies targeting CD55/CD59). Clinical symptoms of PNH include intravascular haemolysis and thrombosis (Endo, Ware, Vreeke, Howard, & Parker, 1996). Whilst there are around 150 GPI-APs, many GPI-APs are immune effectors. This includes CD55 and CD59 which regulate the complement immune system. In PNH, therefore, their widespread deficiency results in complement mediated erythrolysis (Boccuni, Del Vecchio, Di Noto, & Rotoli, 2000). Clinical presentation of symptoms similar to that of PNH have also been observed in patients with germline mutations resulting in systemic loss of the GPI-AP CD59 (Yamashina et al., 1990).

This mutation test has been widely researched in rodents to test novel products for mutagenic effects as part of regulatory safety assessment (Dobrovolsky, Cao, Bhalli, & Heflich, 2020; Olsen et al., 2017). It is also highly suitable for human application as the same biochemical pathway is conserved across mammals (Kawagoe, Takeda, Endo, & Kinoshita, 1994). Typical analysis time is 3-4 hours, not requiring further culturing or sub-cloning of mutant cells and costing as little as £50 per sample. The suitability of the *PIG-A* mutation assay as a tool for human biomonitoring and cancer detection has been examined and reported in the literature and is discussed in detail in this review.

Background mutant frequency in healthy populations

Somatic *PIG-A* mutations can occur at low frequency through spontaneous mutational processes during normal haematopoiesis without inducing PNH-like clinical symptoms. This is perhaps because these mutated, GPI-negative blood cells occur at a lower frequency than is required to activate mass complement mediated lysis (Araten, Nafa, Pakdeesuwan, & Luzzatto, 1999; Hu et al., 2005). These *PIG-A*mutated erythrocytes are present in healthy individuals at low numbers (Cao et al., 2016; Dertinger, Avlasevich, Bemis, Chen, & MacGregor, 2015; Dobrovolsky, Elespuru, Bigger, Robison, & Heflich, 2011; Lawrence, Haboubi, Williams,

Doak, & Jenkins, 2020), allowing for easy observation of any increase in mutant cell levels and association with individual mutagenic exposures. At low levels, such mutant mammalian cells are growth neutral, viable and are easily detectable using flow cytometry (David et al., 2018) (Figure 1).

PIG-A mutations can be measured in all blood cell types and have been reported for erythrocytes, reticulocytes, granulocytes, and lymphocytes, with the majority of the reports using human erythrocytes. A number of publications have measured *PIG-A* mutant erythrocyte levels in healthy populations with values ranging from 2.9 - 5.56 x 10^{-6} mutants per million erythrocytes (Figure 2). These 7 studies all measured *PIG-A* status through flow cytometric analysis, using antibodies that target the GPI-linked protein CD59 (Cao, Wang, Xi, et al., 2020; Cao et al., 2021; Cao et al., 2016; Dobrovolsky et al., 2011), or CD55 and CD59 in combination (Dertinger et al., 2015; Haboubi et al., 2019; Lawrence et al., 2020). Using both CD55 and CD59 in combination may reduce the number of falsely classified mutant cells by eliminating any effect of reduced CD59 expression (Peruzzi, Araten, Notaro, & Luzzatto, 2010). However, as seen in Figure 2, there is no significant difference in mutant cell levels between studies that applied either CD55 alone (blue bars) or in combination with CD59 (red bars) (p=0.057). The green bar on the graph represents the weighted average from these 7 publications which is 4.58 ± 4.2 x 10^{-6} mutants per million erythrocytes. The consistency across studies is remarkable, considering that mutant frequency can be easily inflated by any slight technological variation. The number of mutant cells is subject to technical variation and can be artificially altered in a number of ways. This includes antibody batch to batch variation and supplier differences, sample preparation resulting in shearing of anchored proteins, flow cytometer choice, flow rate, laser strength and gating procedure. Test reproducibility is essential for successful biomarker implementation. It is particularly important if such a test were to be used in the assessment of human genotoxic exposures where a subtle change in mutant frequency, may be indicative of a positive genotoxic response.

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Figure 2. Background levels of phosphatidylinositol glycan class A (*PIG-A*) mutant erythrocytes in healthy populations from published research. Blue bars represent publications that have used antibodies targeting CD55 only and red bars represent publications that have used antibodies targeting CD55 and CD59 in combination. The green bar shows the weighted mean. The bars represent mean values and the error bars represent standard deviation.

