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Cocaine-use disorder and childhood maltreatment are associated with the activation of neutrophils and increased inflammation

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Original Article

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Abstract

Background: Cocaine-use disorder (CUD) has been associated with early life adversity and activated cellular immune responses. Women are most vulnerable to complications from chronic substance disorders, generally presenting an intense feeling of abstinence and consuming significant drug amounts. Here, we investigated neutrophil functional activities in CUD, including the formation of neutrophil extracellular traps (NETs) and related intracellular signalling. We also investigated the role of early life stress in inflammatory profiles. *Methods:* Blood samples, clinical data, and history of childhood abuse or neglect were collected at the onset of detoxification treatment of 41 female individuals with CUD and 31 healthy controls (HCs). Plasma cytokines, neutrophil phagocytosis, NETs, intracellular reactive oxygen species (ROS) generation, and phosphorylated protein kinase B (Akt) and mitogen-activated protein kinases (MAPK)s were assessed by flow cytometry. *Results:* CUD subjects had higher scores of childhood trauma than controls. Increased plasma cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-12, and IL-10), neutrophil phagocytosis, and production of NETs were reported in CUD subjects as compared to HC. Neutrophils of CUD subjects also produced high levels of intracellular ROS and had more activated Akt and MAPKs (p38/ERK), which are essential signalling pathways involved in cell survival and NETs production. Childhood trauma scores were significantly associated with neutrophil activation and peripheral inflammation. *Conclusion:* Our study reinforces that smoked cocaine and early life stress activate neutrophils in an inflammatory environment.

Significant outcomes

- Increased pro-inflammatory cytokines were observed in cocaine-use disorder (CUD).
- CUD subjects had enhanced neutrophil phagocytosis and NETs production.
- CUD neutrophils had high levels of intracellular ROS and activated MAPK.
- Childhood trauma was associated with neutrophil activation and inflammation.

Limitations

- Relatively small sample sizes.
- The study has only included females.
- Potential physiological, behavioural, and lifestyle changes associated with chronic addiction were not explored.

Introduction

Crack cocaine is the alkaloidal freebase smoked cocaine that has been considered as one of the most potent psychoactive substances, with a high potential for relapse and craving (Fischer and Coghlan, 2007). However, the pathophysiology, aetiology, and treatment responses vary based on sex differences (Becker, 2016). It has been shown that women start using cocaine earlier than men; they report higher amounts of cocaine consumption when compared with men; they

report higher craving and withdrawal symptoms to cocaine than men and are more vulnerable to developing cocaine-use disorder (CUD) (Sanvicente-Vieira *et al.*, 2019). From a psychological point of view, early life stress is a recognised risk factor associated with increased vulnerability to drug dependence. Indeed, childhood maltreatment is highly prevalent among cocaine users (Scheidell *et al.*, 2018), and being a predictive risk factor for addiction (Andersen and Teicher, 2009). However, this effect could be apparently true for females but not males (Hyman *et al.*, 2008; Sanvicente-Vieira *et al.*, 2019).

Earlier studies have indicated that CUD may suppress both cellular and humoral immunity (Baldwin *et al.*, 1998). As a consequence, cocaine was associated with increased susceptibility to infections (Baldwin *et al.*, 1998; Restrepo *et al.*, 2007; Francke *et al.*, 2013). In contrast, it has been shown that acute cocaine administration leads to the activation of polymorphonuclear (PMN) neutrophils, with elevated neutrophil-to-lymphocyte ratio, enhanced effector functions, and cytokine production (Baldwin *et al.*, 1997; Soder *et al.*, 2020). The CUD-related immunological changes are evidenced by the interaction of cocaine with dopamine receptors expressed in immune cells (Levite, 2016).

Moreover, CUD is associated with a clear imbalance towards an inflammatory profile. Accordingly, high plasma pro-inflammatory cytokines, such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α, and decreased anti-inflammatory molecules were reported in cocaine users when compared with healthy controls (HCs) (Narvaez *et al.*, 2013; Moreira *et al.*, 2016). Also, high plasma Th1/Th2/Th17-related cytokines have been observed in CUD and associated with withdrawal symptomatology (Levandowski *et al.*, 2016; Zaparte *et al.*, 2019). Short-term exposure to cocaine increased the production of IL-8, a potent PMN chemoattractant and neutrophil-activating factor associated with both acute and chronic lung injury (Baldwin *et al.*, 1997).

Neutrophils participate in the early inflammatory responses and are the most abundant phagocytes in the circulation. These cells are chiefly involved with immunity against bacterial infections as well as sterile inflammation. Their effector responses include the phagocytosis, generation of reactive oxygen species (ROS), and production of neutrophil extracellular traps (NETs) (Brinkmann *et al.*, 2004). NETs are networks of extracellular fibres composed of DNA-containing histones, myeloperoxidase (MPO), and elastase. The formation of NETs by the neutrophils is a crucial mechanism to restrain and kill pathogens. In addition to their microbicidal activities, NETs have also been implicated in tissue injury (Sorvillo *et al.*, 2019; Neumann *et al.*, 2020) and, consequently, in the pathogenesis of various inflammatory conditions including rheumatoid arthritis, diabetes (Fousert *et al.*, 2020), sepsis, and COVID-19 (Veras *et al.*, 2020). Previous studies have shown that cocaine may modulate the neutrophil antibacterial activity by either increasing (Baldwin *et al.*, 1997) or decreasing (Delafuente and Devane, 1991; Mukunda *et al.*, 2000) their functions. Moreover, direct *in vitro* exposure of human neutrophils to cocaine induces the formation of NETs, which may be related to the appearance of cocaine/levamisole-associated autoimmunity in some chronic users (Lood and Hughes, 2017).

Despite this evidence, there is limited data evaluating the effect of cocaine use on innate immune effector responses, particularly its impact on neutrophil activation and NETs formation. Here, we aimed to investigate neutrophil effector functions, including the phagocytosis, NETs production, and related intracellular signalling, as well as the inflammatory profile of women with CUD and healthy non-user controls (HC). In addition, we explored the impact of clinical features and history of childhood maltreatment on effector immune responses. We hypothesise that childhood trauma could be associated with neutrophil activation and peripheral inflammation in CUD.

Methods

Participants

This cross-sectional study included 41 women with CUD and 31 healthy non-user women as HC. Participants with CUD were voluntarily recruited from a public psychiatric hospital detoxification inpatient unit in Southern Brazil. We adopted the following inclusion criteria for CUD group: self-reporting recent use of smoked cocaine before the hospitalisation (patients were in early abstinence), fulfilling the criteria for CUD according to the Diagnostic and Statistical Manual of Mental Disorders version 5 (DSM-5) as the primary diagnostic. The HC group consisted of healthy women, selected by convenience, with no history of any substance use, except for alcohol and nicotine. In both groups, participants did not have active infections, absence of cognitive deficits compromising the ability to answer the questions, and finally, verbal, and written consent to participate in the study. The patients were screened by expert psychiatrists and clinical psychologists. The study was approved by the Ethics Committee from the Pontifical Catholic University of Rio Grande do Sul (PUCRS, # 3.317.295).

