Journal of Cytology and Genetics 2023 VOL. **24** (NS): 87−99

RESEARCH ARTICLE

ASSESSING THE EXPRESSION OF PEPTIDYL ARGININE DEIMINASE TYPE 4 AS A NEUTROPHIL EXTRACELLULAR TRAP MARKER IN SEPSIS AND SEPTIC SHOCK PATIENTS

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(Received 7 October 2023, revised accepted 24 October 2023)

SUMMARY Sepsis is a life-threatening condition characterized by a systemic inflammatory response to infection. Neutrophil extracellular traps (NETs) are an innate immune response that can play a role in the host's defense against infection and the sepsis pathophysiology. Peptidyl arginine deiminase type 4 (PADI4) is a key enzyme involved in NET formation. Our study assessed the concentration of NET released by targeting PADI4 in 90 participants consisting of 30 sepsis patients, 30 septic shock patients and 30 healthy controls by Real-time quantitative polymerase chain reaction (RT-qPCR). We have found that PADI4 levels were significantly elevated in patients with sepsis and septic shock compared to healthy controls. PADI4 levels were also higher in patients with septic shock than in patients with sepsis. Non-survivors had significantly higher PADI4 levels than survivors, especially in septic shock patients. Receiver operating characteristic (ROC) curves showed that PADI4 has the potential to be a valuable diagnostic and prognostic marker for sepsis. Additionally, Pearson's correlation analysis revealed a positive association between PADI4 levels and various clinical parameters. Overall, the results of this study suggest that targeting NETs has the potential to improve clinical outcomes, prognosis and treatment for sepsis.

Keywords: Sepsis, septic shock, neutrophil extracellular traps, peptidyl arginine deiminase type 4.

INTRODUCTION

Sepsis is a critical medical condition characterized by an uncontrolled inflammatory response triggered by infection (Singer et al. 2016). It represents a significant global health concern, contributing substantially to both illness and death worldwide, with an estimated 48.9 million cases and 11 million fatalities recorded in 2017. Sepsis exhibits a pronounced prevalence in low- and middle-income countries, constituting 85% of all reported cases (Rudd et al. 2020). Despite extensive research efforts, the precise pathophysiological mechanisms underlying sepsis remain incompletely understood, posing significant challenges in terms of prevention, diagnosis and treatment. The complexity and multifaceted nature of sepsis pathophysiology involves the activation and dysregulation of numerous

signaling pathways (Jarczak et al. 2021). Neutrophils play a pivotal role in these pathways, as their activation is regarded as a key mechanism contributing to the development of sepsis by releasing neutrophil extracellular traps (NETs) (Brinkmann et al. 2004). These are web-like structures of DNA and antimicrobial proteins that trap and kill pathogens. NETs consist of nuclear and mitochondrial DNA arranged in a web-like structure extruded (Han et al. 2019), interspersed with granular and some cytoplasmic constituents, including myeloperoxidase (MPO), neutrophil elastase (NE) and citrullinated histone H3 (citH3), in response to infections or sterile injury (Denning et al. 2019). NETs play an indispensable role in trapping and eliminating invading pathogens. During NET formation, citrullination assumes particular significance, as it has been implicated in several inflammatory diseases, including sepsis (Mutua et al. 2016). Citrullination entails the post translational modification of peptidyl arginine to peptidyl citrulline in core histones (H3, H4, and H2A), catalyzed by the enzyme peptidyl arginine deiminase type 4 (PADI4) (Leshner et al. 2012). It plays a major role in NET formation by decondensing neutrophil chromatin and forming extracellular traps aimed at pathogens as a host defense mechanism. In contrast to the beneficial functions of NETs, an uncontrolled increase in NET formation, stemming from the failure to clear cell death-derived debris, activates the immune system, potentially leading to multi-organ failure and death (Colon et al. 2019). However, the excessive and uncontrolled production of NETs has been linked to severe organ damage and unfavourable clinical outcomes in various diseases including sepsis (Zhou et al. 2018). Recent studies have identified elevated PADI4 protein levels and higher PAD4 mRNA expression associated with lower intensive care unit (ICU) survival rates (Costa et al. 2018). Therefore, it can be considered that NETs play a pivotal role in inflammation and cell death. This study aims to investigate the activation of neutrophils by releasing NET targeting PADI4 expression levels and correlating with various clinical parameters, including the Acute Physiology and Chronic Health Evaluation II (APACHE II) score, Lactate, Procalcitonin (PCT), Sequential Organ Failure Assessment (SOFA) score, Neutrophil-to-Lymphocyte Ratio (NLR), C-Reactive Protein (CRP) and has the potential to a better understanding of sepsis pathophysiology and improve the ability to assess disease severity, monitor treatment and detect sepsis early, leading to more informed decision-making regarding appropriate treatment strategies.

MATERIALS AND METHODS

The present study was approved by the Institutional Review Board of Deccan College of Medical Sciences, Hyderabad. All participants provided written informed consent and enrolled in this study. A total of 90 male and female participants, aged 18 to 75 y, were enrolled in this study. According to the sepsis-3 criteria (Singer et al. 2016), 30 participants with clinically proven sepsis and 30 participants with septic shock were included. The remaining 30 participants were age- and gender-matched healthy controls. Blood was drawn from the hospitalized participants within 24 h of admission to the ICU. Pregnant women, patients with autoimmune disease, patients with burns, patients in the final stages of a

disease (e.g., end-stage renal disease or any type of cancer), and patients who refused to participate or did not provide consent were excluded from the study.

Sample collection

From all the study participants, 2 ml of venous blood was collected in heparinized coated tubes for Polymorphonuclear leukocytes (PMNs) isolation and was processed within 2 h of sample collection.

Isolation of PMNs

Whole blood was diluted 1:1 with 1 x phosphate buffered saline and carefully layered onto Ficoll-Paque Plus medium in a 15 ml centrifuge tube. The tubes were centrifuged at 400 x g for 30 min to separate the blood components by density gradient centrifugation. The top and middle layers, containing mononuclear cells, plasma, and platelets, were carefully removed and discarded. The bottom layer, containing erythrocytes and granulocytes, was resuspended in 1 x erythrocyte lysis buffer and incubated for 10 min at room temperature. The tubes were then centrifuged at 500 x g for 5 min at 22° C to lyse the erythrocytes. The resulting granulocyte pellet was washed with 1 x PBS and centrifuged at 600 x g for 5 min at 22° C. The final granulocyte pellet was resuspended in 1 x PBS and cell purity was assessed by staining the cells with Leishman stain and observing them under the microscope at a high magnification.

