MONITORING PATIENTS USING CLOZAPINE

Wai Hong Man

Colofon

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- Department of Clinical Chemistry and Haematology of the University Medical Centre Utrecht, the Netherlands
- Department of Psychiatry of the University Medical Centre Utrecht, the Netherlands
- Department of Clinical Pharmacy of the Northwest Ziekenhuisgroep, Alkmaar, the Netherlands
- Department of Clinical Pharmacy of Diakonessenhuis, Utrecht, the Netherlands
- Division of Pharmacoepidemiology and Clinical Pharmacology of the Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, the Netherlands

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MONITORING PATIENTS USING CLOZAPINE

Het monitoren van patiënten die clozapine gebruiken

(met een samenvatting in het Nederlands)

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Promotoren

Prof.dr. A.C.G. Egberts
Prof.dr. W.W. van Solinge

Co-promotoren

Dr. I. Wilting
Dr. E.R. Heerdink

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1

General introduction, objectives and outline of the thesis

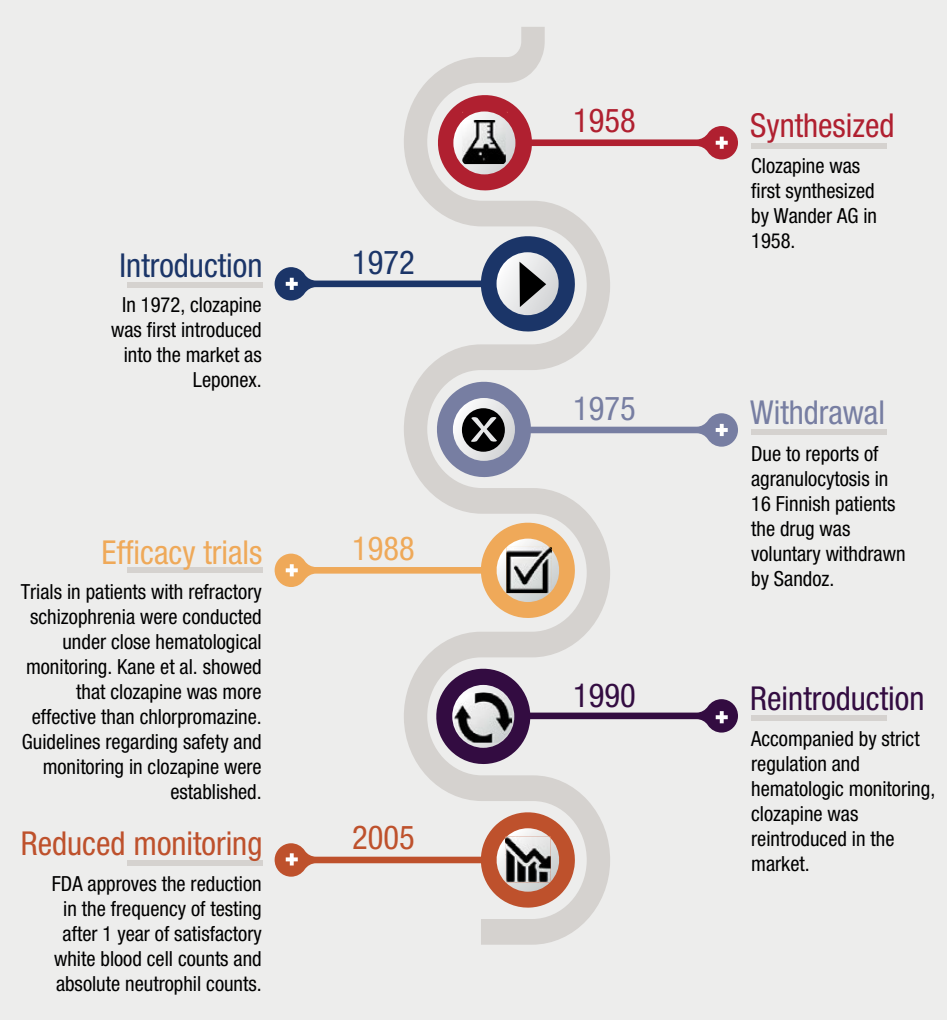
Background

Clozapine has, since its introduction into clinical practice in 1972 (Figure 1), taken a special position being the first antipsychotic agent with a substantially lower risk of extrapyramidal symptoms (EPS) [1–3]. In addition, clozapine is the only antipsychotic with well documented efficacy in patients with refractory schizophrenia [4], and has anti-aggressive [5,6] and anti-suicidal properties [4,7–9]. These clinical advantages were overshadowed by the discovery of fatal agranulocytosis as adverse drug reaction (ADR) in 16 patients from Finland [10]. This resulted in the voluntary withdrawal of clozapine from the market by Sandoz in 1975. In response to pressure from clinicians to reintroduce clozapine, trials in patients with refractory schizophrenia were conducted under close hematological monitoring. These trials showed an improvement in a clinically relevant proportion of patients, confirming its superiority in this patient category [4]. These trials eventually led to the reintroduction of clozapine in 1989, accompanied by strict monitoring regimes including regular white blood cell count monitoring during treatment and strict regulations regarding indications (refractory schizophrenia and suicidal behavior in patients with schizophrenia or schizoaffective disorder). In some countries, patients are even required – as a risk-reduction strategy – to present a normal blood count result prior to subsequent clozapine dispenses at their pharmacy [11,12].
From a pharmacological perspective, clozapine is considered a 'dirty drug' because of its action on many receptors. Clozapine has a relatively high receptor affinity for muscarinic, adrenergic, dopaminergic, histaminergic and serotonergic receptors (Figure 2) [13]. It remains unknown which of these receptors exactly mediate clozapine's clinical efficacy. Yet, it is established that a wide spectrum of different adverse drug reactions (ADRs) of clozapine is mediated by its actions on different receptors. Hence, clinicians are challenged to handle the delicate balance between superior antipsychotic efficacy and patient safety/tolerability: 'clozaphilia' (worshipping and thus overprescribing the drug) should be balanced against 'clozaphobia' (fearing, thus underprescribing and potentially depriving patients from necessary treatment [14]). Monitoring guidelines comprising the prevention and management of ADRs contribute to the possibility of using this for some patients valuable antipsychotic agent without compromising patient safety. The Netherlands Clozapine Collaboration Group ('Clozapine Plus Werkgroep') has developed such a clinical guideline to facilitate optimal use of clozapine in clinical practice [15].

Improving the benefit-risk balance of clozapine by laboratory monitoring

Drug labels often provide instructions on laboratory monitoring to mitigate the benefit-harm balance. However, these instructions are frequently ambiguous, incomplete or lack clinical applicability [16,17]. Conversely, laboratory monitoring regarding

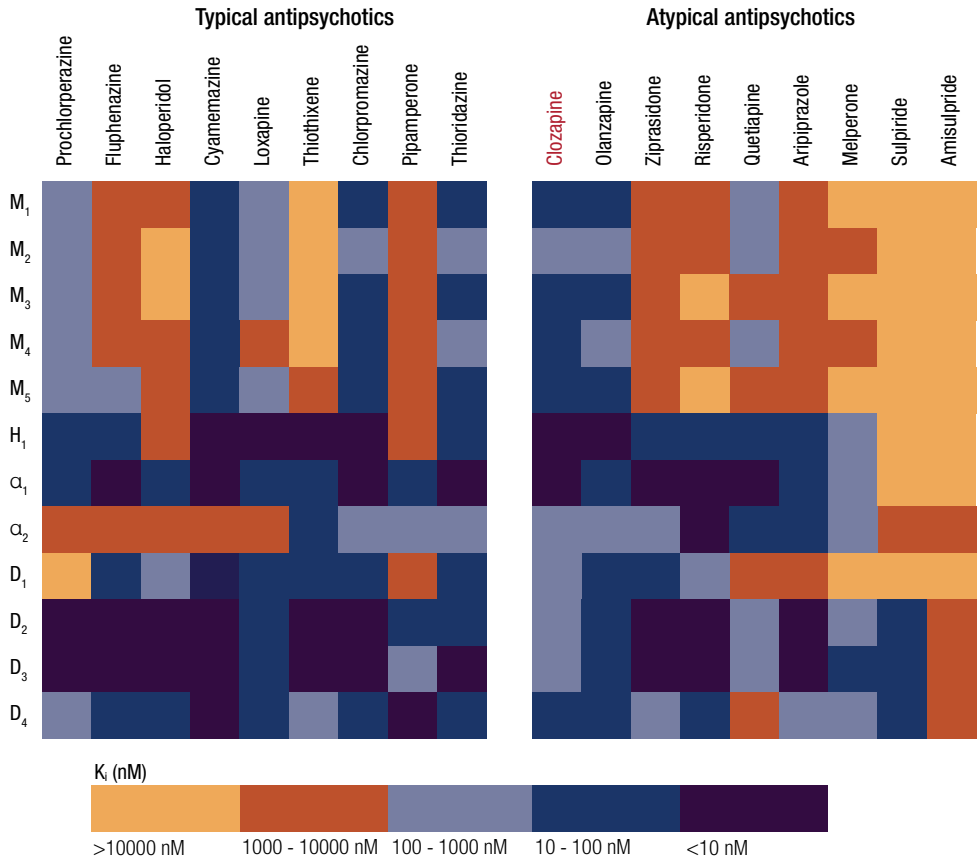
Figure 1. History of clozapine from 1958 – present



blood count and hematology in clozapine therapy is explicitly described and even regarded mandatory. Initiating clozapine therapy necessitates a specific strict monitoring regimen, encompassing regular monitoring of white blood cell count including an absolute neutrophil count – as a mandatory requirement in some countries – in addition to clinical evaluation and monitoring for other somatic complications.

Another laboratory monitoring tool to mitigate the benefit-harm balance of clozapine therapy is therapeutic drug monitoring (TDM) [18], as clozapine is known for its narrow therapeutic range, with wide interindividual variability in metabolism, and both drug-drug interactions and food-drug interactions affecting the dose-concentration relationship. Hence, TDM of clozapine is used to optimize dosage, to minimize concen-

Figure 2. Receptor binding affinity of clozapine in comparison to other antipsychotics



Receptor binding affinity of typical and atypical antipsychotics expressed as heatmap with inhibitory constants (K_i), nM=nanomolar.

tration-dependent toxicity and to evaluate medication adherence. It usually involves measurement of the total (i.e. sum of protein-bound and unbound) plasma concentrations of clozapine. Studies and guidelines mention a therapeutic reference ranging from 350 to 600 ug/L [19], but in some specific conditions the clinical presentation is not correlated with this range.

One of these conditions is inflammation during clozapine therapy. In practice, a fast increase of total clozapine plasma concentrations into toxic ranges has been observed during inflammation, while acute clinical toxicity such as seizures was absent [20–22]. Since clozapine is predominantly bound to the acute phase protein 'alpha-1-acid glycoprotein' (AGP) [21], a possible pharmacological explanation is the absolute increase of protein bound clozapine as a result of enhanced systemic availability of AGP due to inflammation. The protein unbound plasma concentration, that better correlates

to both efficacy and safety, is left unaffected. However, the effect of enhanced AGP plasma concentrations and inflammation on the protein bound and unbound clozapine plasma concentrations has not yet been examined.

A strict monitoring regimen and patient adherence: friend or foe?

Non-adherence is the extent to which patients not take medications as agreed by healthcare providers and patients in the shared decision making process. It can be subdivided into intentional non-adherence where the patient chooses to deviate from the prescribed treatment regimen and into unintentional non-adherence in which the patient may be careless or forgetful about adhering to treatment regimen [23]. Quantitative adherence measures involve direct measures (direct observation of intake) and indirect measures (evidence suggesting medication have been taking such as pill count, drug level monitoring and pharmacy refill records). Subjective measures involve provided testimonies regarding medication intake including self-reports and assessments by others. Due to the non-practical, expensive, invasive, transient or inaccurate nature of mentioned measures, clinicians are challenged to obtain an appropriate and true measurement of medication adherence [24]. This is a relevant topic in the field of psychiatry, since non-adherence is a major issue in the treatment of schizophrenia in general [25]. An estimated 25% of schizophrenia patients are nonadherent to antipsychotic treatment within 7-10 days after initiating therapy [26]. Studies estimate the mean overall prevalence of non-adherence with taking antipsychotics between 40% and 50% [27]. Furthermore, medication discontinuation occurs in approximately 70% of all patients after discharge [25]. Non-adherence may lead to psychiatric deterioration, increased risk of relapses, hospitalization, poorer prognosis and overall treatment costs [28]. Nevertheless, non-adherence was less frequent among users of clozapine compared to users of other antipsychotics (OR=0.08; 95% CI 0.06–0.11) [29]. In addition, users of clozapine had a lower risk (RR=0.45, CI=0.39–0.52) of discontinuation than users of other antipsychotic agents [30]. These higher adherence rates can be partially explained by the mandatory intensive provider–patient visit frequency needed for the required hematologic monitoring. This high visit frequency was shown to be positively correlated with adherence to clozapine [29,31]. Given the risk of non-adherence and its serious consequences, guidelines recommend assessment of medication adherence on a regular basis. There are various tools to assess medication adherence including TDM, as mentioned earlier. TDM, however, only reveals information on medication adherence over the past few days upmost due to the elimination half-life of clozapine and its main metabolite (norclozapine) of 14 and 18 hours respectively [32]. As a consequence, improved medication-taking behavior shortly before or after a provider–patient visit, termed ‘white-coat adherence’, cannot be ruled out with TDM. An objective monitoring tool to quantify medication adherence over a longer time-period could therefore

be of great value but is currently unavailable. In this regard a serendipitous observation by Hoffmann and Lillholm [33] could be of interest. They described enhanced neutrophil fluorescence – termed FL3-fluorescence – in a small study with schizophrenic patients treated with clozapine. FL3-fluorescence is a flow-cytometric parameter that measures nucleated red blood cells and white blood cell viability by propidium iodide (PI) staining. This fluorescence remained elevated for weeks after clozapine cessation.

A patient-centered approach in monitoring bothersome ADRs: sialorrhea as an example

The most important reasons for switching antipsychotic treatment are lack of effectiveness, awareness of illness or occurrence of ADRs. Previous work of our group showed that early switching of antipsychotic treatment was predominantly associated with occurrence of ADRs, whereas switching after a few weeks was more related to lack of effectiveness [34]. Clozapine is indicated in schizophrenia after an adequate trial involving at least two antipsychotic agents with each at least eight weeks treatment and with at least one of them being a second generation agent. The main reason for not using clozapine as a first-line treatment for schizophrenia is its ADR profile. ADRs such as agranulocytosis (0.8%) [35,36] and myocarditis (0.7-1.2%) [37,38] are considered life-threatening thereby alarming clinicians, whereas ADRs including sialorrhea (80-90%) [39], sedation (56-90%) [39] and weight gain (20-64%) [39] are not life-threathening, but experienced as bothersome by patients. In terms of shared decision making for treatment planning it is important to combine the clinician's risk perspective (e.g. primarily compromising patient safety), with patients and relatives' perspective (e.g. primarily tolerability/quality of life). Hodge *et al.* studied the prevalence and severity of ADRs experienced by users of clozapine, which were contrasted with those of clinicians and found an overestimation of these ADRs by clinicians [40]. Clinicians estimated that drowsiness was the most prevalent adverse effect and oversleeping was for patients the most severe adverse effect, whereas patients indicated sialorrhea as the most prevalent (89%) and the most debilitating adverse effect (Table 1) [40]. Comparable results were reported by Qurashi *et al.*, where sialorrhea was considered the most prevalent (84%) and most invalidating adverse effect by patients [41]. In addition, Angermeyer *et al.* reported that both patients and relatives admitted the beneficial features of clozapine over other antipsychotics but differed in their evaluation of ADRs. Patients were complaining about ADRs such as sialorrhea, while relatives were more concerned about weight gain and the clinicians were most concerned of the risk of agranulocytosis [42]. Including patient and relative perspectives on prevention and management of bothersome ADRs such as sialorrhea into the process of shared decision making, might contribute to a better quality of life, increase medication adherence and decrease discontinuation rates.

Table 1. Prevalence and severity of each side-effect of clozapine as obtained from consumers and clinicians [40]

Prevalence	Consumer (%)	Clinician (%)	Severity	Consumer (%)	Clinician (%)
Drooling mouth	89	79	Drooling mouth	73	60
Tiredness	85	88	Putting on weight	57	57
Tension	78	67	Polyuria	50	13
Memory problems	78	88	Tiredness	46	56
Depression	78	71	Sleeping too much	41	63
Putting on weight	78	88	Tension	36	31
Sleeping too much	74	96	Decreased sex drive	35	38
Lack of emotions	70	71	Memory problems	34	41
Polyuria	70	29	Feeling sick	31	28
Feeling sick	67	71	Concentration	30	42
Dizziness	63	83	Constipation	30	30
Concentration	59	83	Dry mouth	29	23
Difficulty staying awake	59	100	Depression	28	32
Dry mouth	56	58	Dizziness	28	36
Constipation	52	67	Lack of emotions	28	34
Increased sweating	52	63	Increased dreaming	28	25
Restlessness	52	71	Increased sweating	25	32
Decreased sex drive	52	63	Difficulty staying awake	24	54
Increased dreaming	48	63	Restlessness	22	26
Sensitivity to the sun	44	38	Anorgasmia	21	24
Difficulty getting to sleep	44	50	Sensitivity to sun	20	16
Pins and needles	44	29	Blurred vision	20	25
Blurred vision	40	58	Difficulty getting to sleep	19	22
Palpitations	37	63	Pins and needles	17	10
Anorgasmia	37	42	Rash	16	0
Shakiness	37	50	Palpitations	15	23
Headaches	33	71	Slowing of movements	14	32
Gynaecomastia	33	38	Gynaecomastia	13	0
Slowing of movements	33	71	Losing weight	13	5
Rash	33	17	Shakiness	13	21
Losing weight	30	17	Itchy skin	13	16
New skin marks	30	38	Headaches	11	22
Itchy skin	26	38	Dyskinesias	11	17
Muscle stiffness	26	67	New skin marks	11	15
Dyskinesias	22	42	Menstrual problems	10	11
Menstrual problems	19	29	Muscle spasms	10	25
Increased sex drive	19	21	Increased sex drive	8	11
Muscle spasms	15	50	Difficulty passing urine	6	13
Diarrhoea	11	46	Muscle spasms	6	22
Periods less frequent	4	33	Diarrhoea	5	18

Thesis objectives

The overall objective of this thesis is to improve the use of clozapine in patients by optimizing monitoring. Specific objectives were:

- To assess the applicability of FL3-fluorescence as a biomarker to monitor long term adherence to clozapine therapy;
- To assess effects of AGP concentration on the unbound fraction of clozapine plasma concentrations in patients;
- To study sialorrhea as a clinical biomarker for ADRs in patients using clozapine.

Thesis outline

Chapter 2 describes laboratory biomarkers that may be beneficial and worthwhile to consider in optimizing monitoring of patients using clozapine in clinical practice from a clinician's perspective. The serendipitous observation of fluorescent neutrophils, called FL3-fluorescence and specifically observed in clozapine users, is further explored in *Chapter 2.1* by studying the association between this FL3-fluorescence and clozapine use in a Dutch patient population. This provides initial information on the concept of neutrophil fluorescence serving as a potential long-term adherence marker for clozapine use. In order to understand the underlying mechanism and to explore neutrophil fluorescence in clozapine users in further detail, the subcellular localization and source of neutrophil fluorescence is extensively studied in *Chapter 2.2*. From this point of view in *Chapter 2.3*, a pharmacokinetic/pharmacodynamic (PK/PD) model relating clozapine exposure to FL3-fluorescence is developed and evaluated. Consequently, a conceptual nomogram for estimation of long-term adherence based upon FL3-fluorescence values and clozapine dose is derived. In *Chapter 2.4*, the effects of AGP on the unbound clozapine plasma concentrations is studied.
In *Chapter 3* the tolerability aspect of clozapine therapy is assessed by focusing on monitoring for one of the ADRs with high impact from a patient's perspective: clozapine-induced sialorrhea. In *Chapter 3.1*, ADR reporting patterns related to drug-induced sialorrhea are compared between clozapine and other antipsychotic agents. Therefore, WHO pharmacovigilance data from VigiBase are used. In *Chapter 3.2*, the efficacy and safety of glycopyrrolate is assessed in patients with clozapine-induced sialorrhea. In *Chapter 3.3*, the extent to which changes in Nocturnal Hypersalivation Rating Scale (NHRS) scores affect patients' perceived burden and treatment satisfaction are studied and assessed.
In the final chapter (*Chapter 4*), the implications and results from the different studies are discussed in a broader perspective. In addition, directions for further research in this area are pointed out.

References

1 de Maio D. Clozapine, a novel major tranquilizer. Clinical experiences and pharmacotherapeutic hypotheses. Arzneimittelforschung 1972; 22: 919–23.

2 Simpson GM, Varga E. Clozapine--a new antipsychotic agent. Curr Ther Res Clin Exp 1974; 16: 679–86.

3 Matz R, Rick W, Thompson H, Gershon S. Clozapine-a potential antipsychotic agent without extrapyramidal manifestations. Curr Ther Res Clin Exp 1974; 16: 687–95.

4 Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. Arch Gen Psychiatry 1988; 45: 789–96.

5 Buckley P, Bartell J, Donenwirth K, Lee S, Torigoe F, Schulz SC. Violence and schizophrenia: clozapine as a specific antiaggressive agent. Bull Am Acad Psychiatry Law 1995; 23: 607–11.

6 Frogley C, Taylor D, Dickens G, Picchioni M. A systematic review of the evidence of clozapine's anti-aggressive effects. Int J Neuropsychopharmacol 2012; 15: 1351–1371.

7 Meltzer HY, Okayli G. Reduction of suicidality during clozapine treatment of neuroleptic-resistant schizophrenia: impact on risk-benefit assessment. Am J Psychiatry 1995; 152: 183–90.

8 Meltzer HY, Alphs L, Green AI, Altamura AC, Anand R, Bertoldi A *et al.* Clozapine treatment for suicidality in schizophrenia: International Suicide Prevention Trial (InterSePT). Arch Gen Psychiatry 2003; 60: 82–91.

9 Walker AM, Lanza LL, Arellano F, Rothman KJ. Mortality in current and former users of clozapine. Epidemiology 1997; 8: 671–7.

10 Idänpään-Heikkilä J, Alhava E, Olkinuora M, Palva I. Letter: Clozapine and agranulocytosis. Lancet 1975; 2: 611.

11 Cetin M, Sayar GH. Risk evaluation and mitigation strategy for clozapine. Klin Psikofarmakol Bul 2016; 26: 1–6.

12 Drug Safety Communication: FDA modifies monitoring for neutropenia associated with schizophrenia medicine clozapine; approves new shared REMS program for all clozapine medicines. US Food and Drug Administration.

13 Meltzer HY. An overview of the mechanism of action of clozapine. J Clin Psychiatry 1994; 55 Suppl B: 47–52.

14 Cetin M. Clozaphobia: Fear of prescribers of clozapine for treatment of schizophrenia. Klin Psikofarmakol Bul 2014; 24: 295–301.

15 Schulte P, Bakker B, Bogers J, Cohen D, van Dijk D. Guideline for the use of clozapine by the Netherlands Clozapine Collaboration Group, version 05-02-2013. www.clozapinepluswerkgroep.nl.

16 Nederlof M, Stoker LJ, Egberts TCG, Heerdink ER. Instructions for clinical and biomarker monitoring in the Summary of Product Characteristics (SmPC) for psychotropic drugs: Overview and applicability in clinical practice. J Psychopharmacol 2015; 29: 1248–54.

17 Geerts AFJ, De Koning FHP, Van Solinge WW, De Smet PAGM, Egberts TCG. Instructions on laboratory monitoring in 200 drug labels. Clin Chem Lab Med 2012; 50: 1351–1358.

18 Kar N, Barreto S, Chandavarkar R. Clozapine monitoring in clinical practice: Beyond the mandatory requirement. Clin Psychopharmacol Neurosci 2016; 14: 323–329.

19 Hiemke C, Bergemann N, Clement HW, Conca A, Deckert J, Domschke K *et al.* Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology: Update 2017. Pharmacopsychiatry 2018; 51: 9–62.