Clinical assessment

During the first week of the detoxification treatment, the Addiction Severity Index, version 6 (ASI-6) was applied to evaluate the substance-use profile and the severity of addiction in women with CUD (Cacciola *et al.*, 2011; Kessler *et al.*, 2012). Also, the severity of withdrawal symptoms was assessed by the Cocaine Selective Severity Assessment (CSSA) (Kampman *et al.*, 1998; Kluwe-Schiavon *et al.*, 2015). Psychiatric comorbidities were investigated using the Structured Clinical Interview for DSM-5. In both samples, the Childhood Trauma Questionnaire (CTQ) (Grassi-Oliveira *et al.*, 2014) was applied to assess the presence of childhood maltreatment. In the HC group, sociodemographic data and substance use were obtained through questionnaires.

Reagents

Phorbol 12-myristate 13-acetate (PMA) and protease-free DNase 1 were obtained from Promega (São Paulo, Brazil). Dextran, cytochalasin D (*Zygosporium mansonii*), lipopolysaccharide (*Escherichia coli:* 0111:B4), and Ficoll-Hypaque-1077 were obtained from Sigma-Aldrich (Sigma, São Paulo, Brazil). EcoRI, HindIII, Hoechst 33342, pHrodo *E. coli* BioParticles conjugates, Probe 5-(and-6)-chloromethyl-2'-7'-dichlorodihydrofluorescein diacetate, and acetyl ester (CM-H2DCFDA) were purchased from Invitrogen (Carlsbad, CA). RPMI 1640 was obtained from Cultilab (São Paulo, SP, Brazil). The following reagents were purchased from BD Biosciences (San Jose, CA): anti-myeloperoxidase (PE), anti-phospho-ERK 1/2 Alexa fluor 488 (FITC), anti-AKT PACIFIC BLUE (bv421), and anti-p38 Alexa fluor 647 (APC), BD Cytofix™ Buffer, BD™ Phosflow Perm Buffer III. Quant-iT™ PicoGreen™ dsDNA Assay Kit was obtained from Life Technologies (São Paulo, SP, Brazil).

Blood collection and isolation of peripheral neutrophils

Peripheral blood (20 mL) was collected in tubes containing the anticoagulant EDTA from all individuals and processed as follows.

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Plasma was collected after centrifugation and stored at −80°C. Erythrocytes were removed using Dextran sedimentation protocol followed by hypotonic lysis. Neutrophils were then isolated with Ficoll-Hypaque-1077 density gradient centrifugation and then resuspended in RPMI 1640 medium. Cells were gated according to granulocyte size on forward and side scatters, followed by CD66b and CD3 expression discrimination, with compatible morphology for granulocytes, and purity around 97% of the cells (Supplemental Figure 1). Cellular viability was always higher than 99%, as examined by Trypan Blue exclusion assay. Cells were freshly analysed after isolation.

Plasma cytokines

Plasma levels of IL-12p70, TNF-α, IL-10, IL-6, IL-1β, and IL-8 were measured using the Human Inflammatory Cytokine Cytometric Bead Arrays (CBAs) kit, following the manufacturer's instruction (BD Biosciences, São Paulo, Brazil). The cytokine levels were measured by flow cytometry using FACSCanto II flow cytometer (Becton Dickinson, CA) and results were analysed using FCAP Array v3.0 software (Soft Flow Inc., Pecs, Hungary). The detection limits for these assays ranged from 2.4 to 4.9 pg/ml. The intra-assay and inter-assay coefficients of variation were <10%.

Neutrophil phagocytosis

The neutrophil phagocytosis was assessed with the PHrodo® *E. coli* BioParticles®, following manufacturer instructions. Briefly, a sample of seeded neutrophils (2×10^6/mL) was pre-treated with 30 uL/mL of Cytochalasin D, as a control for inhibiting phagocytosis, in 24-TPP plates (Techno Plastic Products AG) for 1 h at 37°C in 5% CO_2. The remaining samples of cells were cultured with the medium as a control group. Phagocytosis was induced with 100 ng/mL of LPS. After 1 h of incubation, PHrodo® *E. coli* BioParticles® conjugated solution was added and cultured for another 1 h. Hereafter, data were accessed by flow cytometry FACSCanto II cytometer (BD Biosciences).

Quantification of NETs formation

Purified neutrophils (2×10^6/mL) were stimulated with PMA (50 nM), as classical inducer of ROS-dependent NETosis (Funchal *et al.*, 2015, Barth *et al.*, 2016, Tatsiy and McDonald, 2018). As a control, unstimulated neutrophils were cultured with medium. After 1 h, 20 U/mL from each restriction enzyme (EcoRI and HindIII, 20 U/mL) was added and cultured for another 2 h at 37°C with 5% CO_2. The formation of cell-free DNA was quantified in the culture supernatant using the Quant-iT™ PicoGreen™ dsDNA Assay Kit, according to the manufacturer protocol, using SpectraMax M2/M2e Microplate Readers (Molecular Devices, San Jose, CA).

Immunofluorescence

To visualise and characterise the formation of NETs, neutrophils (2×10^5/300 μL) were incubated with PMA (100 nM) or medium alone for 3 h at 37°C in 5% CO_2 in a six-chamber culture plate with slides inside. After this period, cells were fixed with 4% paraformaldehyde, the slide was removed from the plate and stained with anti-myeloperoxidase PE antibody (1:1000; BD Biosciences) or Hoechst 33342 (1:2000; Invitrogen). Confocal images were taken in an SP8 LIGHTNING microscope.

Intracellular ROS generation

Intracellular ROS generation was determined based on the oxidation of 0.5 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) that produces an intracellular fluorescence. Neutrophils (1×10^6 cells/mL) were incubated with medium only or stimulated with PMA for 1 h at 37°C in 5% CO_2. Cells were then incubated with CM-H2DCFDA probe for 0.5 h at 37°C in 5% CO_2. Cytosolic ROS production was measured by flow cytometry using FACSCanto II flow cytometer (Becton Dickinson) with the BD FACSDiva software and analysed with FlowJo v 7.5 (Tree Star Inc., Ashland, OR). Supplemental Figure 2A shows the intracellular ROS generation in unstimulated and PMA-treated samples.

