Received: 28 September 2019 | Revised: 28 February 2020 | Accepted: 4 March 2020
DOI: 10.1002/prp2.627

ORIGINAL ARTICLE

Neutrophil fluorescence in clozapine users is attributable to a 14kDa secretable protein

Sera A. J. de With[1] | **Wai H. Man**[2,3] | **Coen Maas**[4] | **Maarten ten Berg**[4] | **Wiepke Cahn**[1] | **Arnold C. Koekman**[4] | **Wouter W. van Solinge**[4] | **Tamar Tak**[5]

[1]Department of Psychiatry, University Medical Center Utrecht, Utrecht, The Netherlands

[2]Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht, The Netherlands

[3]Department of Clinical Pharmacy, Meander Medical Center, Amersfoort, The Netherlands

[4]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands

[5]Department of Respiratory Medicine, Laboratory of Translational Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Correspondence
Wouter W. van Solinge, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands.
Email: W.W.vanSolinge@umcutrecht.nl

Abstract

Clozapine is the only antipsychotic agent with demonstrated efficacy in refractory schizophrenia. However, use of clozapine is hampered by its adverse effects, including potentially fatal agranulocytosis. Recently, we showed an association between neutrophil autofluorescence and clozapine use. In this study, we evaluated the subcellular localization of clozapine-associated fluorescence and tried to elucidate its source. Neutrophils of clozapine users were analyzed with fluorescence microscopy to determine the emission spectrum and localization of the fluorescence signal. Next, these neutrophils were stimulated with different degranulation agents to determine the localization of fluorescence. Lastly, isolated neutrophil lysates of clozapine users were separated by SDS-PAGE and evaluated. Clozapine-associated fluorescence ranged from 420 nm to 720 nm, peaking at 500-550 nm. Fluorescence was localized in a large number of small loci, suggesting granular localization of the signal. Neutrophil degranulation induced by Cytochalasin B/fMLF reduced fluorescence, whereas platelet-activating factor (PAF)/fMLF induced degranulation did not, indicating that the fluorescence originates from a secretable substance in azurophilic granules. SDS-PAGE of isolated neutrophil lysates revealed a fluorescent 14kDa band, suggesting that neutrophil fluorescence is likely to be originated from a 14kDa protein/peptide fragment. We conclude that clozapine-associated fluorescence in neutrophils is originating from a 14kDa soluble protein (fragment) present in azurophilic granules of neutrophils. This protein could be an autofluorescent protein already present in the cell and upregulated by clozapine, or a protein altered by clozapine to express fluorescence. Future studies should further explore the identity of this protein and its potential role in the pathophysiology of clozapine-induced agranulocytosis.

Abbreviations: ANC, Absolute Neutrophil Count; CIA, Clozapine-Induced Agranulocytosis; DAPI, 4′,6-diamidino-2-phenylindole; FACS, Fluorescence-Activated Cell Sorting; FAD, Flavin Adenine Dinucleotide; FDA, Food and Drug Administration; fMLF, N-formyl-methionyl-leucyl-phenylalanine; FSC, Forward Scatter; GWAS, Genome-Wide Association Study; H_2O_2, Hydrogen Peroxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HLA-B, Major Histocompatibility Complex, Class I, B; HLA-DQB1, Major Histocompatibility Complex, Class II, DQ beta 1; HOCl, Hypochlorous Acid; MHC, Major Histocompatibility Complex; MPO, Myeloperoxidase; PAF, Platelet Activating Factor; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; SSC, Side Scatter.

Sera A. J. de With and Wai H. Man authors are contributed equally.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2020 The Authors. *Pharmacology Research & Perspectives* published by John Wiley & Sons Ltd, British Pharmacological Society and American Society for Pharmacology and Experimental Therapeutics.

KEYWORDS
azurophilic granules, clozapine, neutrophils

1 | INTRODUCTION

Clozapine is an effective antipsychotic drug, developed in the 1950s. It is the only antipsychotic drug with proven superior efficacy in treatment-resistant schizophrenia[1] and FDA approval for treatment of suicidal ideation and behavior in schizophrenia patients.[2,3] However, despite its effectiveness, physicians are hesitant to prescribe the drug due to the risk of development of severe adverse effects,[4] most notably being clozapine-induced agranulocytosis (CIA) occurring in approximately 0.7% of patients.[5,6] Agranulocytosis is hallmarked by a low absolute neutrophil count (ANC $< 500 \times 10^9$/L), severely weakening the immune system and, if left untreated, can be fatal.[7,8] To prevent this serious adverse effect, a rigorous monitoring program was established. Patients are required to undergo weekly blood draws to monitor neutrophil count for the first 18 weeks of treatment and every 4 weeks thereafter for the remainder of the treatment duration. When the neutrophil count decreases to neutropenia (ANC $< 1500 \times 10^9$/L), treatment with clozapine is discontinued. The monitoring program has successfully lowered the prevalence of clozapine-induced agranulocytosis.[9]