RNA extraction

Total RNA was extracted from PMNs using the guanidinium isothiocyanate method (GITC). Reverse transcription-polymerase chain reaction (RT-PCR) was then used to synthesize cDNA from the isolated RNA. The integrity and size of the cDNA were confirmed by agarose gel electrophoresis. The cDNA was then stored at — 20° C for future use.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Relative expression of PADI4 in each sample was determined using SYBR Green-based Real-time qPCR. 2 µl of cDNA derived from PMNs was added to the mixture containing 10 µl of SYBR green master mix (2 x), 0.5 µl of forward primer, 0.5 µl of reverse primer, and 7 µl of nuclease-free water. PADI4 gene-specific primers were employed in the qPCR study: Forward Primer (FP) (5'CGAAGACCCCCAAGGACT3') and Reverse Primer (RP) (5'AGGACAGTTT GCCCCGTG3') with GAPDH FP (5'CAA CTACATGGTTTACATGTTC3') and RP (5'GCCAGTGGACTCCACGAC3') acting as an endogenous control. Thermocycler settings were as follows: incubation at 95° C for 3 min, then initial denaturation for 30 sec at 95° C, annealing for 30 sec at 56° C, then elongation for 5 min at 72° C for 40 cycles. Samples were quantified in a CFX-96 Real-time system (1000 Tm Thermal cycler, BIORAD) with measurements made in triplicate (mean is taken for consideration). A 15-min melting curve analysis was also performed following reaction cycles to differentiate between amplicons and primer-dimer. Cycle threshold (Ct) values were noted and used for the calculation of fold change compared with endogenous controls using the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001).

Statistical analysis

The data were presented as mean ± standard deviation (SD) and mean difference (MD). All the statistical analyses were performed using

TABLE 1: The baseline characteristics of sepsis and septic shock patients.

Parameters	Sepsis (n = 30)	Septic shock (n = 30)
Age (y) (mean ± SD)	48.7 (11.8)	60.1 (12.2)
Sex [n (%)]		
Male	19 (63%)	20 (67%)
Female	11 (37%)	10 (33%)
Co-morbidities [n (%)]	10	15
Coronary heart disease	2 (6%)	5 (16%)
Diabetes mellitus	4 (13%)	6 (20%)
Chronic obstructive pulmonary disease	5 (16%)	9 (30%)
Hypertension	6 (20%)	7 (23%)
Chronic kidney disease	3 (10%)	4 (13%)
28-day mortality (%)	6 (20%)	11 (37%)
Disease severity (maximum24h) (mean ± SD)		
APACHE II score	15.8 ± 5.1	27.2 ± 6.2
qSOFA score	2.3 ± 0.7	2.7 ± 0.5
SOFA score	4.8 ± 1.9	9.4 ± 3.8
Biochemical parameters (mean ± SD)		
Serum lactate (mmol/L)	1.9 ± 0.9	3.9 ± 1.1
NLR	7.9 ± 2.6	13.1 ± 3.9
WBC (×10^9/L)	8.6 ± 3.1	13.26 ± 3.9
CRP (mg/L)	113 ± 45.1	159 ± 54.5
PCT (ng/L)	23.1 ± 8.2	17.6 ± 5.6

APACHE II, acute physiology and chronic health evaluation; qSOFA, quick sequential organ failure assessment; SOFA, sequential organ failure assessment; NLR, neutrophil to lymphocyte ratio; WBC, white blood cell; CRP, C-reactive protein; PCT, procalcitonin.

GraphPad Prism 8.4.2 (Graphpad Software Inc). A student t-test was used to determine the statistical significance between the two variables. The correlation coefficient (r) was calculated using Pearson correlation. The predictive diagnostic value of PADI4 is expressed as AUC derived from the ROC curve. Results were considered to be statistically significant when p-values were less than 0.05.

OBSERVATIONS

Overall, 60 patients (sepsis and septic shock) were included in this study based on the Sepsis-3 definition. The baseline characteristics of patients were mentioned in Table 1. Neutrophils were isolated and stained with Leishman and then examined under different magnifications and polymorphonuclear morphology was observed in the isolated cell, confirming its neutrophilic

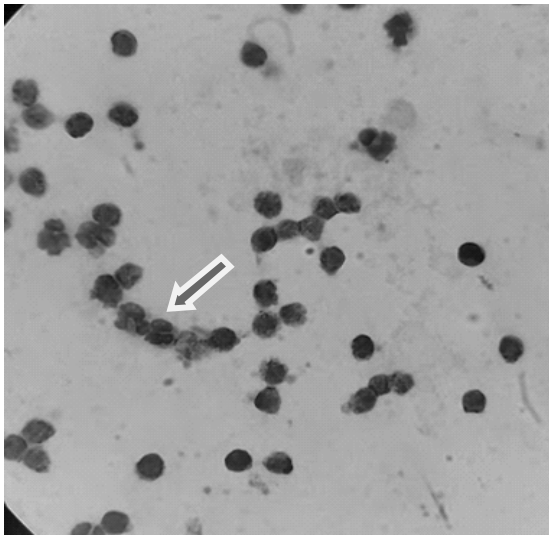
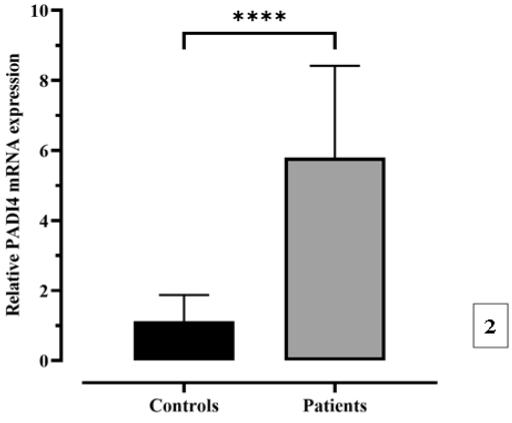
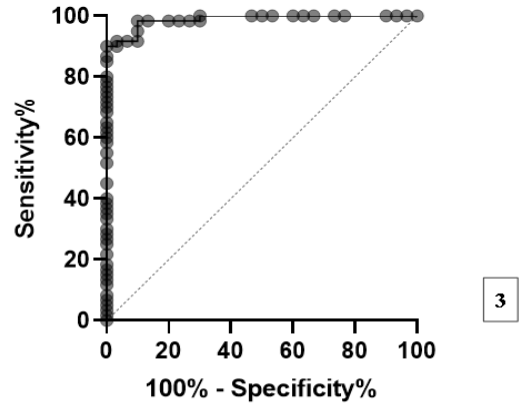
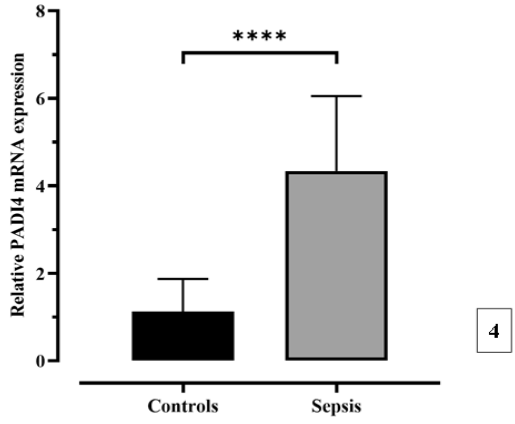