20 Haack MJ, Bak MLFJ, Beurskens R, Maes M, Stolk LML, Delespaul PAEG. Toxic rise of clozapine plasma concentrations in relation to inflammation. Eur Neuropsychopharmacol 2003; 13: 381–5.

21 Leung JG, Nelson S, Takala CR, Gören JL. Infection and inflammation leading to clozapine toxicity and intensive care: a case series. Ann Pharmacother 2014; 48: 801–5.

22 Espnes KA, Heimdal KO, Spigset O. A puzzling case of increased serum clozapine levels in a patient with inflammation and infection. Ther Drug Monit 2012; 34: 489–92.

23 Gadkari AS, McHorney CA. Unintentional non-adherence to chronic prescription medications: how unintentional is it really? BMC Health Serv Res 2012; 12: 98.

24 Nederlof M, Frederix GWJ, Stoker LJ, van Solinge WW, Egberts TCG, Heerdink ER. Patient monitoring: the hidden costs of treatment with antipsychotics. Br J Clin Pharmacol 2016; 82: 570–2.

25 Abdullah-Koolmees H, Gardarsdottir H, Yazir D, Stoker LJ, Vuyk J, Egberts TCG *et al.* Medication discontinuation in patients after discharge from a psychiatric hospital. Ann Pharmacother 2015; 49: 1085–1095.

26 Keith SJ, Kane JM. Partial compliance and patient consequences in schizophrenia: our patients can do better. J Clin Psychiatry 2003; 64: 1308–15.

27 Lacro JP, Dunn LB, Dolder CR, Leckband SG, Jeste D V. Prevalence of and risk factors for medication nonadherence in patients with schizophrenia: a comprehensive review of recent literature. J Clin Psychiatry 2002; 63: 892–909.

28 Laan W, Van Der Does Y, Sezgi B, Smeets HM, Stolker JJ, De Wit NJ *et al.* Low treatment adherence with antipsychotics is associated with relapse in psychotic disorders within six months after discharge. Pharmacopsychiatry 2010; 43: 221–224.

29 Valenstein M, Blow FC, Copeland LA, McCarthy JF, Zeber JE, Gillon L *et al.* Poor antipsychotic adherence among patients with schizophrenia: medication and patient factors. Schizophr Bull 2004; 30: 255–64.

30 Horvitz-Lennon M, Donohue JM, Lave JR, Alegría M, Normand S-LT. The effect of race-ethnicity on the comparative effectiveness of clozapine among Medicaid beneficiaries. Psychiatr Serv 2013; 64: 230–7.

31 Patel NC, Crismon ML, Miller AL, Johnsrud MT. Drug adherence: effects of decreased visit frequency on adherence to clozapine therapy. Pharmacotherapy 2005; 25: 1242–7.

32 Flanagan RJ, Spencer EP, Morgan PE, Barnes TRE, Dunk L. Suspected clozapine poisoning in the UK/Eire, 1992-2003. Forensic Sci Int 2005; 155: 91–9.

33 Hoffmann J, Lillholm A. Neutrophil fluorescence in patients using clozapine. Ned Tijdschr Klin Chem 2000; 25: 108.

34 Hugenholtz GWK, Heerdink ER, Meijer WE, Stolker JJ, Egberts ACG, Nolen WA. Reasons for switching between antipsychotics in daily clinical practice. Pharmacopsychiatry 2005; 38: 122–124.

35 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: 162–7.

36 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.

37 Nederlof M, Benschop TW, De Vries Feyens CA, Heerdink ER. Clozapine re-exposure after dilated cardiomyopathy. BMJ Case Rep 2017; 2017: 10–13.

38 Haas SJ, Hill R, Krum H, Liew D, Tonkin A, Demos L *et al.* Clozapine-associated myocarditis: a review of 116 cases of suspected myocarditis associated with the use of clozapine in Australia during 1993-2003. Drug Saf 2007; 30: 47–57.

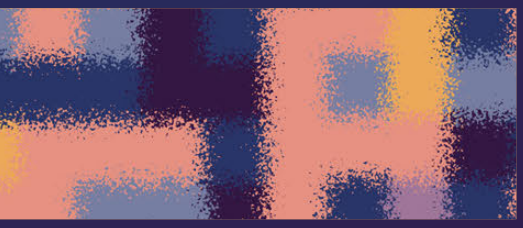
39 Schneider C, Corrigall R, Hayes D, Kyriakopoulos M, Frangou S. Systematic review of the efficacy and tolerability of Clozapine in the treatment of youth with early onset schizophrenia. Eur Psychiatry 2014; 29: 1–10.

40 Hodge K, Jespersen S. Side-effects and treatment with clozapine: A comparison between the views of consumers and their clinicians. Int J Ment Health Nurs 2008; 17: 2–8.

41 Qurashi I, Stephenson P, Chu S, Duffy C, Husain N, Chaudhry I. An evaluation of subjective experiences, effects and overall satisfaction with clozapine treatment in a UK forensic service. Ther Adv Psychopharmacol 2015; 5: 146–50.

42 Angermeyer MC, Löffler W, Müller P, Schulze B, Priebe S. Patients' and relatives' assessment of clozapine treatment. Psychol Med 2001; 31: 509–517.

Biomarkers in clozapine monitoring



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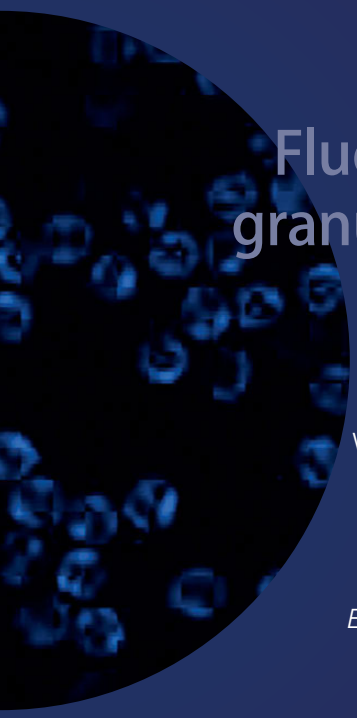


Fluorescence of neutrophil granulocytes as a biomarker for clozapine use

Wai Hong Man, Maarten J ten Berg, Ingeborg Wilting, Albert Huisman, Wiepke Cahn, Jan Willem Douma, Hanneke den Breeijen, Eibert R Heerdink, Toine CG Egberts, Wouter W van Solinge

Eur Neuropsychopharmacol. 2013 Nov;23(11):1408-13.

Abstract

Background

Non-adherence to medication is a major issue in the treatment of schizophrenia in general and in particular for those treated with clozapine. A reliable tool to quantify patients long-term adherence to clozapine is currently unavailable. Enhanced FL3 neutrophil granulocyte fluorescence was serendipitously observed in a small population of schizophrenic patients treated with clozapine. The present study was aimed at assessing the association between clozapine use and FL3-fluorescence.

Methods

A cross-sectional study was performed using data from the Utrecht Patient Oriented Database (UPOD).

Results

A total of 38,390 inpatients were included, of which 124 (0.33%) used clozapine. FL3-fluorescence was significantly higher ($U=240{,}179$, $P<0.001$) in clozapine users (mean (SD)=90.5 (11.8)) than in non-users (mean (SD)=69.8 (3.3)). Observed FL3-fluorescence was found to increase with increasing clozapine dose. The area under the receiver operating characteristic curve was 0.95.

Conclusions

Our results confirm the association between use of clozapine and elevated FL3-fluorescence. Further research is needed to unravel the underlying mechanism and to investigate the true potential of FL3-fluorescence as a clozapine-adherence in clinical practice.

Introduction

Schizophrenia is a disabling chronic form of mental illness affecting about 1% of the world population [1]. Use of antipsychotics is an essential part in the treatment of schizophrenia. Among all antipsychotics, the atypical antipsychotic agent clozapine is the only drug with demonstrated efficacy in patients with refractory schizophrenia and the only agent with demonstrated antisuicidal properties [2–6]. Given these features, clozapine is considered to be of great importance in the treatment of schizophrenia. However, its use is hampered by its adverse effect profile, including the risk for potentially fatal agranulocytosis (0.8% within 4-5 years, with a peak incidence in the first 6-18 weeks of treatment) [5,7,8], necessitating weekly blood monitoring in the first 18 weeks of treatment followed by blood monitoring once every 4 weeks according to European guidelines and regulatory authorities [9].

Apart from the adverse effects of antipsychotic therapy, non-adherence is a major issue in the treatment of schizophrenia. An estimated 25% of the patients is non-adherent within the first 7-10 days of treatment [10] and studies estimate the mean overall prevalence of non-adherence between 40 and 50% [11]. Results of one study reveal that half of the ambulant schizophrenic patients take less than 70% of their medication [12]. A recent study showed that low treatment adherence is associated with a two-fold increased risk for relapse in psychotic disorders within six months after discharge [13]. Physicians apply different strategies to improve treatment adherence including therapeutic drug monitoring (TDM) [14]. TDM consists of determining the ratio of clozapine and N-desmethylclozapine levels, however these measurements have limited value for detection of poor adherence. Due to the elimination half-life of clozapine of about 14 hours [15] and its main metabolite N-desmethylclozapine (half-life of 18 hours [15]), serum level monitoring only provides information on clozapine intake for a few days prior to blood sampling at most. Moreover, merely a suspicion for poor-adherence in the last few days can be raised if a low clozapine:norclozapine ratio is found, because this low ratio could also be explained by rapid metabolizing individuals or drug-drug interactions [16]. Therefore, a biomarker providing long-term information on adherence would be of great value, such as glycosylated hemoglobin (HbA_{1c}), a long-term biomarker of glucose control in diabetes. In this regard a serendipitous observation by Hoffmann and Lillholm [17] could be of interest. They described elevated FL3-fluorescence of neutrophil granulocytes in a small Dutch population of schizophrenic patients treated with clozapine. The objective of the current study is to confirm this observation and to further assess the association between clozapine use and FL3-fluorescence of neutrophil granulocytes.

Experimental procedures

Setting

For this study data from the University Medical Centre Utrecht (UMC Utrecht) were used. The UMC Utrecht is a 1,042-bed academic teaching hospital in the centre of the Netherlands, with annually approximately 28,000 clinical and 15,000 day-care hospitalizations and 334,000 outpatient visits. Data were extracted from the Utrecht Patient Oriented Database (UPOD). UPOD is an infrastructure of relational databases comprising data on patient characteristics, hospital discharge diagnoses, medical procedures, medication orders and laboratory tests for all patients treated at the UMC Utrecht since 2004 [18]. UPOD data acquisition and data management are in line with current Dutch regulations concerning privacy and ethics. The data used for this cross-sectional study were collected for patient care purposes and were used retrospectively. The structure and content of UPOD have been described in more detail elsewhere [18].

UPOD haematology database: FL3-fluorescence of neutrophil granulocytes

Next to clinical laboratory data from the laboratory information system, UPOD contains specific haematology data of automated blood cell analyses performed by the Abbott Cell-Dyn Sapphire automated haematology analyser (Abbott Diagnostics, Santa Clara, CA, USA) used at the UMC Utrecht [19]. A feature of this haematology analyser is that all parameters of the complete blood count (CBC) are measured irrespective of the requested parameter. The analyser is equipped with an integrated 488 nm blue diode laser and uses spectrophotometry, electrical impedance and laser light scattering (multi angle polarized scatter separation, [MAPPS]), to measure the CBC and to classify the white blood cell counts (WBCs). For the WBC differential count the following 5 optical scatter signals are measured for each individual cell: cell size (0° scatter, axial light loss [ALL]), cell complexity and granularity (7° scatter, intermediate angle scatter [IAS]), nuclear lobularity (90° scatter, polarized side scatter [PSS]), depolarization (90° depolarized side scatter [DSS]) and red fluorescence (90° (FL3), 630 ± 30 nm). Per measurement these parameters are stored within UPOD. The FL3-fluorescence parameter measures Nucleated Red Blood Cells and WBC viability by propidium iodide (PI) staining. PI stains nucleic acids and is part of the reagents used by the analyser. PI is capable of crossing the cell-membrane of non-viable WBCs and stains nucleic acids (RNA and DNA). The WBC viability is reported by the analyser as the WVF (the fraction of viable white blood cells within the total population of white blood cells). Older blood samples have a lower WVF. A WVF of 0.95 was considered as the optimal cut-off value

to differentiate between fresh and old samples [20]. FL3-fluorescence measurement of neutrophil granulocytes is performed by the analyser as part of the routine CBC measurement. Analysis of the WBC scatterplots shows that the FL3-fluorescence is only observed in neutrophil granulocytes and not in eosinophilic granulocytes, basophilic granulocytes, lymfocytes and monocytes. Using a PI free WBC reagent we proved that neutrophil FL3-fluoresence was independent of PI. The reliability and validity of all laboratory results was monitored with built in quality flags, daily quality control samples and external quality assesment schemes.

Study population

All inpatients of 18 years or older for whom at least one FL3-fluorescence measurement with a WVF >0.95, and a history of in-hospital prescribed drugs was available between 2007-2010, were eligible for participation. For each patient a random CBC containing one FL3-fluorescence measurement was selected. Inpatients were selected to ensure that the in-hospital prescribed drugs were taken. ICU patients were excluded due to the lack of a well accessible electronic health record.

Outcome

The investigated outcome was FL3-fluorescence of neutrophil granulocytes as measured by the Abbott Sapphire hematology analyser as described above. The date of the selected measurement was termed the index date.

Exposure

Clozapine use on the index date was determined and was defined as having a medication order for the drug starting at least three days prior to the index date and lasting at least until the index date. A medication order was defined as an order for medication administration in the hospital using a computerized physician order entry (CPOE) system. For each medication order for clozapine the dosage (in mg/day) on the day of FL3-measurement was obtained. Finally, it was determined for how many days the patients had used clozapine on the index date.

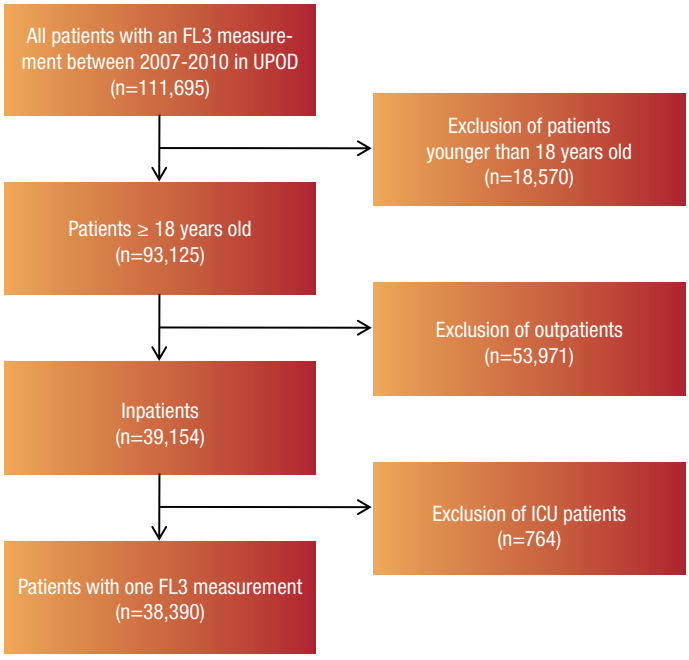
Data analyses

Normality of the FL3-fluorescence data was investigated by visual inspection of the histogram of FL3-fluorescence distribution among clozapine users and non-users, calculation of the mean and median and by the Kolmogorov-Smirnov goodness-of-fit test. The difference in FL3-fluorescence between clozapine users and non-users was investigated, by applying the unpaired Student's t-test in case of normal distribution and Mann-Whitney U-test in case of non-normal distribution. Sensitivity and specificity

Table 1. Patient characteristics

	Total	Clozapine users	Clozapine non-users
Patients, n (%)	38,390 (100%)	124 (0.3%)	38,266 (99.7%)
Male gender, n (%)	18,191 (47%)	80 (65%)	18,111 (47%)
Age, median (SD)	56.3 (89.9)	39.0 (68.1)	56.4 (89.9)

Figure 1. Flowchart of study inclusion



of elevated FL3-fluorescence for clozapine use were determined for different cut-off values (mean plus 0.5SD, 1SD, 2SD, 3SD, 4SD) for the FL3-fluorescence and current use of clozapine were subsequently plotted to generate a receiver operating characteristic (ROC) curve. In clozapine users without elevated FL3-fluorescence, defined as having an FL3-fluorescence below the mean plus three times the standard deviation, an explanation for this observation was sought by examining the drug dose and the duration of treatment.

The relationship between clozapine daily dose at the index date and FL3-fluorescence was investigated. Daily dose at the index was categorized into 5 groups (<25; 25-50; 51-100; 101-200 and >200 mg/day) and the mean FL3-fluorescence with 95% confidence interval per group was calculated. All data analyses were performed using SPSS for Windows version 20.0 (IBM, Chicago, IL, USA). An alpha level of 0.05 was considered statistical significance for all performed statistical tests.

Results

Patients were selected in UPOD as shown in Figure 1. After exclusion of outpatients and ICU patients, a total of 38,390 inpatients were included. Within this group a total of 124 patients (0.32%) used clozapine on the index date (see Table 1).

Figure 2 shows the FL3-fluorescence distribution among clozapine users and the non-users. The mean FL3-fluorescence was 90.5 (SD=11.8) in clozapine users, whereas this was 69.8 (SD=3.3) in non-users. The figure shows two distinct populations amongst the clozapine users, with one population showing complete overlap with the FL3-fluorescence distribution among non-users.

The Kolmogorov-Smirnov goodness-of-fit test showed that the FL3-fluorescence data were non-normally distributed (Z=8.408, P<0.001). Therefore, the Mann-Whitney U test was performed to test the difference in FL3-fluorescence between clozapine users and non-users for statistical significance.

Table 2. Clozapine dose range among the clozapine users

Dose (mg/day)	Number of patients	Mean FL3-fluorescence	SD
< 25	11	73.5	5.3
25	11	79.5	10.0
50	3	82.0	9.0
51-100	3	93.7	9.3
101-200	17	92.5	8.9
>200	59	97.9	6.5
n/a	20	83.1	11.9

From 20 patients the clozapine dose on the index date was not available.

Figure 2. Histogram of FL3-fluorescence among clozapine users and clozapine non-users

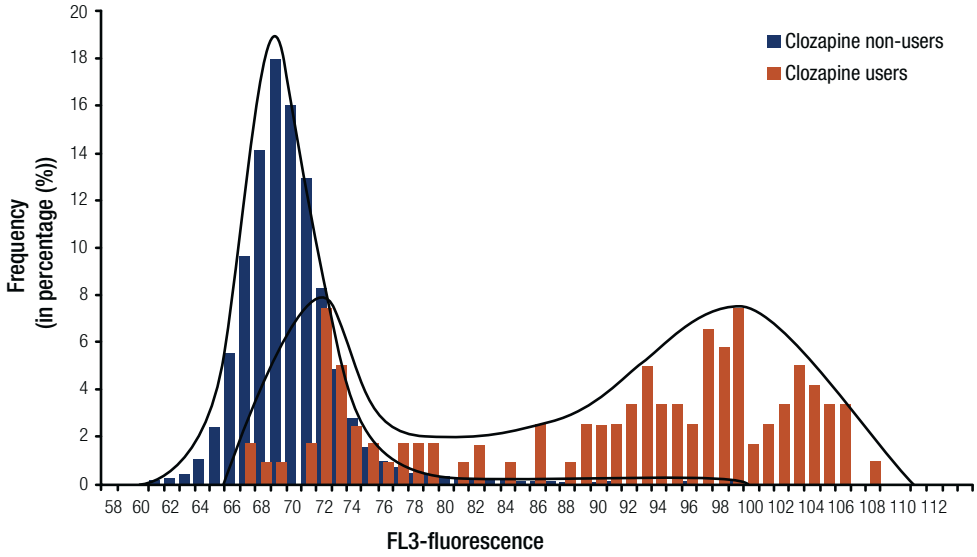


Figure 3. Box-and-whisker plots of stratified clozapine dose *vs.* FL3-fluorescence

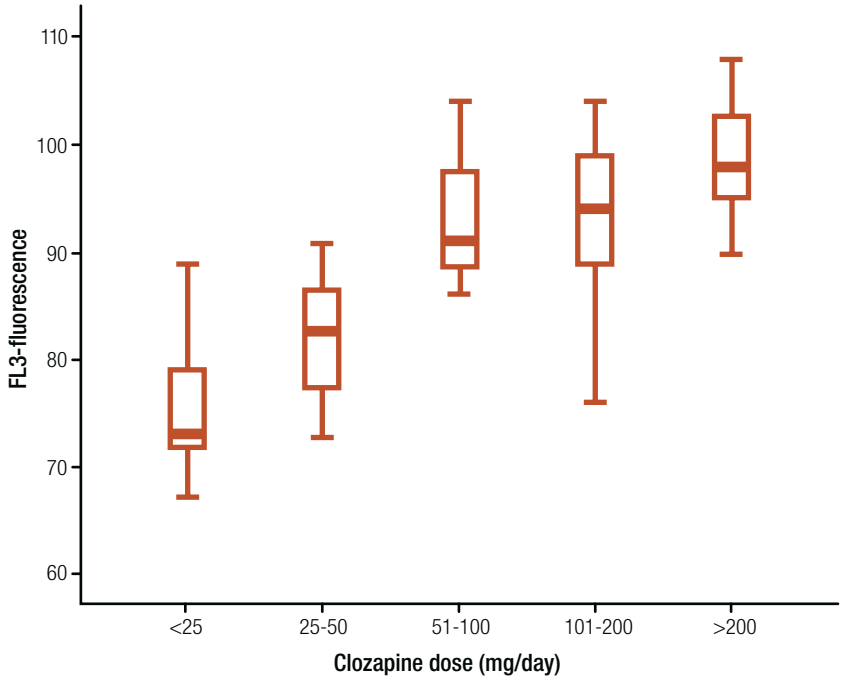
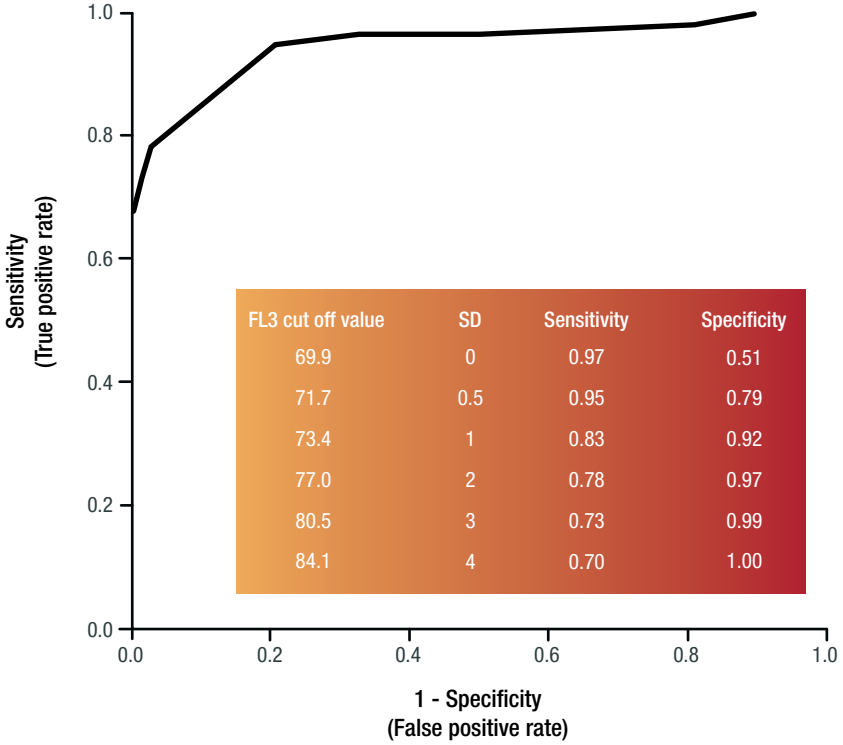


Figure 4. Receiver operating characteristic (ROC) curve of clozapine use *vs* FL3-fluorescence



FL3 cut off value	SD	Sensitivity	Specificity
69.9	0	0.97	0.51
71.7	0.5	0.95	0.79
73.4	1	0.83	0.92
77.0	2	0.78	0.97
80.5	3	0.73	0.99
84.1	4	0.70	1.00

The sensitivity and specificity are displayed with corresponding cut off values. Area under the curve (AUC)=0.95.

