

A study on micronucleus frequency in lymphocytes by flow cytometry to assess genetic damage in Polycystic ovarian syndrome (PCOS)

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Abstract: *Background:* Micronucleus is a small additional nucleus formed due to chromosomal loss or fragmentation and frequently used as a biomarker of genomic damage. Genetic damage and cancer susceptibility are notable concerns in PCOS. The aim of this study was to estimate the frequency of micronuclei in lymphocytes and to determine if it can be used as a biomarker of genomic instability in PCOS through flow cytometric analysis. *Methods:* An observational case control study was conducted among 38 subjects diagnosed with PCOS by Rotterdam's criteria and 38 controls from September 2018 to March 2020 in VIMS & RC. Peripheral venous blood samples were collected from all the subjects for flow cytometric assessment of micronuclei frequency in lymphocytes. Differences between the PCOS group and the control group were examined for statistical significance using two-sample independent t-test. The frequency of micronuclei in lymphocytes was expressed as mean \pm SD. A *P* value of ≤ 0.05 denoted statistically significant difference. *Results:* The mean \pm SD of micronuclei frequencies in lymphocytes was observed to be 1.71 ± 0.88 and 0.43 ± 0.24 (*p*-value < 0.0001) in PCOS and control group, respectively. *Conclusion:* This study is the first to use flow cytometric analysis in subjects with PCOS to detect micronuclei. The findings indicate that women with PCOS have an increased genomic instability as assessed by micronuclei frequency and thus it can be used a biomarker for genetic damage.

Keywords: Micronucleus, Polycystic Ovarian Syndrome, Flow Cytometry, Biomarker, Genetic Damage

Introduction

Polycystic Ovarian Syndrome (PCOS) is a complex and multifactorial endocrine disorder which has a malicious influence on women of reproductive age group [1-2]. It globally affects about 4% to 20% of women [3]. Chronic anovulation, excessive androgen production, and the development of polycystic ovaries are the key features of this disorder. PCOS is known to coexist with endometriosis and these women have an increased probability of developing endometrial cancer due to absence of ovulation and long-term exposure to oestrogen [1, 4]. There is a threefold increase of endometrial cancer and two-fold increase of ovarian cancer in PCOS women [5]. PCOS is known to have a strong familial aggregation and multiple candidate genes has been investigated for the same. However, its heritability is still unclear and said to arise from complex genetic and environmental factors [6-7].

Early genetic alterations are assessed by utilizing a biomarker termed as the micronucleus. Micronuclei are minuscule structures that arise from a fragment of acentric chromosome or from whole chromosomes that fall behind during anaphase and fail to merge with the chief nucleus during telophase [8-9]. A nuclear covering appears around it and resembles the main nucleus except that it is smaller in size [10]. Presence of micronuclei is a biological indicator of genomic toxicity and occurrence of unstable chromosomes [11].

Their appearance is an account of rise in carcinogen manifested tissues, much prior than the existence of the actual symptom in the body [12]. Scoring of micronuclei in lymphocytes is commonly used to show genetic damage in various disorders and used

as a predictive biomarker of cancer risk [13-14]. Cytokinesis-block micronucleus (CBMN) assay is immensely used to assess genetic damage in various cells, especially in lymphocytes [11]. Even though this assay is reliable, it is a strenuous and time-consuming method where 1000 – 2000 cells should be scored manually and automated slide-scoring may give false positive scores [15]. As a result, there have been some attempts to use flow cytometry to automate micronuclei scoring in human cells. Flow cytometric analysis is a high throughput procedure that allows rapid acquisition of micronuclei frequency in a short time span [16]. The current study is the first to use flow cytometric analysis to detect micronuclei in PCOS.

DNA damage has been linked to an increased incidence of PCOS, particularly in women who have obesity and metabolic syndrome [17-18]. Since unstable genes and impaired DNA may lead to gynaecological cancers in women with PCOS [17], presence of micronuclei can be useful in identifying women with PCOS who are at risk of genetic damage and cancer susceptibility. Hence, this study aimed to assess possible genetic damage in women with PCOS by flow cytometric analysis of micronuclei.