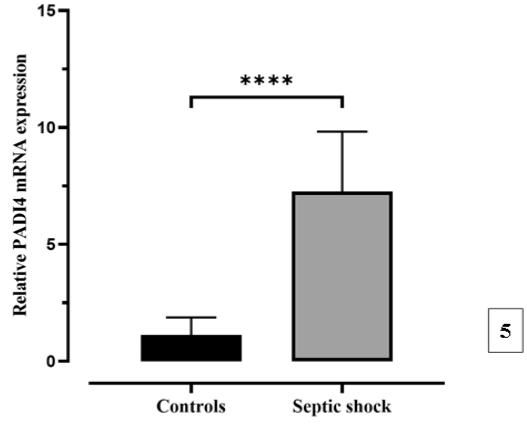
Fig. 1: Neutrophils isolated from blood by density gradient method and stained with Leishman. The arrow indicates a polymorphic nucleus.

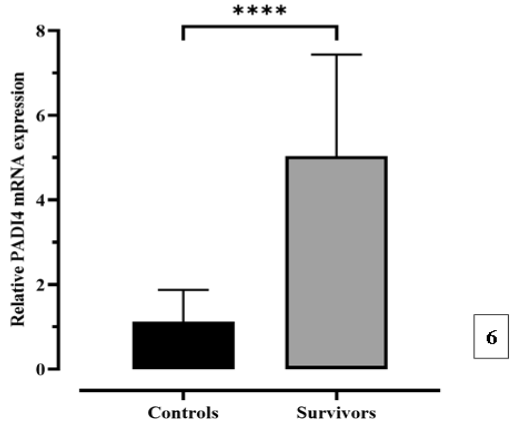
identity at 100 x magnification (Fig. 1). PADI4 is upregulated in patients as compared to healthy controls The mRNA expression levels of PADI4 in neutrophils derived from patients were significantly higher compared to healthy individuals (MD: 4.68 ± 0.48; 95% CI: 3.78 to 5.61; ****p<0.0001) (Fig. 2). The ROC analysis showed the area under the curve was 0.97 with a significant diagnostic value (95% CI: 0.97 to 1.03; ****p<0.0001) (Fig. 3). In sepsis patients, the mRNA expression levels of PADI4 were notably increased in comparison with healthy ones (MD: 3.21 ± 0.34; 95% CI: 2.53 to 3.18; ****p<0.0001) (Fig. 4). Similarly, PADI4 was significantly upregulated in septic shock patients when compared with healthy persons (MD: 6.13 ± 0.48; 95% CI: 5.12 to 0.16; ****p<0.0001) (Fig. 5). Additionally, in survivors, the mRNA expression levels of PADI4 were elevated in comparison with healthy individuals (MD: 3.91 ± 0.45; 95% CI: 3.03 to 4.18; ****p<0.0001) (Fig. 6). Similarly, PADI4 was significantly high in nonsurvivors when compared with those in healthy condition (MD: 6.63 ± 0.43; 95% CI: 5.72 to 7.16; ****p<0.0001) (Fig. 7). Relative expression of PADI4 mRNA in neutrophils from different patient groups The expression analysis of PADI4 showed significantly upregulated expression in septic shock patients as compared to sepsis patients (MD: 2.92 ± 0.52; 95% CI: 1.78 to 4.42; ***p<0.001) (Fig. 8). The ROC analysis showed the area under the curve was 0.803 with a significant diagnostic value (95% CI: 0.68 to 0.92; ***p<0.0001) (Fig. 9). To determine the mortality prediction, we compared PADI4 levels between survivors and nonsurvivors, which showed significantly upregulated expression in nonsurvivors as compared to survivors (MD: 2.63 ± 0.64; 95% CI: 1.32 to 4.02; ***p<0.001) (Fig. 10). The ROC analysis showed the area under the curve was 0.79 with a significant diagnostic value (95% CI: 0.65 to 0.93; ***p<0.001) (Fig. 11). PADI4 expression levels are elevated in nonsurvivors of sepsis and septic shock PADI4 expression level showed a significant elevation in nonsurvivors of sepsis patients as compared with survivor patients (MD: 1.67 ± 0.742; 95% CI: 0.14 to 3.12; *p= 0.02) (Fig. 12). The ROC analysis showed 0.79 as the area under the curve with a significant diagnostic value (95% CI: 0.62 to 0.92; *p<0.02) (Fig. 13). Similarly, PADI4 was significantly upregulated in septic shock nonsurvivors when compared with survivors (MD: 2.46 ± 0.74; 95% CI: 0.73 to 4.12; **p=0.02) (Fig. 14). The ROC analysis showed 0.76 as the area under the curve with a significant diagnostic value (95% CI: 0.63 to 0.92; *p=0.01) (Fig. 15). Comparison of PADI4 levels in men and women expression level showed a nonsignificant association between men and women