To date, the etiology of CIA remains unknown. There are two hypotheses involving the etiology of CIA. First, CIA is thought to have an immunological component[8,10]; when patients with a history of CIA are challenged with clozapine a second time, they develop CIA more often and faster, suggesting sensitization of the immune system.[10] Additionally, there are some (inconsistent) findings of antibodies against myeloperoxidase (MPO)—an enzyme secreted by neutrophils—, also suggesting immune mediation of the response. Second, clozapine can be oxidized by a combination of MPO and H_2O_2 to a reactive nitrenium ion that is thought to have a direct toxic effect on the neutrophil, although this has only been shown in vitro.[11-14]

The two hypotheses are not mutually exclusive; it is possible that the reactive clozapine nitrenium ion irreversibly binds to neutrophils, resulting in altered membrane structure and therefore can act as a hapten in the production of antibodies.[15]

Still, it remains unknown how CIA would affect only some patients and not all. An explanation for this might lay in the identification of a genetic component to CIA: a recent genome-wide association study (GWAS) has identified two alleles with genome-wide significance associated with CIA.[16] These two alleles (HLA-DQB1 and HLA-B) reside in the major histocompatibility complex (MHC), a region coding for the human leucocyte antigens (HLA), proteins essential for antigen presentation to the adaptive immune system and subsequent clearance of pathogens. Both proteins can probably be expressed on neutrophils,[17,18] however, the mechanism by which these alleles predispose for CIA remains unknown. In addition, the specificity and sensitivity of the genetic markers associated with CIA up to now do not make them suitable to use for a clinical predictor test. Such a (genetic) predictor test would be of great clinical significance.

Understanding of the mechanisms involved in the pathogenesis of CIA is of obvious clinical importance. Recently, our group found a significant association between neutrophil fluorescence and clozapine use. In this study we showed that neutrophil fluorescence was significantly higher in clozapine users than in nonusers.[19] Although the autofluorescence was observed in neutrophils of all clozapine users and therefore not necessarily related to CIA, studying the origin of this fluorescence could give insight in the way clozapine affects the neutrophil and if and how this could be related to CIA.

The present study aimed to explore clozapine-associated fluorescence of neutrophils in further detail by evaluating its subcellular localization and elucidating its source. We show that the fluorescence spectrum of clozapine-associated autofluorescence of neutrophils ranges from 420 to 720nm with a peak near 520 nm and that the fluorescence follows a granular pattern. Additionally, stimulation and degranulation of the neutrophils induced a significant decrease in fluorescence signal, also suggesting a granular localization of the fluorescence. Lysates of these neutrophils show a fluorescent signal at 14kDa indicating the signal is originating from a protein or protein fragment of 14kDa.

2 | MATERIALS AND METHODS

2.1 | Reagents and antibodies

Shock buffer consisted of 155 mmol/L Ammonium Chloride, 10 mmol/L Potassium Bicarbonate and 0.1 mmol/L EDTA. The SDS lysis buffer contained 0.5% (w/v) SDS, 0.05 M Tris·Cl, and 1 mmol/L fresh dithiothreitol (DTT). Phosphate-buffered saline (PBS) was supplemented with 0.32% trisodium citrate and 10% pasteurized plasma solution (Sanquin, Amsterdam, the Netherlands) to create PBS2+. Functional assays were performed in HEPES buffer (20 mmol/L HEPES, 132 mmol/L NaCl, 6 mmol/L KCl, 1 mmol/L MgSO4, and 1.2 mmol/L KH2PO4), supplemented with 5 mmol/L glucose, 1.0 mmol/L CaCl2, and 0.5% Human Serum Albumin. Antibodies for CD14 (PE-Cy™7, clone M5E2, dilution 1:50), CD16 (APC H7, clone 3G8, dilution 1:50), and CD63 (PE, clone H5C6, 1:50) were obtained from BD Biosciences (San Diego, CA, USA). Platelet-activating factor 16 (PAF) was from Calbiochem (Darmstadt, Germany), and formyl-methionyl-leucyl-phenylalanine (fMLF) and cytochalasin B were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 | Subjects and study design

Remnant blood samples of psychiatric patients using clozapine (N = 7) were collected at the psychiatry outpatient clinic of the University Medical Center Utrecht (UMC Utrecht). Samples consisted of residuals

from monthly checkups and were anonymized for the purpose of the study. Therefore, the study was exempted from obtaining written informed consent according to Dutch law. The study was approved by the local Medical Research and Ethics Committee and Biobank. Patients using clozapine with a dose higher than 100 mg/day for longer than 1 month were included in the study, insuring clozapine-induced fluorescence had reached detectable and stable levels.[19]

Healthy volunteer samples were obtained from the bloodbank Mini Donor Dienst of the UMC Utrecht after written informed consent was obtained in accordance to the declaration of Helsinki.