Expression of Akt and mitogen-activated protein kinases (MAPKs) ERK and p38

The expression of phospho-ERK 1/2, phospho-p38, and phospho-AKT in neutrophils was measured by flow cytometry using BD Phosflow (BD Biosciences) according to manufacturer's instructions for human blood. Briefly, neutrophils (2×10^6 cells/300 μL) were incubated with medium only or stimulated with PMA for 10 min at 37°C in 5% CO_2. Cells were then fixed in BD Cytofix™ Buffer for 10 min at 37°C, washed, and permeabilizated with Phosflow Perm Buffer III for 30 min on ice. Neutrophils were washed twice and stained with anti–phospho-ERK 1/2 Alexa fluor 488 (FITC), anti-AKT PACIFIC BLUE (bv421), and anti-p38 Alexa fluor 647 (APC) for 30 min on ice. Finally, data were accessed by flow cytometry using FACSCanto II cytometer (Becton Dickinson) with BD FACSDiva software, and posterior analyses were made with FlowJo v 7.5 (Tree Star Inc., Ashland, OR, USA). Supplemental Figure 2B and 2C shows the relative expression (as determined by mean fluorescence intensity, MFI) of p-AKT, p-ERK, and p-P38 in unstimulated (CN) and PMA-treated samples.

Statistical analyses

All variables were checked for normality of distribution using the Shapiro–Wilk test. For categorical variables, differences between groups were compared using chi-square (X^2) or Mann–Whitney tests. In the CUD group, Spearman correlations were performed to investigate the influence of substance use characteristics e.g., crack cocaine use in the last 30 days, years of crack cocaine use, the severity of substance use (ASI-drugs score), and severity of withdrawal symptoms (CSSA scores) with the biological measures. Significant correlations ($p < 0.05$) were further tested by adjusting for age, body mass index (BMI), and CTQ scores using linear regression models or partial correlations.

Plasma inflammatory cytokines levels were compared between CUD and HC groups through Generalized Linear Models (GzLM) using gamma distribution with the log link function. In addition, generalised estimating equation (GEE) models were performed to analyse the interaction effect of group (CUD × HC) and treatment (unstimulated × stimulated) with investigated biological parameters (NETs, ROS generation, expression of ERK, AKT, p38, and neutrophil phagocytosis). Linear distribution was selected, and robust estimation with an unstructured working correlation matrix was set. Bonferroni post hoc test was used to compare means between groups and adjust the observed significance level considering multiple contrasts being tested. Age, BMI, and CTQ scores were included as covariates in GzLM and GEE analyses (Wald

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Table 1. Demographic and clinical characteristics

	Control ($n = 31$)	Cases ($n = 41$)	Statistics	p-value
Age (years)	33.00 [29.0–39.0]	36.00 [19.0–63.0]	$U = 736.500$	0.250
BMI	22.98 [16.7–35.6]	22.90 [15.8–29.8]	$U = 499.500$	0.462
Childhood maltreatment (scores)	30.00 [25.0–62.0]	48.00 [27.0–88.0]	$U = 809.500$	<0.001
Marital status (single)	21 (67.7)	25 (78.1)	0.415	0.519
Chronic diseases				
Hypertension	0	2 (5.6)	1.775	0.495
Diabetes	0	3 (8.3)	2.704	0.243
Heart disease	0	2 (5.6)	1.775	0.495
Stroke	0	1 (2.8)	0.874	0.999
Epilepsy	0	4 (11.1)	3.663	0.118
Tuberculosis	0	1 (2.8)	0.874	0.999
Hepatitis	1 (3.2)	3 (8.3)	2.704	0.243
Liver disease	0	2 (5.6)	1.775	0.495
Kidney disease	0	2 (5.6)	1.775	0.495
Pulmonary disease	5 (16.1)	12 (33.3)	2.604	0.159

Data are presented as *n* (%) or as median [minimum–maximum].

X^2) to adjust for potential confounders. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0. The significance level was set at $\alpha = 0.05$ (two-tailed).

Results

Demographic and clinical characteristics

Demographic and clinical data are shown in Table 1. Cases and controls were similar regarding age ($U = 736.500$, $p = 0.250$), BMI ($U = 499.500$, $p = 0.462$), and marital status ($X^2 = 0.415$, $p = 0.519$). In contrast, CUD subjects had higher scores of childhood trauma (CTQ: $U = 809.500$, $p < 0.001$) as compared with the HC group. Supplemental Table 1 describes the substance use profile of women with crack cocaine addiction. All cases were diagnosed with current CUD following the DSM-5 criteria. In the last 30 days prior to blood collection, the use of crack cocaine and sedatives was daily (median 30 days of use). Women with CUD had 9 years of crack cocaine use, while the use of alcohol, cannabis, and sedatives was less frequent and for a shorter period (from 1 to 3 years).

Inflammatory profile in CUD

First, we investigated whether CUD was associated with peripheral inflammation by assessing inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-12p70) and IL-10 in plasma samples. Subjects with CUD had higher levels of IL-12p70 (Wald $X^2 = 3.969$, $p = 0.046$), IL-10 (Wald $X^2 = 14.974$, $p < 0.001$), TNF-α (Wald $X^2 = 14.826$, $p < 0.001$), IL-1β (Wald $X^2 = 45.984$, $p < 0.001$), IL-6 (Wald $X^2 = 52.687$, $p < 0.001$), and IL-8 (Wald $X^2 = 55.462$, $p < 0.001$) compared with the HC group (Fig. 1) – controlling for age, BMI, and CTQ scores.

Neutrophils from cocaine users exhibited increased phagocytosis

Next, we examined neutrophil phagocytosis as an important effector function involved in antimicrobial immunity. Phagocytosis was assessed at baseline (unstimulated) or following stimulation with LPS. Cytochalasin D was also used as an inhibitor of phagocytosis. A significant GROUP effect (i.e., CUD versus HC) for the phagocytosis index was observed (Wald $X^2 = 4.106$, $p = 0.043$). In addition, these analyses revealed statistically significant TREATMENT (Wald $X^2 = 61.310$, $p < 0.001$) as well as GROUP × TREATMENT interaction (Wald $X^2 = 52.846$, $p < 0.001$), Fig. 2. Pairwise comparisons between HC and CUD groups statistically significant differences in unstimulated ($p = 0.001$), LPS ($p = 0.028$), and cytochalasin D groups ($p = 0.001$), but not in the cytochalasin D-treated cells before the LPS stimulation ($p = 0.405$). Unstimulated CUD neutrophils had increased phagocytosis than unstimulated HC group ($p = 0.001$), but in similar magnitude to LPS-treated cultures. Moreover, within-group comparisons indicated that LPS stimulation in HCs yielded a robust phagocytosis ($p < 0.001$) that was abrogated by cytochalasin D treatment ($p < 0.001$). There was no significant difference between the Cyto D group and Cyto D pre-treated before the LPS stimulation group in HCs ($p = 0.132$) or CUD ($p = 0.513$). These data indicate that CUD neutrophils were activated at a baseline level and associated with impaired stimulation.