Material and Methods

Study population: A total of 76 women attending the Obstetrics and Gynaecology outpatient department (OPD) in Vydehi Institute of Medical Sciences & Research Centre (VIMS & RC), Bangalore, India between September 2018 to March 2020 were included in the study. 38 women diagnosed with PCOS based on the Rotterdam's 2003 criteria [19] and 38 age matched controls with regular menstrual cycles and normal ovaries were recruited. Women clinically diagnosed with congenital adrenal hyperplasia, Cushing's syndrome, androgen secreting tumours, functional hypothalamic amenorrhoea and Smokers were excluded from the study. This observational case control study included women aged between 18 to 45 years. The study was carried out after the ethical clearance (VIEC/2018/APP/048) was obtained from the Institutional Ethics Committee. Written informed consent was obtained from all the women included in the study. This study adheres to the Declaration of Helsinki.

Sample size calculation: For the present study, sample size was calculated by R software using historical data of micronuclei [20] with mean (SD) of 0.74 (0.34) and 1.19 (0.57) in PCOS group and control group, respectively. Considering a significance level of 0.05 and 95% power, and using two-sided two-sample t test, the current study required to enrol approximately 29 subjects in each group (Total of 58). Considering a drop-out rate of 20%, Considering a drop-out rate of 20%, a total of 76 subjects (38 in each group) were recruited for the present study.