2.3 | Confocal microscopy of neutrophils

Blood samples of patients (N = 2) and healthy volunteers (N = 2) were used to prepare blood films for evaluation with confocal microscopy. First, the samples were fixed with 4% paraformaldehyde in PBS. The fixed cells were transferred and attached to a glass slide by Cytospin centrifugation (Thermo Scientific, Waltham, MA, USA) for evaluation with a Zeiss LSM710 (Carl Zeiss, Sliedrecht, the Netherlands) confocal laser scanning microscope. Using a multilaser flow cytometer, we observed clozapine-associated fluorescence to be stronger when excited with a 405 nm laser instead of a 488 nm laser as published previously[19] (Figure S1). Therefore, clozapine-associated fluorescence emission spectrum was examined using lambda stacks and violet laser light exciting at 405nm, measuring fluorescence emission intensity from 420 to 720 nm in 3.2 nm steps. Fluorescence was imaged using excitation of 405 nm violet laser light and the standard DAPI (4',6-diamidino-2-phenylindole) filter set.

2.4 | Neutrophil sorting, immunostaining, and degranulation assays

Whole blood samples from 5 patients and 2 healthy volunteers were treated with erythrocyte shock buffer and stained with CD14 and CD16. Neutrophils were sorted based on FSC/SSC (forward-scattered light; FSC and side-scattered light; SSC) and $CD16^{high}$/$CD14^{neg}$ with a BD FACSAria III™ (BD Biosciences, San Jose, CA, USA). Samples were typically > 99% pure as determined by re-analysis with the BD FACSAria III™ and microscopic evaluation of May-Grünwald Giemsa-stained cytospins. Sorted neutrophils were kept on ice in HEPES buffer or preincubated for 5 minutes with either cytochalasin B (5 µg/mL) or PAF (10^{-6} M) followed by stimulation with 10^{-6} M fMLF for 15 minutes to determine localization of the fluorescence signal.

The remainder of the sample was immunostained for CD63 in PBS2 + for 20 minutes to confirm successful neutrophil degranulation.

After fixation with 1% formaldehyde, samples were analyzed on a Beckman Coulter Gallios™ within 24 hours. Fluorochromes were chosen for minimal bleed-through from clozapine signal.

2.5 | Isolation of neutrophils (for use in SDS-PAGE assay)

Mononuclear cells were depleted from whole blood by centrifugation (1500 rpm for 5 minutes (450G)) over isotonic Ficoll (GE Healthcare Life Sciences) to obtain a neutrophil-containing erythrocytes layer. Isotonic lysis of remaining erythrocytes took place for 5 minutes at room temperature with shock buffer. Neutrophils were washed and resuspended in shock buffer, followed by boiling the samples for 5 minutes.

2.6 | SDS-PAGE assays

Neutrophil lysates were prepared from the isolated neutrophils using sodium dodecyl sulfate (SDS) lysis buffer. Samples prepared with identical neutrophil numbers were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bolt™ 4%-12% Bis-Tris Plus gel (Thermo Scientific, Waltham, MA, USA) following manufacturer's instructions. Gels were scanned at different wavelengths using a Typhoon 9400 Scanner (GE Healthcare, UK).

2.7 | Data and FACS analyses

Images were analyzed with ImageJ 1.47T (National Institutes of Health, Bethesda, MD, USA) and FACS data were analyzed with FCS Express 5.01 (De Novo Software, Glendale, CA, USA). Statistical analysis was performed with the free software environment R.[20] A Wilcoxon signed-rank test was used to compare groups, a *P*-value of .05 or lower was considered statistically significant.

3 | RESULTS

3.1 | Localization of clozapine-associated fluorescence

To confirm previous findings of autofluorescence, specifically observed in neutrophils of clozapine users,[19] we started by determining the emission spectrum of clozapine-associated neutrophil fluorescence. Clozapine-associated fluorescence showed a broad emission spectrum, with peak emission around 520 nm (Figure 1). Therefore, we chose to use FL-10 fluorescence channel of the FACS (excitation 405 nm, emission window ranging from 530 to 570 nm) in our current study. It should be noted that the emission spectrum of most molecules is independent of the excitation wavelength and excitation with either 405 or 488 nm will most probably result in a similar emission spectrum.[21]

In accordance with our previous findings, we observed a high fluorescence intensity in patient samples signal when compared to

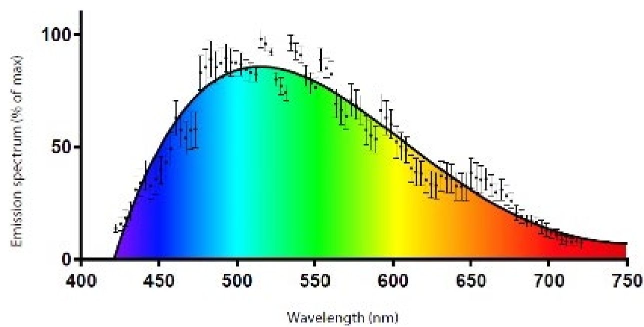

FIGURE 1 Fluorescence spectrum of clozapine-associated autofluorescence in neutrophils. The emission spectrum of clozapine-associated autofluorescence in neutrophils was determined with a confocal scanning microscope in lambda mode (Zeiss LSM710) measuring emission from 420 to 720nm in 3.2nm steps. The (unstained) sample was excited with laser light at 405nm. An emission peak was found near 520 nm indicating green fluorescence. Depicted dots and error bars within the figure are averages and 95% standard deviations, respectively (n = 3)

controls (Figure 2). Moreover, microscopy images showed that fluorescence was specifically located in the neutrophils of the patient samples. The fluorescence followed a perinuclear uneven staining pattern, reminiscent of granular staining.