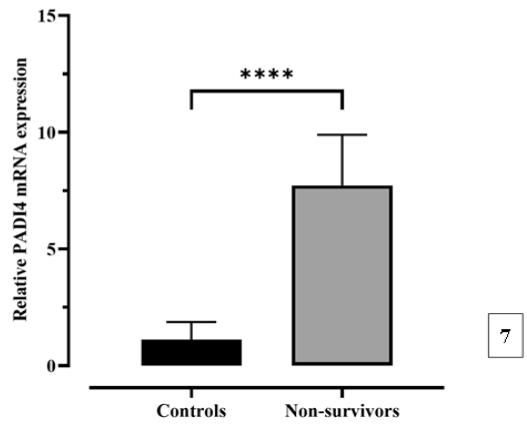




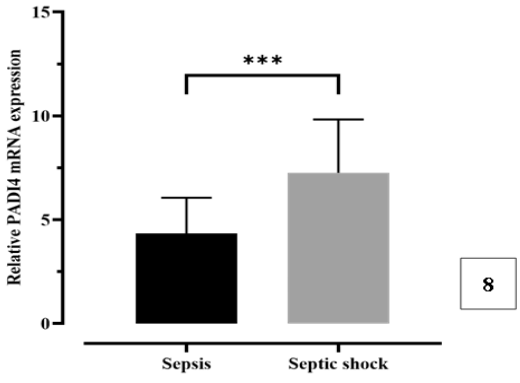


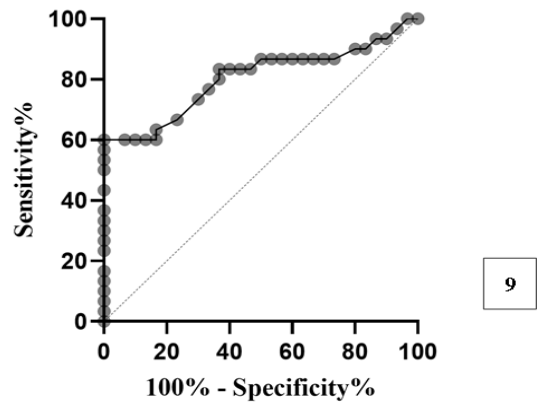


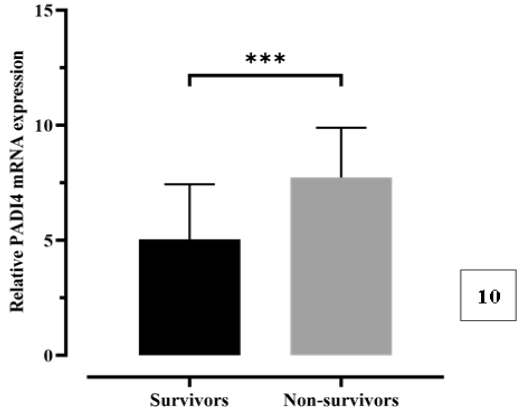


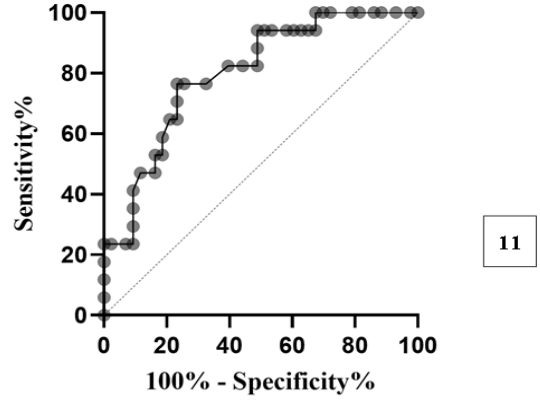


Figs 2−7: Relative expression levels of PADI4 in various groups. 2. Control versus patients. 3.The ROC curve in diagnosing patients from healthy controls based on PADI4 levels. 4. Control versus sepsis patients. 5. Control versus septic shock patients. 6. Control versus survivors. 7. Control versus nonsurvivors (****P < 0.0001).



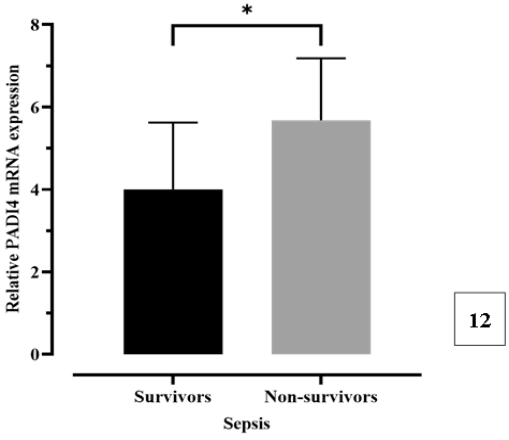






Figs 8−11: PADI4 mRNA relative expression levels. 8. Sepsis versus septic shock patients. 9. The ROC curve in diagnosing septic shock patients from sepsis patients. 10. Survivors versus nonsurvivors. 11. ROC curve in predicting mortality (***P < 0.001).

among sepsis survivors (MD: - 0.61 ± 0.68; 95% CI: - 2.06 to 0.81; p= 0.38) (Fig. 16). In sepsis nonsurvivors, expression level also showed a nonsignificant association between men and women (MD: - 1.8 ± 1.12; 95% CI: - 4.32 to 1.24; p= 0.17) (Fig. 17). Similarly, nonsignificant association between men and women among septic shock survivors (MD: - 1.96 ± 1.16; 95% CI: -4.43 to 0.51; p= 0.31) (Fig. 18). In septic shock nonsurvivor, expression levels also showed a nonsignificant association between men and women (MD: 0.18 ± 1.12; 95% CI: -2.32 to 2.72; p= 0.82) (Fig. 19). Correlation analysis of PADI4 with various clinical parameters in sepsis and septic shock patients Pearson's correlation analysis showed a significant positive association between PADI4 and NLR in sepsis patients (95% CI: 0.2921 to 0.7923; r=0.60 ***p<0.001) (Fig. 20). In addition, septic shock patients showed a significant positive association between PADI4 and SOFA score (95% CI: 0.5481 to 0.8787; r=0.76 ***p<0.001) (Fig. 21)