Sample collection and processing:

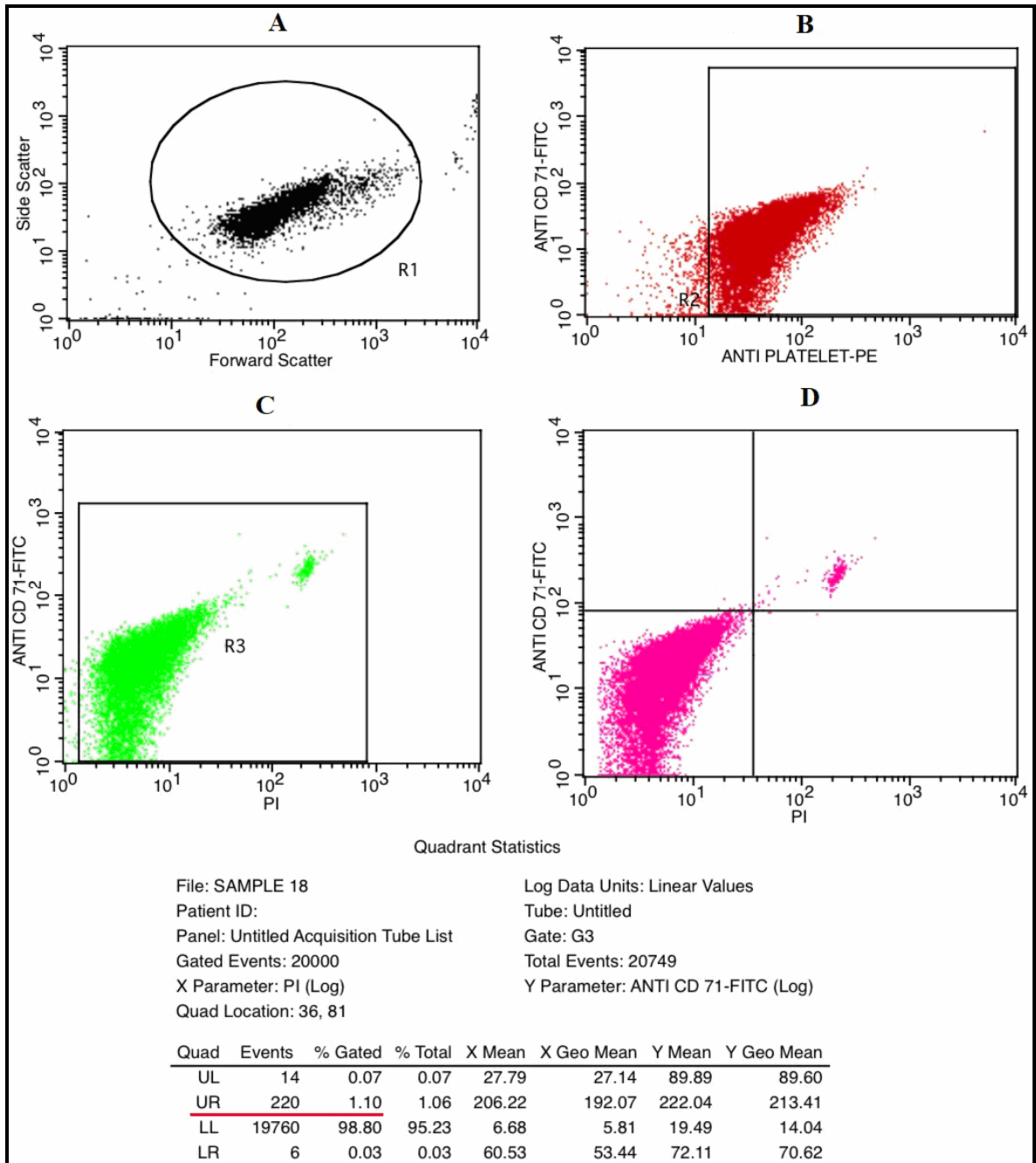
- 5 ml peripheral venous blood was collected from all the subjects for flow cytometric assessment of lymphocytes.
- The collected blood samples were kept at room temperature for 30 minutes in gyratory shaker for proper mixing of blood cells
- The samples were fixed in ultracold methanol (-80°C) in 5 ml storage vials. For the fixation, 50ul of blood was added to a polypropylene centrifuge tube filled with 2 ml ultracold methanol, mixed and stored at -80°C until analysis. Fixation was done for overnight at -80°C in deep freezer.
- After overnight fixation, each tube in turn was tapped sharply and centrifuged at 1800 rpm for 5 minutes at room temperature.
- The blood cells were washed with 2ml of ice-cold bicarbonate buffer (0.9% NaCl, 5.3 ml NaHCO₃ [pH 7.5]) and centrifuged again to remove excess of methanol.
- The cells were further incubated in bicarbonate buffer containing RNase A and anti-CD71-FITC antibody (CD 71 is the main antibody that will bind to the lymphocytes and FITC is fluorescence iso-thio cyanide for staining the lymphocytes green) at 4°C for 45 min in dark.
- The cells were again washed with 1ml of bicarbonate buffer and stained with CD-61 PE antibody (CD 61 is an antibody that binds to platelets and other monocytes and PE is phycoerythrin for staining them red) and resuspended in 1.25ug/ml of