3.2 | Fluorescence intensity decreases after neutrophil degranulation

To demonstrate that the fluorescence signal originates from granules isolated neutrophils from patient samples were stimulated with degranulation-inducing agents PAF/fMLF and cytochalasin B/fMLF. The strong degranulation-inducing agent cytochalasin B/fMLF stimulates the neutrophils to release all types of granules (secretory, gelatinase containing, specific and azurophilic), achieving complete degranulation, while the moderately degranulation-inducing agent PAF/fMLF induces release of most types of granules, except azurophilic granules.[22]

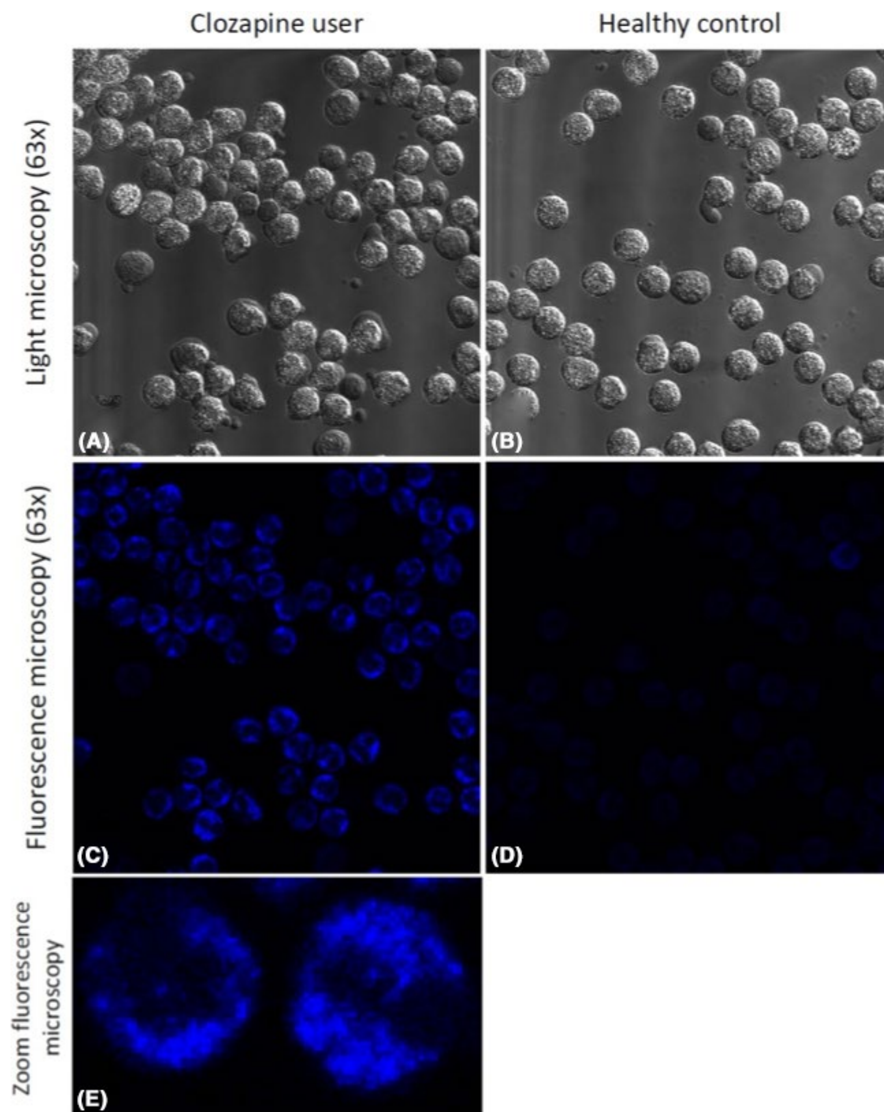

FIGURE 2 Confocal fluorescence microscopy images of unstained blood samples from a clozapine user and a healthy control. Blood samples of a clozapine user (A,C,E) and a healthy control (B,D) were fixed with 4% paraformaldehyde in PBS and attached to a glass slide for confocal microscopy imaging. Samples were excited at 405 nm violet laser light and fluorescence was detected using the standard DAPI (4′,6-diamidino-2-phenylindole) filter set (C,D,E). Neutrophils of clozapine user showed high fluorescence intensity, whereas this signal was absent in the control sample. The fluorescent neutrophils show a perinuclear-staining pattern (E)

Before and after stimulation, neutrophil autofluorescence (Figures 3 and 4) and a protein marker (CD63, a lysosomal protein residing in the azurophilic granules[23]) for degranulation of the neutrophil azurophilic granules were monitored. A significant increase in the degranulation marker ($P < .015$) was found, indicating successful stimulation (Figure 4A).