We have assessed repeated measurements of individuals over time to provide further evidence relating to the reproducibility of the assay and the stability of the erythrocyte *PIG-A* mutant phenotype. Figure 3A shows repeat measurements from the same 4 individuals (2 females and 2 males) over a period of 17 weeks (see (Lawrence, 2020 #4) for methods). Although intra-individual variation exists, erythrocyte*PIG-A* mutant frequency remains relatively stable. For example, the mutant frequency of Participant 2 remains relatively high between 7 and 14 x 10^{-6} mutants (average 10.53 ± 2.16 x 10^{-6} mutants) whilst the mutant frequency of Participant 4 is consistently below 5 x 10^{-6} mutants (average 3.56 ± 1.22 x 10^{-6} mutants). This is similar to what has been previously published where Cao and colleagues repeatedly measured the RBC *PIG-A* mutant frequency of 3 individuals and discovered that whilst 2 subjects had MF's consistently below 5 x 10^{-6} mutants, one subject's MF always remained around 15 x 10^{-6} mutants (Cao et al., 2016). The average coefficient of variation (CV) (indicator of intra-individual variation) of the 4 individuals presented here was 35.2%. This is lower than the intra-individual variation observed in granulocytes by Rondelli *et al* where 32 healthy subjects were tested for *PIG-A*status on 3 separate occasions which produced a CV of 44.3% (Rondelli et al., 2013). Figure 3B further shows the erythrocyte *PIG-A*mutant frequency for 9 healthy volunteers at 2 different time points. The duration between each time point ranged from 92 to 516 days with an average duration of 270 days. Participant HV112 had consistently high mutation frequency of 9.61 – 10.75 x 10^{-6} mutant erythrocytes and HV038 mutant frequency remained low between 2.14 and 2.43 x 10^{-6} mutants over this period. This data further demonstrates the general stability of *PIG-A* mutant phenotype over a longer period in self-reported healthy volunteers.

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Figure 3: (A) Repeat erythrocyte phosphatidylinositol glycan class A (*PIG-A*) mutant frequency measurements for 4 participants over a 17-week period. (B) Measurement of *PIG-A* mutant frequency for 9 healthy volunteers on two separate occasions. The time between each blood sample acquisition ranged from 92 to 516 days with an average time of 270 days. Methods published previously (Lawrence, 2020 #23).

Factors that influence *PIG-A* mutant frequency

Several human *PIG-A* studies have also looked to identify individual factors that may result in mutant frequency variation. The fact that humans have higher *PIG-A* mutant frequencies compared to laboratory rodents (Olsen et al., 2017) could be due the fact that humans are long-lived and are exposed to a wider range of lifestyle genotoxins compared to laboratory confined rodents. Some of the human studies mentioned above explored whether mutant frequency differs between sexes, producing contrasting results (Figure 4). Dobrovolsky and colleagues found that females had significantly higher erythrocyte mutant frequencies compared to males (p=0.025), although only 26 females were included in this cohort (Dobrovolsky et al., 2011). Conversely, Cao and colleagues measured higher mutant erythrocytes in males compared to females (p<0.0001) (Cao et al., 2016). The other publications presented in Figure 2 (Cao, Wang, Xi, et al., 2020; Cao et al., 2021; Haboubi et al., 2019; Lawrence et al., 2020) as well as the study by Dertinger and colleagues (Dertinger et al., 2015) (not shown in Figure 4) revealed no significant differences in erythrocyte *PIG-A*mutant frequencies between sexes. A study measuring reticulocyte*PIG-A* identified a higher level of mutant reticulocytes in males compared to females (Torous et al., 2020). Even though the reason behind these sex differences is unclear it may be due to the different ethnicities studied in these particular two groups with one research group measuring *PIG-A* mutation status in an ethnic Chinese population (Cao et al., 2016) and the other in a mainly Caucasian/Hispanic population (Dobrovolsky et al., 2011).This suggested difference in gender may also be due to limited sample sizes and lack of integration of alternative factors (known or unknown) that can confound mutation status at the *PIG-A* locus.

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Figure 4: Sex differences in erythrocyte phosphatidylinositol glycan class A (*PIG-A*) mutant frequency for 5 publications. Solid bars represent the mean mutant frequency and error bars show standard deviation. The white numbers on each bar represent the number of healthy participants in each category.

As DNA damage and subsequent mutations can accumulate with age due to age-related reductions in DNA repair capacity and the lifelong accumulation of such DNA damage, the potential impact of age has been investigated by several research groups. A significant association between older age and increased mutant frequencies was found in three cohorts interrogating erythrocytes (Dertinger et al., 2015; Haboubi et al., 2019; Lawrence et al., 2020) and one interrogating reticulocytes (Torous et al., 2020), whilst other research groups either found minimal or no correlation with age. However, when Cao and colleagues measured the utility of this assay to identify mutagenicity in a cohort occupationally exposed to polycyclic aromatic hydrocarbons, the statistical analysis performed accounted for the impact of age on mutant frequency, suggesting there may be an age effect in this ethnic Chinese population (Cao et al., 2021). We may expect a correlation between mutation accumulation and age, with previous publications demonstrating an increased in single-base substitution mutational burden with age (Robinson et al., 2021). However, the studies published to date do not include a sufficient number of participants at either end of the age spectrum to fully inform us whether an effect exists on an epidemiological scale.