Clozapine use was significantly associated with elevated FL3-fluorescence level (U=240,179, P<0.001). Within the group of 124 clozapine users 33 (26.6%) patients did not show an elevated FL3-fluorescence level. Within these 33 clozapine users 25 patients had used clozapine for less than two weeks on the index date and/or were using clozapine at low doses (maximum of 50 mg/day) for indications other than schizophrenia (i.e. Parkinson's disease). No detailed dose information at index date was available for seven clozapine users.

The dose-response is presented in Table 2 showing the doses of clozapine on the index date among the clozapine users sorted from low to high dose with coherent FL3-fluorescence values. Figure 3 displays the median FL3-fluorescence stratified to different clozapine dose ranges. Within the group of 38,266 control patients, 371 (1%) showed an elevated FL3-fluorescence level.

Figure 4 displays the ROC-curve for clozapine use based on different cut-off values for an elevated FL3-fluorescence. The area under the ROC-curve (AUC) was found to be 0.95. An FL3-fluorescence that was higher than the mean plus three times the standard deviation (80.5) was chosen as a cut-off value, with a sensitivity and specific-

ity of respectively 0.73 and 0.99 (Figure 4).
Since many clozapine users without an elevated FL3-fluorescence used clozapine at low doses (below 50 mg/day) on the index date (n=25), a ROC-curve was drawn excluding those patients using clozapine at a dose of less than 50 mg/day. This ROC-curve had an AUC of 0.99. At the cut-off point of 80.5, this resulted in a sensitivity and specificity of respectively 0.94 and 0.99.

Discussion

The results of this study confirm the association between clozapine use and FL3-fluorescence as reported previously by Hoffmann and Lillholm [17]. FL3-fluorescence was significantly elevated in the group of clozapine users compared to non-users. The distribution of FL3-fluorescence among clozapine users showed two peaks, indicating two distinct populations amongst those using clozapine. The first peak overlaps the distribution peak of the non-users. Patients displaying FL3 signals in the first peak appeared to be clozapine users who are still in the initiation phase of clozapine therapy and/or using clozapine at low dose (<50 mg/day), while the second peak depicts the patients with elevated FL3-fluorescence, using clozapine for longer than two weeks at a dose higher than 50 mg/day.
Stratifying for clozapine dose resulted in an increasing trend of the FL3-fluorescence with rising clozapine dose (dose-response curve). From the observed dose-response relationship, we can determine that elevated FL3-fluorescence is only observed above certain clozapine doses (approximately above 25-50 mg/day). We found a high AUC of the ROC-curve, this was even higher when excluding the low dose (<50 mg/day) clozapine users and patients having used clozapine for less than two weeks.
The reported FL3-fluorescence is the mean FL3-fluorescence of all individual neutrophil granulocytes measured in an individual patient. Therefore, the reported FL3-fluorescence is not dependent on the neutrophil granulocyte count. Moreover, we examined the association between neutrophil granulocyte count and FL3-fluorescence for non-users, low and high dose clozapine users, and found no correlation (data not shown).
The strengths of the study include its large population size and information on FL3-fluorescence and exposure to clozapine. According to our knowlegde, this is the first study to investigate the association between clozapine use and FL3-fluorescence in a large number of inpatients. In addition, we used data from the UPOD database, containing complete and validated automated data [18].
Our study has several limitations that need to be adressed. First, exposure was studied in terms of current clozapine use, defined as the amount of clozapine prescribed per day based on active medication orders. Hence, exposure in our study is based on assumptions of correct and adequate drug intake. It is well established that clozapine pharmacokinetics are affected by various factors such as smoking, concomitant drug

use and cytochrome P450 1A2 polymorphisms [21–25]. It would be most ideal if actual daily dosing information and clozapine serum levels would be available. Therefore, we only included inpatients because the medication intake of this patient population are strictly monitored, thus implicating a high medication adherence, however this is not guaranteed.

Second, the FL3-fluorescence over time, could not be studied since we used a cross-sectional design. Investigating the time of onset of the increased FL3-fluorescence is relevant because it could provide information about the underlying mechanism, i.e. a cause in peripheral blood or a cause in cell maturation process in the bone marrow. Time-studies are part of our current laboratory studies into this phenomenon. The association between current use of clozapine and FL3-fluorescence in our study is observed using the Abbott Cell-Dyn Sapphire automated haematology analyser which is used in daily routine laboratory practice using the analysers standard FL3 channel. This makes FL3-fluorescence a potential easily available biomarker, at no extra costs. FL3-fluorescence as measured by the Abbott Cell-Dyn Sapphire is a parameter unique for this analyser. To our knowledge, currently there are no haematology analysers from other manufacturers on the market that measure the same fluorescence parameter.

Although no other studies on the association of FL3-fluorescence and clozapine use have been published, the effects of antipsychotics on neutrophil maturity have been described in literature. Results of one study revealed that the mean lobe numbers of neutrophils in patients receiving antipsychotic drugs including clozapine were significantly lower compared to healthy volunteers. The authors suggest that this mean lobe number could be used as a marker for predicting patients at risk from agranulocytosis [26]. However, this method is difficult to implement in a routine clinical setting and dificult to standardize and quantify.

We have set the optimal cut-off point for an elevated FL3-fluorescence at the mean plus three times the standard deviation, based on a high specificity and a relatively lower sensitivity, because we think a false negative result (higher specificity) would be less aggravating than a false positive result (higher sensitivity). More specifically, we would rather miss a poor adherent clozapine user than accusing an adherent user falsely, as the latter would irretrievably damage the relationship between the patient and the clinician.

Exclusion of clozapine users having used clozapine for less than 2 weeks and/or using clozapine at doses below 50 mg/day, resulted in an improvement of sensitivity (0.94). For clozapine users using maintenance doses higher than 50 mg/day as is in schizophrenia and using for longer than 2 weeks a sensitivity of 0.94 and specificity of 0.99 are feasible.

The association between FL3-fluorescence and clozapine dose is intriguing and leaves little doubt that clozapine use causes an increased FL3-fluorescence. However, it is unknown what the underlying mechanism is. FL3-fluorescence is the result of

DNA-staining and is used by the Sapphire to determined the white cell viability. Hence, high FL3-fluorescence could implicate cell death. However, Hoffman *et al.* suggested that the phenomenon is due to autofluorescence [17]. The underlying mechanism is subject of current research by our group. With this study a first step towards a potential biomarker providing information on long-term adherence to clozapine treatment was made by confirming the association between clozapine use and FL3-fluorescence of neutrophil granulocytes in a large sample of patients. This provides a basis of further research into FL3-fluorescence as a potential biomarker for patient adherence to clozapine treatment.

Acknowledgements

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References

1 Bromet EJ, Fennig S. Epidemiology and natural history of schizophrenia. Biol Psychiatry 1999; 46: 871–81.

2 Chakos M, Lieberman J, Hoffman E, Bradford D, Sheitman B. Effectiveness of second-generation antipsychotics in patients with treatment-resistant schizophrenia: a review and meta-analysis of randomized trials. Am J Psychiatry 2001; 158: 518–26.

3 Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. Arch Gen Psychiatry 1988; 45: 789–96.

4 Meltzer HY, Alphs L, Green AI, Altamura AC, Anand R, Bertoldi A *et al.* Clozapine treatment for suicidality in schizophrenia: International Suicide Prevention Trial (InterSePT). Arch Gen Psychiatry 2003; 60: 82–91.

5 Reid WH, Mason M, Hogan T. Suicide prevention effects associated with clozapine therapy in schizophrenia and schizoaffective disorder. Psychiatr Serv 1998; 49: 1029–33.

6 Pickar D, Owen RR, Litman RE, Konicki E, Gutierrez R, Rapaport MH. Clinical and biologic response to clozapine in patients with schizophrenia. Crossover comparison with fluphenazine. Arch Gen Psychiatry 1992; 49: 345–53.

7 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: 483–8.

8 Dibonaventura M, Gabriel S, Dupclay L, Gupta S, Kim E. A patient perspective of the impact of medication side effects on adherence: results of a cross-sectional nationwide survey of patients with schizophrenia. BMC Psychiatry 2012; 12: 20.

9 Schulte PFJ. Risk of clozapine-associated agranulocytosis and mandatory white blood cell monitoring. Ann Pharmacother 2006; 40: 683–8.

10 Keith SJ, Kane JM. Partial compliance and patient consequences in schizophrenia: our patients can do better. J Clin Psychiatry 2003; 64: 1308–15.

11 Lacro JP, Dunn LB, Dolder CR, Leckband SG, Jeste D V. Prevalence of and risk factors for medication nonadherence in patients with schizophrenia: a comprehensive review of recent literature. J Clin Psychiatry 2002; 63: 892–909.

12 Cramer JA, Rosenheck R. Compliance with medication regimens for mental and physical disorders. Psychiatr Serv 1998; 49: 196–201.

13 Laan W, van der Does Y, Sezgi B, Smeets HM, Stolker JJ, Wit NJ de *et al.* Low treatment adherence with antipsychotics is associated with relapse in psychotic disorders within six months after discharge. Pharmacopsychiatry 2010; 43: 221–4.

14 Mennickent S, Sobarzo a, Vega M, de Diego M, Godoy G, Rioseco P *et al.* Determination of clozapine in serum of patients with schizophrenia as a measurement of medication compliance. Int J Psychiatry Clin Pract 2010; 14: 41–6.

15 Flanagan RJ, Spencer EP, Morgan PE, Barnes TRE, Dunk L. Suspected clozapine poisoning in the UK/Eire, 1992-2003. Forensic Sci Int 2005; 155: 91–9.

16 Couchman L, Morgan PE, Spencer EP, Flanagan RJ. Plasma clozapine, norclozapine, and the clozapine:norclozapine ratio in relation to prescribed dose and other factors: data from a therapeutic drug monitoring service, 1993-2007. Ther Drug Monit 2010; 32: 438–47.

17 Hoffmann J, Lillholm A. Neutrophil fluorescence in patients using clozapine. Ned Tijdschr Klin Chem 2000; 25: 108.

18 ten Berg MJ, Huisman A, van den Bemt PML a, Schobben AF a M, Egberts ACG, van Solinge WW. Linking laboratory and medication data: new opportunities for pharmacoepidemiological research. Clin Chem Lab Med 2007; 45: 13–9.

19 Groeneveld KM, Heeres M, Leenen LPH, Huisman A, Koenderman L. Immunophenotyping of posttraumatic neutrophils on a routine haematology analyser. Mediators Inflamm 2012; 2012: 509513.

20 Huisman A, Stokwielder R, van Solinge WW. Mathematical correction of the invitro storage--related increase in erythrocyte mean cell volume of an automated hematology analyzer--the Cell-Dyn 4000. Lab Hematol 2004; 10: 68–73.

21 Masellis M, Basile VS, Özdemir V, Meltzer HY, Macciardi FM, Kennedy JL. Pharmacogenetics of antipsychotic treatment: lessons learned from clozapine. Biol Psychiatry 2000; 47: 252–266.

22 McEvoy JP, Freudenreich O, Wilson WH. Smoking and therapeutic response to clozapine in patients with schizophrenia. Biol Psychiatry 1999; 46: 125–9.

23 Hasegawa M, Gutierrez-Esteinou R, Way L, Meltzer HY. Relationship between clinical efficacy and clozapine concentrations in plasma in schizophrenia: effect of smoking. J Clin Psychopharmacol 1993; 13: 383–90.

24 Taylor D. Pharmacokinetic interactions involving clozapine. Br J Psychiatry 1997; 171: 109–12.

25 van der Weide J, Steijns LS, van Weelden MJ. The effect of smoking and cytochrome P450 CYP1A2 genetic polymorphism on clozapine clearance and dose requirement. Pharmacogenetics 2003; 13: 169–72.

26 Delieu JM, Badawoud M, Williams MA, Horobin RW, Duguid JK. Antipsychotic drugs result in the formation of immature neutrophil leucocytes in schizophrenic patients. J Psychopharmacol 2001; 15: 191–4.

2.2

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

Jytte de With*, Wai Hong Man*, Coen Maas, Maarten J ten Berg, Wiepke Cahn, Arnold C Koekman, Wouter W van Solinge, Tamar Tak

*Joined first author

Abstract

Background

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.

Methods

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 the fluorescence. Lastly, isolated neutrophil lysates of clozapine users were separated by SDS-PAGE and evaluated.

Results

Clozapine-associated fluorescence ranged from 420nm to 720nm, peaking at 500-550nm. 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 or peptide fragment.

Conclusions

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.

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 < 500x109/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 < 1500x109/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 [10,11]; 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 [12]. 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 have a direct toxic effect on the neutrophil, though this has only been shown in vitro [13–16].

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 [17].

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 [18]. 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 [19,20], 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 non-users [21]. As this fluorescence was specifically observed in neutrophils, studying the origin of this fluorescence could give insight in the way clozapine affects the neutrophil and induces 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.

Method

Reagents and antibodies

Shock buffer consisted of 155 mM Ammonium Chloride, 10 mM Potassium Bicarbonate and 0.1 mM EDTA. The SDS lysis buffer contained 0.5% (w/v) SDS, 0.05 M Tris·Cl and 1 mM 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 (20mM HEPES, 132mM NaCl, 6mM KCl, 1mM $MgSO_4$, 1.2mM KH_2PO_4), supplemented with 5mM glucose, 1.0mM $CaCl_2$ 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), formyl-methionyl-leucyl-phenylalanine (fMLF) and cytochalasin B were purchased from Sigma-Aldrich (St. Louis, MO, USA).

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 Centre Utrecht (UMC Utrecht). Samples consisted of residuals from monthly check-ups 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 100mg/day for longer than one month were included in the study, insuring clozapine induced fluorescence had reached detectable and stable levels [21]. 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.

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 multi-laser flow cytometer, we observed clozapine-associated fluorescence to be stronger when excited with a 405nm laser instead of a 488nm laser as published previously [21]. 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 720nm in 3.2nm steps. Fluorescence was imaged using excitation of 405 nm violet laser light and the standard DAPI (4',6-diamidino-2-phenylindole) filter set.

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 pre-incubated 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.

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

Mononuclear cells were depleted from whole blood by centrifugation (1500 rpm for 5 min (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.

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).

Data and FACS analyses

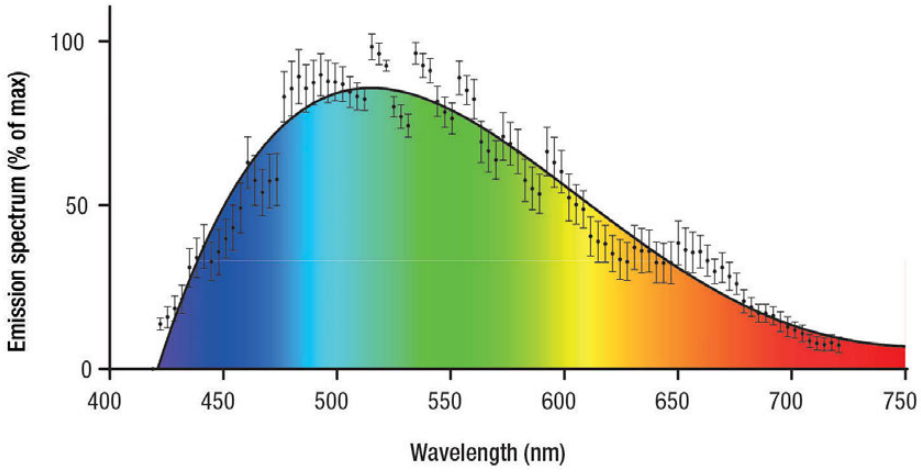
Images were analyzed with ImageJ 1.47T (National Institutes of Health, Bethesda, MD, USA) and FACS data were analyzed with FCS Express 5.01 (De Novo Software, Glendale, CA, USA). Statistical analysis was performed with the free software environment R [22]. A Wilcoxon signed-rank test was used to compare groups, a p-value of 0.05 or lower was considered statistically significant.

Results

Localization of clozapine-associated fluorescence

To confirm previous findings of autofluorescence, specifically observed in neutrophils of clozapine users [21], we started by determining the emission spectrum of clozapine-associated neutrophil fluorescence. Clozapine-associated fluorescence showed a broad emission spectrum, with peak emission around 520nm (Figure 1). Therefore, we chose to use FL-10 fluorescence channel of the FACS (excitation 405nm, emission window ranging from 530-570nm) 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 488nm will most probably result in a similar emission spectrum [23].

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 (Zeiss LSM710) in lambda mode, 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).

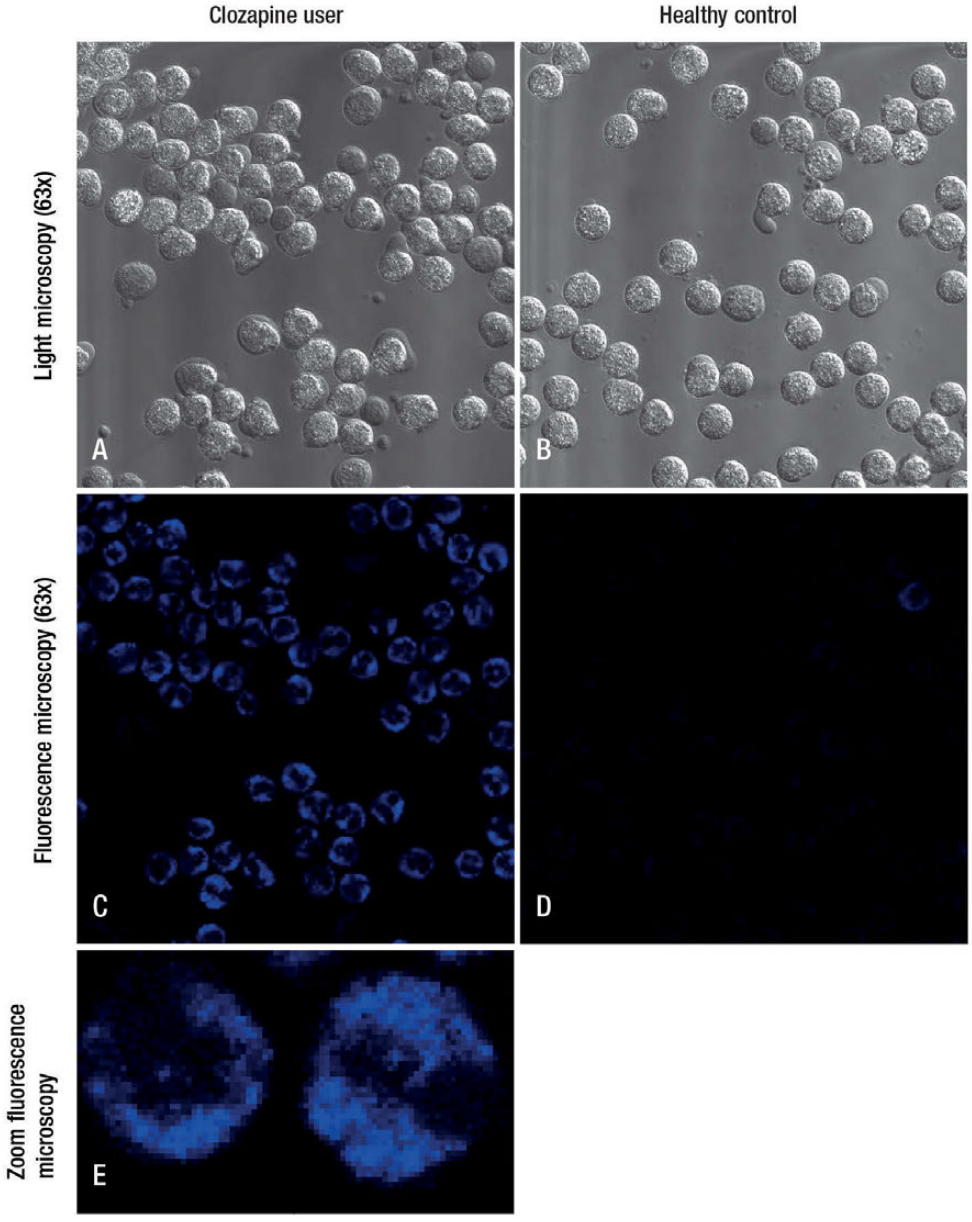
In accordance with our previous findings, we observed a high fluorescence intensity in patient samples signal when compared to controls (Figure 2). Moreover, micro scopy images showed that fluorescence was specifically located in the neutrophils of the patient samples. The fluorescence followed a perinuclear uneven staining pattern, reminiscent of granular staining.

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 [24].

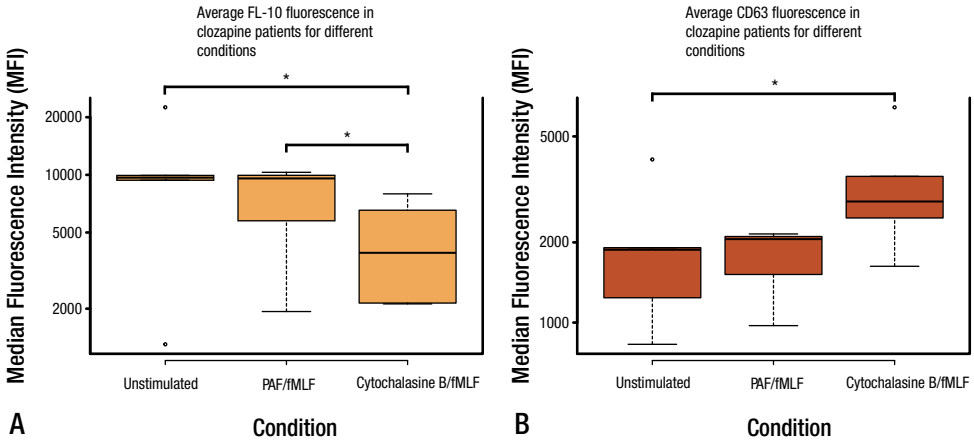
Before and after stimulation, neutrophil autofluorescence (Figure 3 and 4) and a protein marker (CD63, a lysosomal protein residing in the azurophilic granules [25]) for degranulation of the neutrophil azurophilic granules were monitored. A significant increase in the degranulation marker ($p<0.015$) was found, indicating successful stimulation (Figure 3).

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).

Figure 3. Effects of neutrophil stimulation on neutrophil fluorescence and CD63 expression in clozapine users



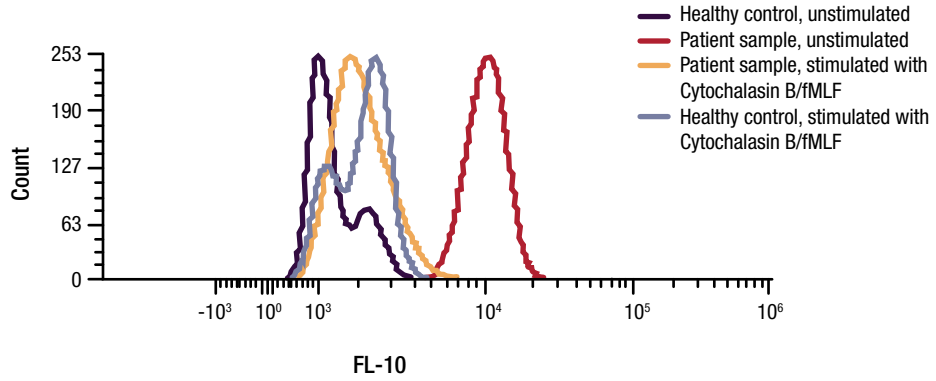
3A: Neutrophil stimulation of clozapine users (N=7) with cytochalasin B/fMLF significantly reduced fluorescence intensity (p=0.015), while stimulation with PAF/fMLF did not (p=0.81). 3B: this chart shows a significant increase in CD63 (p=0.015) – the degranulation marker of azurophilic granules – after incubation with degranulation agents (Cytochalasin B and PAF), indicating successful neutrophil stimulation and degranulation.

A significant decrease in clozapine associated fluorescent signal after stimulation with cytochalasin B/fMLF (p=0.015) was observed, but not after stimulation with PAF/fMLF (p=0.81) (Figure 3B). This finding suggests that the fluorescent signal originates from the azurophilic granules.