CUD is associated with increased NETs formation

NETs were investigated here as another effector function, associated with microbicidal actions as well as sterile inflammation and tissue injury (Sorvillo *et al.*, 2019; Neumann *et al.*, 2020). We observed a significant GROUP effect (CUD versus HC, Wald $X^2 = 6.122$, $p = 0.013$), as well as a significant influence of TREATMENT (Wald $X^2 = 5.188$, $p = 0.023$). Moreover, the

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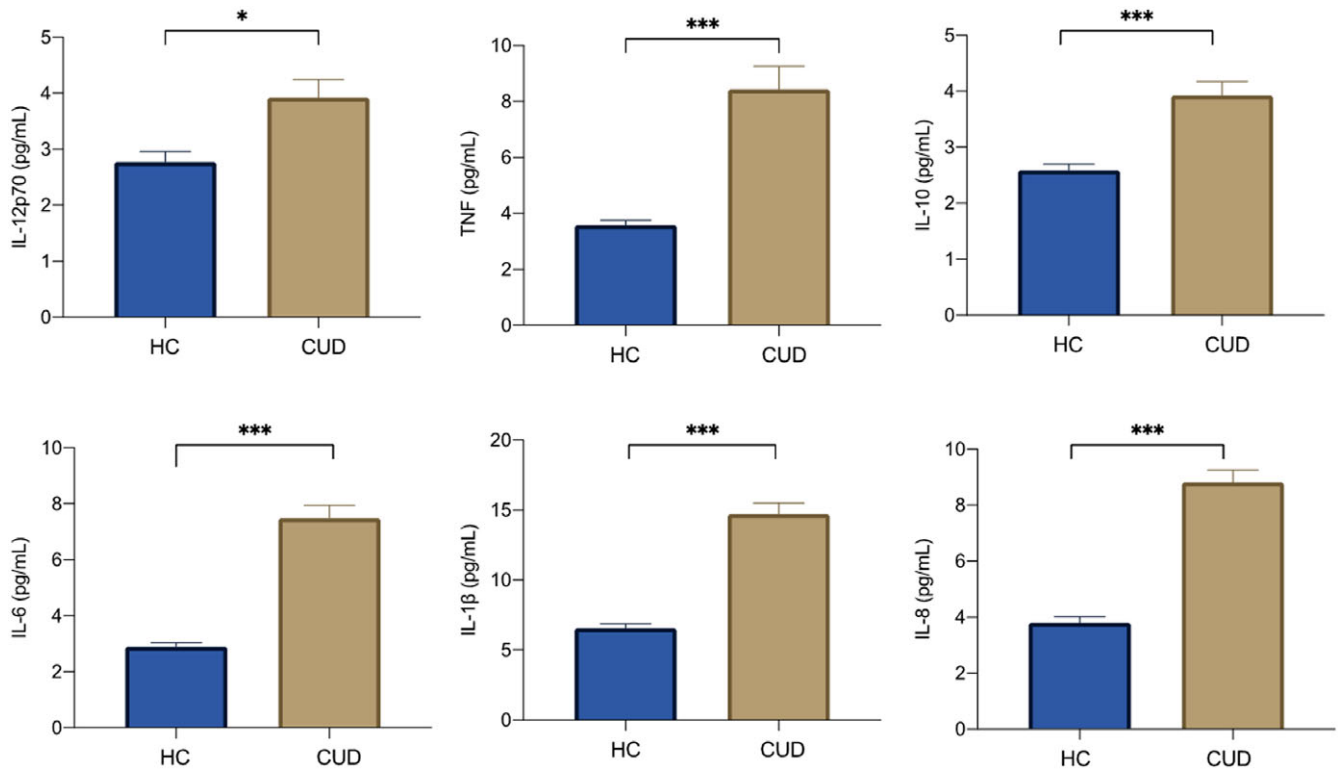

Fig. 1. CUD was associated with an inflammatory profile. Plasma samples were collected from healthy controls (HC) ($n = 29$) and cocaine used disorder (CUD, $n = 32$) subjects. Cytokines were quantified by Cytokine Beads Assays (CBAs) through flow cytometry. Data are shown as mean concentration (pg/mL) ± SEM. Statistically significant differences are indicated: $^{*}p < 0.05$; $^{***}p < 0.0001$.

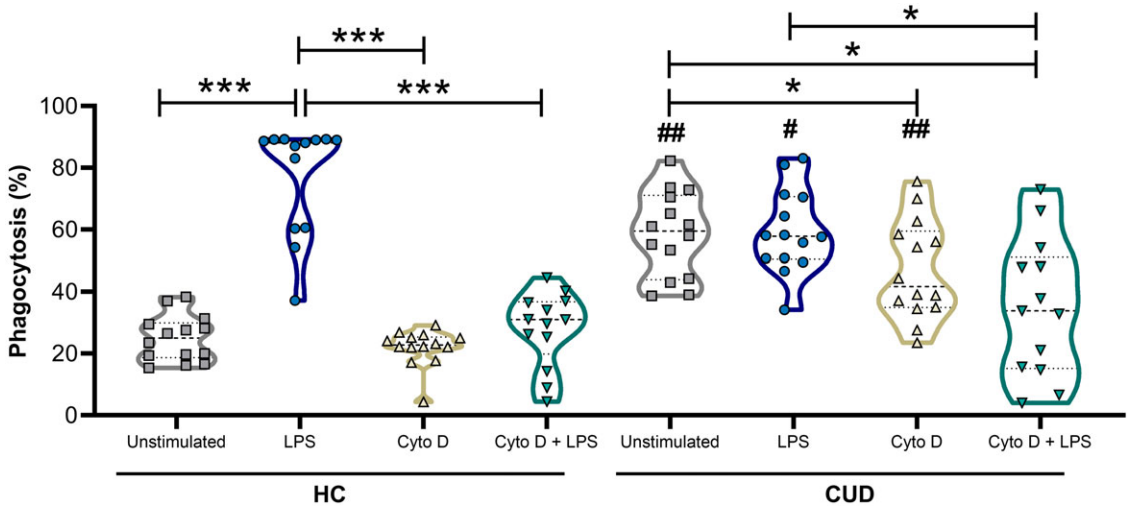

Fig. 2. Neutrophils with increased phagocytosis in cocaine-use disorder. Phagocytosis was assessed with pHrodo *E. coli* BioParticles. Legends: Cyto D, cytochalasin D; CUD, cocaine-use disorder; HC, healthy controls; LPS, lipopolysaccharide. Data are shown as phagocytosis index (%). Statistically significant differences are indicated: within-groups were informed as $^{*}p < 0.05$, $^{***}p < 0.001$; and between groups (HC as relative control) $^{\#}p < 0.05$, $^{\#\#}p = 0.001$.

interaction term GROUP × TREATMENT was also found statistically significant (Wald $X^2 = 8.320$, $p = 0.004$, Fig. 3). Unstimulated neutrophils of CUD subjects produced higher NETs at baseline than HC group ($p < 0.001$). We next assessed the response to PMA, which represents a potent NET inducer and has been extensively used to characterise NET formation (Funchal *et al.*, 2015; Barth *et al.*, 2016). The PMA stimulation yielded a robust formation of NETs in controls only ($p < 0.001$). In addition, CUD unstimulated cells had similar NETs production compared to the HC cells stimulated with PMA ($p = 0.661$), indicating that neutrophils in CUD are already activated at baseline, producing NETs.