***PIG-A* mutation test in human biomonitoring**

Other factors that may influence mutation levels in participants include smoking, diet and medication usage. A summary of studies assessing different exposures on erythrocyte *PIG-A* mutant levels is shown in Table I. Although a significant increase in *PIG-A* mutant cells was observed in smokers of one study (Haboubi et al., 2019), and an association between the number of *PIG-A* mutant cells and cigarette-pack-years was seen in another study (Cao et al., 2016), other research groups failed to identify an association between cigarette smoking and mutant cell levels. Whilst the genotoxic nature of cigarette smoking has been extensively studied (Alsaad et al., 2019; Mohammed, Hussen, Rashad, & Hasheesh, 2020), the lack of association with*PIG-A* mutant frequency may be due to the limited number of smokers recruited in some studies, the lack of information on cigarette-pack-years or the influence of confounding factors including gender, age, diet, inflammation and DNA repair, on mutant cell number.

The effect of diet and lifestyle has been studied in a single, large cohort with results indicating that high dietary intake of vegetables and exercising for more than one hour per week, may reduce mutant cell levels (Lawrence et al., 2020). In addition, aspirin intake may be protective against an increase in *PIG-A* mutation levels. Whilst the anti-cancer effects of aspirin have been well documented (Jiang, Swacha, Aung, & Gekara, 2023), the specific interaction of dietary intake and erythrocyte mutant levels remains unclear. Cancer treatment i.e., chemo or radiotherapy, may also change mutation levels. One study measured granulocyte *PIG-A* mutant levels in breast cancer patients undergoing radiotherapy but found no change in mutant cell number. However, the authors state that as the irradiation administered concerned only a part of the sternum and ribs, the probability of a granulocyte precursor gaining a mutation could have been too low to significantly impact blood cell *PIG-A* mutation levels (Bonetto et al., 2021). Another study measuring erythrocyte mutant cell levels in a cohort of patients undergoing chemotherapy +/- radiotherapy could not evaluate the effect of treatment as no pre-treatment blood samples were obtained. Furthermore, these patients had various types of malignancy and were undergoing different treatment regimens (Horibata, Ukai, Ishikawa, Sugano, & Honma, 2016). Another study identified only 1/10 patients undergoing chemotherapy had a notable (3-fold) increase in erythrocyte *PIG-A* mutant cell levels (Dobrovolsky et al., 2011). Reticulocyte *PIG-A* mutant cell levels have been shown to increase in testicular cancer patients (n=3) after cisplatin-based treatment, but it took longer to observe *PIG-A* mutant reticulocytes compared to micronucleated reticulocytes (Torous et al., 2023). Although we would expect systemic genotoxic agents such as chemotherapy to induce mutant levels, the time between treatment and mutant manifestation is critical. One of the downfalls in the literature to date in measuring *PIG-A*mutation in patients undergoing treatment, is the lack of pre-treatment samples to allow for meaningful comparisons. In addition, lack of associations between chemotherapy and mutant cell levels may be due to the long-term nature of *PIG-A* mutant manifestation, where the genotoxic effect is not detectable within the study timeframe (Dobrovolsky et al., 2011). By maximising study durations and obtaining samples at the optimum time point (considering cell life span and/or maturation time from haematopoietic stem cell), this assay could potentially inform us as to which patients are more sensitive to treatment and may therefore respond better to therapy. A test battery would also be most beneficial in measuring chemotherapy sensitivity, as effects may be observed sooner with the micronucleus assay than the*PIG-A* test, which in turn may be more informative on the accumulation of genotoxic exposure and long-term effects (Torous et al., 2020).