Neutrophil fluorescence originates from a 14kDa protein or peptide fragment

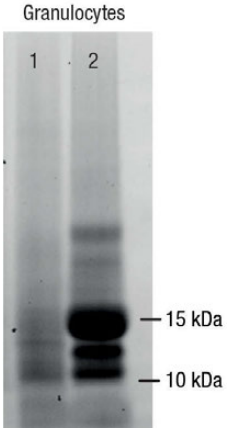
To identify the source of the observed fluorescence, isolated neutrophil lysates were separated by SDS-PAGE and were evaluated under a laser scanner (Figure 5). The gels were excited at 532nm and emission was detected at 555nm. A fluorescent 14kDa band was detected in the patient samples, whereas this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

Figure 4. 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 (purple 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 (yellow).

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).

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 532nm and emission was detected at 555nm. A fluorescent 14kDa 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 14kDa protein or protein fragment.

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 [21] 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.

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) [26]. MPO is thought to play a role in the formation of the clozapine nitrenium ion, [15,27], an ion possibly toxic to neutrophils [13,14]. Interestingly, MPO is most abundantly present in azurophilic granules of neutrophils – the same type of granules involved in observed clozapine-associated fluorescence –. Therefore, clozapine-associated fluorescence might be linked with the formation of the clozapine nitrenium ion and indirectly with CIA.

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 a non-peptide, 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 auto-fluorescent protein, or a combination of the two. As mentioned before, clozapine can be oxidized and catalyzed by peroxidases such as MPO, located in the azurophilic granule, into a reactive nitrenium metabolite potentially causing cell damage. Hence, an explanation for the fluorescence could be the alteration of an endogenous protein into a fluores-

cent protein, induced by intracellular oxidized clozapine. Moreover, 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 (which we did not observe).
It remains difficult, however, to pinpoint one protein responsible for the fluorescence. It is possible that the fluorescence arises from a protein of 14kDa, residing in the granules. However, it is also possible that the protein is altered by a reaction with clozapine (or one of its metabolites) additionally, the protein could be degraded by the enzymes in the granule.
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 auto-fluorescent when oxidized [28]. There are approximately 90 flavoproteins known in humans [29]. 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. Additionally, it has been shown that certain flavoproteins are involved in clozapine oxidation such as NADPH, mediated by NADPH oxidase, an enzyme that together with MPO forms the major oxidation system in neutrophils and can generate a superoxide (O^-_2) that is converted to H_2O_2 [16,30]. Consequently, MPO catalyzes the formation of hypochlorous acid (HOCl) from H_2O_2 and chloride [16,30]. Eventually, hypochlorous acid oxidizes clozapine to the reactive nitrenium metabolite that is irreversibly bound to cells [13–16].
The fluorescence could also be originating from (a part of) MPO. MPO is built up from two light chains and two heavy chains that are linked by sulfur bridges. These sulfur bridges are cleaved by DTT in the lysis buffer during SDS-PAGE analysis, leading to the availability of the light and heavy chains in the SDS gel. Interestingly, the light chain is approximately 14kDa [31]. Given that MPO is secreted during neutrophil degranulation [32], the observed fluorescent 14kDa band could be originating from MPO.
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. There have been lists of granular proteins published [33]. We have used one of these lists to select proteins of approximately 14kDa. 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)*. The first is a protein (BPI3) expressed in mouse brain [34], but its function in humans remains unknown. The latter, NADH dehydrogenase (ubiquinone) is an enzyme complex involved in the respiratory chain [35]. Moreover, NADH dehydrogenase may have a role in triggering apoptosis and appears to be a potent source of reactive oxygen species by transferring one electron from reduced flavin mononucleotide ($FMNH_2$) to oxygen [36–38]. In this regard, observed clozapine-associated fluorescence, if indeed appears to be originating from (upregu-

lated) ubiquinone, could be involved as a part in the CIA puzzle since ubiquinone plays a role in triggering apoptosis. This is, however highly speculative and future studies have to reveal the nature of this protein.

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 approximately 230 hours [39]. A marker to evaluate adherence would be highly beneficial in this specific patient population, as medication non-adherence is the major cause of treatment failure in schizophrenia patients [40].

More importantly, it is possible that the clozapine-associated fluorescence in neutrophils provides a piece of the CIA puzzle. Considering the half-life of FL3-fluorescence, 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 [13,16].

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, possibly by intracellular superoxide production, 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 an episode of agranulocytosis showed enhanced apoptosis expression markers and intracellular superoxide production compared to clozapine users without agranulocytosis [41].

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 in clozapine-induced agranulocytosis and leukopenia.

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References

1 Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic: A double-blind comparison with chlorpromazine. Arch. Gen. Psychiatry. 1988; 45: 789–796.

2 Food and drug administration USA. FDA label Clozapine. 1989.

3 Meltzer HY, Alphs L, Green AI, Altamura AC, Anand R, Bertoldi A *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.

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.

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.

7 Griffith RWW, Saameli K. Letter: Clozapine and agranulocytosis. Lancet 1975; 2: 657.

8 Idänpään-Heikkilä J, Alhava E, Olkinuora M, Palva I. Letter: Clozapine and agranulocytosis. Lancet 1975; 2: 611.

9 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: 3–7.

10 Regen F, Herzog I, Hahn E, Ruehl C, Le Bret N, Dettling M *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.

11 Hinze-Selch D, Becker EW, Stein GM, Berg PA, Mullington J, Holsboer F *et al.* Effects of clozapine on in vitro immune parameters: A longitudinal study in clozapine-treated schizophrenic patients. Neuropsychopharmacology 1998; 19: 114–122.

12 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: 180–6.

13 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: 1375–82.

14 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: 1463–75.

15 Guest I, Sokoluk B, MacCrimmon J, Uetrecht J. Examination of possible toxic and immune mechanisms of clozapine-induced agranulocytosis. Toxicology 1998; 131: 53–65.

16 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.

17 Johnston A, Uetrecht J. Current understanding of the mechanisms of idiosyncratic drug-induced agranulocytosis. Expert Opin Drug Metab Toxicol 2014; 72: 1–15.

18 Goldstein JI, Fredrik Jarskog L, Hilliard C, Alfirevic A, Duncan L, Fourches D *et al.* Clozapine-induced agranulocytosis is associated with rare HLA-DQB1 and HLA-B alleles. Nat Commun 2014; 5: 4757.

19 Fanger NA, Liu C, Guyre PM, Wardwell K, O'Neil J, Guo TL *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: 4128–35.

20 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.

21 Man WH, Ten Berg M, Wilting I, Huisman A, Cahn W, Douma JW *et al.* Fluorescence of neutrophil granulocytes as a biomarker for clozapine use. Eur Neuropsychopharmacol 2013; 23: 1408–13.

22 R Core Team. R: A Language and Environment for Statistical Computing. 2013.

23 Kln P, Wirz J. Photochemistry of Organic Compounds: From Concepts to Practice. John Wiley & Sons, Ltd: Chichester, UK, 2009.

24 Bentwood BJ, Henson PM. The sequential release of granule constitutents from human neutrophils. J Immunol 1980; 124: 855–62.

25 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.

26 Klebanoff SJ. Myeloperoxidase: friend and foe. J Leukoc Biol 2005; 77: 598–625.

27 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.

28 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.

29 Macheroux P, Kappes B, Ealick SE. Flavogenomics--a genomic and structural view of flavin-dependent proteins. FEBS J 2011; 278: 2625–34.

30 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.

31 Nauseef WM, Malech HL. Analysis of the peptide subunits of human neutrophil myeloperoxidase. Blood 1986; 67: 1504–7.

32 King CC, Jefferson MM, Thomas EL. Secretion and inactivation of myeloperoxidase by isolated neutrophils. J Leukoc Biol 1997; 61: 293–302.

33 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: 711–21.

34 Gong Y, Wu J, Qiang H, Liu B, Chi Z, Chen T *et al.* BRI3 associates with SCG10 and attenuates NGF-induced neurite outgrowth in PC12 cells. BMB Rep 2008; 41: 287–93.

35 Brandt U. Energy converting NADH:quinone oxidoreductase (complex I). Annu Rev Biochem 2006; 75: 69–92.

36 Ohnishi T. Iron-sulfur clusters/semiquinones in Complex I. Biochim Biophys Acta - Bioenerg 1998; 1364: 186–206.

37 Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417: 1–13.

38 Jaskova K, Pavlovicova M, Jurkovicova D. Calcium transporters and their role in the development of neuronal disease and neuronal damage. Gen Physiol Biophys 2012; 31: 375–382.

39 Man WH, Pérez-Pitarch A, Wilting I, Heerdink ER, van Solinge WW, Egberts ACG *et al.* Development of a nomogram for the estimation of long-term adherence to clozapine therapy using neutrophil fluorescence. Br J Clin Pharmacol 2018.

40 Valenstein M, Blow FC, Copeland LA, McCarthy JF, Zeber JE, Gillon L *et al.* Poor antipsychotic adherence among patients with schizophrenia: medication and patient factors. Schizophr Bull 2004; 30: 255–64.

41 Fehsel K, Loeffler S, Krieger K, Henning U, Agelink M, Kolb-Bachofen V *et al.* Clozapine induces oxidative stress and proapoptotic gene expression in neutrophils of schizophrenic patients. J Clin Psychopharmacol 2005; 25: 419–26.

2.3

Development of a nomogram for the estimation of long-term adherence to clozapine therapy using neutrophil fluorescence

Wai Hong Man, Alejandro Pérez-Pitarch, Ingeborg Wilting, Eibert R Heerdink, Wouter W van Solinge, Toine CG Egberts, Alwin DR Huitema

Br J Clin Pharmacol. (in press)

Abstract

Background

Previously, we have reported an association between clozapine use and elevated FL3 neutrophil fluorescence, a flow-cytometric parameter for cell viability. Here, we developed and evaluated a pharmacokinetic–pharmacodynamic (PK/PD) model relating FL3-fluorescence to clozapine exposure and derived a nomogram for estimation of long-term adherence.

Methods

Data from 27 patients initiating clozapine were analyzed using nonlinear mixed effects modelling. A previously described pharmacokinetic model for clozapine was coupled to a FL3 fluorescence model. For this an effect compartment with clozapine concentrations as input and a first order decay rate as output was linked with an E_{max} model to FL3-fluorescence. FL3-fluorescence was simulated for clozapine doses of 50, 150 and 400 mg daily (N=10,000) to establish the nomogram. Finally, true simulated adherence (% of daily doses taken over 100 days) was compared to nomogram-estimated adherence to evaluate the performance of the nomogram.

Results

The half-life of FL3-fluorescence was estimated at 228 hours (coefficient of variation 35%). Median absolute prediction errors (PE) of the nomogram in case of fully random adherence for 50, 150 and 400 mg ranged from -0.193% to -0.525%. The nomogram performed slightly worse in case of nonrandom adherence (median PE up to 5.19%), but was still clinically acceptable. Compliance patterns containing longer drug holidays revealed that the nomogram adequately estimates compliance over approximately the last 3 weeks prior to FL3-measurement.

Conclusions

Our nomogram could provide information regarding long-term adherence based on prescribed clozapine dose and FL3-fluorescence. Future studies should further explore the clinical value of this biomarker and nomogram.

Introduction

Schizophrenia is a severely disabling chronic form of mental illness with a prevalence of about 1% of the world population [1]. Pharmacotherapy with antipsychotics forms the cornerstone of treatment. Within this group, clozapine is the only drug with demonstrated efficacy in treatment-resistant schizophrenia [2–5]. Moreover, it is the only agent with demonstrated anti-suicidal properties [6].

Known for its serious adverse effects including agranulocytosis, use of clozapine is limited to those suffering from treatment-resistant schizophrenia. Non-adherence is a major issue in the treatment of schizophrenia. An estimated 25% of the patients is partially compliant within the first 7–10 days of treatment [7] and studies estimate the mean overall prevalence of non-adherence between 40% and 50% [8]. Results of one study revealed that half of the ambulant schizophrenic patients take less than 70% of their medication [9]. Another study showed that low treatment adherence is associated with a two-fold increased risk for relapse in psychotic disorders within six months after discharge [10]. More specifically, gaps in antipsychotics coverage of 10 days based on dispensing data was associated with an increased risk of psychiatric hospitalization [11,12], reflecting the potential forgiveness of antipsychotic agents. Compared to other atypical antipsychotics, higher rates of adherence were observed with clozapine, explained by superior efficacy or the requirements for close monitoring [13]. An earlier cohort study showed that 11% of patients registered to receive clozapine did not initiate therapy, while 30% discontinued therapy after having received clozapine for at least 1 week [14].

Given the strong relationship between serum concentrations and clinical effect of clozapine, therapeutic drug monitoring (TDM) is a valid tool to optimise dosing [15]. TDM may also be used to assess adherence to therapy [16], however, due to relatively short elimination half-life of clozapine of about 14 hours [17] and its main metabolite N-desmethylclozapine (half-life of 18 hours [17]), serum concentration monitoring only provides information on clozapine intake for a few days prior to blood sampling at most. Therefore, a biomarker providing long-term information on adherence would be of great value.

Neutrophil granulocyte fluorescence that is measured at the FL3 channel on a flow cytometer, in this article termed 'FL3-fluorescence', is used as a flow-cytometric parameter that measures nucleated red blood cells and white blood cell viability by propidium iodide (PI) staining. Elevated FL3-fluorescence has been serendipitously observed in patients using clozapine [18]. In a previous study, our group has assessed the association between clozapine use and FL3-fluorescence [19]. FL3-fluorescence was significantly higher in clozapine users than in non-users. FL3-fluorescence was found to increase with increasing clozapine dose and at a given clozapine dose this fluorescence intensity seemed to reach a maximum. Also, the degree of elevation in FL3-fluorescence was most likely associated with duration of clozapine therapy. To

determine whether FL3-fluorescence could serve as a potential biomarker to estimate patients' long-term adherence to clozapine, information regarding the time course of FL3-fluorescence in relation to clozapine therapy is the next step. The objectives of the current study were to describe the association between FL3-fluorescence and clozapine therapy in time using PK/PD modelling and to establish a nomogram for estimating long-term adherence based on FL3 values.

Methods

Patient population and data collection

Data were extracted from the Utrecht Patient Oriented Database (UPOD). UPOD is an infrastructure of relational databases comprising data on patient characteristics, hospital discharge diagnoses, medical procedures, medication orders and laboratory tests for all patients treated at the University Medical Center Utrecht (UMC Utrecht) since 2004 [20]. UPOD data acquisition and data management were in line with current Dutch regulations concerning privacy and ethics. The data used for this cohort study were collected for patient care purposes and were used retrospectively. Because no extra material, for example, blood samples, is taken from patients, there is not a requirement to obtain informed consent from individual patients or seek IRB approval for every study protocol. The structure and content of UPOD have been described in detail elsewhere [20].

The study population comprised of all psychiatric inpatients of the UMC Utrecht aged 16 years or older having been prescribed clozapine between February 2004 and September 2013 with at least two FL3-fluorescence measurements available (see below). Clozapine use was defined as having a medication order for clozapine. A medication order was defined as an order in the computerised physician order entry (CPOE) system. Clozapine medication orders, co-medication, clozapine serum levels, indications for clozapine prescriptions and comorbidity were retrieved from the medical record.

Definition clozapine starters and stable users

Start of clozapine was defined as a first clozapine prescription with no evidence of a clozapine prescription for at least one month before, followed by a gradual dose escalation resulting in a dose of at least 50 mg once daily within the subsequent seven days. Participants were followed until they were defined as stable users or stopped clozapine therapy. Stable clozapine users were defined as patients with a medica-

tion order for clozapine where the prescribed dose remained unchanged for at least five consecutive days or information in the patient records explicating continuing of same dose for at least five days, as it was expected that clozapine pharmacokinetics reached steady state after five days.

FL3-fluorescence of neutrophil granulocytes

UPOD contains specific haematology data of automated blood cell analyses performed by the Abbott Cell-Dyn Sapphire automated haematology analyser (Abbott Diagnostics, Santa Clara, CA, USA) [21–23]. A feature of this haematology analyser is that all parameters of the complete blood count (CBC) are measured irrespective of the requested parameter. The analyser is equipped with an integrated 488 nm blue diode laser and uses spectrophotometry, electrical impedance and laser light scattering (multi angle polarised scatter separation, [MAPPS]), to measure the CBC and to classify the white blood cell counts (WBCs). For the WBC differential count the following five optical scatter signals are measured for each individual cell: cell size (0°scatter, axial light loss [ALL]), cell complexity and granularity (7° scatter, intermediate angle scatter [IAS]), nuclear lobularity (90° scatter, polarised side scatter [PSS]), depolarization (90° depolarised side scatter [DSS]) and red fluorescence (90° (FL3), 630 ± 30 nm). This FL3-fluorescence parameter measures Nucleated Red Blood Cells and WBC viability by propidium iodide (PI) staining. PI stains nucleic acids and is part of the reagents used by the analyser. PI is capable of crossing the cell-membrane of non-viable WBCs and stains nucleic acids (RNA and DNA). The WBC viability is reported by the analyser as the white cell viability factor (WVF). In the current analysis, only FL3-fluorescence values associated with a WVF of ≥0.95 were included in order to differentiate between fresh and old samples [24]. Because the clinical relevance of the FL3 parameter is unknown, this parameter was not reported to the physician but still included in the UPOD database.

Pharmacokinetic-pharmacodynamic model development

Nonlinear mixed-effect modelling was performed using NONMEM software (Version 7.2.0; ICON Development Solutions, Ellicott City, MD, USA). NONMEM runs were executed using Pirana modelling and simulation workbench for NONMEM [25]. The first-order conditional estimation with interaction (FOCE-I) method was used. The minimum value of the objective function (-2 times the log likelihood, OFV), typical goodness-of-fit diagnostic plots, evaluation of the precision of model parameter and variability estimates were used for model selection during the model-building process. Data processing and preparation of plots was performed in R (version 3.2.1).

Clozapine plasma-concentration data were not available for this study so a previously published model was employed [26]. Population PK parameters were fixed to those in the original model and population PK predictions were used as a driving force for the pharmacodynamics (PD) model.
A PK-PD model was built to describe the relation between clozapine plasma concentrations and FL3-fluorescence over time. During PD model building-process, different drug effect models and drug effect delay models were tested.
Inter-individual variability (IIV) for PD parameters was modelled assuming a log normal distribution:

$$P_i = \theta_P \cdot exp(\eta_{P,i})$$

where P_i is the individual parameter, θ_P is the typical value of the parameter and $\eta_{P,i}$ is the random effect for that parameter with a mean of 0 and variance of ω_P^2. Additive, proportional, additive-proportional and exponential error models where tested to describe residual error in FL3-fluorescence prediction.
The evaluation of the model was carried out by means of an internal validation process. Non-parametric bootstrap resampling (n=1,000) was applied to the final model and the 95% confidence intervals (95%CI) for the estimates were calculated. The mean and 95%CI (percentiles 2.5 and 97.5%) of these 1,000 estimates were compared to those obtained in the original population set.

Development of adherence nomogram

The final PK/PD model was implemented in R as a set of ordinary differential equations. Inter-individual and residual variability was taken into account in these simulations. A 100-day study was simulated with 10,000 patients in each study arm. Three study-arms were included: clozapine 50, 150 and 400 mg daily. The main outcome was FL3-fluorescence values at the end of study period. A 100-day period was chosen so that steady state was reached across model-compartments. In order to simulate a wide variety of treatment adherence patterns, a random clozapine-intake probability (between 0-1 from a uniform distribution) was generated for each patient. Then, this probability was used to determine whether each patient did or did not take its clozapine dose for each scheduled dose administration.
Simulation results were explored to find the relations between daily clozapine dose, treatment adherence and FL3-fluorescence values at steady state. A graphical nomogram was then designed for estimation of predicted treatment adherence (PTA) expressed as proportion (%) daily doses taken over 100 days.
The ABC taxonomy, as described by the EMERGE steering committee [27], was followed

for describing and defining adherence to clozapine use by means of A. initiation, B. implementation and C. persistence of therapy.

Evaluation of the nomogram

The predictive performance of the nomogram was also investigated through simulations. The same study design as used in nomogram development was employed. For each simulated patient, nomogram-based PTA values were compared to true treatment adherence (TA) to evaluate predictive performance. The median absolute prediction error (PE) and IQR were calculated for each dosing level. PE was calculated as:

$$PE = PTA - TA$$

As an exploration of model robustness in adverse conditions, the nomogram was also tested assuming nonrandom adherence as might be expected in clinical practice. In these simulations, on the last day before FL3-fluorescence analysis, patients with adherence levels equal or above 80% took the prescribed dose, and those patients with adherence below 80% took double the prescribed dose. In addition, we simulated three specific nonrandom adherence patterns (A, B and C) to evaluate the predictive performance of the nomogram. Details of these patterns are described in the figure legend of Figure 5.

Table 1. Patient characteristics

	Included patients (N=27)
Gender, n (% male)	19 (70.4)
Age in years, mean (SD)	33.1 (15.4)
Weight in kg, mean (SD)	70.5 (13.8)
BMI in kg/m^2, mean (SD)	22.1 (3.3)
Clozapine dose in mg/day during steady state, mean (SD)	175.5 (90.6)
Indication of clozapine therapy	
Schizophrenia, n (%)	18 (66.7)
Schizoaffective disorder, n (%)	3 (11.1)
Psychotic disorder NOS, n (%)	3 (11.1)
Delirium in pre-existing Lewy body dementia, n (%)	1 (3.7)
Schizophreniform disorder, n (%)	1 (3.7)
Depressive disorder with psychotic features, n (%)	1 (3.7)

Results

Data

A total of 27 patients (N=181 FL3 measurements) starting clozapine therapy were followed up in time after initiation of clozapine therapy. Patient characteristics are shown in followed-up Table 1.

Pharmacokinetic-pharmacodynamic model development and evaluation

Among the tested pharmacodynamic-model structures to describe FL3-fluorescence, the final model (schematic representation on Figure 1) consisted of an effect compartment linked to FL3-fluorescence by an E_{max} effect model. FL3-fluorescence was described by:

$$FL3 = FL3_0 \cdot \left(1 + \frac{E_{max} \cdot C_e}{Ce_{50} + C_e} \right)$$

in which, $FL3_0$ denotes the baseline value of FL3-fluorescence, E_{max} denotes the maximal effect (maximal increase of FL3-fluorescence relative to $FL3_0$), Ce_{50} denotes the concentration of clozapine in the effect compartment which produces 50% of maximal effect and C_e denotes the concentration of clozapine in the effect compartment, which was described by:

$$\frac{dC_e}{dt} = C_p - k_{out} \cdot C_e$$

In this equation, C_p denotes clozapine plasma-concentration and k_{out} denotes the effect compartment elimination rate constant. k_{out} was parameterised as:

$$k_{out} = \frac{ln(2)}{Te_{1/2}}$$

where $Te_{(1/2)}$ denotes elimination half-life in effect compartment, which was estimated. Residual error in FL3-fluorescence was modelled using:

$$FL3_{obs} = FL3 \cdot exp\ (\varepsilon_{exp,FL3})$$

In which $FL3_{obs}$ is the observed FL3-fluorescence, $FL3$ is the model predicted

FL3-fluorescence and $\varepsilon_{exp,FL3}$ represents the exponential random error for FL3. It was assumed that ε is normally distributed with the mean 0 and variance σ^2.