NETs formation was visualised and further characterised by confocal microscopy (Fig. 4). Neutrophils were stained for DNA (Hoescht) and MPO. While unstimulated, neutrophils from the HC group had an integral nucleus structure (Fig. 4A). Following

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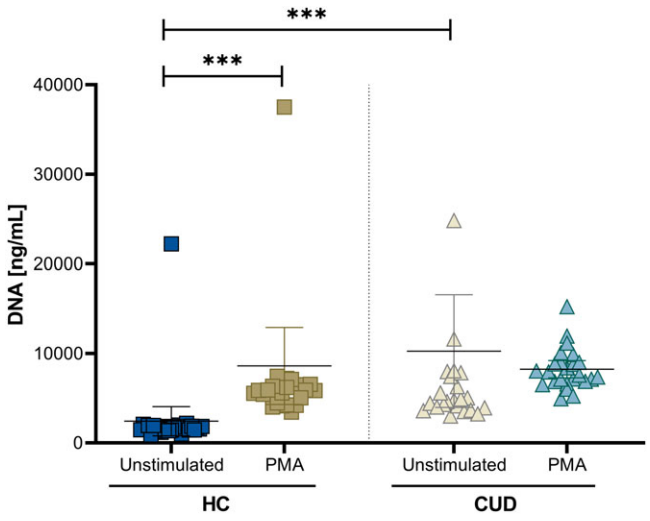

Fig. 3. High baseline but impaired LPS-induced formation of NETs in CUD. Neutrophil production of NETs was quantified in culture supernatants using Quant-iT™ PicoGreen™ dsDNA Assay Kit. Data were performed in triplicates of each group and represent as mean of extracellular DNA concentration (pg/mL) and 95% CI. Legends: PMA, Phorbol Myristate Acetate. Statistically significant differences are shown as ***$p < 0.001$.

PMA stimulation, the HC neutrophils nucleus lost its shape, the granule membranes disintegrated, cell membrane collapsed, and NETs formation occured (Fig. 4B). Unlike HC neutrophils, the CUD cells presented NETs formation at baseline (i.e., neutrophils with medium, Fig. 4C) and following PMA stimulation (Fig. 4D).

Increased intracellular ROS generation in CUD

We next investigated intracellular ROS generation as an important pathway involved in production of NETs as well as in antimicrobial effector function. Therefore, we used PMA as a classical ROS-dependent stimulus (NADPH oxidase activation) to induce NETosis (Tatsiy and McDonald, 2018). We have thus determined both baseline (unstimulated) and stimulated (PMA) intracellular ROS generation. We observed significant GROUP (Wald $X^2 = 12.419$, $p < 0.001$) and TREATMENT effects (Wald $X^2 = 61.011$, $p < 0.001$). Moreover, the interaction term GROUP × TREATMENT was also found statistically significant (Wald $X^2 = 16.921$, $p < 0.001$, Fig. 5). Neutrophils from both groups had increased intracellular ROS formation after PMA stimulation (both $p < 0.001$). In addition, the unstimulated and stimulated neutrophils from CUD presented heightened ROS formation than the HC group ($p = 0.005$ comparing unstimulated cells, and $p < 0.001$ after treatment with PMA).

The use of crack-cocaine induces AKT and MAPK phosphorylation

Akt and MAPK phosphorylation was investigated here as important intracellular pathways involved in cell survival (Akt) and NETs generation. The activated p-p38, p-ERK 1/2, and p-AKT levels were determined by flow cytometry. Concerning AKT activation (Fig. 6A), we observed a statistically significant GROUP effect (CUD versus HC, Wald $X^2 = 22.030$, $p < 0.001$), with higher phosphorylation in the CUD group compared with HCs ($p < 0.001$). Also, a significant influence of TREATMENT (Wald $X^2 = 48.862$, $p < 0.001$) was detected. Both groups presented a more phosphorylated AKT after PMA treatment ($p < 0.001$). There were no significant GROUP × TREATMENT interactions (Wald $X^2 = 0.373$, $p = 0.541$).

ERK 1/2 analysis showed similar results (Fig. 6B). We detected a significant GROUP effect (CUD versus HC, Wald $X^2 = 11.480$, $p = 0.001$), with significantly higher levels of p-ERK in cells of CUD subjects than those of controls. Moreover, neutrophils treated with PMA had higher p-ERK compared with unstimulated cells in both groups (Wald $X^2 = 45.370$, $p < 0.001$). There were no significant GROUP × TREATMENT interactions (Wald $X^2 = 1.642$, $p = 0.200$).

Nonetheless, considering p38 activation (Fig. 6C), a significant interaction term was observed (Wald $X^2 = 15.057$, $p < 0.001$), as well as a significant TREATMENT effect (Wald $X^2 = 49.588$, $p < 0.001$). Concerning unstimulated neutrophils, CUD subjects had higher intracellular levels of p-p38 levels than HC ($p = 0.031$). Otherwise, similar phosphorylation was detected after PMA treatment comparing these two groups ($p = 0.685$). In addition, there was more p-p38 in neutrophils treated with PMA compared with unstimulated cells in both CUD ($p = 0.048$) and controls ($p < 0.001$).

The relationship between substance-use characteristics and biological measurements

Exploratory analyses in the CUD group revealed main associations between clinical severity (years of drug use, ASI and CSSA) and biological variables. First, a negative correlation was observed concerning years of crack cocaine use and activation of AKT under PMA stimulation ($r = -0.652$, $p = 0.012$). However, this result did not remain statistically significant after adjustment for potential confounders in the linear regression model ($\beta = -0.480$, $p = 0.375$). Second, the severity of substance use (ASI drugs) was negatively correlated to NETs formation ($r = -0.482$, $p = 0.009$). This association did not remain significant after adjusting for age, BMI, and CTQ scores ($\beta = -0.420$, $p = 0.080$).

The relationship between childhood trauma and biological assessments

Exploratory analyses have also revealed significant associations between CTQ and biological assessments. CTQ scores were found to be positively correlated with NETs production (unstimulated, $\rho = 0.58$, $p < 0.001$). These associations remained significant following adjustments for BMI and age (partial correlations). A median split of CTQ (median = 37.5) was performed to demonstrate the magnitude of this effect, comparing subjects with lower (<37.5) and higher (≥37.5) CTQ scores. Subjects with HIGH CTQ produced significantly more (51.9%) PMA-induced NETs (8455.34 ± 689.84) comparing with those with LOW CTQ (5565.32 ± 385.97), $p < 0.001$.

CTQ was found to be negatively correlated with LPS-induced phagocytosis ($\rho = -0.63$, $p = 0.002$). These associations remained significant following adjustments for BMI and age (partial correlations). Subjects with HIGH CTQ had much lower LPS-induced phagocytosis (57.23) comparing with LOW CTQ group (78.8), $p < 0.05$.