Work carried out by Cao and colleagues has assessed the DNA damage potential of multiple exposures including azathioprine treatment in a cohort of inflammatory bowel disease (IBD) patients (Cao, Wang, Liu, et al., 2020) and occupational exposure to lead (Cao, Wang, Xi, et al., 2020), polycyclic aromatic hydrocarbons (PAHs) (Cao et al., 2021; Xi et al., 2023) and benzene (Cao et al., 2023). Whilst IBD patients had higher mutant cell levels than healthy controls, azathioprine treatment did not affect erythrocyte *PIG-A* mutant frequency (Cao, Wang, Liu, et al., 2020). Interestingly, *PIG-A* mutant frequency was higher in workers occupationally exposed to lead (Cao, Wang, Xi, et al., 2020), PAHs (Cao et al., 2021) and benzene (Cao et al., 2023) (compared to controls). The important work carried out in workers exposed to benzene demonstrated that even below the occupational exposure limit (OEL) of 1ppm, workers had elevated levels of erythrocyte *PIG-A*mutant cells and lymphocyte micronuclei compared to controls, with the authors proposing a new OEL of 0.07ppm (Cao et al., 2023). This study highlights the limitations of current DNA

damage tests particularly in the occupational exposure setting. In addition, measuring urinary or airborne levels of genotoxins in this manner should be widely implemented in studies where the exposure is known, with these biomarkers of exposure complementing the *PIG-A* biomarker of effect. In order to avoid exposing workers to such health hazards, the*PIG-A* test should be included in a battery of genotoxicity assays as well as quantifying genotoxin levels to re-define OELs and ensure safe work environments. Whilst these tests will be useful as biomarkers of exposure, the potential long-term carcinogenic effects can be elucidated through longitudinal epidemiological studies.

The importance of studying large cohorts is paramount when developing a biomonitoring tool. Disparate or non-reproducible results in some publications could be due to the small number of individuals studied. Whereas studies examining *PIG-A* MF in hundreds of patients may be more informative, population scale studies including multi-ethnicities, wide age ranges and different exposures (diet, cigarette smoking, medication and occupational genotoxins) are required. These studies will require an international, collaborative effort, but are crucial to the understanding of what drives mutant cell levels, and a necessity for biomarker implementation.

Table I: The effect of different exposures on phosphatidylinositol glycan class A (*PIG-A*) mutant frequency.

Exposure	**Cell type**	**Test groups**	**Effect of exposure**	**Reference**
Cigarette smoking	Erythrocytes	Non-cancer control cohort non-smokers (n=274) and smokers (n=26)	No difference in *PIG-A* mutant cell levels (p=0.186)	(Lawrence et al., 2020)
	Erythrocytes	Male healthy volunteers (n=129)	No difference between smoker and non-smoker (p=0.8594). No association between *PIG-A* and smoking duration (p=0.0541). Association between PIG-A mutant frequency and cigarette pack/years (p<0.0001).	(Cao et al., 2016)

Exposure	**Cell type**	**Test groups**	**Effect of exposure**	**Reference**
	Erythrocytes	Non-cancer control cohort non-smokers (n=247) and smokers (n=29)	Smokers had *PIG-A* mutant frequencies of over double that of non-smokers (p=0.011) with mutant frequencies of 5.82 (95% CI 2.79–9.52) and 2.8 (95% CI 2.49–3.57) respectively.	(Haboubi et al., 2019)
Depleted Uranium	T-Lymphocytes	Gulf war I veterans (n=35) Low-uU (n=22) Vs High-uU (n=13)	No significant difference Low-uU mean mutant frequency =18.13±4.85 High-uU mean mutant frequency = 9.45 ± 0.81 P = 0.08	(McDiarmid et al., 2011)
Chemotherapy	Erythrocytes	Patients with different cancer types (n=10)	Pre-treatment *PIG-A* mutant frequency was the same as healthy controls. Minimal changes in mutant frequency during and post therapy except for one patient undergoing Cisplatin and Etoposide therapy who had 3x increase in *PIG-A* mutant levels.	(Dobrovolsky et al., 2011)

Exposure	**Cell type**	**Test groups**	**Effect of exposure**	**Reference**
Chemotherapy +/- Radiotherapy	Erythrocytes	Healthy volunteers (n=10) and cancer patients undergoing chemotherapy +/- radiotherapy (n=27)	Healthy volunteer *PIG-A* mutant frequency range = 0.00–5.00 $\times$ 10^{-6} and cancer patients = 0.00–49.67 x 10^{-6}. No pre-treatment blood samples taken so difficult to determine effect of therapy on mutant levels.	(Horibata et al., 2016)
Radiotherapy	Granulocyte	Patients undergoing therapy for breast cancer (n=30). Five patients had previously received chemotherapy.	Lower mutant frequency during (p=0.0035) and after radiotherapy treatment (p=0.006) compared to pre-treatment.	(Bonetto et al., 2021)
Azathioprine (AZA)	Erythrocyte	AZA treated inflammatory bowel disease (IBD) patients (n=36) and healthy controls (n=36).	IBD patients exhibited a higher MF (6.10 $\pm$ 4.44 $\times$ 10^{-6}) than healthy volunteers (4.97 $\pm$ 2.74 $\times$ 10^{-6}) (P = 0.0489). No association between AZA treatment and MF.	(Cao, Wang, Liu, et al., 2020)
Lead	Erythrocyte	Workers occupationally exposed to lead (n=267) and healthy volunteers from previous study (n=217).	*PIG-A* MFs were significantly higher in lead-exposed workers (10.90 $\pm$ 10.7 $\times$ 10-6) than in a general population studied previously (5.25 +- 3.6 x 10-6) (p <0.0001).	(Cao, Wang, Xi, et al., 2020)