A significant decrease in clozapine-associated fluorescent signal after stimulation with cytochalasin B/fMLF ($P = .015$) was observed, but not after stimulation with PAF/fMLF ($P = .81$) (Figure 4B). This finding suggests that the fluorescent signal originates from the azurophilic granules.

3.3 | Neutrophil fluorescence originates from a 14kDa protein or peptide fragment

To identify the source of the observed fluorescence, isolated neutrophil lysates were separated by SDS-PAGE and were evaluated under a laser scanner (Figure 5). The gels were excited at 532 nm and emission was detected at 555 nm. A fluorescent 14 kDa band was detected in the patient samples, whereas this band was absent in the healthy control samples. Neutrophil fluorescence is likely to be originated from a 14 kDa protein or protein fragment.

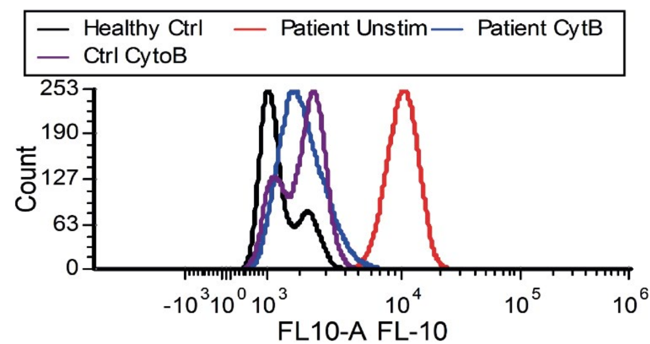

FIGURE 3 Flowcytometry histograms of neutrophil fluorescence before and after neutrophil stimulation using Cytochalasin B/fMLF. Neutrophil autofluorescence was measured in a patient and a control sample in the FL10 channel (excitation: 405nm, emission: 550/40nm). Among the unstimulated samples (black and red) an increased fluorescence is seen only in the patient sample (red). Histograms show a decrease in neutrophil fluorescence after stimulation of the patient sample (blue)

4 | DISCUSSION

With this study we have further explored clozapine-associated neutrophil fluorescence in clozapine users to gain more insight into the source and localization of the signal. We have confirmed previous findings of enhanced fluorescence following clozapine exposition[19] and further defined the fluorescent emission spectrum of clozapine-associated neutrophil autofluorescence. Microscopy showed that the fluorescence follows a granular staining pattern. Stimulating neutrophils from clozapine users to release their granules showed a significant decrease in signal with the release of azurophilic granules. Lastly, with SDS-PAGE of the neutrophil lysates we detected a 14kDa fluorescent band, suggesting that the fluorescent peptide originates from a 14kDa protein or protein fragment.

4.1 | Clozapine-associated fluorescence originates from the granules

The granular pattern of clozapine-associated fluorescence and the signal reduction after release of the granules suggest that the fluorescence arises from the granules in the neutrophil. The fact that the fluorescence signal decreases significantly after cytochalasin B exposure and not after PAF indicates that this signal mainly arises from the azurophilic granules. This azurophilic granule contains a number of oxidizing agents, including myeloperoxidase (MPO).[24] MPO is thought to play a role in the formation of the clozapine nitrenium ion,[12,25] an ion possibly toxic to neutrophils,[12,13] and most abundantly present in azurophilic granules of neutrophils. Therefore, clozapine-associated fluorescence might be linked with the formation of the clozapine nitrenium ion and indirectly with CIA.

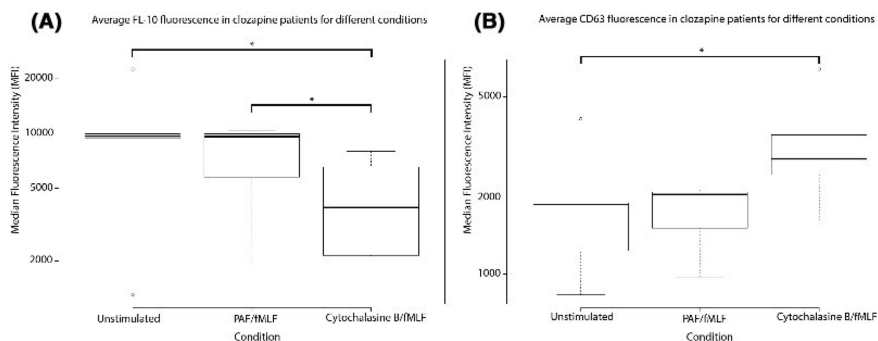

FIGURE 4 Effects of neutrophil stimulation on neutrophil fluorescence and CD63 expression in clozapine users. (A) Neutrophil stimulation of clozapine users (N = 7) with cytochalasin B/fMLF significantly reduced fluorescence intensity ($P = .015$), while stimulation with PAF/fMLF did not ($P = .81$). (B) Shows a significant increase in CD63 ($P = .015$)—the degranulation marker of azurophilic granules—after incubation with degranulation agents (Cytochalasin B and PAF), indicating successful neutrophil stimulation and degranulation

4.2 | The fluorescent molecule is most likely (bound to) a 14 kDa granular protein

The results from the performed SDS-PAGE analyses showed that clozapine-associated fluorescence originates from a protein (or protein fragment) of approximately 14kDa. As clozapine itself is not a peptide, but a small molecule, it is not the direct origin of the fluorescent band. The signal must therefore be originating from a protein with added clozapine (or metabolite), from increased expression of an autofluorescent protein, or a combination of the two. The decrease in fluorescence signal after neutrophil stimulation suggests that the signal originates from a soluble protein, as a membrane bound protein would show an increased fluorescence on the outer membrane of the cell, but not a loss in overall fluorescence (which we did not observe).