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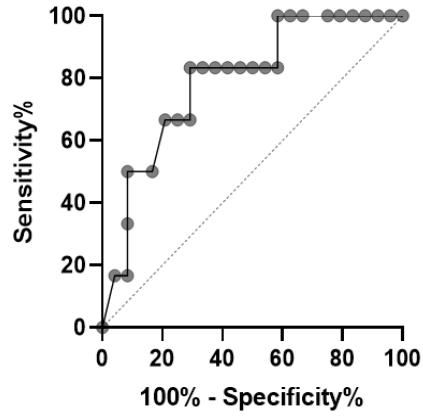

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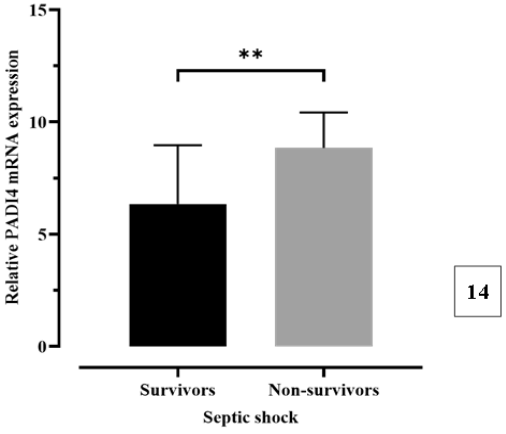

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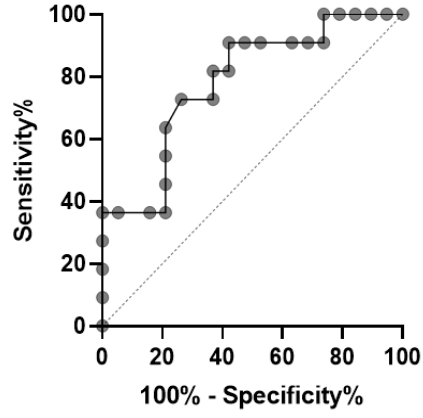

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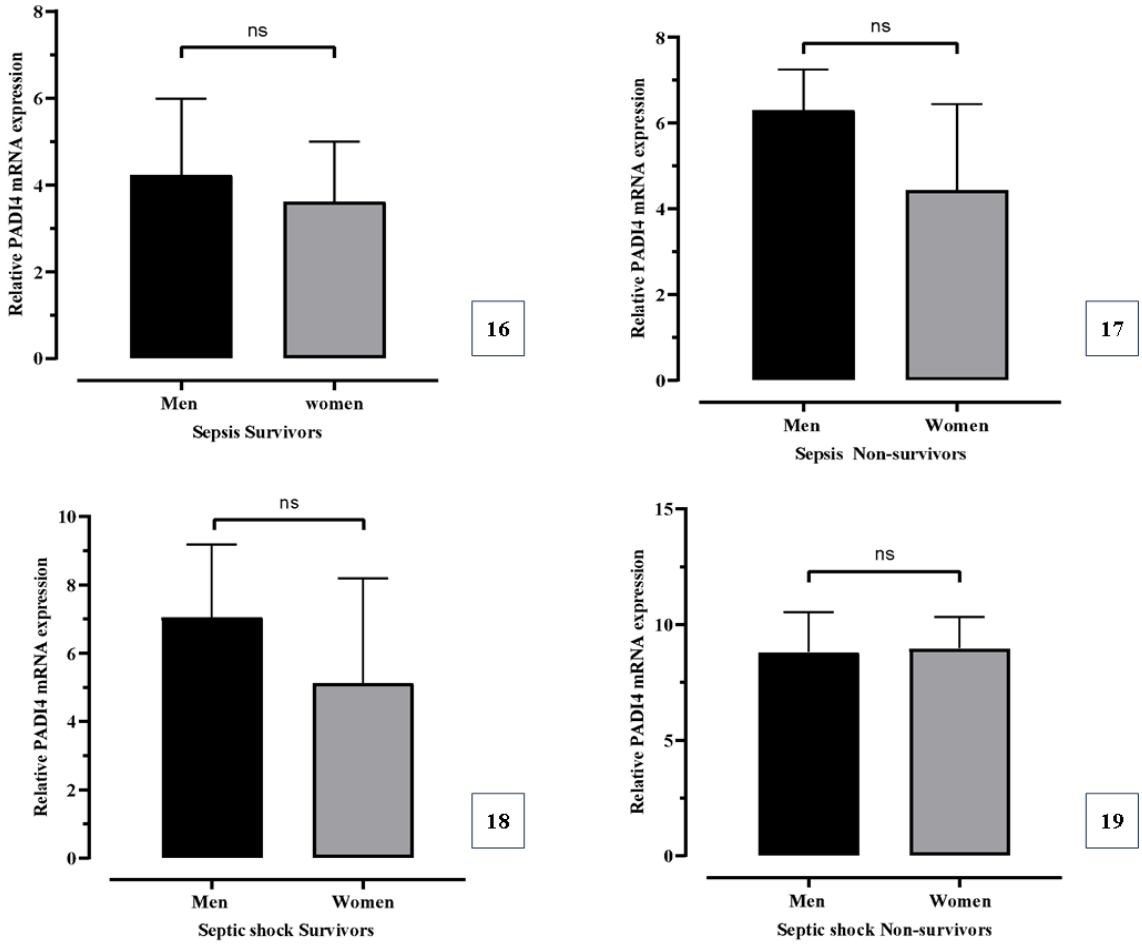
Figs 12−15: PADI4 mRNA relative expression levels. 12. Sepsis survivors versus nonsurvivors (*P < 0.05). 13. ROC curve in predicting mortality among sepsis patients. 14. Septic shock survivors versus nonsurvivors (**P < 0.01). 15. ROC curve in predicting mortality among septic shock patients.