Exposure	**Cell type**	**Test groups**	**Effect of exposure**	**Reference**
Polycyclic Aromatic Hydrocarbons (PAH)	Erythrocyte	PAH exposed BBQ restaurant workers (n=70) and healthy controls (n=56). Urinary PAH metabolites measured to determine individual exposure.	PAH exposed group had higher *PIG-A* MFs than healthy controls ($p<0.001$). A higher *PIG-A* MF was associated with higher PAH exposure determined by urinary metabolites ($p=0.006$).	(Cao et al., 2021)
	Erythrocyte	PAH exposed coke oven workers (n=364), newly employed non-exposed controls (n=34) and control group from non-industrially polluted area (n=273).	Coke oven workers had higher *PIG-A* MF's of 21.01 ± 25.1 x 10^{-6} compared to 4.3 ± 3.02 x 10^{-6} for newly employed non-exposed group and 5.45 ± 4.56 x 10^{-6} for the larger control group from non-industrial city.	(Xi et al., 2023)
Benzene	Erythrocyte	Benzene exposed chemical plant workers (n=104) and controls (n=273) from previous publication (Cao et al., 2016 and Cao et al., 2021).	Benzene exposed workers had higher *PIG-A* MF's of $15.96 \pm 14.41 \times 10^{-6}$ compared to controls who had an average MF of 5.46 +- 4.56 x 10^{-6} ($p<0.001$). Observed a significant association between *PIG-A* MF and airborne benzene exposure levels ($r=0.501$, $p<0.001$) measured by gas chromatography.	(Cao et al., 2023)

The *PIG-A* mutation test in disease

Whilst the applicability of this mutation assay has mostly been investigated in humans exposed to defined mutagenic agents, the application of the *PIG-A* test in disease detection has also been explored. Reports by Nichols et al., showed that patients with pancreatic cancer had elevated levels of *PIG-A* mutant erythrocytes (5.775×10^{-6} (95% CI 4.777-10) compared to non-cancer controls (4.211×10^{-6} (95% CI 1.39-5.16)) (Nichols et al., 2023). Although this difference was subtle, it may indicate an underlying individual susceptibility to mutation and hence risk of malignancy. In a larger cohort studied by Haboubi et al, oesophageal adenocarcinoma patients had elevated levels of mutant erythrocytes (9.75 x 10^{-6} (95% CI 4.36-17.52)) compared to healthy volunteers (2.8 x 10^{-6} (95% CI 2.21-3.57)), patients with gastro-oesophageal reflux disease (3.44 x 10^{-6} (95% CI 1.56-5.43)) and patients with the pre-malignant condition Barrett's oesophagus (4.35 x 10^{-6} (95% CI 2.49-6.09)). Moreover, the number of*PIG-A* mutant cells was associated with tumour staging and metastatic disease. Elevated *PIG-A* mutant frequency was identified as risk factor for oesophageal cancer independent of age and gender using general linear model analyses (Haboubi et al., 2019). Two publications have assessed *PIG-A* mutant cells in IBD patients. Firstly, Baig and colleagues found no significant increase in mutant reticulocytes in paediatric IBD patients although 10 patients with higher micronucleus frequencies all had established disease (Baig et al., 2020). Secondly, Cao et al., found significantly elevated erythrocyte mutant cell number in adult Chinese IBD patients, although these were all undergoing azathioprine treatment (Cao, Wang, Liu, et al., 2020). Whilst the mechanistic link between solid tumour development and induction of circulating *PIG-A* mutation levels remain unclear, with inflammatory driven malignancies such as oesophageal cancer and IBD, circulating blood cells (RETs or immature red blood cells) may be exposed to inflammatory mediators and subsequently accumulate DNA damage which results in a *PIG-A* mutant phenotype in erythrocytes.