We cannot exclude the possibility that neutrophil fluorescence results from oxidized flavoproteins. These are proteins containing a flavin adenine dinucleotide (FAD) group, making the protein autofluorescent when oxidized.[26] It is possible that one of these oxidized flavoproteins is responsible for the clozapine-associated fluorescence observed by our group, since the emission/excitation spectrum (measured at 488/530nm) is very similar to the spectrum we have observed.

It is also possible that clozapine (or one of its metabolites) alters an existing protein to exhibit a fluorescent signal. Since our results indicate the signal originates from the granules, it must be a protein present in the granules. A potential origin of the fluorescent 14kDa band is the light chain of MPO, which is 14kDa under reducing conditions like in an SDS-PAGE gel,[27] resides in neutrophil azurophilic granules and secreted during neutrophil degranulation.[28] We have used a published list of granular proteins[29] to select proteins of approximately 14kDa (Supplement Material, Table 1). If we assume that the fluorescence is arising from the azurophilic granules and the protein is not degraded by enzymes, the list of candidate proteins is reduced to Brain Protein I3 and NADH dehydrogenase (ubiquinone). Although it is tempting to hypothesize of the possible role of these proteins, this would be speculative and future studies have to reveal the nature of this protein.

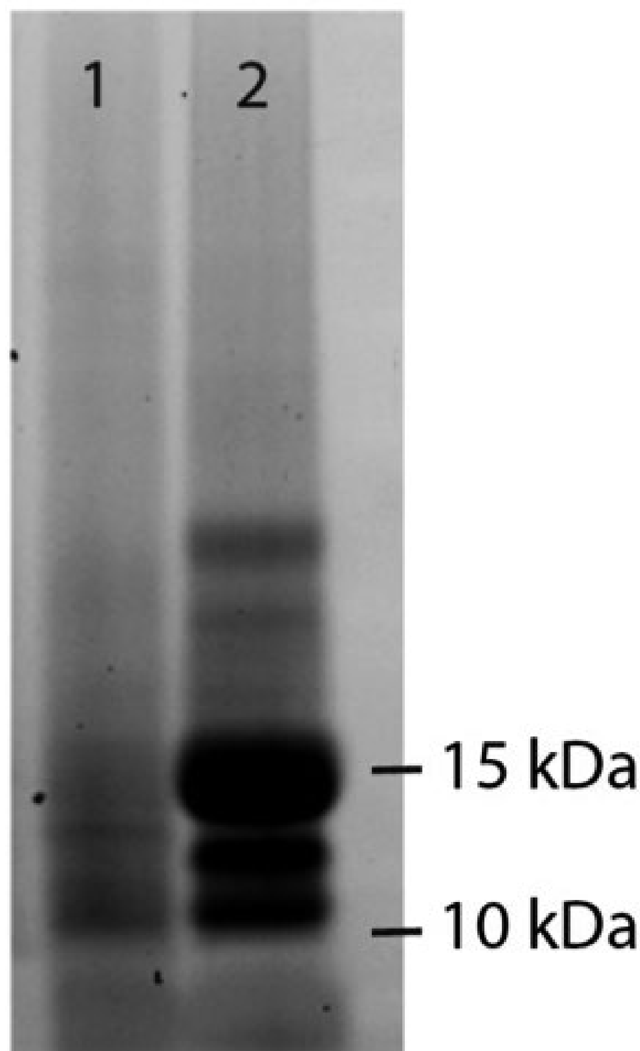

FIGURE 5 SDS-PAGE of isolated peripheral blood mononuclear cells and neutrophil lysates from samples of clozapine users and healthy controls. Isolated granulocytes of a healthy control (sample 1) and a clozapine user (sample 2) were separated by SDS-PAGE and evaluated under a laser scanner (excited at 532nm, emission at 555nm). A fluorescent protein band of nearly 14 kDa was observed in clozapine sample (sample 2), whereas this band was absent in the healthy control (sample 1)

4.3 | Implications of the fluorescence finding

The finding of clozapine-associated fluorescence of neutrophils from clozapine users could be a mere coincidence without clinical meaning. It is, however, likely that this finding could be exploited in clinical practice. The signal could be used as a biomarker for long-term adherence, as the half-life of enhanced fluorescence in clozapine users was estimated on 230 hours.[30] A marker to evaluate adherence would be highly beneficial in this specific patient population, as medication nonadherence is the major cause of treatment failure in schizophrenia patients.[31]