CTQ scores were also found to correlate with the following plasma cytokines: TNF-α ($\rho = 0.51$, $p < 0.001$), IL-6 ($\rho = 0.48$, $p < 0.001$), IL-1β ($\rho = 0.59$, $p < 0.001$), IL-8 ($\rho = 0.60$, $p < 0.001$), and IL-10 ($\rho = 0.45$, $p = 0.001$). These associations remained significant following adjustments for BMI and age (partial correlations). Again, higher cytokine levels were found in the HIGH CTQ group comparing with LOW CTQ (all $p < 0.01$): TNF-α

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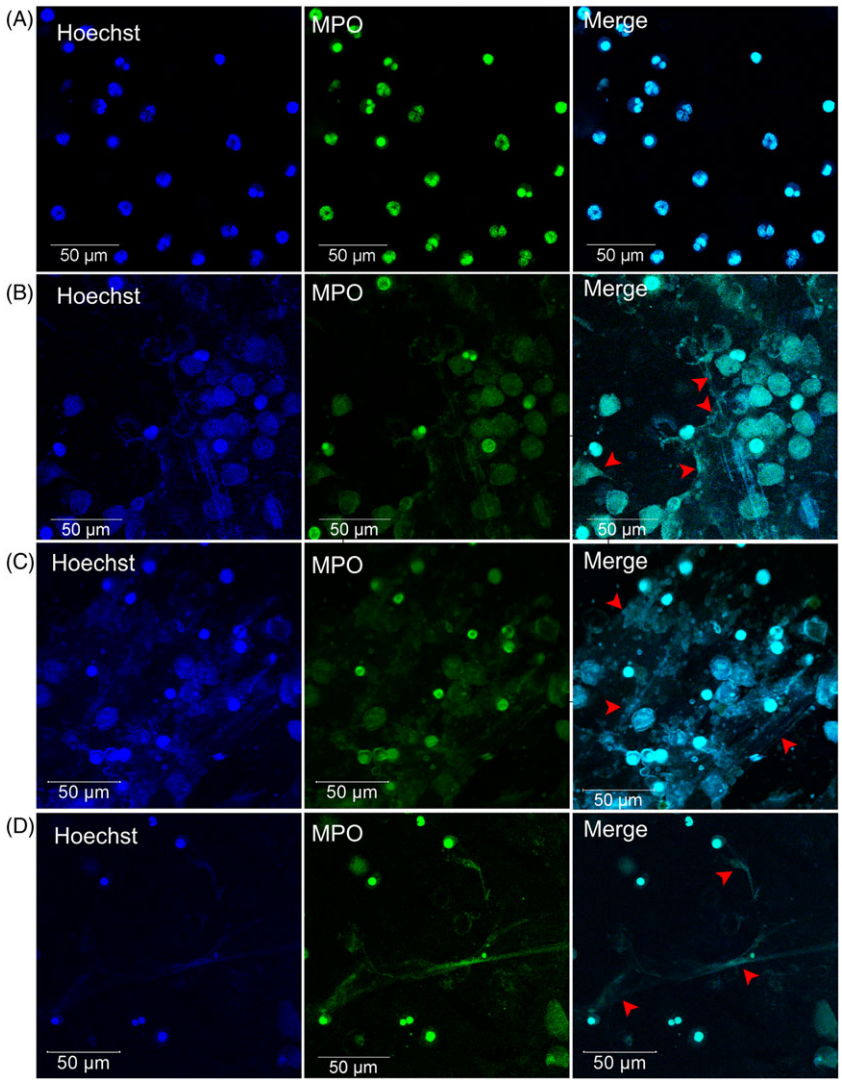

Fig. 4. Increased NETs formation at baseline in CUD. Isolated neutrophils were cultured with medium (**A:** HC, **C:** CUD) or stimulated with Phorbol Myristate Acetate (PMA, 100 nM) for 3h (**B:** HC, **D:** CUD). NETs are identified by extracellular DNA co-localized with MPO (indicated by red arrows). Images are representative of two independent experiments and were taken in confocal microscope. Scale bars = 50 µm.

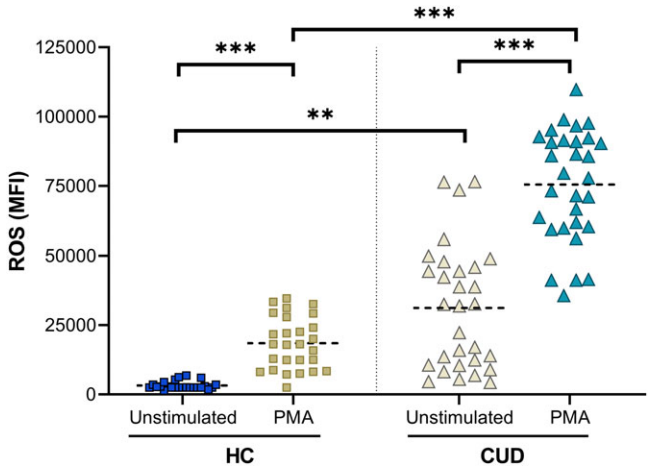

Fig. 5. Neutrophils display increased intracellular ROS generation in CUD. Circulating neutrophils were cultured with medium alone (unstimulated) or stimulated with PMA (100 nM). Following 1h of cell culture, intracellular ROS generation was determined by the oxidation of 0.5 µM CM-H2DCFDA to yield an intracellular fluorescent compound. Data were performed in triplicates of each group and represented as mean fluorescence intensity. Statistically significant differences are shown as $^{*}p < 0.05$, $^{**}p = 0.005$ and $^{***}p < 0.001$.

(median, 5.54 versus 3.67), IL-6 (median, 5.96 versus 2.96), IL-1β (mean, 12.54 versus 8.14), IL-8 (mean, 8.20 versus 4.64), and IL-10 (mean, 3.52 versus 2.79).

Discussion

Neutrophils are essential effector cells of innate immunity. However, when overactivated, they constitute a crucial inflammatory disease agent (Kolaczkowska and Kubes, 2013; Furman *et al.*, 2019). Cocaine smokers are known to have a high susceptibility to infection, perhaps because of exposure to pathogenic agents and drug components (including mixtures) that act directly or indirectly in immunity (Friedman *et al.*, 2003; Cabral, 2006; Brunt *et al.*, 2017). Among all users, women are the prominent group with intense craving sensation (Elman *et al.*, 2001) and consume more significant amounts of crack stones daily than men (Bertoni *et al.*, 2014). Therefore, we performed a comprehensive assessment of neutrophil-related inflammation, effector functions and signalling pathways, while also assessing important CUD-related clinical variables. The novelty of this study is the comprehensive analysis of neutrophil functions in CUD women, including formation of NETs, intracellular signalling pathways as well as peripheral inflammation.