Technical differences

When measuring such scarce cells as *PIG-A* mutant erythrocytes, it is important to note that slight methodological variations can significantly alter results. For example, using antibodies that target the GPI-AP's CD55 and CD59 in combination may reduce the number of false positive mutant cells compared to targeting only one GPI-AP (see Figure 2). Alternatively, GPI anchor status can be measured using a fluorophore conjugated variant of proaerolysin. Proaerolysin is an inactive form of the bacterial toxin aerolysin which binds to GPI anchors, permitting direct assessment of *PIG-A* status. Whilst a FITC-conjugated form of proaerolysin is commercially available (Cedarlane, Canada), most research groups have chosen to use antibodies that target GPI-AP's.

Furthermore, the type and/or performance of the flow cytometer used may impact results. Previous work carried out in our lab revealed that variations between cytometers exists. For example, the Facs Aria I (BD Biosciences) on average scored a higher number of mutant cells than the Navios flow cytometer (Beckman Coulter) although there was a strong correlation between the two machines (Haboubi et al., 2019). Flow cytometer performance characteristics that may impact mutant cell scoring include laser strength and alignment, flow speed, stream characteristics and cleanliness of inner tube networks. These factors can be mitigated with routine maintenance and quality control. Whilst it is difficult to account for slight variations that could significantly influence classification of *PIG-A* phenotype and hence falsely classify a mutant cell, *in vitro* cell lines with known*PIG-A* status can be used as a quality control. For example, we have generated a *PIG-A* mutant L5178Y mouse lymphoma cell line by treating wild type cells with the methyl methanesulfonate and using magnetic bead enrichment followed by fluorescence activated cell sorting (FACS) to create a cell line consisting of ˜96%*PIG-A* mutant cells which is routinely used to check for flow cytometer variations (Haboubi et al., 2019).

The gating strategy applied to categorise mutant cells is also very important to consider. Inflating the lower fluorescence limit for mutant classification could falsely classify erythrocytes with lower (but not absent) GPI-AP staining as *PIG-A* mutant. These intermediate cells may have less GPI anchors or GPI-AP's due to incomplete staining, shearing off of GPI-AP's during sample processing or may pass through the laser stream at an angle where fewer GPI-APs are exposed and so less fluorophores are excited by the corresponding laser.

This is particularly important when measuring *PIG-A* status of biconcave erythrocytes. The gating strategy usually involves the gating of singlet erythrocytes using anti-CD235a (erythrocyte specific marker) followed by the gating of *PIG-A* mutant erythrocytes using an unstained or mutant mimic control (also known as an instrument calibration standard) with this methodology being adopted by most research groups (Cao et al., 2016; Dertinger et al., 2015; Dobrovolsky et al., 2011; Haboubi et al., 2019; Lawrence et al., 2020). As this *PIG-A* erythrocyte test only requires a finger prick volume of whole blood, re-running samples from individuals with known mutant frequency can also act as an additional quality control.

Although this test is quick to perform, batching samples would be most beneficial. Not only would this save time and money, but it would also allow the transport of samples from regional/remote locations to a central processing laboratory. However, the stability of *PIG-A*status post blood-draw has not been measured over a prolonged period. Within our research group we have demonstrated that erythrocyte*PIG-A* mutant frequency is stable up to 72 hours post venepuncture when collected in K_2 EDTA coated vacutainers and stored at room temperature (unpublished, data not shown). Dertinger and colleagues have demonstrated *PIG-A* mutant stability of over a week when the sample was kept at 4°C (Dertinger, 2015 #21). After this time, the mutant frequency increases, potentially due to loss of membrane integrity, GPI anchors and/or GPI-APs as cells start to die. Cryopreservation medium for human samples similar to that supplied by Litron Laboratories (New York, USA) for mouse and rat blood could help to make sample batching of human blood a reality.

Cell types investigated for *PIG-A* status

There are multiple different reports evaluating which human cell type is most suitable for the *PIG-A* assay, including the use of erythrocytes, reticulocytes (RETs), granulocytes, and peripheral blood mononuclear cells (PBMCs) (Table II). The most frequently used cell type for measuring *PIG-A* mutations is erythrocytes as only a finger prick volume of whole blood (3-10μl) is required to measure the*PIG-A* status of over a million cells. This is crucial to identify such rare events as *PIG-A* mutant cells, where millions of cells need to be interrogated. Furthermore, with the addition of the anti-CD235a antibody, blood pre-processing is minimised, and the high throughput technology of flow cytometry is exploited. Alongside RBC*PIG-A* analysis, some groups have also measured the percentage of reticulocytes (%RET) to account for abnormalities in haematopoietic function (Cao et al., 2016; Dertinger et al., 2015). However, no compromise in %RET has been observed to date (Dertinger et al., 2015; Xi et al., 2023).