Figure 1. Schematic representation of the model

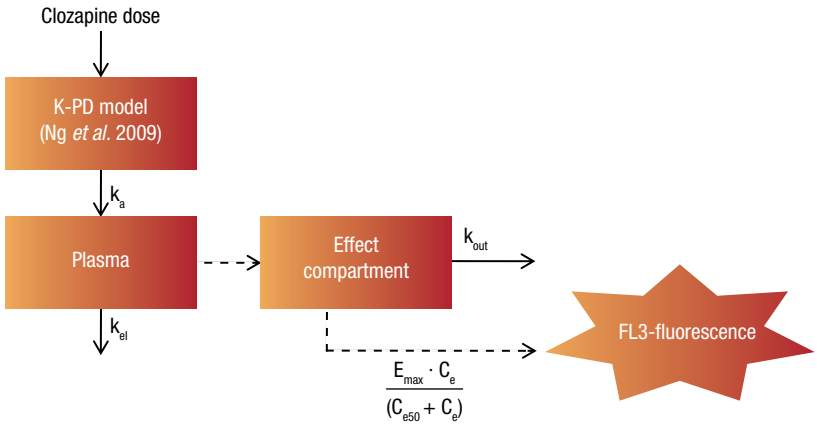
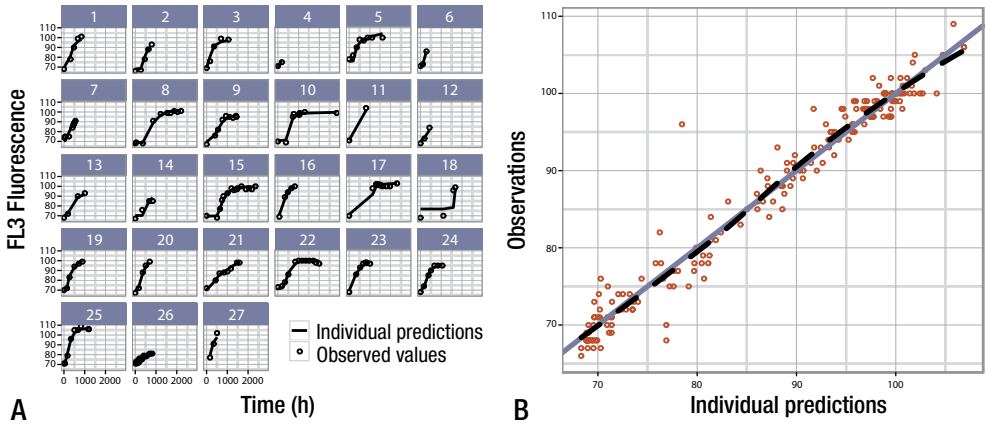


Table 2. Final model parameter estimates and bootstrap results

Parameter	Estimate	RSE (%)	Bootstrap results*	
			Median	95% CI
$FL3_0$	70.3	1	70.2	69.0-71.5
E_{max}	0.578	10	0.599	0.518-0.701
$T_{e1/2}$ (h)	228	35	205	131-400
C_{e50}	57.0	14	58.2	45.9–78.7
CV% IIV $FL3_0$	3.08	22	2.98	0.732–4.50
CV% IIV E_{max}	9.05	49	10.07	2.07–18.2
CV% RSE_{exp}	3.32	25	2.98	1.92-4.24

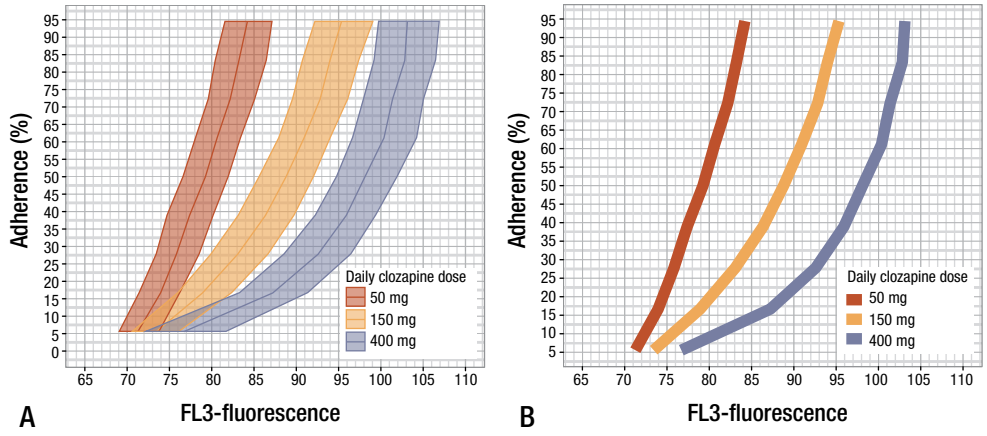
*Bootstrap results are derived from 667 successful bootstrap runs. RSE: relative estimation error; 95% CI: 95% confidence interval derived from the 2.5 and 97.5 percentiles of parameter estimates in successful bootstrap runs; $FL3_0$: baseline FL3-fluorescence value; E_{max}: maximum increase in $FL3_0$ as a consequence of clozapine administration relative to $FL3_0$, Ce_{50}: clozapine concentration in effect compartment necessary to achieve 50% of the maximum increase in FL3-fluorescence; CV% IIV: parameter inter-individual variability expressed as coefficient of variation (%); CV% RSE_{exp}: residual exponential error expressed as coefficient of variation (%).

Figure 2. Goodness of fit plots



2A: individual time course of observed (DV) and model derived individual predictions (IPRED) for FL3-fluorescence; 2B: DV *vs* IPRED, blue line shows line of identity and the striped line is the trend line showing the relation between DV and IPRED

Figure 3. Simulation results and proposed nomogram



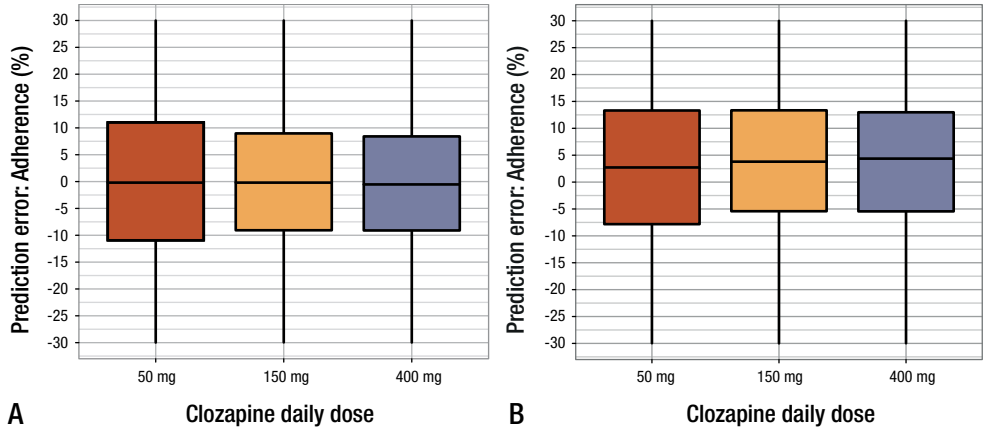
3A: Simulation results: 100-day study with 10,000 patients in each study arm (clozapine 50, 150 and 400 mg daily). FL3-fluorescence values at the end of study period (steady state) are plotted against treatment adherence values. Results have been stratified per dosing level (red: 50 mg QD; yellow: 150 mg QD; blue: 400 mg QD). Solid lines and shaded areas represent FL3-fluorescence median and interquartile range, respectively, across treatment adherence values for different dosing arms. 3B: Proposed nomogram for treatment adherence estimation, defined by a representation of solely median FL3-fluorescence values as were also depicted in Figure 3A.

The model adequately described the data as can be seen in the goodness-of-fit plots: Figures 2A shows the individual time course of observed (DV) and model derived individual predictions (IPRED) of FL3-fluorescence values, and Figure 2B displays the relationship between DV and IPRED.
Model parameter estimates, estimation errors and bootstrap results are shown in Table 2. All parameters were estimated with adequate precision and results of the bootstrap analysis were in close agreement to the final parameter estimates.

Development of nomogram

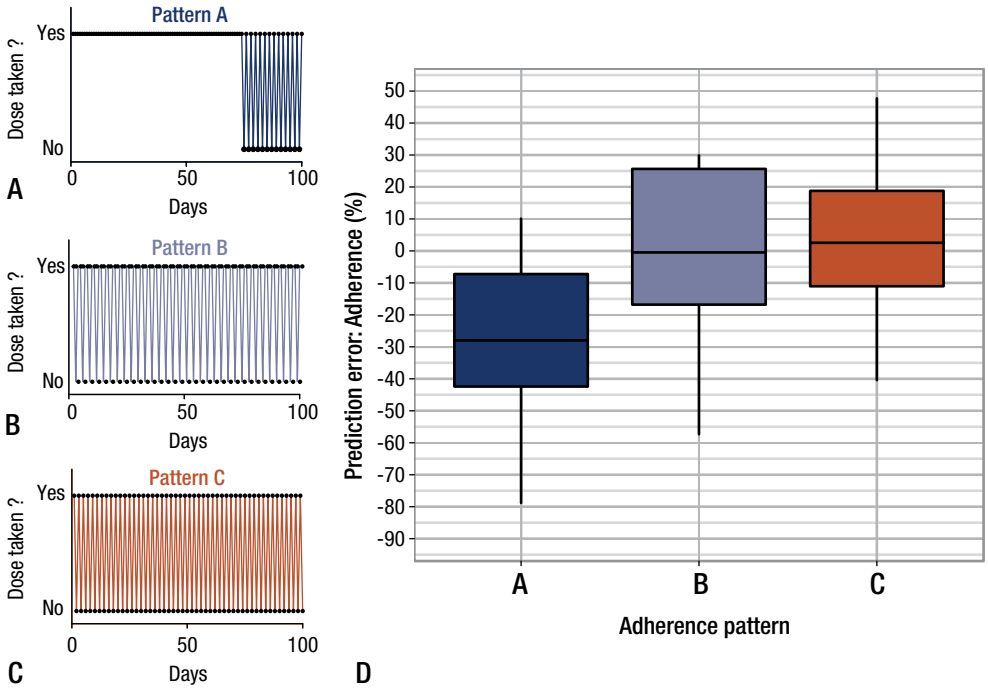
Results for the 100-day simulation study with 10,000 patients in each study arm (clozapine 50, 150 and 400 mg daily, fully random adherence) are presented in Figure 3A. The relationship between FL3-fluorescence and treatment adherence in patients receiving 50 mg clozapine QD was almost linear. At higher doses (150 and 400 mg), this relationship was strongly nonlinear.
In order to develop a nomogram for clozapine adherence prediction, both daily dose and FL3-fluorescence values must be taken into account as evidenced in Figure 3A. Hence, the nomogram consisted of the representation of median FL3-fluorescence values across adherence values, based on the simulation results derived from the developed PK-PD model (Figure 3B). The interpolation of FL3-fluorescence value on the corresponding daily dose line outputs the predicted treatment adherence (PTA).

Figure 4. Prediction error of the nomogram



4A: Prediction error when nomogram is tested in patients having random adherence; 4B: Prediction error when nomogram is tested with patients having nonrandom adherence (on the last day before FL3-fluorescence analysis, patients with adherence levels equal or above 80% took the prescribed dose, and those patients with adherence below 80% took double the prescribed dose). Box and whisker plots depict the median and interquartile range (IQR) of the prediction error.

Figure 5. Nonrandom adherence patterns and prediction error of the nomogram



5A: Pattern A: until day 75 all doses were taken in. From day 75 a dose was not taken in every two days. Thus, the overall adherence rate is nearly 90%; 5B: Pattern B: Throughout all 100 days, starting from day 1 a dose was not taken in every three days. Thus, the overall adherence rate is nearly 66%.; 5C: Pattern C: Throughout all 100 days, starting from day 1 a dose was not taken in every two days. Thus, the overall adherence rate is nearly 50%.; 5D: Prediction error when the nomogram is tested in patients following pattern A,B and C.

Evaluation of the nomogram

The performance of the nomogram for the 100-day simulation study with 10,000 patients in each study arm (clozapine 50, 150 and 400 mg daily, fully random adherence) is presented in Figure 4A. Median absolute prediction errors (PE) and the IQR (%) for the 50, 150 and 400 mg arms were -0.193 (IQR -14.1–14.2) , -0.193 (IQR -10.9–10.7 and -0.525 (IQR -12.1–11.8), respectively. No relevant bias was shown in nomogram predictions under these conditions.

In case of nonrandom adherence (e.g. patients with low adherence take a double dose just before measurement) the performance of the nomogram is shown in Figure 4B. Under these conditions the median absolute PE and IQR (%) were 4.07 (IQR -9.50–18.7), 4.37 (IQR -7.05–16.6) and 5.19 (IQR -8.43–17.6). In addition, we tested a few typical adherence patterns to calculate the performance of the nomogram in each

specific pattern in the 150 mg daily study arm, as depicted in Figure 5. Pattern 5A reflects a nonuniform pattern across the whole study period. Until day 75 all doses were taken in and from then on each 2 days a dose was not taken in, resulting in an overall adherence rate of nearly 90%. Pattern 5B depicts a uniform pattern across the study period, with one dose being omitted each 3 days (overall adherence rate of nearly 66%). Lastly, pattern 5C depicts also a uniform pattern, with one dose being omitted each 2 days (overall adherence rate of 50%). Median absolute PE and IQR (%) for pattern 5A, 5B and 5C were respectively -27.97 (IQR -42.42–-7.23), -0.47 (IQR -16.79–25.65) and 2.54 (IQR -11.05–18.78).

Discussion

Previously, we performed a cross-sectional observational study reporting a significant association between clozapine use and FL3-fluorescence [19]. FL3-fluorescence was elevated in clozapine users and was found to increase with increasing clozapine dose. However, the timing of onset and maximal increase of FL3-fluorescence after initiation of clozapine therapy was not quantified. This information would contribute to the evaluation of FL3-fluorescence being a potential long-term adherence biomarker. For this, we developed a PK/PD model based on additional data. In the results of our data-analysis two essential factors arose.

First, a significant nonlinear relationship appears to exist between clozapine dose and FL3-fluorescence evidenced by the E_{max} model that describes this relationship. Moreover, FL3-fluorescence based estimations of adherence can only be performed taking the clozapine dose into account. As depicted in Figure 3, the association between adherence and FL3-fluorescence at a clozapine dose of 50 mg/day displays an almost linear relationship, indicating that the nomogram becomes less discriminative at lower clozapine dose, resulting in increased prediction errors (Figure 4).

Second, the half-life of FL3-fluorescence elevation after clozapine exposure appeared to be approximately 230 hours (CV 35%), which would imply that nearly 48 days (5 times the half-life) are needed to reach steady state condition of elevated FL3-fluorescence. This long half-life makes FL3-fluorescence an appropriate indicator for long-term adherence.

As depicted in Figure 5, in case of nonrandom specific adherence patterns, the performance of the nomogram is not expected to change when the adherence pattern is more or less uniform across the whole study period of 100 days, evidenced by the patterns in Figure 5B and Figure 5C. More specifically, random omission of intake leading to short drug holidays (period of consecutive omitting clozapine intake) do not affect the performance of the nomogram, in accordance with the long half-life of FL3-fluorescence. This would also implicate that long drug holidays, thereby forcing clozapine intake to follow a nonuniform pattern across the study period, affect the per-

formance of the nomogram. That is, the longer the drug holiday, the more time is given for enhanced FL3 to normalise towards baseline, eventually affecting the predictive performance of the nomogram negatively. In other words, if the patterns considerably changes during the study period, results will depend mainly on the pattern in the period preceding the FL3 measurement as evidenced by Figure 5A.

Compared to the average complete over the 100-day study period, the nomogram gives a biased estimate of adherence. However, an adequate estimation of adherence of the three weeks preceding the FL3 measurement is obtained. These results indicate that FL3 measurement could be considered as a potential mid-term adherence marker estimating adherence over a time period of approximately 3 weeks.

In our dataset we only had data from patients available who started clozapine therapy. Including patients who discontinued therapy would have provided an improved estimate of the half-life of elevated FL3-fluorescence, which is the key parameter for establishment of adherence based on this parameter. Unfortunately, FL3 measurements in patients discontinuing clozapine were scarce in our dataset, since in clinical practice blood counts are not routinely monitored after cessation of therapy.

The findings described above are consistent with our previous cross-sectional observational study, in which clozapine users who did not show elevated fluorescence appeared to be in the clozapine initiation phase and thus had not yet reached steady state of elevated FL3-fluorescence.

Clozapine doses of 50, 150 and 400 mg/day were chosen for the simulation study, related to clinical practice, since these dosages distinguish geriatric and psychiatric indications, are prescribed as maintenance dose in initial clozapine therapy and prescribed as effective chronic maintenance dose.

The exact mechanism of FL3-fluorescence increase on clozapine use remains unknown. Considering the half-life and thus the period needed to reach steady state (approximately 48 days) of FL3-fluorescence, the physiology of elevated FL3-fluorescence might be linked to incorporation of this signal in the bone marrow. Indeed, FL3-fluorescence is observed in neutrophil granulocytes and it is established that duration of myelopoiesis takes time in terms of weeks [28].

A limitation of our study is our assumption that patients included in this study were fully adherent to clozapine. However, all participants were inpatients and clozapine intake took place under surveillance.

Another limitation is the lack of potential relevant covariates including smoking status and plasma concentrations. However, the smoking status might have an impact on clozapine exposure, but its influence on FL3-fluorescence is unknown. Furthermore, it should be noted that the nomogram does not take parameter uncertainty into account. Full evaluation of the predictive performance can only be performed with additional data from patients with known and preferably also suboptimal adherence.

An advantage of FL3-fluorescence is that this flow-cytometric marker is determined automatically on the Abbott Cell-Dyn Hematology analyser when a blood count is being

measured, which is often the case in clozapine users. For this reason, no additional costs or sample preparation are needed to obtain FL3-fluorescence. Nevertheless, this specific flow-cytometric analyser is not available in every clinical setting. Thus, signal output of other flow-cytometry channels from other flow-cytometry analysers should be examined and interpreted differently.

To date, as far as we know, a biomarker that could estimate long term clozapine adherence – likewise Glycated Haemoglobin (HbA1c) provides long term information about glucose control - has not been established yet. Previously, other long half-life markers are described in the assessment of adherence, including very low-dose phenobarbital (to investigate adherence in patients on reducing doses of methadone) [29], bromide in hypertensive patients [30] and methotrexate polyglutamates in juvenile idiopathic arthritis and juvenile dermatomyositis [31]. In contrary to FL3 fluorescence, mentioned markers were quite time-, cost- and work-consuming, therefore hampering use in clinical practice. However, just like FL3 fluorescence, so called 'drug holidays' cannot be assessed due to the long half-life of the markers. Moreover, levels of such markers would not indicate when the last dose was taken or reflect irregular intake pattern exactly.

The developed nomogram could be of value in the forensic psychiatry. Monitoring of adherence to clozapine in this setting is of high importance, especially in patients that can become a danger to their environment in case of non-adherence, including patients admitted under involuntary commitment or by authorisation of the court. In these patients, assurance of adequate medication intake, supported by monitoring tools such as the developed nomogram, can contribute to better diagnosis and prognosis as clinicians are provided information on adherence patterns so that they can anticipate and prevent relapses. Moreover, this is valuable due to the lack of a depot formulation for clozapine.

As a suggestion for future studies, we propose to conduct a prospective validation study first comparing FL3 measurements with other forms of adherence measurements (e.g. MEMS). This would ultimately show the predictive performance of the current nomogram and will serve as external model evaluation. Eventually, this could be followed by a prospective validation in clinical setting linking estimated adherence to clinical outcome measures.

Conclusion

With our proposed nomogram clinicians are offered an additional tool to obtain indicative information regarding long term clozapine adherence. Together with TDM the use of the nomogram could depict any possible non-adherence in a broader context. Future studies should point out the extent of uniformity in neutrophil fluorescence signal on several different analysers compared to FL3-fluorescence signal obtained with

the Abbott Cell-Dyn. Also, a prospective follow-up study comparing true adherence (measured with micro-electromechanical systems or other adherence mechanisms) with the nomogram estimated adherence should be conducted to further evaluate our established model and nomogram for use in clinical practice.

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References

1 Bromet EJ, Fennig S. Epidemiology and natural history of schizophrenia. Biol Psychiatry 1999; 46: 871–81.

2 Chakos M, Lieberman J, Hoffman E, Bradford D, Sheitman B. Effectiveness of second-generation antipsychotics in patients with treatment-resistant schizophrenia: a review and meta-analysis of randomized trials. Am J Psychiatry 2001; 158: 518–526.

3 Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic: A double-blind comparison with chlorpromazine. Arch. Gen. Psychiatry 1988; 45: 789–796.

4 Pickar D, Owen RR, Litman RE, Konicki E, Gutierrez R, Rapaport MH. Clinical and biologic response to clozapine in patients with schizophrenia. Crossover comparison with fluphenazine. Arch Gen Psychiatry 1992; 49: 345–353.

5 Reid WH, Mason M, Hogan T. Suicide prevention effects associated with clozapine therapy in schizophrenia and schizoaffective disorder. Psychiatr Serv 1998; 49: 1029–1033.

6 Meltzer HY, Fatemi H. Suicide in schizophrenia: The effect of clozapine. Clin Neuropharmacol 1995; 18: S18–S24.

7 Keith SJ, Kane JM. Partial Compliance and Patient Consequences in Schizophrenia: Our Patients Can Do Better. J Clin Psychiatry 2003; 64: 1308–1315.

8 Lacro JP, Dunn LB, Dolder CR, Leckband SG, Jeste D V, Pharm D *et al.* Prevalence of and risk factors for medication nonadherence in patients with schizophrenia: A comprehensive review of recent literature. J Clin Psychiatry 2002; 63: 892–909.

9 Cramer JA, Rosenheck R. Compliance With Medication Regimens for Mental and Physical Disorders. Psychiatr Serv 1998; 49: 196–201.

10 Laan W, van der Does Y, Sezgi B, Smeets HM, Stolker JJ, Wit NJ de *et al.* Low treatment adherence with antipsychotics is associated with relapse in psychotic disorders within six months after discharge. Pharmacopsychiatry 2010; 43: 221–4.

11 Weiden PJ, Kozma C, Grogg A, Locklear J. Partial compliance and risk of rehospitalization among California Medicaid patients with schizophrenia. Psychiatr Serv 2004; 55: 886–91.

12 Caseiro O, Pérez-Iglesias R, Mata I, Martínez-Garcia O, Pelayo-Terán JM, Tabares-Seisdedos R *et al.* Predicting relapse after a first episode of non-affective psychosis: A three-year follow-up study. J Psychiatr Res 2012; 46: 1099–1105.

13 Valenstein M, Copeland L, Blow FC, McCarthy JF, Zeber JE, Gillon L *et al.* Pharmacy data identify poorly adherent patients with schizophrenia at increased risk for admission. Med Care 2002; 40: 630–9.

14 Taylor DM, Douglas-Hall P, Olofmjana B, Whiskey E, Thomas A. Reasons for discontinuing clozapine: Matched, case-control comparison with risperidone long-acting injection. Br J Psychiatry 2009; 194: 165–167.

15 Spina E, Avenoso A, Facciolà G, Scordo MG, Ancione M, Madia G *et al.* Relationship between plasma concentrations of clozapine and norclozapine and therapeutic response in patients with schizophrenia resistant to conventional neuroleptics. Psychopharmacology 2000; 148: 83–9.

16 Mennickent S, Sobarzo , Vega M, de Diego M, Godoy G, Rioseco P *et al.* Determination of clozapine in serum of patients with schizophrenia as a measurement of medication compliance. Int J Psychiatry Clin Pract 2010; 14: 41–6.

17 Flanagan RJ, Spencer EP, Morgan PE, Barnes TRE, Dunk L. Suspected clozapine poisoning in the UK/Eire, 1992-2003. Forensic Sci Int 2005; 155: 91–9.

18 Hoffmann J, Lillholm A. Neutrophil fluorescence in patients using clozapine. Ned Tijdschr Klin Chem 2000; 25: 108.

19 Man WH, ten Berg M, Wilting I, Huisman A, Cahn W, Douma JW *et al.* Fluorescence of neutrophil granulocytes as a biomarker for clozapine use. Eur Neuropsychopharmacol 2013; 23: 1408–1413.

20 ten Berg MJ, Huisman A, van den Bemt PML, Schobben AFM, Egberts ACG, van Solinge WW. Linking laboratory and medication data: new opportunities for pharmacoepidemiological research. Clin Chem Lab Med 2007; 45: 13–9.

21 Groeneveld KM, Heeres M, Leenen LPH, Huisman A, Koenderman L. Immunophenotyping of posttraumatic neutrophils on a routine haematology analyser. Mediators Inflamm 2012; 2012: 509513.

22 Kang SH, Kim HK, Ham CK, Lee DS, Cho HI. Comparison of four hematology analyzers, CELL-DYN Sapphire, ADVIA 120, Coulter LH 750, and Sysmex XE-2100, in terms of clinical usefulness. Int J Lab Hematol 2008; 30: 480–486.

23 Muller R, Mellors I, Johannessen B, Aarsand AK, Kiefer P, Hardy J *et al.* European multicenter evaluation of the Abbott Cell-Dyn sapphire hematology analyzer. Lab Hematol 2006; 12: 15–31.