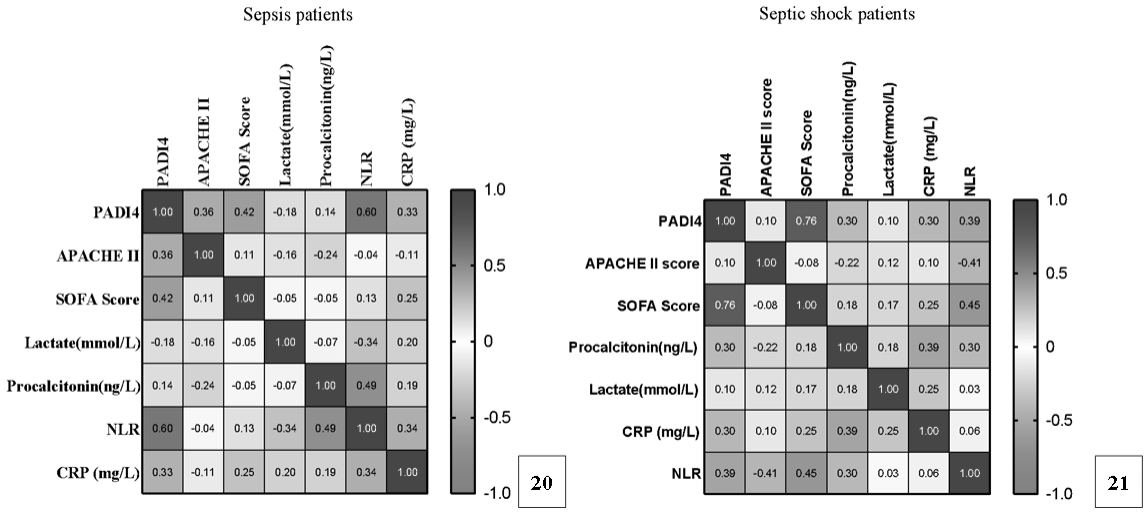
DISCUSSION

In the current study, we have observed a significant elevation in the concentration of NETs among sepsis and septic shock patients in comparison to healthy individuals. This increase in NET levels is associated with the upregulated mRNA expression levels of the PADI4 gene that are associated with high inflammatory responses and can lead to multi-organ failure. Recent studies have also highlighted the role of PADI4 in multi-organ failure and increased mortality in septic patients (Leshner et al. 2012, Yahagi et al. 2019, Zhou et al. 2018). Accordingly, nonsurvivors in our study exhibited elevated NET concentration as compared to survivors. These findings emphasize NET formation by PADI4 as a predictor of mortality exhibiting risk of organ dysfunction. NETosis, a highly regulated



Figs 16−19: PADI4 mRNA relative expression levels in men and women. 16. Sepsis survivors men versus women. 17. Sepsis nonsurvivors men versus women. 18. Septic shock survivors men versus women. 19. Septic shock nonsurvivors men versus women (ns, nonsignificant).

biological process, culminates in cell death and is triggered by specific stimuli. Notably, stimuli like phorbol myristate acetate (PMA) or cholesterol crystals initiate late suicidal NETosis through reactive oxygen species (ROS) dependent pathway (Mutua et al. 2016). Conversely, complement receptors, Toll-like receptors (TLR-2, TLR-9), induce vital NETosis through a ROS-independent pathway (Chen et al. 2021). Importantly, both processes rely on the activation of PADI4 that catalyzes the conversion of peptidyl arginine to citrulline on histones, leading to chromatin decondensation and subsequent NET release (Koushik et al. 2017). Previous studies have identified NETs in bronchoalveolar lavage fluid from septic patients, suggesting that neutrophils continue to undergo NETosis after transmigration (Feng et al. 2021). Furthermore, serine proteases released during NETosis, such as proteinase-3, cathepsin G and neutrophil elastase,



Figs 20−21: Heat map summarizing Pearson correlation coefficients (r) for PADI4 and various clinical parameters. 20. In sepsis patients. 21. In septic shock patients.

can degrade critical surfactants, D and A (Shen et al. 2021), which are essential for inflammation resolution (Li et al. 2018). Neutrophil elastase can also increase epithelial permeability through actin cytoskeleton alterations in epithelial cells (Shen et al. 2021). Moreover, NETs can activate macrophages and dendritic cells, eliciting the release of inflammatory cytokines, including IL-1β, TNF-α, IL-8, and IL-6, which further contribute to organ damage and recruit additional neutrophils to affected organs (Domer et al. 2021, Shen et al. 2021). It has also been reported that PADI4 appears to enhance neutrophil accumulation by modulating CXCR2 expression, potentially influencing disease progression (Liu et al. 2021). The heightened organ damage observed in septic shock patients due to increased inflammatory cytokine production in these organs promotes neutrophil accumulation and subsequent high rates of NET formation (Costa et al. 2021). Accordingly, we have observed significantly higher PADI4 expression levels in septic shock patients when compared to sepsis patients.

Previous research has established associations between PADI4 and various diseases, such as cancer, multiple sclerosis (MS), ulcerative colitis (UC), rheumatoid arthritis (RA), etc. Ongoing interest remains in elucidating how citrullination, mediated by PADI4, influences the NET formation and is possibly involved in disease progression (Bagheri-Hosseinabadi et al. 2023, Koushik et al. 2017, Smith et al. 2022, Sun et al. 2023). Lethal hypercitrullination, potentially induced by perforin or the membrane-attack complex during neutrophil killing by natural killer cells or cytotoxic T cells, has been proposed as a mechanism leading to excessive citrullination in RA patients (Koushik et al. 2017). Furthermore, a previous study explored the exacerbation of kidney ischemia-reperfusion injury due to PADI4 activation. Inhibition of PADI4 or the degradation of NETs significantly mitigated systemic inflammation and organ dysfunction, ultimately improving sepsis outcomes (Zhou et al. 2018). Silencing PADI4 led to

a notable reduction in the expression of inflammation-related cytokines. In a rat model of hemorrhagic shock, PADI4 silencing attenuated local inflammatory responses. Additionally, the PADI4 inhibitor, Cl-Amidine, alone reduced mortality rates in septic animals, and this effect was further enhanced when combined with antibiotics (Koushik et al. 2017). These results emphasize the potential utility of PADI4 inhibitors as a therapeutic option, particularly in sepsis. Future research avenues should explore the long-term effects of PADI4 inhibition and determine the optimal timing and duration of such interventions. However, there is a lack of clear consensus in the literature regarding the physiological equivalence by gender. To address this limitation, we evaluated PADI4 expression based on gender and found that PADI4 levels did not significantly differ between men and women, suggesting that PADI4 is not a gender-specific marker in distinguishing septic shock from sepsis. Nonetheless, the physiological functions of PADI4 remain largely elusive, and our understanding of the role of neutrophil PADI4 in sepsis beyond NET formation remains limited. Further research is necessary to unravel the precise mechanisms through which PADI4 within NETs influences the course of sepsis. Lastly, our study highlights the positive association between PADI4 levels and various clinical parameters, including APACHE II score, Lactate, PCT, SOFA score, NLR and CRP. The PADI4 relationship with these parameters may provide valuable insights into the mechanistic links between PADI4, NET formation and disease progression.