It is possible that the clozapine-associated fluorescence in neutrophils provides a (small) piece of the CIA puzzle. Considering the half-life of FL3 fluorescence, and the long lag between intake of clozapine and neutrophil fluorescence being observed, the fluorescence could be indication of an early effect on the maturation process of a neutrophil in the bone marrow. It is possible that clozapine affects the maturation process of the neutrophil, resulting in slightly damaged or activated neutrophils, or in neutrophils that survive for a shorter period in the bloodstream, thus requiring an increased production. Previous studies showing direct toxicity of the clozapine nitrenium ion to neutrophils and bone marrow precursors are in agreement with this hypothesis.[12,15]

We hypothesize that clozapine alters the balance between production and degradation of neutrophils in a subtle way. This

fragile balance in turn might be more easily disturbed, leading to clozapine-induced agranulocytosis in only a small number of patients. This hypothesis is supported by Fehsel et al who reported that blood of CIA patients who underwent agranulocytosis showed enhanced apoptosis expression markers and intracellular superoxide production compared to clozapine users without agranulocytosis.[32]

This study has further explored clozapine-associated neutrophil autofluorescence by investigating the location and origin of this fluorescence. We conclude that the fluorescence originates from neutrophil azurophilic granules. Additionally, this fluorescence originates from a protein or protein fragment of approximately 14kDa. These findings could provide a piece of the clozapine-induced agranulocytosis puzzle, however, future studies are necessary to further explore the clinical importance of clozapine-associated neutrophil fluorescence and its role in clozapine-induced agranulocytosis and leukopenia.

ACKNOWLEDGMENTS

Our gratitude goes to Dr EB Compeer and Drs. S van Staveren for their help with the confocal microscope. We would like to thank Prof. RA Ophoff for helpful discussions.

AUTHOR CONTRIBUTIONS

All authors contributed substantially to the scientific process leading up to the writing of the present article. Authors SdW, WHM, CM, WvS, and TT made the conception and design of the study. The performance of the data analysis/interpretation was done by SdW, WHM, CK, CM, and TT. SdW and WHM wrote the first draft of the manuscript. Critical revisions of the manuscript were made by MtB, WC, CM, TT, and WvS. All authors contributed to and have approved the final manuscript. The authors declare no conflict of interest and this study was not funded by any sources.

DATA ACCESSIBILITY

There is no public data available at the moment.