24 Huisman A, Stokwielder R, van Solinge WW. Mathematical correction of the invitro storage--related increase in erythrocyte mean cell volume of an automated hematology analyzer--the Cell-Dyn 4000. Lab Hematol 2004; 10: 68–73.

25 Keizer RJ, Karlsson MO, Hooker A. Modeling and Simulation Workbench for NONMEM: Tutorial on Pirana, PsN, and Xpose. CPT pharmacometrics Syst Pharmacol 2013; 2: e50.

26 Ng W, Uchida H, Ismail Z, Mamo DC, Rajji TK, Remington G *et al.* Clozapine exposure and the impact of smoking and gender: a population pharmacokinetic study. Ther Drug Monit 2009; 31: 360–366.

27 Vrijens B, De Geest S, Hughes DA, Przemyslaw K, Demonceau J, Ruppar T *et al.* A new taxonomy for describing and defining adherence to medications. Br J Clin Pharmacol 2012; 73: 691–705.

28 Cowland JB, Borregaard N. Isolation of neutrophil precursors from bone marrow for biochemical and transcriptional analysis. J. Immunol. Methods. 1999; 232: 191–200.

29 Wolff K, Hay AA, Raistrick D, Feely M. Use of 'very low-dose phenobarbital' to investigate compliance in patients on reducing doses of methadone (detoxification). J Subst Abuse Treat 1993; 10: 453–458.

30 Braam RL, Van Uum SHM, Lenders JWM, Thien T. Bromide as marker for drug adherence in hypertensive patients. Br J Clin Pharmacol 2008; 65: 733–736.

31 Hawwa AF, AlBawab A, Rooney M, Wedderburn LR, Beresford MW, McElnay JC *et al.* Methotrexate polyglutamates as a potential marker of adherence to long-term therapy in children with juvenile idiopathic arthritis and juvenile dermatomyositis: an observational, cross-sectional study. Arthritis Res Ther 2015; 17: 295.

2.4

The unbound fraction of clozapine significantly decreases with elevated plasma concentrations of the inflammatory acute-phase protein alpha-1-acid glycoprotein

Wai Hong Man, Ingeborg Wilting, Eibert R Heerdink,
Gerard WK Hugenholtz, Maarten J ten Berg, Wouter W van Solinge,
Toine CG Egberts, Erik M van Maarseveen

Abstract

Background

During inflammation, a substantial rise of the total (unbound plus protein-bound) clozapine plasma concentration into the toxic range has been observed, often prompting a dose reduction of clozapine. It has been suggested that elevated alpha-1-acid glycoprotein (AGP) concentrations during inflammation cause increased clozapine - plasma AGP binding, resulting in elevated total clozapine plasma concentrations, but not in a significant change in unbound concentrations. As the protein-unbound plasma concentration of clozapine is responsible for the pharmacological and toxicological effects, this would negate the need for clozapine dose-adjustments. The objective of this study was to investigate the association between AGP plasma concentrations and the clozapine unbound fraction.

Methods

The study consisted of an 'in vitro' spiking experiment and a patient study. In the spiking experiment, 0.025 mL and 0.060 mL AGP solution (20 mg/mL) was added to plasma sample aliquots of patients on clozapine (n=3). In addition, a patient study was performed to determine the association between AGP plasma concentration and the clozapine unbound fraction in samples originating from patients using clozapine. AGP plasma concentrations above 1.2 g/mL were considered as elevated. In all samples the total and unbound clozapine plasma concentrations were determined by liquid chromatography-tandem mass spectrometry. Data from the spiking experiment were analyzed with a paired t-test. Data from the patient study were analyzed with an unpaired t-test and linear regression analysis.

Results

The AGP spiking experiment showed significantly lower mean unbound clozapine fractions following 0.025 and 0.060 mL AGP spikes (respective relative reductions of 28.3%, p=0.032 and 43.4%, p=0.048). In the patient study, total clozapine plasma concentrations were 10% higher in the elevated AGP samples (n=6) compared to normal AGP samples (n=20) (525 ng/mL *vs* 479 ng/mL, mean difference=47 ng/mL [95% CI: -217, 310], p=0.72). However, samples with elevated AGP concentrations had on average a 26% relatively lower mean unbound fraction in comparison to samples with normal AGP concentrations (1.22% *vs* 1.65%, mean difference=-0.43% [95%CI: -0.816; -0.0443], p=0.03). Linear regression analysis showed a weak correlation between AGP concentration and unbound fraction (r^2=0.214, p=0.017).

Conclusions
The spiking experiment and patient study consistently showed that elevated AGP plasma concentrations were significantly associated with a lower clozapine unbound fraction.
This is most likely explained by increased systemic availability of AGP as a result of inflammation. Therefore, adjusting clozapine dose solely based on total plasma concentrations during inflammation is not recommended.

Introduction

Clozapine is an atypical antipsychotic agent indicated for use in refractory schizophrenia. It is the only antipsychotic with documented efficacy in this patient group, but it is also associated with potentially life-threatening side effects including agranulocytosis and myocarditis [1,2]. Moreover, clozapine is known for its narrow therapeutic range with wide interindividual variation in the dose-concentration relationship due to variability in metabolism, drug-drug as well as food-drug interactions. Therapeutic drug monitoring (TDM) is therefore considered useful and involves measurement of the total (i.e. sum of the protein-bound and unbound) plasma concentration of clozapine with previous studies indicating a reference concentration ranging from 350 to 600 ng/mL [3].
In patients with inflammation, a three- to five-fold rise in the total clozapine plasma concentrations into toxic ranges has been observed in several case reports [4–14]. This rise in total clozapine plasma concentration is currently mechanistically unexplained [15,16]. Two mechanisms related to drug distribution and drug metabolism have been proposed. First, clozapine is highly (95%) bound in plasma to the acute phase protein alpha-1-acid glycoprotein (AGP) [17,18]. In patients with inflammation systemic AGP concentrations are significantly elevated [19]. This may result in elevated total clozapine plasma concentrations, due to drug accumulation in plasma owing to increased binding to systemic AGP. The unbound (and pharmacological active) concentration, on the other hand, is suggested to remain unchanged by this alleged AGP-associated effect [18]. This could explain the observed discrepancy between high and theoretically toxic total clozapine plasma concentrations and absence of toxic side effects in patients with inflammation. The other proposed mechanism is the production of interleukin-6 (IL-6) which is transiently increased during inflammation. IL-6 is claimed to inhibit the metabolism of clozapine by downregulation of the expression of CYP1A2 and CYP3A4 [13], potentially leading to elevated total clozapine plasma concentrations. In this hypothesis, unbound clozapine concentration would also rise, and patients would then be at an increased risk for clinical signs of toxicity.
Because of the elevated total clozapine plasma concentration, clinical guidelines

currently advise halving the clozapine dose in case of inflammation [20]. Nevertheless, if the unbound plasma concentration is unaffected during inflammation because only the total plasma concentration increases, a strong dose reduction would increase the risk of psychiatric deterioration. Elucidation of the above described discrepancy can contribute to safer and more effective clozapine therapy. To our knowledge, no studies have yet examined the relation between AGP plasma concentration and the clozapine unbound fraction. The objective of the current study was to assess this association.

Methods

Setting and study samples

The current study consisted of 1) a spiking experiment and 2) a prospective patient study. Inclusion of study samples took place at the University Medical Center Utrecht, an academic teaching hospital in the center of The Netherlands, with annually approximately 28,000 clinical, 15,000 day-care hospitalizations and 334,000 outpatient visits, and at the Diakonessenhuis, a community hospital in Utrecht, Zeist and Doorn with annually approximately 24,000 clinical, 23,000 day-care hospitalizations and 380,000 outpatient visits. The Institutional Review Board of the UMC Utrecht determined that the study was not subject to the Medical Research Involving Human Subjects Act, and the UMC Utrecht Biobank approved use of anonymized remnant material for this study.

In the spiking experiment, blood sample aliquots (0.65 mL) from three randomly selected patients using clozapine were spiked with 0.025 mL (AGP spike 1) and 0.060 mL (AGP spike 2) of 20 mg/mL AGP (Sigma-Aldrich) stock solution. The AGP stock solution of 20 mg/mL was prepared in PBS buffer. Aliquots were then incubated for one hour at room temperature in order to establish an equilibrium. The total and unbound clozapine concentrations were determined in the pre-spiked and post-spiked sample aliquots. All samples were prepared and determined in duplicate.

For the prospective patient study, remnant EDTA plasma samples from all patients using clozapine aged 18 years or older were collected following routine TDM of total clozapine plasma concentrations at the University Medical Center Utrecht and the Diakonessenhuis Utrecht from October 2017 to December 2017. Samples were excluded if the blood sample was drawn within 8 hours after the last clozapine intake or in case of a suspicion of intentional clozapine intoxication, ensuring that an adequate trough concentration had established and that only samples with non-intentional elevated concentrations were included. As anonymized remnant samples were used, multiple samples originating from the same patient were allowed for inclusion.

Samples were aliquoted and stored as plasma EDTA at −80°C for the measurement

of total clozapine concentrations and at –20°C for the AGP determination. For the measurement of unbound plasma concentration, aliquot samples were stored as ultra-filtrate at –20°C after the protein filtering step, as described below.

Measurements of total and unbound clozapine plasma concentrations

Total and protein unbound clozapine plasma concentrations were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the laboratory of clinical pharmacy and pharmacology of the UMC Utrecht. To determine unbound clozapine plasma concentrations, plasma samples were ultra-centrifuged with a filter as an additional sample preparation step before LC-MS/MS quantitation. The LC mobile phases consisted of MS grade water mixed with formic acid (0.1%) and acetonitrile mixed with formic acid 0.1%, both ordered at Sigma-Aldrich (Saint Louis, Missouri, USA). The LC-MS/MS system included a Thermofisher Hypersil GOLD PFP 50x2,1 mm column (Waltham, Massachusetts, USA) pressurized by a Surveyor MS Plus Pump (Waltham, Massachusetts, USA). Signal was detected with a Surveyor Autosampler plus (Waltham, Massachusetts, USA) and a Thermofisher TSQ Quantum access mass spectrometer with a Heated Electrospray Ionization Probe (Waltham, Massachusetts, USA). Data gathering and system control were performed by Xcalibur software (HPLC) and Thermo Scientific Trace Finder software (MS). Using Merck Millipore centrifree YM-30 cat4104 Amicon filters (Darmstadt, Germany), ultra-centrifugation took place at 2500 RMP at 25 °C for 30 minutes using a Hettich Rotina 380R bucket swing centrifuge (Geldermalsen, The Netherlands). The ultra-filtrate and samples were stored in Eppendorf (Nijmegen, The Netherlands) micro seed containers of 1,5 ml. The linearity of the assay ranged from 2.5 ng/mL–570 ng/mL with R^2 values exceeding 0.99. Spiked quality control samples at low, medium and high concentrations were within 15% of the nominal values. The LC–MS/MS method was validated in accordance with the EMA Guideline on bioanalytical method validation [21]. Clozapine unbound fraction was calculated by dividing unbound plasma concentration by the total plasma concentration.

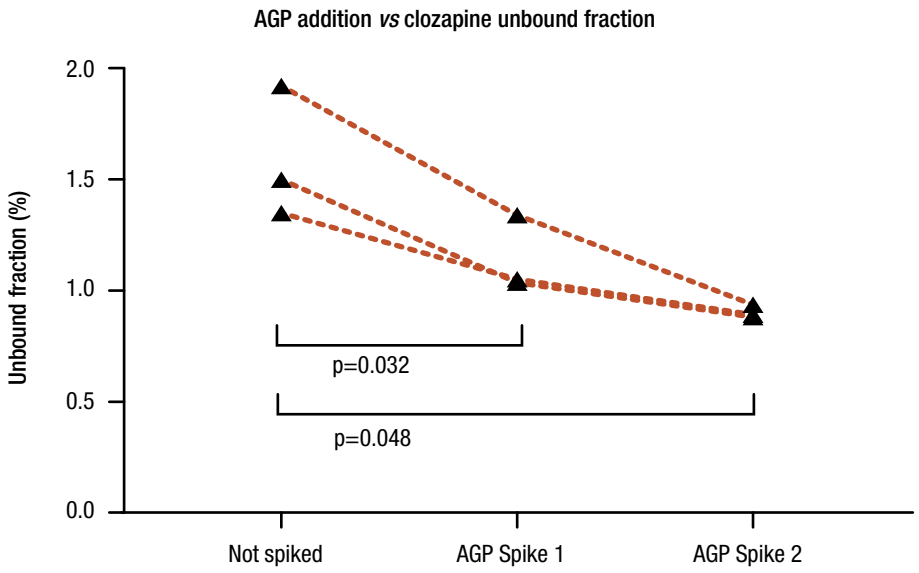
Measurement of AGP plasma concentrations

AGP plasma concentrations were determined using an immunoturbidimetric assay according to manufacturer's instruction (Roche) at the Clinical Laboratory Chemistry and Hematology at Leiden University Medical Center (LUMC). Spiked quality control samples at low, medium and high concentrations were within 15% of the nominal values. Samples with an AGP concentration ≥ 1.2 g/L and thus exceeding the upper limit of the reference concentration of normal physiologic AGP were assumed to be elevated.

Data analysis

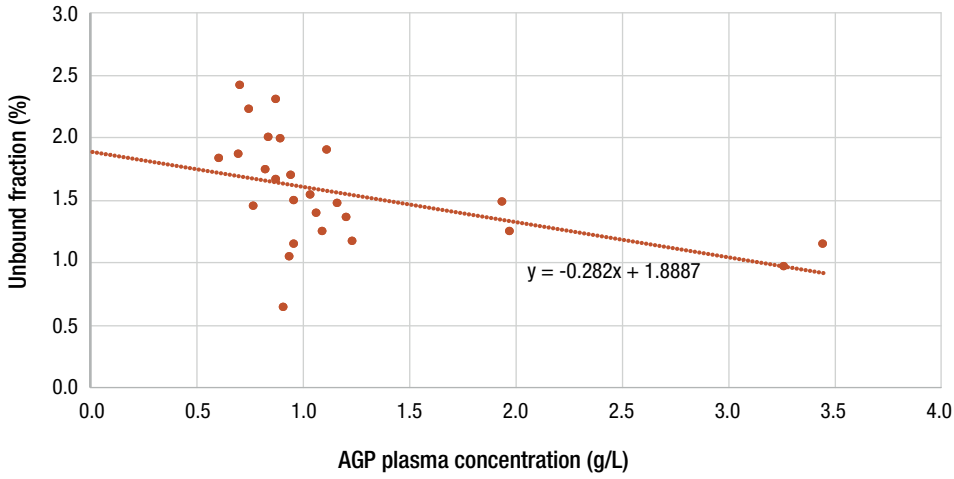
Differences in mean unbound fraction between the pre-spiked and spiked samples calculated from the spiking experiment were analyzed using a paired t-test (Graphpad Prism 7.02 (La Jolla, California, USA)). Differences in mean unbound fraction and in total plasma concentrations between the normal and elevated AGP groups in the patient study were analyzed using an unpaired t-test (Graphpad Prism 7.02 (La Jolla, California, USA)). These results were plotted in scatter graphs and box-and-whisker plots using Graphpad Prism 7.02 (La Jolla, California, USA). A linear regression analysis was performed to assess the contribution of AGP to the clozapine unbound plasma concentration using SPSS for Windows version 24.0 (IBM, Chicago, IL, USA). An alpha level of 0.05 was considered statistically significant for all performed statistical tests.

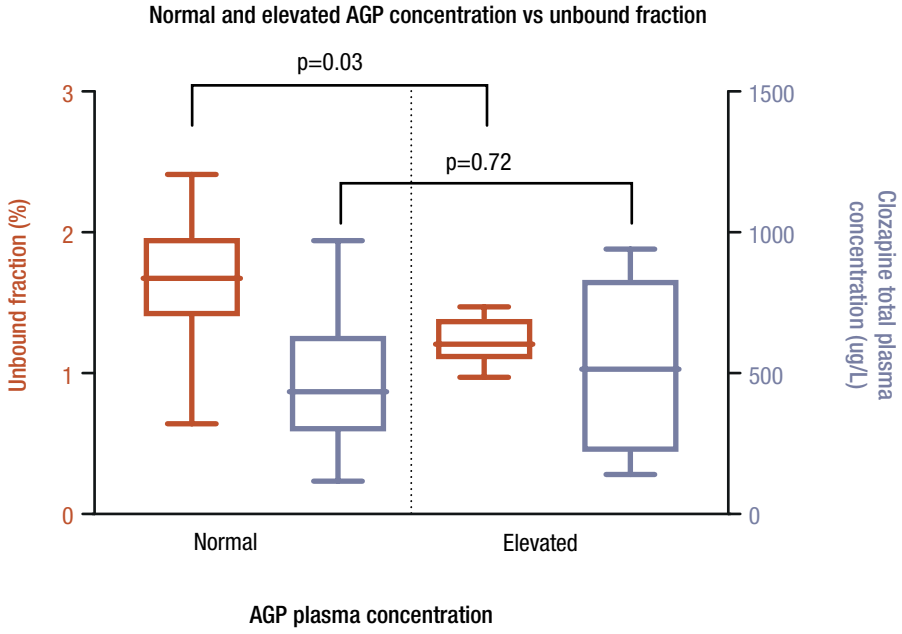
Figure 1. The relation between AGP addition and clozapine unbound fraction



Spiking experiment displaying clozapine unbound fraction (Fu) after in vitro addition of AGP in three patient samples. Each group has three samples (indicated with triangles, individual samples are connected with dotted lines). After each in vitro AGP addition the unbound fraction gradually decreases in each patient.

Figure 2. AGP plasma concentrations *vs* clozapine unbound fraction in the patient study





The relation between AGP plasma concentrations and the clozapine unbound fraction in the patient study are displayed in a scatter plot (above) and in box-whisker plots (beneath). In the box-whisker plots a distinction between normal (0.6–1.2 g/L) and elevated (>1.2 g/L) AGP plasma concentrations was made and grouped (depicted in red). Depicted in blue are the clozapine total plasma concentrations. A significant lowered mean unbound fraction was found in the elevated AGP group in comparison to the normal AGP group. An increase in mean total clozapine plasma concentration was found in the elevated AGP group, but was not statistically significant.

Results

Spiking experiment

In all three patients the unbound fraction gradually decreased after each AGP addition (Figure 1). After adding 0.025 mL AGP solution (AGP spike 1), the unbound fraction relatively decreased with 30.7% in patient A (from 1.50% to 1.04%), with 30.2% in patient B (from 1.92% to 1.34%) and with 22.2% in patient C (from 1.35% to 1.05%) in comparison to the aliquots not spiked with AGP. The mean relative decrease in unbound fraction (28.3%) was statistically significant (p=0.032). After adding 0.060 mL AGP solution (AGP spike 2), the unbound fraction relatively decreased with 41.3% in patient A (from 1.50% to 0.88%), relatively decreased with 51.0% in patient B (from 1.92% to 0.94%) and relatively decreased with 34.1% in patient C (from 1.35% to 0.89%), in comparison to aliquots not spiked with AGP. This mean relative decrease in unbound fraction (43.4%) was also significant (p=0.048).

Patient study

A total of 26 remnant samples from patients using clozapine were collected and included in the study. Study samples with elevated AGP concentrations (n=6) showed a 25% significantly lower mean unbound fraction (1.22% versus 1.65%, mean difference=-0.43% [95%CI: -0.816; -0.0443], p=0.03) in comparison to samples with normal AGP concentrations (n=20). Linear regression analysis showed a significant and moderate correlation between AGP concentration and unbound fraction ($y=-0.281x + 1.888$, $r^2=0.214$, p=0.017; Figure 2), indicating a relative decrease of approximately 17.5% in mean clozapine unbound fraction for a one-unit (g/L) increase in AGP plasma concentration. Total clozapine plasma concentrations did not significantly differ between study samples with elevated AGP and those with normal AGP concentrations (525 ng/mL *vs* 479 ng/mL, mean difference=47 ng/mL [95%CI: -217; 310], p=0.72).

Discussion

Our spiking experiment as well as our patient study showed that elevated AGP plasma concentrations are associated with a lower clozapine unbound fraction. According to the spiking experiment, AGP addition significantly lowered the unbound fraction in vitro. This finding was further supported by the results of the patient study that demonstrated a lower unbound fraction in the elevated AGP group compared to the normal AGP group. We also observed higher total plasma concentrations in the elevated AGP

group in comparison to the normal AGP group, however this was not statistically significant. This could be explained by the small number of patients included in the elevated AGP group (n=6) as compared to the larger normal AGP group (n=20). In addition, guided by TDM, clinicians could have reduced the clozapine dose anticipating high plasma concentrations due to inflammation, resulting in lowered total plasma concentrations within the therapeutic window for the total concentration.

In case of inflammation, high concentrations of AGP can lead to a lowered unbound fraction of clozapine. This is most likely due to increased AGP-clozapine binding causing clozapine to accumulate in plasma resulting in elevated total clozapine plasma concentrations, while the unbound concentration is pharmacokinetically unaffected by AGP as it is determined by dose and clearance. Because the protein-unbound plasma concentration of clozapine is responsible for the pharmacological effects and thus efficacy and toxicity, it would be incorrect to reduce the clozapine dose based on total clozapine plasma concentrations, as currently advised by clinical guidelines [20].

To our knowledge, this is the first study ever performed that actually determined clozapine unbound fraction in patients using clozapine with normal and elevated AGP concentrations. The major strength of our study is that the results of the spiking experiment and patient study both independently and interchangeably explain the mechanistic pharmacology behind this phenomenon.

A few limitations need to be addressed regarding our current study. First, quantitation of clozapine plasma concentrations was performed in patient samples originating from anonymized remnant material. Therefore, we were unable to account for confounding factors including smoking, caffeine use, interacting co-medication and non-adherence, nor to include information regarding clozapine dose (adjustments) in our study. These factors are of high influence on the clozapine total plasma concentration and could explain the non-significant difference between mean total concentrations between normal and elevated AGP samples, as discussed earlier. Second, multiple study samples could be originated from the same patient, since sample inclusion was based upon different blood drawings instead of subjects. Therefore, a crossover analysis could not be performed. Third, aliquots from the spiking experiments were incubated at room temperature after AGP addition, therefore not representing the human body temperature. This could affect the unbound fraction, since the equilibrium between protein-bound and unbound concentrations is dependent of temperature [22]. Lastly, the individual's intraindividual course of clozapine plasma concentrations were not followed-up during the inflammatory course. Intraindividual variations are therefore not included in our analysis.

Based on the findings of our study it would be wise to consider monitoring protein-unbound plasma concentrations of clozapine routinely and adjust clozapine dose upon clinical course and signs, rather than adjust dose based upon total clozapine plasma concentrations in case of inflammation. Although we here provide the first evidence for altered clozapine pharmacokinetics due to AGP, follow-up in depth pharmacolog-

ical and clinical studies are needed. Future studies are preferably of larger sample size to further examine the effects of AGP during inflammation on clozapine total and protein unbound plasma concentrations, also taking into account of multiple moments of sampling within the full time-course of inflammation.

Conclusion

Our spiking experiment demonstrated a decrease in clozapine unbound fraction following in vitro addition of AGP. In our patient study, clozapine unbound fraction was found to be significantly decreased during inflammation due to elevated AGP concentrations. This study postulates the new paradigm to adjust clozapine dose based on unbound plasma concentrations in conditions with elevated AGP plasma concentrations including inflammation, taking into account of a lowered unbound fraction.

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References

1 Asenjo Lobos S, Komossa K, Hunger H, Schmid F, Schwarz S, Leucht S. Clozapine versus other atypical antipsychotics for schizophrenia (Review). Cochrane Database Syst Rev. 2010 11):CD006633

2 Leucht S, Cipriani A, Spineli L, Mavridis D, Orey D, Richter F *et al.* Comparative efficacy and tolerability of 15 antipsychotic drugs in schizophrenia: a multiple-treatments meta-analysis. Lancet 2013; 382: 951–62.

3 Hiemke C, Bergemann N, Clement HW, Conca A, Deckert J, Domschke K *et al.* Consensus Guidelines for Therapeutic Drug Monitoring in Neuropsychopharmacology: Update 2017. Pharmacopsychiatry 2018; 51: 9–62.