Some groups favour the use of alternative blood cells to measure*PIG-A* mutation as mature erythrocytes that lack the complement inhibitors CD55 and CD59 may undergo complement mediated lysis (Ruiz-Arguelles & Llorente, 2007). Although we believe this effect to be minimal, evidence suggests mutant erythrocytes may be subject to modest selective pressure (Dertinger et al., 2015). Despite this disadvantage to using erythrocytes over RETs, the population of reticulocytes found in human peripheral blood is low in comparison, and therefore the use of erythrocytes is much more rapid, and simple to carry out. Furthermore, Dertinger and colleagues have noted that inter-individual variation of RET *PIG-A* mutant frequency may be as high as 30-fold (Dertinger et al., 2015). However, RETs may be the most desirable cell type for analysis when measuring mutant induction post exposure as such an effect may be observed sooner than with RBCs which need to enucleate and mature to yield a mutant phenotype post exposure. Importantly, *in vitro Pig-A* analysis in the L5178Y cell line has shown a phenotypic expression period of 8 days post treatment (David et al., 2018).

The utility of granulocytes in measuring *PIG-A* mutation status has also been explored. As with RETs, mutation induction post exposure may be observed sooner than with RBCs. However, the short life span of granulocytes (1-2 days) provides only a short window in which mutant cell levels can be accurately measured following a mutagenic event. Although granulocyte mutant cell levels are present at approximately the same frequency as erythrocyte mutant cells in healthy volunteers (Rondelli et al., 2013), using granulocytes to measure *PIG-A*mutation requires blood pre-processing and a more complex flow cytometry gating strategy (Bonetto et al., 2021; Peruzzi et al., 2010; Rondelli et al., 2013). Peripheral blood mononuclear cells (PBMCs) have also been interrogated for *PIG-A* mutation status. Like granulocytes, this process requires cell isolation from whole blood prior to flow cytometry. Two studies carried out by McDiarmid et al., and Ware et

al., detected higher background mutant frequencies in PBMCs (compared to healthy volunteer erythrocytes published elsewhere) of 18.13 x 10^{-6} (McDiarmid et al., 2011) and 18 x 10^{-6} mutants (Ware, Pickens, DeCastro, & Howard, 2001) respectively. This technique also required the subculture of PBMCs under Aerolysin selection. Although there are advantages and disadvantages to using each cell type (Table II), the decision of which type to use should be based on the type and requirements of the study.

Table II: Advantages and disadvantages of exploiting different blood cell types for the phosphatidylinositol glycan class A (*PIG-A*) mutation assay.

Cell type	**Advantages**	**Disadvantages**
Erythrocytes	Small volume of blood (3-10μl) required so suitable for finger prick test and multiple sampling. Doesn't require any blood pre-processing so less hands on time. Reticulocyte percentage (%RET) in whole blood can be analysed simultaneously as a measure of haematopoietic function. Can detect an accumulation of DNA damage from repeat dosing over a prolonged period; long term effect.	Cannot confirm mutant phenotype by DNA sequencing. Unsuitable for cryopreservation and batching samples from remote locations. *PIG-A* mutant erythrocytes may be subject to complement mediated lysis.
Reticulocytes	May be able to observe mutant induction sooner post exposure than erythrocytes. Reticulocyte percentage (%RET) in whole blood can be analysed simultaneously as a measure of haematopoietic function. *PIG-A* mutant reticulocytes would not be sensitive to complement mediated lysis.	Proportion of reticulocytes is low in whole blood compared to erythrocytes so would require larger blood volume.
Granulocytes	May be able to observe mutant induction sooner post exposure than erythrocytes. Allows for mutation confirmation using DNA sequencing.	Requires blood pre-processing and a more complex flow cytometry gating strategy. Short life span of 1-2 days provides a small window of opportunity to capture mutant cells.
Peripheral blood mononuclear cells	Allows for mutation confirmation using DNA sequencing.	Published methodology includes subculture of PBMCs under Aerolysin selection. Requires larger volume of blood. Current studies show higher background level of mutant cells compared to erythrocytes.

Future directions

More research is required to assess the effectiveness of detecting genotoxic exposures in humans using the *PIG-A* assay, especially in different blood cell types (Bonetto et al., 2021). International collaborative

projects are required and are anticipated to provide further evidence of the application of the human *PIG-A* assay. Studies are necessary to understand the role of this reporter gene in detecting genotoxin exposure in order to interpret future research findings. We also need to assess the long term follow up of patients following baseline *PIG-A* measurement to link the level of mutant frequency to development of later cancers and other chronic disease. This has proved to be fascinating in the lymphocyte MN field (Bonassi et al., 2007) and can be carried out using long-term epidemiological studies.