ORCID

Sera A. J. de With https://orcid.org/0000-0003-3825-3348

REFERENCES

1. Kane J. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. *Arch Gen Psychiatry*. 1988;45:789-796. https://doi.org/10.1001/archpsyc.1988.01800330013001
2. Administration U states F and D. FDA Label Clozapine. 1989.
3. Meltzer HY, Alphs L, Green AI, et al. Clozapine treatment for suicidality in schizophrenia: International Suicide Prevention Trial (InterSePT). *Arch Gen Psychiatry*. 2003;60:82-91.
4. Horvitz-Lennon M, Donohue JM, Domino ME, Normand SL. Improving quality and diffusing best practices: the case of schizophrenia. *Health Aff*. 2009;28:701-712. https://doi.org/10.1377/hlthaff.28.3.701
5. Atkin K, Kendall F, Gould D, Freeman H, Liberman J, O'Sullivan D. Neutropenia and agranulocytosis in patients receiving clozapine in the UK and Ireland. *Br J Psychiatry*. 1996;169:483-488. https://doi.org/10.1192/bjp.169.4.483
6. Alvir JM, Lieberman JA, Safferman AZ, Schwimmer JL, Schaaf JA. Clozapine-induced agranulocytosis. Incidence and risk factors in the United States. *N Engl J Med*. 1993;329:162-167. https://doi.org/10.1056/NEJM199307153290303
7. Idänpään-Heikkilä J, Alhava E, Olkinuora M, Palva I. Letter: clozapine and agranulocytosis. *Lancet (London, England)*. 1975;2:611.
8. Honigfeld G, Arellano F, Sethi J, Bianchini A, Schein J. Reducing clozapine-related morbidity and mortality: 5 years of experience with the Clozaril National Registry. *J Clin Psychiatry*. 1998;59(Suppl 3):3-7.
9. Regen F, Herzog I, Hahn E, et al. Clozapine-induced agranulocytosis: evidence for an immune-mediated mechanism from a patient-specific in-vitro approach. *Toxicol Appl Pharmacol*. 2016;316:1-29. https://doi.org/10.1016/j.taap.2016.12.003
10. Hinze-Selch D, Becker EW, Stein GM, et al. Effects of clozapine on in vitro immune parameters: a longitudinal study in clozapine-treated schizophrenic patients. *Neuropsychopharmacology*. 1998;19:114-122. https://doi.org/10.1016/S0893-133X(98)00006-2
11. Maggs JL, Williams D, Pirmohamed M, Park BK. The metabolic formation of reactive intermediates from clozapine, a drug associated with agranulocytosis in man. *J Pharmacol Exp Ther*. 1995;275:1463-1475.
12. Williams DP, Pirmohamed M, Naisbitt DJ, Maggs JL, Park BK. Neutrophil cytotoxicity of the chemically reactive metabolite(s) of clozapine: possible role in agranulocytosis. *J Pharmacol Exp Ther*. 1997;283:1375-1382.
13. Guest I, Sokoluk B, MacCrimmon J, Uetrecht J. Examination of possible toxic and immune mechanisms of clozapine-induced agranulocytosis. *Toxicology*. 1998;131:53-65.
14. Manu P, Sarpal D, Muir O, Kane JM, Correll CU. When can patients with potentially life-threatening adverse effects be rechallenged with clozapine? A systematic review of the published literature. *Schizophr Res*. 2012;134:180-186. https://doi.org/10.1016/j.schres.2011.10.014
15. Liu ZC, Uetrecht JP. Clozapine is oxidized by activated human neutrophils to a reactive nitrenium ion that irreversibly binds to the cells. *J Pharmacol Exp Ther*. 1995;275:1476-1483.
16. Johnston A, Uetrecht J. Current understanding of the mechanisms of idiosyncratic drug-induced agranulocytosis. *Expert Opin Drug Metab Toxicol*. 2014;72:1-15. https://doi.org/10.1517/17425255.2015.985649
17. Fanger NA, Liu C, Guyre PM, et al. Activation of human T cells by major histocompatability complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid. *Blood*. 1997;89:4128-4135.
18. Vono M, Lin A, Norrby-Teglund A, Koup RA, Liang F, Loré K. Neutrophils acquire the capacity for antigen presentation to memory CD4 $^{+}$ T cells in vitro and ex vivo. *Blood*. 2017;129:1991-2001. https://doi.org/10.1182/blood-2016-10-744441
19. Man WH, ten Berg M, Wilting I, et al. Fluorescence of neutrophil granulocytes as a biomarker for clozapine use. *Eur Neuropsychopharmacol*. 2013;23:1408-1413. https://doi.org/10.1016/j.euroneuro.2013.08.005
20. R Core Team. *R: A Language and Environment for Statistical Computing*. 2013.
21. Kln P, Wirz J. *Photochemistry of Organic Compounds: From Concepts to Practice*. Chichester, UK: John Wiley & Sons, Ltd; 2009. https://doi.org/10.1002/9781444300017
22. Bentwood BJ, Henson PM. The sequential release of granule constitutents from human neutrophils. *J Immunol*. 1980;124:855-862.
23. Naegelen I, Beaume N, Plançon S, Schenten V, Tschirhart EJ, Bréchard S. Regulation of neutrophil degranulation and cytokine secretion: a novel model approach based on linear fitting. *J Immunol Res*. 2015;2015:1-15. https://doi.org/10.1155/2015/817038

24. Klebanoff SJ. Myeloperoxidase: friend and foe. *J Leukoc Biol*. 2005;77:598-625. https://doi.org/10.1189/jlb.1204697
25. Williams DP, Pirmohamed M, Naisbitt DJ, Uetrecht JP, Park BK. Induction of metabolism-dependent and -independent neutrophil apoptosis by clozapine. *Mol Pharmacol*. 2000;58:207-216.
26. Kindzelskii A, Petty HR. Fluorescence spectroscopic detection of mitochondrial flavoprotein redox oscillations and transient reduction of the NADPH oxidase-associated flavoprotein in leukocytes. *Eur Biophys J*. 2004;33:291-299. https://doi.org/10.1007/s00249-003-0361-4
27. Lobach AR, Uetrecht J. Involvement of myeloperoxidase and NADPH oxidase in the covalent binding of amodiaquine and clozapine to neutrophils: implications for drug-induced agranulocytosis. *Chem Res Toxicol*. 2014;27:699-709. https://doi.org/10.1021/tx500019u
28. Nauseef WM, Malech HL. Analysis of the peptide subunits of human neutrophil myeloperoxidase. *Blood*. 1986;67:1504-1507.
29. Rørvig S, Østergaard O, Heegaard NHH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. *J Leukoc Biol*. 2013;94:711-721. https://doi.org/10.1189/jlb.1212619
30. Man WH, Pérez-Pitarch A, Wilting I, et al. Development of a nomogram for the prediction of long-term adherence to clozapine therapy using neutrophil fluorescence. (in submission). 2017.
31. Valenstein M, Blow FC, Copeland LA, et al. Poor antipsychotic adherence among patients with schizophrenia: medication and patient factors. *Schizophr Bull*. 2004;30:255-264.
32. Fehsel K, Loeffler S, Krieger K, et al. Clozapine induces oxidative stress and proapoptotic gene expression in neutrophils of schizophrenic patients. *J Clin Psychopharmacol*. 2005;25:419-426.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: de With SAJ, Man WH, Maas C, et al. Neutrophil fluorescence in clozapine users is attributable to a 14kDa secretable protein. *Pharmacol Res Perspect*. 2020;e00627. https://doi.org/10.1002/prp2.627