Propidium iodide dye (stains the DNA) prepared in bicarbonate buffer for 20mins.

Sample analysis: The samples were analysed by fluorescence automatic cell sorter (FACS Calibur) by using FL-1 (for CD-71 FITC antibody), FL-2 (CD-61 PE) and FL-3(For PI)

channels. 20,000 cells per sample were selected to count the frequency of micronuclei in blood cells and was analysed by BD Cell Quest Pro ver.6.0 software. The gating strategy for gating micronuclei was performed as shown in Figure 1.

Fig-1: Flow Cytometric Assessment of Micronuclei. A: Forward and side scatter histogram where subcellular-sized particles were excluded and R1 represents particles gated/included. B: R2 gate includes cells that are stained by CD 71 FITC (lymphocytes) and CD 61 PE (platelets). C: R3 gate represents excluded cells that express platelet-specific antigen, CD61 and includes only lymphocytes. D: Representative box plot of lymphocyte population: upper right quadrant – micronucleated lymphocytes.



Statistical analysis: The data was analysed using R software and expressed as mean ± SD. Differences between the test group and the control group were examined for statistical significance using two-sample independent t-test. A P value of ≤0.05 denoted the presence of a statistically significant difference.

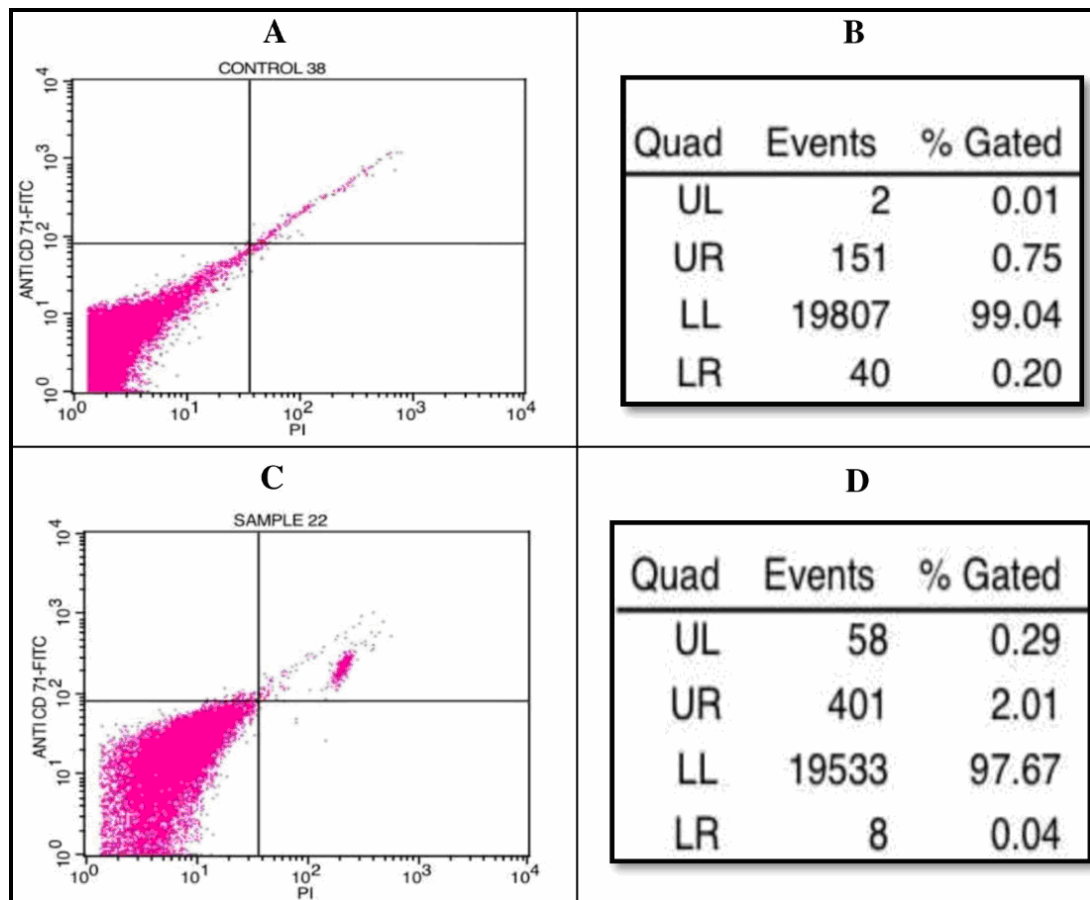
Results

Based on two-sample independent t-test, micronuclei frequency in lymphocytes was found to differ significantly in subjects with PCOS and the control group as shown in Table 1. An example of representative box plot of lymphocyte population where frequency of micronucleated lymphocytes can be noted in upper right quadrant is depicted in Figure 2.

Table-1: Descriptive statistics of micronuclei in test and control group

Groups	Age (Mean ± SD)	Mean micronuclei frequency (Mean ± SD)	p-value
Test group (N = 38)	28.24 ± 4.33 years	1.71 ± 0.88	<0.0001
Control group (N = 38)	26.53 ± 4.40 years	0.43 ± 0.24	

Fig-2: Representation of box plot of lymphocyte population obtained from flow cytometric analysis of the samples. A – box plot of a control sample, B – upper right (UR) quadrant represents the frequency of MN in control sample, C – box plot of subject with PCOS sample, D - UR quadrant represents the frequency of MN in the PCOS subject sample. It can be noted that the frequency of MN in PCOS sample is elevated when compared to the control sample.



In subjects with PCOS, the mean micronuclei frequency of lymphocytes was compared in those below the age of 25 years and the ones above the age of 25 as represented in table 2. The mean

±SD was found to be 1.44 ± 1.07 in PCOS subjects aged ≤ 25 and 1.80 ± 0.80 in those whose age was > 25.