Moreover, it is not only crucial to determine the individual consequences of high levels of *PIG-A* mutant cells but also to confirm the degree to which GPI anchor deficiency is comparable to*PIG-A* mutation. Confirmation of *PIG-A* mutation has been carried out in GPI-deficient human T-lymphocytes (Ware et al., 2001) and granulocytes (Araten et al., 1999). Base-pair substitutions, small frameshift insertions and large deletions were identified in GPI-deficient T-lymphocytes isolated using aerolysin selection (Ware et al., 2001). *PIG-A* mutations including base-pair changes leading to single amino acid replacements were identified in granulocytes isolated by flow sorting from healthy volunteers. All mutations were found to either interfere with protein function or lead to protein truncation (Araten et al., 1999). Although confirmatory mutant sequencing is impossible in anucleate erythrocytes, periodic assessment in alternative blood cell types may provide information regarding the nature of mutation and also the type of genotoxic exposure.

Although there is still some uncertainty about whether erythrocytes are a suitable cell population for a human *PIG-A* assay, the proof-of-concept studies in the current literature indicates that the assay could be helpful for monitoring populations exposed to potential genotoxins. Possible clinical applications of the assay may include the diagnosis of DNA repair-deficient cancer-prone 'mutator' phenotypes or monitoring chemotherapy patients for drug-induced mutation as a predictor of susceptibility to the formation of secondary tumours.

Another area for refinement for this test is the need for standard protocols for not only sample preparation but also data analysis. When measuring such rare cells, any subtle changes in the staining protocol or data acquisition, for example defining mutant gates on the flow cytometer can have an impact on results. Whilst the published*PIG-A* mutant data in healthy controls is remarkably consistent, a standard protocol would potentially minimise inter-laboratory variation and allow for comparison of results between research groups. Furthermore, the true potential of the *PIG-A* assay will be observed when we can overcome the issue of sample batching and are able to provide this test in low-income countries where mutagenic hazards and exposure-related diseases are often more common.

Combining this assay with others focussed on different mutational endpoints, such as the MN test or Comet assay, can enhance its effectiveness (Cao et al., 2023). Mutagenic exposures that act through different mechanisms and may give contrasting results in tests that measure a particular endpoint. A combination of tests including CBMN (Fenech et al., 2011), Comet (Milic et al., 2021) hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Townsend et al., 2018) andγH2AX assay (Kopp, Khoury, & Audebert, 2019) may improve test sensitivity and inform us about the nature of exposures. Research in our group indicates that the correlation between individual *PIG-A*mutant frequency and lymphocyte MN levels is not good with individuals often having high levels of *PIG-A* mutation or MN, but not vice versa (Figure 5). This suggests that using only one test is not sufficient to detect elevated levels of DNA damage in all individuals. Furthermore, when more than one DNA damage endpoint (*PIG-A* , micronuclei or COMET) has been measured in humans in response to a genotoxic exposure, positive associations have not been found with all endpoints. This could be due to the test used; for example, the RBC*PIG-A* assay may represent an accumulation of exposure over a prolonged period given the time taken for immature RBCs or haematopoietic stem cells to mature into *PIG-A* mutant RBCs. Given that the DNA damage evaluated in the COMET assay can be repaired, it is perhaps not surprising that not all genotoxins (i.e., lead) produce a positive response (Cao, Wang, Xi, et al., 2020). Aneugenic or clastogenic type DNA damage that can be observed in the lymphocyte micronucleus assay is essentially a snapshot of damage detected at the time of blood sampling which may otherwise be repaired *in vivo* so represents a shorter time window between exposure and measurable blood cell effects. This short time frame of opportunity may also be the case for the COMET assay. Together with the mechanistic differences of these endpoints and the differences in fate of the mutated cells, proves the need

for the combination of complementary assays to comprehensively recognise genotoxic exposures (Torous et al., 2023).

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Figure 5: Erythrocyte phosphatidylinositol glycan class A (*PIG-A*) mutant frequency and lymphocyte micronucleus levels for 141 individuals (R=0.2124, p=0.0114).

Conclusion

As evidenced by the current literature, the *PIG-A* mutation test is a promising tool for human biomonitoring and disease. With further technological development, test battery development and international collaboration, we expect this simple, high-throughput assay to advise us on both harmful and beneficial exposures to genotoxins and chemo-preventative agents. This will ultimately lead to improved lifestyle choices, reduced occupational hazards and even earlier diagnosis of high-risk cancer patients and other cancer associated inflammatory diseases such as IBD.

Conflicts of interest

The authors declare no conflicts of interest

Author contributions

RL, KM and HN carried out and wrote the manuscript under supervision by GJ. HH, LT, SD and LW contributed ideas and edited the manuscript.

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