In conclusion, our study provides significant insights into the role of PADI4-mediated NET formation in sepsis and its prognostic, diagnostic and therapeutic implications. Further research is required for advancing sepsis management and improving patient outcomes.

Authors' contributions Bushra and AAK designed the research; SB conducted experiment; SIA collected samples required for the study; Bushra analysed the data, wrote manuscript and prepared figures; SCP and AAK supervised. All authors read and approved the manuscript.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

Consent to participate All authors have seen and agree with the content of this manuscript. All authors agree with the publication of this manuscript.

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Journal of Cytology and Genetics 2023 VOL. **24** (NS): 101-104

RESEARCH ARTICLE

Short communication:

A REPORT ON MALE MEIOSIS IN *MELICA SCABERRIMA* (POACEAE) FROM WESTERN HIMALAYA

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(Received 2 February 2023, revised accepted 22 May 2023)

SUMMARY Male meiosis in 7 accessions of *Melica scaberrima* (Steud.) Hook. f. from western Himalayan region has been analysed. In all accessions, each PMC showed 7 bivalents at meiosis I. However, in 3 of 7 accessions collected from Har Ki Dun Valley, 1 or 2 supernumerary chromosomes (B–chromosomes) have been observed at meiosis I. This is the first report for *M. scaberrima.* These supernumerary chromosomes are found to be absent in 4 accessions collected from Assi Ganga Valley. There appears to be some correlation between chiasma frequencies to the presence of B–chromosomes. It has been inferred that there is a tendency of lowering the chiasma frequency in accessions with Bs as compared to those without Bs. Pollen fertility in all accessions has been found to be 98–100%.

Keywords: *Melica scaberrima*, cytology, male meiosis, B–chromosomes, chiasma frequency.

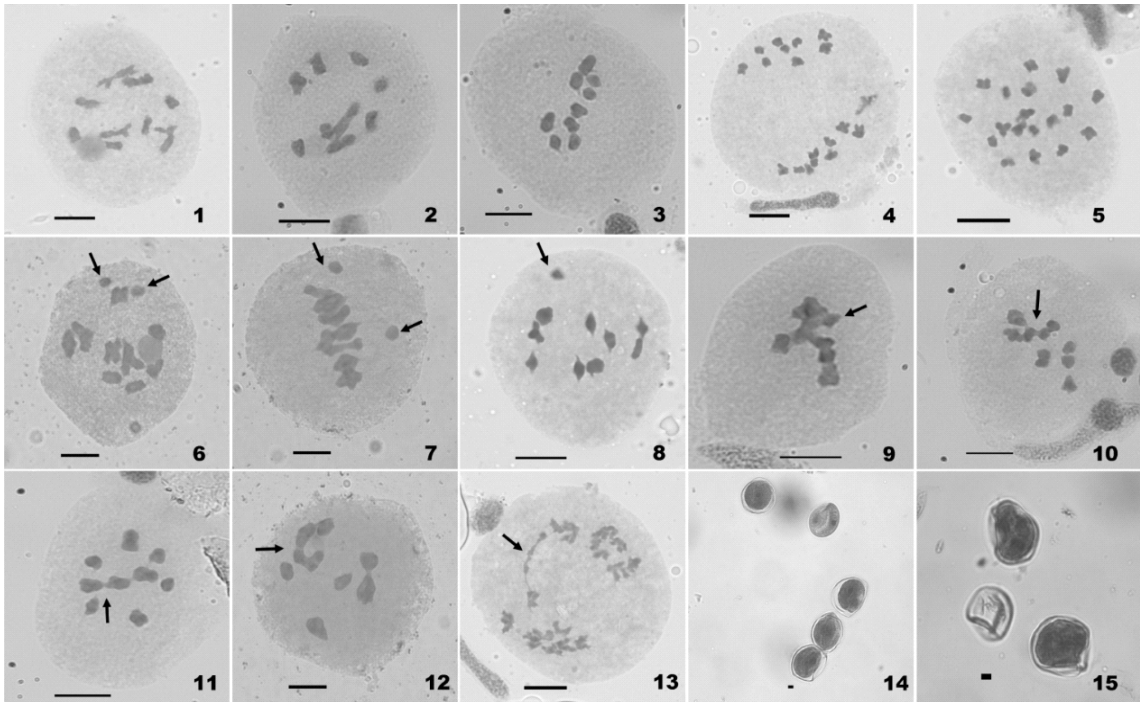
Melica L. comprises 92 species the world over (Soreng et al. 2022). While enumerating grasses from India, Kellogg et al. (2020) enlisted 5 species namely, *M. nutans* L., *M. onoei* Franch. & Sav., *M. persica* Kunth, *M. scaberrima* (Steud.) Hook. f. and *M. secunda* Regel. The chromosome number for *Melica* species was presented for the first time by Avdulov (1928) through karyotypic analysis in 4 species 2n = 18 with uniform count. Currently, an Index to plant chromosome number databases enlist chromosome numbers for 42 *Melica* species (Goldblatt & Johnson 1979, Rice et al. 2015, Kamari et al. 2017, Jha et al. 2019). Previsosly, among them only 4 species, *M. nutans, M.onoei, M. persica* and *M. scaberrima* were subjected to cytological studies (Mehra & Sharma 1972, 1975, 1977, Sharma & Kumar 1980, Gohil & Koul 1986, Yang 2004, Kumari & Saggoo 2016). Due to morphological similarities and affinities with sedges (enclosed leaf sheaths) and festucoid grasses (true grass) its systematic position rem-ained puzzled within grasses, and karyosys-tematic studies advocated its distinct nature by presenting a unique basic number of x = 9 and characteristically large–sized chromosomes (Avdulov 1928, 1931). Earlier, Mehra & Sharma (1972, 1975) (India: Uttrarakhand, Nainital, Cheena slopes) and Sharma & Kumar (1980) (India: Himachal Pradesh, Shimla, Mashobra) have studied *M. scaberrima* and reported the chromosome number of 2n = 18. While consi-dering the importance of cytogeographic studies in plants and an endeavour to explore inter- and

intraspecific chromosomal variation in melic grasses, plants of *M. scaberrima* found within an altitude range of 2100–2400 m in Uttarkashi district were subjected to cytological analysis. And this study is an attempt to prepare the comprehensive report on cytological data of Indian melic grasses. The present study deals with male meiosis and pollen fertility in 7 accessions of *M. scaberrima* from western Himalayan region.