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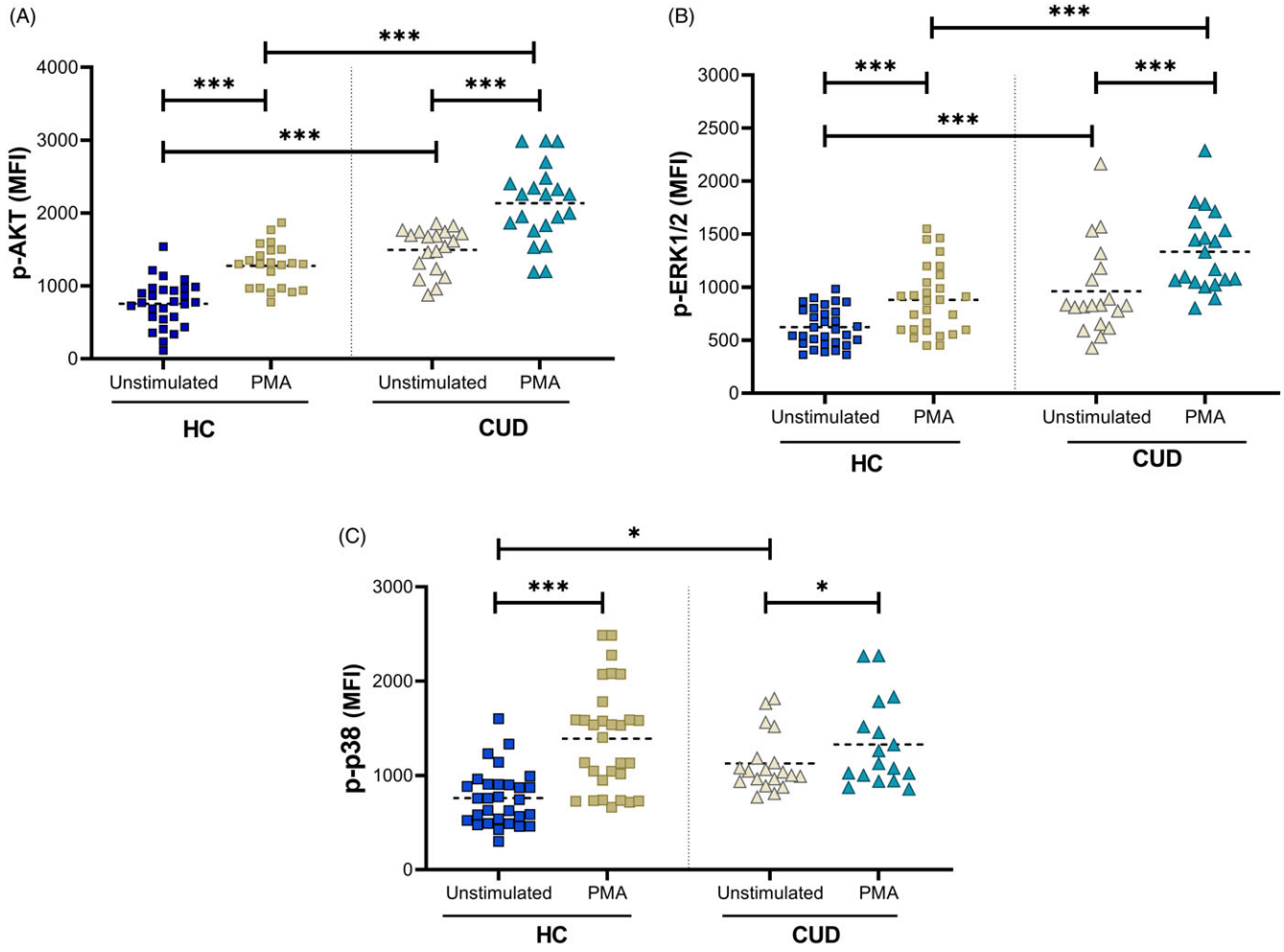

Fig. 6. CUD had activated AKT and MAPK at baseline and following stimulation. Neutrophils from HC ($n = 29$) and CUD ($n = 17$) groups were cultured with medium alone (unstimulated) or stimulated with PMA (100 nM) for 10 min at 37°C with 5% CO_2. Phosphorylated MAPKs were determined by flow cytometry. Data were performed in triplicates of each group and represented as mean fluorescence intensity. Statistically significant differences are shown as $^{*}p < 0.05$ and $^{***}p < 0.001$.

First, we demonstrated that CUD was associated with high levels of pro-inflammatory cytokines (IL-12p70, TNF-α, IL-6, IL-1β, and IL-8) as well as IL-10. These data are in line with previous studies indicating an immunological imbalance in CUD (Fox *et al.*, 2012; Levandowski *et al.*, 2014; Zaparte *et al.*, 2015; Levandowski *et al.*, 2016). IL-8 acts as chemokine in inflammation, promoting neutrophil influx to sites of infection and activating neutrophils (Bernhard *et al.*, 2021). IL-6 is a proposed biomarker for sepsis and septic shock, also related to its prognosis (Song *et al.*, 2019). Although IL-12 is critically involved with neutrophil activation and resistance to pathogens (Mehrpouya-Bahrami *et al.*, 2021), it has also been associated with polymicrobial sepsis (Moreno *et al.*, 2006). TNF-α is a pro-inflammatory cytokine that was found previously higher in cocaine users, being identified as predictor of a lifetime use of cocaine and withdrawal symptom severity (Levandowski *et al.*, 2014; Araos *et al.*, 2015). Therefore, acute abstinence is associated with higher levels of inflammatory-related cytokines, which may play a role in reinforcing negative behaviour effects of CUD, of note in craving and negative mood (Fox *et al.*, 2012). Compelling evidence indicates the roles of peripheral pro-inflammatory cytokines on depression, by altering brain metabolism of neurotransmitters involved in regulating mood (i.e., dopamine, glutamate, and serotonin) as well as by impairing neuroplasticity and cognition (Bauer and Teixeira, 2019). Also, circulating cytokines can reach the brain and cross the blood–brain barrier (BBB), activating microglia and hence contributing to neuroinflammation (Torres-Platas *et al.*, 2014; Miller and Raison, 2016) – another important mechanism involved in the pathophysiology of psychiatric disorders. During early abstinence, many subjects present a prodrome of cognitive and behavioural changes that are strikingly similar to those of sickness behaviour syndrome due to increased levels of pro-inflammatory cytokines (Dantzer *et al.*, 2008). Therefore, considering drug abuse from a chronic stress perspective, cytokine-related damage observed in chronic stress, neuroinflammation, and drug abuse may lead to sickness behaviour syndrome-like symptoms and open the door for relapse (Agarwal *et al.*, 2022). The elevated inflammatory profile may thus influence peripheral leukocytes as well as brain cells involved with changes in behaviour and related symptomatology in CUD. Meanwhile, IL-10 has anti-inflammatory and regulatory roles, contributing either to the pathogen clearance or by preventing tissue damage (Rasquinha *et al.*, 2021). In this study, higher levels of IL-10 could be produced as a potential negative feedback loop to refrain pro-inflammatory cytokines.