Category	Variables	Number (%)	Mean micronuclei frequency in cervical smear
Age	≤ 25	10 (26.3)	1.44 ± 1.07
	> 25	28 (73.6)	1.80 ± 0.80

Discussion

PCOS manifests in the form of reproductive concerns due to menstruation disruption, hyperandrogenism, as well as the metabolic repercussions like insulin resistance, increased risk of obesity, cardiovascular disorders and Type 2 Diabetes mellitus [1]. Both genetics and environmental factors play a crucial role in this heterogenous condition. The reproductive and metabolic characteristics that characterize PCOS as a condition may be due to underlying genetic variability [21]. Multiple candidate genes have been investigated including those involved in insulin signalling, susceptibility to type 2 diabetes mellitus and obesity, steroid hormone synthesis and action, carbohydrate metabolism, gonadotrophin regulation and action [7].

Studies have shown that the number of micronuclei increases steadily with age [22-23]. Similarly, in the present study, the mean micronuclei in lymphocytes were found to be increased in women with PCOS who 25 years were above i.e., 1.80 ± 0.80 . The mean micronuclei frequency in women aged below 25 years was 1.44 ± 1.07 . Increase of MN with age is due to a combination of factors which can be acquired mutations in genes involved in DNA repair and chromosome segregation or numerical and structural aberrations in chromosomes caused by a wide range of unhealthy lifestyle factors [22].

The international collaborative project on micronucleus frequency in human populations (HUMN) was established to gather information on micronucleus frequencies in various cell types in human populations in the year 1999 [24] and after several years and with over a database of over 7000 patients, the CBMN technique and scoring criteria has now been standardized, with the effects of age, gender, and smoking status. It was also concluded that a higher MN frequency in lymphocytes predicts DNA damage and cancer risk in human populations. An extension of this

project, known as the HUMN_{XL} (micronucleus assay for exfoliated cells) was established in 2011 [25] and in the year 2019, this project further associated micronuclei frequencies with several cancers, infertility, spontaneous abortions, obesity and diabetes mellitus [26].

The formation of micronuclei in lymphocytes hence is extensively used in as a biomarker of chromosomal damage and cancer risk [11, 13]. In cell cultures, flow cytometry is used to detect micronuclei frequency generated by ionising radiation and chemicals [27-28]. In this method, a fluorescent dye is utilized to selectively identify the cell population of interest. Fluorescently tagged cells can also be sorted into different tubes, depending on their size and fluorescence signal by a process called fluorescence-activated cell sorting (FACS) [29]. Even though earlier approaches had difficulty distinguishing micronuclei from cellular debris in cell populations, later developments addressed these issues [30].

Flow cytometry provides the benefit of assessing a high number of cells and detecting micronuclei rapidly [15]. Studies have shown elevated levels of micronuclei in lymphocytes in women with PCOS [17, 20, 31]. In the present study, a significant increase in mean micronuclei frequencies as assessed by flow cytometry was found to be elevated in lymphocytes of test group (1.71 ± 0.88) when compared to controls (0.43 ± 0.24). A similar study which included 36 women with PCOS and 29 women in control group, the mean MN frequency was found to be 4.1 ± 1.0 and 2.1 ± 0.6 in the test and control group, respectively [20, 32].

This increased micronucleus frequency in PCOS might be attributed to entire chromosomal loss since X chromosome accounts for more than half of all spontaneous micronuclei in women. Reduced developmental competence of oocytes in

PCOS may be caused by abnormalities in meiosis and genes involved in chromosome alignment and spindle dynamics which also indicates formation of micronuclei [33]. PCOS has a genetic aetiology that is uncertain. Although the mode of inheritance has not been thoroughly established, a family history of PCOS is extremely prevalent among PCOS patients, implying a major genetic component [6]. Therefore, analysis of frequencies of micronuclei by flow cytometry in lymphocytes can be used as a biomarker of genetic damage in PCOS.

Conclusion

The present study shows that DNA damage is increased in women with PCOS as assessed by presence of micronuclei using flow cytometry.

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While other methods of detection of micronuclei rely on labour-intensive and microscopy-based techniques, flow cytometric method of micronucleus scoring is rapid and high number of cells can be counted automatically. The present study showed elevated micronuclei in PCOS indicating probable DNA damage in these women.

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Conflicts of interest: There are no conflicts of interest.

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