Wild plants of *M. scaberrima* were collected from Assi Ganga Valley (30°54'09" N 78°31'09" E), Dodital region, 2900–3000 m (Accessions PUN62902 and PUN62908), on way to Darwa, 3100–3400 m (Accessions PUN63088 and PUN63120), Har Ki Dun Valley (31°07'01" N 78°20'58" E), Taluka, 2100 m (Accession PUN 63096), Osla, 2800 m (Accession PUN60476) and Renugaad, 2950 m (Accession PUN61017) in Uttarakhand State of India. Identifications of plants were confirmed by comparing herbarium specimens housed in Herbarium, Botanical Survey of India, Dehra Dun (BSD) and Vouchers have been deposited in the Herbarium, Department of Botany, Punjabi University, Patiala (PUN).

For meiotic study, young flag leaf enclosed panicles were fixed in Carnoy's fixative (6:3:1 ethanol: chloroform: acetic acid) for 48 h and transferred to 70% ethanol and stored in a refrigerator at 4º C. Meiotic preparations were made by squashing anthers from the unopened florets in 1% acetocarmine. Pollen fertility was estimated through stainability test by squashing the mature anthers in a mixture of glycerol and 1% acetocarmine (1:1). Well-filled pollen grains with completely stained nuclei and cytoplasm were scored as fertile, while partially stained and shrivelled ones as sterile. PMCs with well-spread bivalents/chromosomes and pollen grains were selected for photomicrographs using a Nikon Digital Eclipse 80i and Leica Qwin microscopes equipped with a digital imaging system.

Meiosis in 7 accessions of *M. scaberrima* has been studied. At diakinesis and metaphase I, each PMC shows 9 bivalents (Figs 1–3). Meiosis is normal with 9:9 equal segregation (Figs 4, 5). However, in 3 of 7 accessions (PUN60476, PUN61017 and PUN63096) in addition to 9 bivalents, 1 or 2 B–chromosomes were observed in 7.41–13.64% PMCs at diakinesis and metaphase I (Figs 6–8). In the other 4 accessions (PUN62902, PUN62908, PUN63088 and PUN63120) no such supernumerary chromosomes were found. To affirm any correlation between presence of Bs on chiasma frequency, 4 accessions, PUN60476, PUN61017, PUN62902 and PUN63096 were subjected to chiasma frequency analysis. Chiasma frequency per PMC (17.86 ± 0.36) and per bivalent (1.98) is higher in accession, PUN62902 lacking B–chromosome as compared to accessions possessing Bs (PUN 61017: 16.92 ± 1.73; 1.88). Similarly, PMCs of accession, PUN61017 lacking Bs show higher chiasma frequency per PMC (17.65 ± 0.70) and per bivalent (1.96) than PMCs possessing Bs (15.37 ± 2.26; 1.71). This reveals that there is a negative correlation between the presence of Bs and chiasma frequency. In addition, some meiotic abnormalities have been noticed in accessions with B–chromosomes. They include, chromatin stickiness (Fig. 9), formation of inter–bivalent connections (Figs 10–12) and chromatin bridges (Fig. 13) in a frequency of 4.48–7.41%, 7.46–



Figs 1–15: Male meiosis in *M. scaberrima.* 1, 2. Meiocyte showing 9 bivalents at diakinesis (PUN62902, PUN63088). 3. Meiocyte showing 9 bivalents at M I (PUN63088). 4. Meiocyte at M II (PUN60476). 5. Meiocyte at M II (Polar view) (PUN60476). 6, 7. Meiocytes showing 2 B–chromosomes (arrowed) (PUN61017, PUN63096). 8. Meiocyte at M I showing B–chromosome (arrowed) (PUN60476). 9. Meiocyte showing chromatin stickiness (arrowed) (PUN63096). 10–12. Meiocytes showing inter-bivalent connections (arrowed) (PUN61017, PUN63096). 13. Meiocyte showing a chromatin bridge at A I (arrowed) (PUN60476). 14, 15. Fertile and sterile pollen grains (PUN61017, PUN62902). Scale bar = 10 µm.

18.52% and 5.56–16.67% respectively. In pollen fertility study, 98–100% of pollen grains have been found to be fertile (Figs 14, 15).

The present report of n = 9 is in agreement with the previous chromosome counts made by Mehra & Sharma (1972, 1975) and Sharma & Kumar (1980). However, the report of super-numerary chromosomes seen in 3 of 7 accessions, is the first record for *M. scaberrima.* Further studies are needed to find the presence of B–chromosomes in the unexplored populations of this species from other geographical areas.

ACKNOWLEDGEMENTS

Authors wish to thank the University Grants Commission (UGC) and Department of Science and Technology, New Delhi for financial support under FIST of DSA–I, II schemes and UGC for the award of UGC–BSR–Fellowship to JS (Award letter no. 15610/ Research/ 03/06/2015). Thanks are also due to Scientist in-charge, Botanical Survey of India, NRC, Dehra Dun for Herbarium facility; Head, Department of Botany, Punjabi University, Patiala, for providing necessary library and internet facilities and Principal, Mata Sundri University Girls College, Mansa for laboratory

facilities.

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Place of publication	Bengaluru
Periodicity of publication	Yearly (may be issued in two parts)
Printer, publisher and editor	B. H. M. Nijalingappa
Nationality	Indian
Address	261, 7th Main Road, Hampinagara, Vijayanagar II Stage, Bengaluru 560 104
Names and addresses of individuals who own the newspapers and partners or shareholders holding more than one per cent of the total capital	Society of Cytologists and Geneticists (Registered under the Bombay Public Trust Act XXIX of 1950) with Registration No. G. 399 (DWR)

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