Phagocytosis was evaluated here as an important effector function of neutrophils. Smoked cocaine induced different outcomes

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in vivo, either acting as a stimulating factor for phagocytosis, as neutrophils from CUD patients were activated at baseline and following stimulation *in vitro*. Cytochalasin D inhibits the actin polymerisation, and it was efficient to impair the phagocytosis, of note in HC who were less activated at baseline than CUD patients. Further studies are necessary to explore the influence of CUD on different phagocytes (e.g., macrophages) as well as on antigen presentation to T cells.

NETs constitute an essential cellular response, involving DNA coated to granule proteins such as elastase and MPO, mediated against infections (Brinkmann *et al.*, 2004). Lood and colleagues identified that direct exposure to cocaine (or to its contaminant, levamisole) *in vitro* induces the formation of NETs in neutrophils from healthy donors (Lood and Hughes, 2017). Accordingly, we evaluated the impact of smoked cocaine in modulating NETs formation. With two different approaches, we demonstrated that CUD subjects had activated neutrophils producing high amounts of NETs, with similar baseline levels to those found in the stimulated HC cells. These CUD NETs contain both the DNA and MPO co-localized (Fig. 4). The MPO, expressed on NETs DNA decondensed chromatin fibres, is an antimicrobial protein and outbreaks from neutrophil azurophilic granules (Brinkmann and Zychlinsky, 2012; Saitoh *et al.*, 2012; Mutua and Gershwin, 2020; Yousefi *et al.*, 2020). The visible smaller number of cells (Fig. 4C) is likely to be derived from NETosis. One critical gap in our study is that we could not discriminate direct immune-related effects of cocaine. Future studies shall investigate whether our findings are replicated by direct action of purified cocaine on peripheral PMNs. In addition, we did not investigate the pyrolysis products generated by smoking cocaine (Cone *et al.*, 1994) and their potential cytotoxic effects on PMNs.

Previous studies have indicated that ROS is required for NETosis when cells are stimulated with PMA (Parker *et al.*, 2012; Yang *et al.*, 2016). Here, we found that cells from CUD subjects had increased intracellular ROS than HC, corroborating with a previous work (Lood and Hughes, 2017). In addition, NETs formation requires additional signalling including the activation of Akt and MAPKs ERK/p38. Indeed, the activation of Akt and MAPKs constitute downstream signals of NADPH Oxidase-derived ROS production (Hosseinzadeh *et al.*, 2016; Liu *et al.*, 2020; Wright *et al.*, 2021). Cells from CUD subjects had increased activation of Akt and MAPKs, which are essential steps involved in cell proliferation and survival (Akt) as well as in the NETs production. However, further pharmacological experiments are necessary to confirm the potential roles of ROS, Akt, and MAPKs in triggering NETosis in the CUD. For instance, the abrogation of NADPH/NOX, MAPKs, or PAD4 with specific inhibitors would have yielded more confirmatory data concerning the signalling pathways involved with enhanced NETosis in CUD.

Early life stress is recognised as a risk factor for the development of neuropsychiatric disorders and drug abuse in the adulthood. Childhood maltreatment is highly prevalent among cocaine users (Scheidell *et al.*, 2018), being a predictive risk factor for addiction. For instance, childhood sexual abuse exposure in women increases the risk of alcohol or illicit drug dependence 6.6-fold (Kendler *et al.*, 2000). CUD subjects with a history of childhood neglect exhibited more severe depressive and abstinence symptoms during drug withdrawal (Sanvicente-Vieira *et al.*, 2019). Furthermore, childhood maltreatment has been associated with chronic low-grade inflammation related with CUD in adulthood (Levandowski *et al.*, 2016), as cytokine imbalance elevates the chance of drug use in adulthood women (Coelho *et al.*, 2014). Crack cocaine users with and without prior ELS history may be clinically and immunologically distinct. Indeed, CUD subjects with a history of childhood maltreatment showed a greater increase of TNF-α levels following 2 weeks of crack cocaine abstinence (Levandowski *et al.*, 2016). In line with previous studies, we report here that CUD subjects show higher CTQ than HC, which were significantly associated with neutrophil activation and peripheral inflammation. Therefore, CUD and childhood trauma may have synergistic immunological effects by promoting inflammation and impairing cellular immunity to viral infections (Roth *et al.*, 2002; Slopen *et al.*, 2012; Tyrka *et al.*, 2013).

Finally, it is important to discuss some limitations of this study. First, the sample size is moderate but similar to previous studies in this area (Levandowski *et al.*, 2016; Zaparte *et al.*, 2019). It is also necessary to be careful in extrapolating our findings to males. For instance, women present higher vulnerability, worse clinical outcomes, and faster escalation to drug dependence (Sanvicente-Vieira *et al.*, 2019). We did not explore the potential immunomodulatory effects of long-term polydrug use profiles of CUD subjects. For instance, heavy alcohol and marijuana consumption may impact cell-mediated immunity and host defenses. Dysfunctional adaptive immune system was noted in alcohol-use dependents, exhibiting more activated CD8+ T cells compared to healthy subjects (Zuluaga *et al.*, 2017). Moreover, acute alcohol withdrawal has been associated with increased levels of pro-inflammatory cytokines (Adams *et al.*, 2020). It should be noted that we have explored here the systemic chronic effects of crack-cocaine addiction on isolated PMNs. In this context, there are several potential indirect effects associated with chronic addiction – including physiological changes (ex., endocrine changes), behavioural (e.g., stress), and lifestyle – that were not explored here. Considering drug abuse from a chronic stress perspective, a significant activation of the hypothalamic–pituitary–adrenal axis has been associated with craving and early relapse among individuals with substance-use disorders (Sinha *et al.*, 2003; Ligabue *et al.*, 2020). Increased cortisol levels have potent immunoregulatory actions, ranging from immunosuppressive to activated immune responses (Cain and Cidlowski, 2017). The constant exposure to high cortisol levels may lead to steroid resistance. In the lack of adequate inhibitory control of cortisol, the consequence is increased immune signalling as shown by increased levels of activated cells and proinflammatory cytokines (Bauer and Teixeira, 2019). Finally, individuals with CUD may develop negative health behaviours (e.g., a sedentary lifestyle, poor diet, poor sleep, and smoking) that can contribute to uncontrolled inflammation and depression. Taken together these limitations, it is important to highlight that this is an associative cross-sectional study, and inferences on the direct immunological effects of cocaine on the course of the addition cannot be made.

In conclusion, our study reinforces that CUD and early life stress are associated with activated neutrophils in an inflammatory environment. This could be of clinical relevance, as years of smoked cocaine was found to be negatively correlated to enhanced intracellular signalling, and severity of substance use (ASI) was correlated to NETs formation.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/neu.2023.11

Author contributors. RGO designed the study. AZ collected the blood samples. GAF performed the experiments. GAF, RGO, and MEB wrote the main manuscript text and prepared the figures. JBS and TWV helped with the

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statistical analyses. The manuscript was reviewed by all authors. All authors have contributed to the article and approved the submitted version.

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Conflict of interest. None.

Ethical statement. The protocol of this study was reviewed and approved by the Ethics committee of PUCRS. Each patient signed an informed consent form approved by the local institutional Review Board.

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