4 Hefner G, Shams MEE, Unterecker S, Falter T, Hiemke C. Inflammation and psychotropic drugs: the relationship between C-reactive protein and antipsychotic drug levels. Psychopharmacology 2016; 233: 1695–705.

5 Pfuhlmann B, Hiemke C, Unterecker S, Burger R, Schmidtke A, Riederer P *et al.* Toxic clozapine serum levels during inflammatory reactions. J Clin Psychopharmacol 2009; 29: 392–4.

6 ten Bokum EM, van de Oever HLA, Radstake DWS, Arbouw MEL. Clozapine intoxication due to cessation of smoking and infection. Neth J Med 2015; 73: 345–7.

7 Matthews CJ, Hall TL. A clozapine conundrum: clozapine toxicity in an acute medical illness. Australas Psychiatry 2014; 22: 543–5.

8 Takahashi T, Masuya Y, Ueno K, Watanabe K, Takahashi M, Morita S *et al.* Clozapine-related negative myoclonus associated with urinary tract infection: a case report. J Clin Psychopharmacol 2015; 35: 205–6.

9 Yadav DS, Singh S. Toxic levels of clozapine in a patient with acute urethritis. Prog Neurol Psychiatry 2013; 17: 14–16.

10 Raaska K, Raitasuo V, Arstila M, Neuvonen PJ. Bacterial pneumonia can increase serum concentration of clozapine. Eur J Clin Pharmacol 2002; 58: 321–2.

11 de Leon J, Diaz FJ. Serious respiratory infections can increase clozapine levels and contribute to side effects: a case report. Prog Neuropsychopharmacol Biol Psychiatry 2003; 27: 1059–63.

12 Jecel J, Michel TM, Gutknecht L, Schmidt D, Pfuhlmann B, Jabs BE. Toxic clozapine serum levels during acute urinary tract infection: a case report. Eur J Clin Pharmacol 2005; 60: 909–10.

13 Haack MJ, Bak MLFJ, Beurskens R, Maes M, Stolk LML, Delespaul PAEG. Toxic rise of clozapine plasma concentrations in relation to inflammation. Eur Neuropsychopharmacol 2003; 13: 381–5.

14 Diaz FJ, de Leon J, Josiassen RC, Cooper TB, Simpson GM. Plasma clozapine concentration coefficients of variation in a long-term study. Schizophr Res 2005; 72: 131–5.

15 Leung JG, Nelson S, Takala CR, Gören JL. Infection and inflammation leading to clozapine toxicity and intensive care: a case series. Ann Pharmacother 2014; 48: 801–5.

16 Sambhi RS, Puri R, Jones G. Interaction of clozapine and ciprofloxacin: a case report. Eur J Clin Pharmacol 2007; 63: 895–6.

17 Schaber G, Stevens I, Gaertner HJ, Dietz K, Breyer-Pfaff U. Pharmacokinetics of clozapine and its metabolites in psychiatric patients: plasma protein binding and renal clearance. Br J Clin Pharmacol 1998; 46: 453–9.

18 Espnes KA, Heimdal KO, Spigset O. A puzzling case of increased serum clozapine levels in a patient with inflammation and infection. Ther Drug Monit 2012; 34: 489–92.

19 Hochepied T, Berger FG, Baumann H, Libert C. Alpha(1)-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties. Cytokine Growth Factor Rev 2003; 14: 25–34.

20 Schulte P, Bakker B, Bogers J, Cohen D, van Dijk D. Guideline for the use of clozapine by the Netherlands Clozapine Collaboration Group. 2013.

21 European Medicines Agency. Guideline on validation of bioanalytical methods. EMEA/CHMP/EWP/192217/2009. 2009.

22 Nilsson LB. The bioanalytical challenge of determining unbound concentration and protein binding for drugs. Bioanalysis 2013; 5: 3033–50.

Monitoring and management of clozapine-induced sialorrhea

3.1

Reporting patterns of sialorrhea comparing users of clozapine to users of other antipsychotics: a disproportionality analysis using VigiBase

Wai Hong Man, Ingeborg Wilting, Patrick C Souverein,
Ronald HB Meyboom, Toine CG Egberts, Eibert R Heerdink

Abstract

Background

Sialorrhea is a common and socially invalidating adverse drug reaction (ADR). In patients using clozapine of which the exact mechanism is unknown. The objective of this study was to assess ADR reporting patterns of sialorrhea in users of clozapine compared to users of other antipsychotics by determining differences in relative reporting frequency, reporter type and receptor affinity profile.

Methods

A case/non-case disproportionality analysis using data from the WHO global individual case safety report (ICSR) database VigiBase (1968-2016) was performed; data were restricted to reports involving antipsychotics. Reports of antipsychotics with 'salivary hypersecretion' as ADR were considered as cases and those with ADRs other than 'salivary hypersecretion' were defined as non-cases. Relative reporting frequencies were expressed as reporting odds ratios (RORs) and multivariate logistic regression was performed with the drug-ADR pair as unit of analysis to estimate RORs with 95% confidence intervals. A stratified analysis by reporter type (health-care professional and consumer) was performed. A heat map with receptor binding affinities was generated for each antipsychotic to assess whether these could explain differences in relative reporting frequency.

Results

A total of 1,169,254 drug-ADR pairs from 425,304 unique ICSRs were identified. Of these reports 20.8% involved users of clozapine (N=243,564) and 79.2% involved users of other antipsychotics (N=925,690). Health-care professionals and consumers reported a total of 146,794 (60.3%) respectively 5521 (2.3%) drug-ADR pairs related to clozapine. Sialorrhea was relatively more frequently reported in users of clozapine (N=2732 (1.1%)) compared to users of other antipsychotics (N=2911 (0.31%)) (ROR=3.60; 95% CI 3.41–3.79) and was reported more often by consumers (ROR=19.8; 95% CI 15.1–25.9) compared to health-care professionals (ROR=2.44; 95% CI 2.27–2.63). The relative high sialorrhea reporting rate found in clozapine might be related to muscarinic M_4 and dopamine D_1 agonism, as indicated by the generated heat map.

Conclusion

Sialorrhea with clozapine use was reported almost four times more often than with other antipsychotic use and was reported eight times more often by patients than by health-care professionals. The etiology of sialorrhea might be related to dopamine D_1 and muscarinic M_4 agonism based on the relative high reporting rate found in clozapine reports and our heatmap. Our results underline the importance for health-care professionals to incorporate sialorrhea into the shared decision process when considering initiation and evaluation of clozapine therapy.

Introduction

Clozapine is an antipsychotic drug with distinct therapeutic advantages, including superior efficacy over other antipsychotics in refractory schizophrenia [1]. On the other hand, clozapine may cause many adverse drug reactions (ADRs) as a result of its broad pharmacological profile and it is well known for its specific safety problems such as life-threatening agranulocytosis and myocarditis [2]. A frequently occurring non-life threatening, but socially invalidating ADR in patients using clozapine is sialorrhea or excessive salivation [3]. The prevalence of sialorrhea among patients using clozapine has been estimated at 30-80%, rendering it the second most frequently occurring ADR of clozapine [4]. Sialorrhea often occurs shortly after initiation of clozapine therapy and it is most prominent during sleep, resulting in amongst others wet pillows and skin problems [2–6]. In several case reports it has also been linked to clinical complications including parotitis, sleeping disorders and aspiration pneumonia [6–8]. Moreover, sialorrhea can be socially invalidating thereby negatively affecting the quality of life. This might also negatively impact medication adherence [4,9] and as such indirectly lead to psychiatric deterioration [10].

It has been established that sialorrhea is a frequent ADR occurring during clozapine therapy. However, its relative reporting frequency compared to other antipsychotic agents has, up to present, not been evaluated. In addition, the exact mechanism behind this ADR remains unknown. In this study, we evaluated ADR reporting patterns of sialorrhea in users of clozapine compared to users of other antipsychotics by determining differences in reporting frequency, reporter type and receptor affinity profile.

Methods

Setting

This study was conducted using reports in the World Health Organization (WHO) global Individual Case Safety Report (ICSR) database, VigiBase [11]. In 1968, in the aftermath of the thalidomide disaster in the early 1960s, the WHO program for International Drug Monitoring was established. In each country participating in this program, a national centre for pharmacovigilance collects and manages suspected adverse drug reaction (ADR) case reports. These ICSRs are sent to the Uppsala Monitoring Centre (UMC), the WHO Collaborating Centre for International Drug Monitoring, that stores the reports in the WHO global ICSR database, VigiBase. ICSRs in VigiBase include patient demographics, suspected drugs, suspected ADRs, and additional information relevant to the case [11]. By May 2016, the VigiBase database contained over 12 million case reports originating from more than a hundred national pharmacovigilance centres from all over the world. Suspected ADRs are coded at the originating national centre, either according to the WHO Adverse Reaction Terminology (WHO-ART) or the Medical Dictionary for Regulatory Activities (MedDRA). In VigiBase both WHO-ART and MedDRA are used. Suspected drugs are classified in VigiBase according to the Anatomical Therapeutic Chemical (ATC) classification. In the current study the MedDRA hierarchy was used for the evaluation of reported ADRs in the ICSRs.

Study population

All reports in VigiBase related to clozapine (N05AH02) or other antipsychotics (ATC codes N05A, excluding lithium (N05AN01) notified since the establishment of VigiBase in 1968 until May 2016 were selected. Causality assessment of the evaluated ADR reports was not performed, meaning reports were included not taking into account the reported level of causality. Reports without a valid MedDRA code were excluded. Duplicate drug-ADR pairs within each unique ICSR report were merged into one record.

Design and outcome

Drug-ADR pairs involving all antipsychotics were divided into reports involving clozapine or other antipsychotics. To assess the association between involvement of clozapine in comparison to other antipsychotics and sialorrhea, a case/non-case approach [12] was used among reports with antipsychotics termed as the suspected drug.

Cases were defined as drug-ADR pairs associated with antipsychotic use mentioning the ADR 'Salivary hypersecretion' (MedDRA code 10039424, preferred term). The non-case group consisted of pairs involving an antipsychotic agent mentioning ADRs other than 'Salivary hypersecretion'.
Exposure was defined as the identification of clozapine (N05AH02) being marked as the suspected drug in an ADR report, while non-exposure to clozapine was defined as the presence of an antipsychotic agent other than clozapine (ATC codes N05A, excluding lithium (N05AN01) being marked as the suspected drug in an ADR report.
Report data were stratified by reporter types health-care professionals and consumers. Health-care professionals included reporter types 'Physician', 'Pharmacist', 'Other Health Professional', 'General Practitioner' and 'Specialist Physician'. Drug-ADR pairs reported by consumers included 'Consumer/Non Health Professional' marked as reporter type.
To evaluate a possible relation between receptor binding affinities and sialorrhea RORs, firstly a selection of fifteen antipsychotic agents with the highest ADR absolute counts was made in order to maintain an appropriate calculated ROR. These fifteen agents were then ranked based upon the lower limit on the 95% confidence interval for the ROR. All different salt formations or dosage forms of each active pharmaceutical ingredient (API) were merged and counted as a whole, because the route of administration is not expected to affect the risk of sialorrhea. That is, sialorrhea is expected to occur after systemic exposure of the causative agent. Consequently, a selection of receptors that are frequently affected by antipsychotics was made and consisted of the dopamine D_1, D_2, D_3 and D_4; adrenergic α_1 and α_2; histamine H_1; muscarinic M_1, M_2, M_3, M_4 and M_5; and serotonin $5HT_{1A}$, $5HT_{1B}$, $5HT_{2A}$, $5HT_{2C}$, $5HT_6$ and $5HT_7$ receptors [13]. The binding affinities (K_i) of each receptor were acquired by previous studies in literature were categorized in 5 ranges: 1-10 nM, 10-100 nM, 100-1000 nM, 1000-10000 nM and >10000 nM. These ranges were depicted in a gradient color. A visual rendition of these receptor binding affinities, with distinction of (partial) agonistic and antagonistic properties of the agent, was created for each ranked antipsychotic agent, thus forming a heat map.

Potential confounding and effect modifying variables

Age, sex, reporting year and reporter type were recorded and explored for potential confounding or effect modification. Age was categorized into four categories: < 40 years, 40 to 65 years, 65 to 80 years and 80 years or older. Time periods were categorized in decennia, from 1968 onwards. A stratified analysis was performed by reporter types: health-care professionals and consumers.

Data analysis

Characteristics of the cases and controls were analyzed using t-test and X^2 tests with respect to the age, gender, reporter type, and time categories. The association between sialorrhea and presence of each ADR of clozapine was expressed as reporting odds ratios (ROR) as a measure of disproportionality between clozapine versus other antipsychotics, accompanied with 95% confidence intervals (CI). The ROR was based on a 2-by-2 contingency table:

$$ROR = (a/c)/(b/d) = ad/bc$$

in which a and c denote the cases among the clozapine and other antipsychotic pairs respectively; b and d denote the non-cases among clozapine and other antipsychotic pairs respectively.
The ROR calculation for each antipsychotic other than clozapine within the selection of fifteen antipsychotics follows the same approach as the 2-by-2 contingency table, as described above but instead of clozapine, counts of the antipsychotic agent in particular were used. The unit of analysis was the drug–ADR pair, rather than the unique ICSR report itself.
A multivariate regression analysis was performed to assess confounding and effect modifications (see above). A variable was termed confounder if it independently changed the ROR with more than 10%. All confounders were incorporated into the multi-variable model. The generated heat map was evaluated visually to determine any possible relation between receptor binding affinities and sialorrhea report ranking.

Results

By May 2016 VigiBase contained 12,612,671 ICSRs filed, representing a total of 46,894,844 drug-ADR pairs. Antipsychotics were termed as the suspected drug in 1,169,254 drug-ADR pairs (2.49%). Of these 20.8% (N=243,564) were related to clozapine and 79.2% (N=925,690) were related to other antipsychotics. Whilst clozapine-ADR pairs were attributed to males (61.1%) a proportion of 50.0% of all other antipsychotic-ADR pairs was attributed to females (Table 1). Health-care professionals were the largest contributors of the clozapine-ADR pairs (60.3%), while consumers only reported 2.3%.
A total of 5,643 (0.48%) drug-ADR pairs were identified as cases and 1,163,611 pairs were non-cases. Of all cases, a total of 2,732 were attributed to clozapine pairs and a total of 2,911 to other antipsychotic pairs (Figure 1), resulting in sialorrhea being reported nearly four times more often (ROR=3.60 (95% CI 3.41–3.79)) in users of clozapine than in users of other antipsychotics (Figure 2). When stratified by reporter

type, sialorrhea was reported by consumers nearly 20 times more often in clozapine compared to other antipsychotics (ROR=19.8 (95% CI 15.1–25.9)), whereas this ratio was found nearly eight times lower in health-care professionals (ROR=2.44 (95% CI 2.27–2.63)).

To broaden the view on all antipsychotics and their relation to sialorrhea reporting frequencies, all drug-sialorrhea pairs were counted and ranked based on their lower limit of the 95% CI for the ROR. As a next step, the binding affinities of several receptors were displayed, if available, for each antipsychotic agent. Based on this generated heat map regarding receptor affinities, sialorrhea reporting rates might seem to be related to dopamine D_1 and muscarinic M_4 receptor agonism as clozapine, being the one with highest ROR, is the only antipsychotic agent with agonistic properties on these receptors (Figure 2).

Table 1. Characteristics of reported suspected drug-ADR pairs

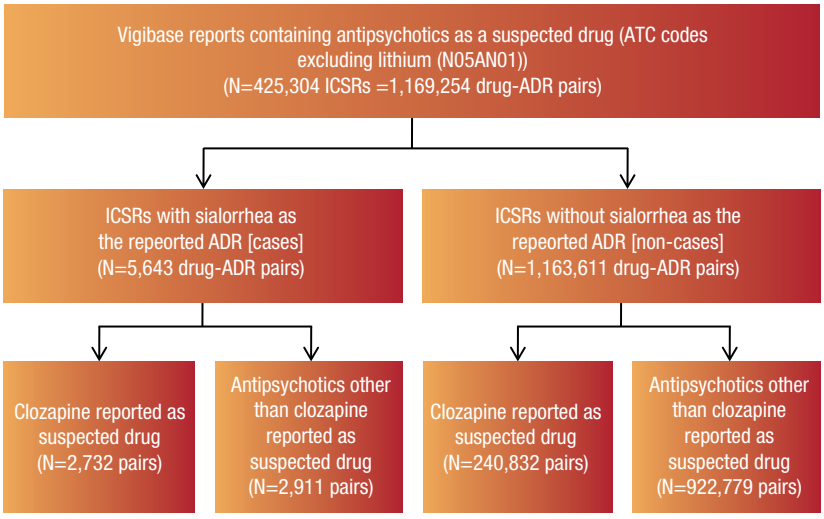
Variable	Clozapine ADRs (N=243,564 (20.8%))	Other antipsychotic ADRs (N=925,690 (79,2%))
Age, mean (SD)	43.4 (15.4)	44.04 (19.1)
<40 years, n (%)	92,278 (37.9)	293,255 (31.7)
40-65 years, n (%)	100,626 (41.3)	297,704 (32.2)
65-80 years, n (%)	17,375 (7.1)	70,182 (7.6)
> 80 years, n (%)	3,611 (1.5)	33,793 (3.7)
Unknown, n (%)	29,674 (12.2)	230,756 (24.9)
Gender		
Male, n (%)	148,832 (61.1)	415,289 (44.9)
Female, n (%)	89,267 (36.7)	462,571 (50.0)
Unknown, n (%)	5,465 (2.2)	47,830 (5.1)
Reporter		
Health-care professional, n (%)	146,794 (60.3)	428,869 (46.3)
Consumer, n (%)	5,521 (2.3)	221,145 (23.9)
Other, n (%)	66,416 (27.3)	135,711 (14.7)
Unknown, n (%)	24,833 (10.2)	139,965 (15.1)
Reporting year		
2010–2016, n (%)	127,435 (52.3)	586,875 (63.4)
2000–2009, n (%)	77,712 (31.9)	243,818 (26.3)
1990–1999, n (%)	37,679 (15.5)	57,929 (6.3)
1980–1989, n (%)	471 (0.2)	22,887 (2.5)
1970–1979, n (%)	267 (0.1)	12,523 (1.4)
1968–1969, n (%)	0 (0.0)	1,658 (0.2)

N=count, %=percent, SD=standard deviation

Table 2. Stratified analysis by reporter types health-care professionals and consumers

	Sialorrhea reported by health-care professionals	Sialorrhea reported by consumers	Overall
Clozapine (N of total)	1,290 / 146,794	79 / 5,521	2,732 / 243,564
Other antipsychotic (N of total)	1,553 / 428,869	162 / 221,145	2,911 / 925,690
ROR (95% CI)	2.44 (2.27–2.63)	19.8 (15.1–25.9)	3.60 (3.41–3.79)

Figure 1. The study flow diagram



Discussion

Sialorrhea is reported nearly four times more frequently in reports with clozapine as suspected drug in comparison to other antipsychotics. This relative reporting rate was found eight times higher in consumers compared to health-care professionals (Table 2). The mechanistic etiology of sialorrhea might be explained by muscarinic M_4 and dopamine D_1 receptor agonism based on our generated heat map involving receptor binding affinities.

Our results indicated that sialorrhea is observed more frequently in clozapine in comparison to other antipsychotics. Visual evaluation of our generated heat map might indicate an association between receptor profile and sialorrhea reports.

It is established that both parasympathetic and sympathetic stimulation evoke secretion

Figure 2. Lower limit ROR based ranking order of antipsychotics with respect to sialorrhea and corresponding receptor binding affinity heat map

Antipsychotic agent	$ROR_{overall}$ (95% CI)	N_{case} (% of total (N=5,643))	D_1	D_2	D_3	D_4	α_1	α_2	M_1	M_2	M_3	M_4	M_5	H_1	$5HT_{1A}$	$5HT_{1B}$	$5HT_{2A}$	$5HT_{2C}$	$5HT_6$	$5HT_7$
Clozapine	3.60 (3.41–3.79)	2,732 (48.4%)													P					
Haloperidol	2.14 (1.95–2.34)	524 (9.3%)																		
Fluphenazine	2.32 (1.90–2.83)	98 (1.7%)																		
Zuclopentixol	2.35 (1.86–2.96)	73 (1.29%)																		
Amisulpride	1.75 (1.40–2.18)	81 (1.3%)		P																
Risperidone	1.13 (1.05–1.22)	788 (14.0%)																		
Chlorpromazine	0.88 (0.71–1.10)	83 (1.5%)																		
Aripiprazole	0.50 (0.44–0.58)	202 (3.6%)	P	P	P	P									P			P		
Olanzapine	0.43 (0.39–0.48)	339 (6.0%)																		
Asenapine	0.45 (0.32–0.65)	30 (0.53%)																		
Thioridazine	0.49 (0.32–0.74)	22 (0.4%)																		
Paliperidone	0.37 (0.30–0.46)	85 (1.51%)																		
Prochlorperazine	0.33 (0.22–0.48)	25 (0.44%)																		
Ziprasidone	0.26 (0.19–0.34)	49 (0.87%)													P					
Quetiapine	0.087 (0.072–0.11)	110 (1.95%)													P					

Receptor affinity (K_i) of antagonists

> 10,000 nM	1000–10,000 nM	100–1000 nM	10–100 nM	1–10 nM	Unknown

Receptor affinity (K_i) of agonists

> 10,000 nM	1000 - 10,000 nM	100–1000 nM	10–100 nM	1–10 nM	Unknown

P=partial agonist; D_1=dopamine receptor D_1; D_2=dopamine receptor D_2; D_3=dopamine receptor D_3; D_4=dopamine receptor D_4; α_1=alpha-$_1$ adrenergic receptor; α_2=alpha-$_2$ adrenergic receptor; M_1=muscarinic acetylcholine receptor M_1; M_2=muscarinic acetylcholine receptor M_2; M_3=muscarinic acetylcholine receptor M_3; M_4=muscarinic acetylcholine receptor M_4; M_5=muscarinic acetylcholine receptor M_5; H_1=histamine H_1 receptor; $5HT_{1A}$=serotonin-$_{1A}$ receptor; $5HT_{1B}$=serotonin-$_{1B}$ receptor; $5HT_{2A}$=serotonin-$_{2A}$ receptor; $5HT_{2C}$=serotonin-$_{2C}$ receptor; $5HT_6$=serotonin-$_6$ receptor; 5HT7=serotonin-$_7$ receptor.

of saliva trough muscarinic and alpha/beta adrenergic receptors, respectively [14]. In literature, various pharmacological mechanisms behind the etiology of sialorrhea have been proposed. In accordance with our findings, suggesting that the high sialorrhea report rate particularly found in clozapine reports might be the result of muscarinic M_4 agonism, direct muscarinic agonists and indirect muscarinic stimulants are suggested to increase saliva flow by stimulating muscarinic M_3 and M_4 receptors [10,15]. This is in line with findings that smoking is associated with enhanced salivary flow rate [16] by stimulating muscarinic receptors as a result of high nicotine exposure. This supports the suggestion that sialorrhea could be mediated by muscarinic M_4 agonism. In addition, in a pre-clinical study it was shown that salivation occurs through direct activation of the $\alpha_3\beta_4$ nicotinic receptor subtype [17].

In case of antipsychotics, beside of direct agonism of mentioned muscarinic receptors, antagonism of α_2-adrenergic receptors could also result in sialorrhea [15]. Moreover, it has been suggested that a reduced swallowing reflex contributes to sialorrhea [18]. The swallowing reflex is modulated by dopamine D_1 receptors and substance P [19]. Hence, inhibition of dopamine D_1 receptor impairs this reflex by reducing substance P content in the larynx and pharynx where the swallowing reflex is triggered [20]. This is why sialorrhea is also a symptom in Parkinson's disease, where dopaminergic impairment is important in the etiology [21].

From the results of our stratified analysis, we observed a discrepancy in ADR reporting in consumers compared to health-care professionals. More specifically, there were more reports on sialorrhea involving clozapine reported by consumers than health-care professionals after stratification by reporter type. None of such large discrepancy between these reporter types was found for other antipsychotic agents. This implicates differences in interests and focus of ADR reporting among consumers and health-care professionals when it comes to clozapine therapy. From the view of health-care professionals sialorrhea in clozapine therapy seemed to be one of the ADRs that are of less concern compared to life-threatening ADRs including myocarditis and hematological adverse effects. For patients themselves ADRs impairing activities of daily life and quality of life appear to be relatively more important.

Within the group of antipsychotics, clozapine accounts for nearly half of all reported drug-ADR pairs involving sialorrhea (Table 1). This is a markedly high proportion, certainly because clozapine is reserved as a drug of last resort and therefore overall prescribing rates are expected to be lower than for other antipsychotics [22]. This high reporting rate may be partially explained by the fact that clozapine is subject to mandatory hematological and serum level monitoring in certain countries, including the UK and the US. As a consequence, this could lower the reporting-threshold and raise awareness to report clozapine related ADRs. In addition, US pharmaceutical companies were previously obliged to maintain a clozapine registry as part of a post-marketing drug surveillance program. Post-marketing ADRs were assessed and reported by US manufacturers on behalf of consumers. This could explain why some

of the ADRs, including neutropenia, leukocytosis and eosinophilia (data not shown), labelled as reported by consumers, are unlikely direct reports by consumers, as these require objective professional examination. The adjusted stratum-specific ROR for the reporter type 'consumers' should therefore be interpreted with caution.

A strength of our study is the data source that is used. VigiBase is the largest available pharmacovigilance database and therefore enables to perform disproportionality analyses with high statistical power. It also enables to evaluate unexpected or unknown reported ADRs.

Moreover, the disproportionality analysis is a validated method in drug safety research and surveillance [23]. However, several limitations in our study need to be addressed. First, it should be noted that VigiBase includes two report formats: 'International Drug Information System' (INTDIS) and the E2B standard. For our stratified analysis ICSRs with both reports formats were included in the estimation of reporter type proportion. In addition, since consumer reports in the INTDIS format are marked as 'Other', while in the E2B format they are marked as 'Consumer/Non-health professional', a sensitivity analysis was performed to estimate the proportion of consumer reports by limiting selection to only E2B reports. This did not substantially change the reporting ratios in consumers (ROR=19.8 (95% CI 15.1–25.9)) nor in health-care professionals (ROR=2.52 (95% CI 2.32–2.73)). Second, data were obtained through spontaneous reporting without additional clinical assessment or qualitative verifications by the authors. Although assessment of the quality regarding reports is included in VigiBase, this parameter was not included in our dataset. In this context, clinical details such as severity of drooling and clozapine dosage were not included in our analyses.

Third, antipsychotic agents are introduced into medical practice at different times dependent on the country, potentially leading to selection bias. fourth, given the high incidence of sialorrhea in clozapine users, as described in previous studies [4], this incidence is not expected to be reflected in our data as underreporting of this ADR is most likely. Fifth, the Weber effect, in which severe ADRs and ADRs not listed in the summary of product characteristics are relatively more often reported in newer agents. Lastly, as discussed earlier, clozapine therapy is subject to hematological monitoring programs that are mandatory in certain countries thereby increasing the number of healthcare workers and the number of patient visits, potentially reflected in disproportionate higher incidence of reports and indirectly of sialorrhea reports.

Conclusion

Sialorrhea was reported almost four times more frequently in users of clozapine than in users of other antipsychotics. The reporting rate was found eight times higher in consumers in comparison to health-care professionals, implicating a large underreporting of sialorrhea by health-care professionals and a difference in concerns and

focus for reporting this adverse event. High sialorrhea reporting rates might seem to relate to muscarinic M_4 and dopamine D_1 receptor agonism. This study underlines the importance for health-care professionals to incorporate sialorrhea as a relevant topic of discussion into the shared decision process with patients when initiating clozapine therapy.

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References

1 Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. Arch Gen Psychiatry 1988; 45: 789–96.

2 Grohmann R, Rüther E, Sassim N, Schmidt LG. Adverse effects of clozapine. Psychopharmacology 1989; 99 Suppl: S101-4.

3 Praharaj SK, Arora M, Gandotra S. Clozapine-induced sialorrhea: pathophysiology and management strategies. Psychopharmacology 2006; 185: 265–73.

4 Bird AM, Smith TL, Walton AE. Current Treatment Strategies for Clozapine-Induced Sialorrhea. Ann Pharmacother 2011; 45: 667–675.

5 Lahdelma L, Appelberg B. Clozapine-Induced Agranulocytosis in Finland, 1982–2007. J Clin Psychiatry 2012; 73: 837–842.

6 Lieberman J a, Safferman a Z. Clinical profile of clozapine: adverse reactions and agranulocytosis. Psychiatr Q 1992; 63: 51–70.

7 Robinson D. Possible association of parotitis with clozapine. Am J Psychiatry 1995; : 297–298.

8 Van Os J. A salience dysregulation syndrome. Br J Psychiatry 2009; 194: 101–103.

9 Krivoy A, Malka L, Fischel T, Weizman A. Predictors of clozapine discontinuation in patients with schizophrenia. 2008; : 311–315.

10 Sockalingam S, Shammi C, Remington G. Clozapine-induced hypersalivation : A review of treatment strategies. Can J psychiatry; 52: 377–384.

11 Lindquist M. VigiBase, the WHO Global ICSR Database System: Basic Facts. Drug Inf J 2008; 42: 409–419.

12 Egberts ACG, Meyboom RHB, van Puijenbroek EP. Use of measures of disproportionality in pharmacovigilance: three Dutch examples. Drug Saf 2002; 25: 453–8.

13 Stahl SM. Stahl's essential psychopharmacology: Neuroscientific basis and practical applications. 2008.

14 Proctor GB. The physiology of salivary secretion. Periodontol 2000 2016; 70: 11–25.

15 Miranda-Rius J, Brunet-Llobet L, Lahor-Soler E, Farré M. Salivary secretory disorders, inducing drugs, and clinical management. Int J Med Sci 2015; 12: 811–824.

16 Macgregor IDM. Smoking, saliva and salivation. J Dent 1988; 16: 14–17.

17 Iida T, Ono K, Inagaki T, Hosokawa R, Inenaga K. Nicotinic receptor agonist-induced salivation and its cellular mechanism in parotid acini of rats. Auton Neurosci Basic Clin 2011; 161: 81–86.

18 Davydov L, Botts SR. Clozapine-induced hypersalivation. Ann Pharmacother 2000; 34: 662–5.

19 Tadori Y, Kobayashi H. [The therapeutic plasma concentrations of antiparkinson dopamine agonists and their in vitro pharmacology at dopamine receptors]. Nihon Shinkei Seishin Yakurigaku Zasshi 2014; 34: 127–32.

20 Jia YX, Sekizawa K, Ohrui T, Nakayama K, Sasaki H. Dopamine D1 receptor antagonist inhibits swallowing reflex in guinea pigs. Am J Physiol 1998; 274: R76-80.

21 Srivanitchapoom P, Pandey S, Hallett M. Drooling in Parkinson's disease: A review. Park Relat Disord 2014; 20: 1109–1118.

22 Tungaraza TE, Ahmed W, Chira C, Turner E, Mayaki S, Nandhra HS *et al.* Prescribing pattern of clozapine and other antipsychotics for patients with first-episode psychosis: a cross-sectional survey of early intervention teams. Ther Adv Psychopharmacol 2017; 7: 103–111.

23 Montastruc JL, Sommet A, Bagheri H, Lapeyre-Mestre M. Benefits and strengths of the disproportionality analysis for identification of adverse drug reactions in a pharmacovigilance database. Br J Clin Pharmacol 2011; 72: 905–908.

3.2

The effect of glycopyrrolate on nocturnal sialorrhea in patients using clozapine: a randomized, crossover, double-blind, placebo-controlled trial

Wai Hong Man, Jantine CA Colen - de Koning, Peter FJ Schulte, Wiepke Cahn, Ingrid M van Haelst, Jeroen Doodeman, Toine CG Egberts, Eibert R Heerdink, Ingeborg Wilting

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Abstract

Background

Clozapine-induced (nocturnal) sialorrhea (CIS) is one of the most frequent adverse events of clozapine treatment. Symptomatic management of CIS usually consists of off-label treatment with anticholinergic agents. The objective of this study was to evaluate the efficacy and safety of glycopyrrolate in psychiatric patients with CIS.

Methods

In a double-blind randomized crossover trial patients suffering from CIS (n=32) were randomized to treatment with glycopyrrolate one mg or placebo. This double-blinded phase was followed by an optional open-label extension phase with glycopyrrolate two mg. Exposure periods consisted of six consecutive days and were separated with one wash out week.

The primary outcome was clinical improvement of CIS assessed by the Patient Global Impression of Improvement (PGI-I).

Results

The proportion of patients with a clinical improvement according to PGI-I did not significantly differ between one mg and placebo (18.8% versus 6.3%, p=0.289), but glycopyrrolate two mg once daily *vs* placebo did (43.5% versus 6.3%, p=0.039). Glycopyrrolate was not associated with severe adverse events and worsening of cognitive adverse events.

Conclusions

Glycopyrrolate one mg was not superior to placebo, while two mg showed a significant clinical improvement of nocturnal CIS compared to placebo. Glycopyrrolate seemed to be a tolerable anticholinergic agent in the treatment of CIS.

Introduction

Clozapine is the only antipsychotic agent with demonstrated efficacy in patients with refractory schizophrenia and with demonstrated antisuicidal properties [1,2]. However, its use is hampered by its safety profile, including serious potential life-threatening adverse effects such as agranulocytosis and myocarditis [3–5], but also effects impairing tolerability to the drug such as clozapine-induced (nocturnal) sialorrhea (CIS) [6]. The prevalence of CIS in patients using clozapine is estimated at 30-90%, rendering it the second most common adverse event of clozapine after sedation [3,5]. Sialorrhea often occurs shortly after initiation of clozapine therapy and most patients exhibit sialorrhea only during sleep.

The impact of CIS is generally underestimated. It reduces quality of life and can lead to complications including parotitis, mucositis, sleep disorders and aspiration pneumonia [5,7,8]. Moreover, CIS can be socially incapacitating and can result in poor medication adherence or even patient initiated discontinuation of clozapine, potentially leading to severe psychiatric deterioration [9].

The exact mechanism behind CIS is unknown. CIS is considered a paradoxical effect, because due to the anticholinergic properties of clozapine itself, a reduction of saliva secretion would be more expected. Multiple hypotheses exist to explain this paradoxical effect involving differences in affinity for muscarinic 3 (M_3) and muscarinic 4 (M_4) receptors. Clozapine tends to have stronger agonistic effects on M_4 receptors than on the M_3 receptors thereby inducing saliva secretion [10]. Moreover, it has been hypothesized that clozapine may reduce the swallowing reflex and thereby contributing to CIS [11].

Various anticholinergic agents have been suggested as treatment options for CIS, however their use is hampered by central anticholinergic adverse effects including memory impairment and other cognitive deficits [12–14]. Glycopyrrolate is an anticholinergic agent with a quaternary ammonium structure, limiting its passage across the blood-brain barrier, thereby greatly reducing the risk for central anticholinergic adverse effects [15]. The effect of glycopyrrolate on CIS has been studied in one randomized controlled trial so far, comparing a fixed oral dosage of one mg of glycopyrrolate with two mg of biperiden, twice daily [14]. Glycopyrrolate reduced sialorrhea significantly more than biperiden with less effect on cognitive function [14]. However, glycopyrrolate has not been compared to placebo. Furthermore, beneficial effects of glycopyrrolate on CIS have been reported in case reports and case-series [16–18]. In addition, glycopyrrolate has been shown to be effective in treating sialorrhea in patients with Parkinson's disease and in children with neurological disorders [19–21]. However, at present there are – as far as we know – no published studies comparing glycopyrrolate with placebo on clozapine induced nocturnal sialorrhea.

The objective of this study was to determine the effect of oral glycopyrrolate on clozapine induced nocturnal sialorrhea compared with placebo in psychiatric patients.

Materials and Methods

Study design

This study was designed as a multicenter, randomized, double-blind, placebo-controlled crossover trial with an extended open label phase. The double-blind phase consisted of baseline measurements and two intervention weeks separated by a one-week washout period (Figure 1).
During the intervention each participant was randomly assigned to receive either once daily glycopyrrolate one mg before bedtime or placebo before bedtime. Randomisation was performed by computer-generated random allocations at the clinical trial support unit of the Department of Clinical Pharmacy at the University Medical Center Utrecht. Allocation concealment was ensured through the use of sequentially numbered, opaque, sealed envelopes.
Both investigators and patients were blinded to the sequence of interventions during the double-blind phase. Participants were instructed to take the study medication for six consecutive days during intervention. Participants who tolerated the medication in the intervention weeks (defined as no worsening of adverse events compared to baseline) and who were willing to participate in the open label extension phase, were instructed to take two mg glycopyrrolate (unblinded) once daily for six consecutive days after one wash-out week.
After each week, during the weekly consultation, questionnaires were completed by the participants in the presence of the researcher (total of six times).
At the end of follow-up, the treating physician discussed with the patient the possibility of continuing the use of glycopyrrolate. The physician took into account in this discussion whether the patient had experienced any benefit from the glycopyrrolate and whether the patient tolerated glycopyrrolate during the study, including the open label phase.

Inclusion and exclusion criteria

Patients using clozapine for at least 30 days in the same dose and who were suffering from CIS were eligible to participate.
Inclusion criteria were 1) having a diagnosis meeting DSM-IV criteria of a psychiatric disorder 2) using clozapine in a dosage that had remained unchanged for at least 30 days prior to inclusion, 3) aged between 18 and 65 years, 4) suffering from nocturnal sialorrhea defined as a score ≥ 2 on the Patient Global Impression of Severity (PGI-S) scale, 5) having no change in dosages of co-medication potentially influencing salivary flow (clonidine, sulpiride, moclobemide) for one month prior to inclusion, 6) able to

answer questionnaires during a weekly consultation with the researcher, and 7) willing and, according to the treating physician, able to give informed consent.
Exclusion criteria were 1) known hypersensitivity to glycopyrrolate, sorbic acid or saccharine sodium, 2) a comorbidity associated with sialorrhea (e.g. Parkinson's disease, cerebral palsy), 3) having the following comorbidities: inadequately treated constipation, urine retention, bladder obstruction, 4) concomitant use of anticholinergic agents: tricyclic antidepressants or anticholinergics (atropine, ipratropium bromide, trihexyphenidyl, biperiden, scopolamine, oxybutynin), 5) concomitant use of medications that potentially interact with glycopyrrolate (potassium chloride slow-release tablets, digoxin, corticosteroids), 6) pregnancy or lactation, 7) a history of myasthenia gravis, cardiac arrhythmia, symptomatic coronary insufficiency, glaucoma, pyloris stenosis, paralytic ileus, prostate hypertrophy, renal failure, 8) inability to autonomous intake of medication and 9) an abnormal electrocardiogram.
At baseline, blood was drawn and an electrocardiogram was recorded in order to assess participant eligibility. Medication dispensing data from the community pharmacy and patient records from the general practitioner were collected. All participants had to give written informed consent to participate before study inclusion.

Settings

Recruitment took place between April 2013 and June 2015 at the University Medical Center Utrecht, an academic teaching hospital in the center of The Netherlands, with annually approximately 28,000 clinical, 15,000 day-care hospitalizations and 334,000 outpatient visits, and at the Mental Health Services North-Holland North, Heerhugowaard (a mental health care institute located in North Holland, covering a population of 225,000 inhabitants). This study was approved by the Medical Ethical Committee at the University Medical Center Utrecht. All study procedures were carried out in accordance with the Declaration of Helsinki and standards of Good Clinical Practice. The study was registered at www.clinicaltrialsregister.eu (EudraCT number: 2012-002299-15).

Interventions

During the double-blind phase the intervention consisted of five ml (one mg) glycopyrrolate oral solution of 0.2 mg/mL or five mL placebo approximately within one hour before bedtime during consecutive six days. Based on prior studies with glycopyrrolate on sialorrhea a one mg dose each dose point was considered an effective and tolerable dose [20].

The trial medication was prepared by the Department of Clinical Pharmacy of the University Medical Center Utrecht and dispensed by the clinical pharmacy department of the University Medical Center Utrecht and Medical Center Alkmaar.
The placebo oral solution consisted of the same compounds as the glycopyrrolate oral solution with the exception of the active substance glycopyrroniumbromide. The other components were kept in the placebo in order to ensure similarity in taste and appearance. In order to assure blinding of investigators both placebo and verum were provided with identical opaque plastic droplet bottles. During the open label phase, intervention involved the intake of ten ml (two mg) glycopyrrolate oral solution of 0.2 mg/mL during six consecutive days.

Outcomes

The primary outcome was clinical improvement of nocturnal sialorrhea measured by the 7-point Patient Global Impression of Improvement (PGI-I) scale.

Table 1. Outcome measures

Scale	Question	Score	Description
PGI-I	Check the one number that best describes how the symptoms of your nocturnal CIS are now, compared with how they were before you began taking medication in this study.	1	Very much improved
		2	Much improved
		3	A little improved
		4	No change
		5	Minimally worse
		6	Much worse
		7	Very much worse
PGI-S	Check the one number that best describes the severity of impact concerning your nocturnal CIS.	1	No problems with nocturnal CIS
		2	A little affected
		3	Quite much affected
		4	Much affected
		5	Very much affected
MSQ	How satisfied or dissatisfied are you, in general, with clozapine as treatment of your mental disorder at this moment?	1	Extremely dissatisfied
		2	Very dissatisfied
		3	Dissatisfied
		4	Somewhat satisfied
		5	Satisfied
		6	Very satisfied
		7	Extremely satisfied
NHRS	Check the one number that best describes the severity of symptoms of nocturnal CIS.	0	Absent
		1	Minimal: minor signs of saliva on the pillow in the morning
		2	Mild: major signs of saliva on the pillow in the morning
		3	Moderate: sialorrhea wakes the patient up once during the night
		4	Moderate/severe: sialorrhea wakes the patient up twice during the night
		5	Severe: sialorrhea wakes the patient up at least three times during the night

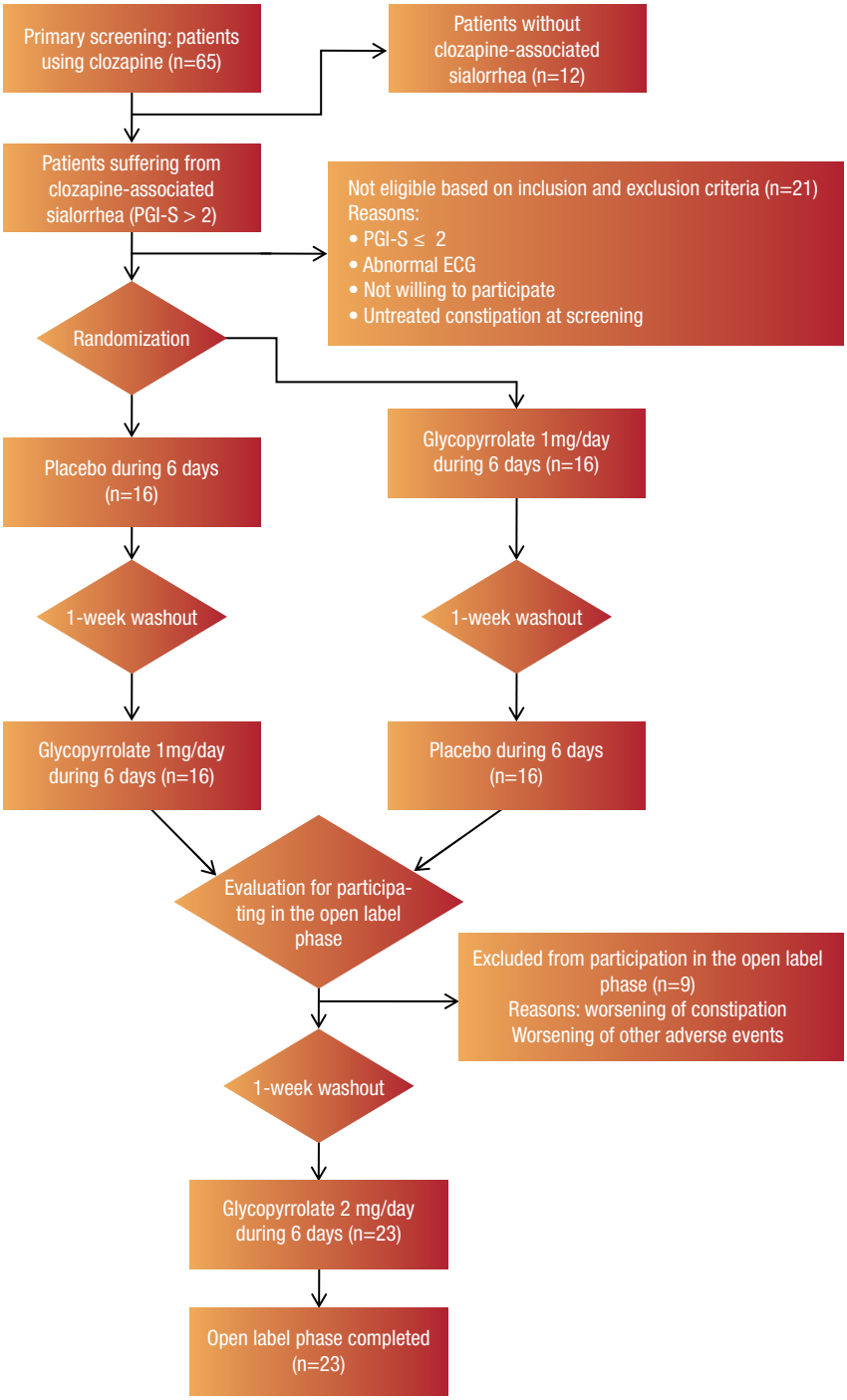
Clinically significant improvement was defined as a score 1 ('very much better') or 2 ('much better') on the PGI-I scale (Table 1). In addition, the PGI-S, the Nocturnal Hypersalivation Rating Scale (NHRS) and the Medication Satisfaction Questionnaire (MSQ) (Table 1) were used to determine respectively the severity of CIS, the extent of occurrence of nocturnal sialorrhea and the participants' satisfaction with clozapine as treatment of participant's psychiatric disorder. During the study period of six weeks these patient reported outcomes were collected by the researcher each week during the weekly visit.

Furthermore, participants were asked to identify their treatment preference at the end of the double-blinded phase and also at the end of the open label phase. In terms of patient safety monitoring, blood was also drawn in the last week of inclusion and occurrence of possible side effects and especially constipation was evaluated with questionnaires during the weekly visit by the researcher. Occurrence or worsening of constipation was monitored extensively using specific constipation assessment questionnaires during the weekly visits as clozapine is also related to increased incidence of constipation, consequently enhancing the risk of an ileus.

Table 2. Patient characteristics at baseline

	Double-blinded (N=32)	Double-blinded and open label (N=23)
Male gender n (%)	21 (65.6%)	13 (56.5%)
Age in years, mean (SD)	38.9 (11.2)	39.2 (12.1)
Weight in kg, mean (SD)	82.4 (16.9)	80.7 (17.2)
Smoking n (%)	9 (28.1%)	5 (21.7%)
Alcohol consumption in alcohol units per week, median (IQR)	0 (4)	0 (4)
Use of recreational drugs, n (%)	4 (12.5%)	3 (13.0%)
Clozapine dose in mg/day, median (IQR)	250 (200)	250 (100)
Duration of clozapine therapy in months), mean (SD)	89.9 (63.2)	85.7 (68.4)
Indication of clozapine therapy		
Schizophrenia, n (%)	23 (71.9%)	
Schizoaffective disorder bipolar type, n (%)	4 (12.5%)	
Psychotic disorder not otherwise specified, n (%)	3 (9.4%)	
Bipolar I disorder, n (%)	2 (6.3%)	
Heart rate in bpm, mean (SD)	90.9 (10.0)	91.0 (10.5)
Systolic blood pressure in mmHg, mean (SD)	123.5 (12.6)	124.8 (12.9)
Diastolic blood pressure in mmHg, mean (SD)	84.1 (11.5)	85.2 (12.4)
Sodium serum level in mmol/L, median (IQR)	139 (2)	139 (2)
Potassium serum level in mmol/L, mean (SD)	4.1 (0.3)	4.1 (0.3)
Creatinine serum level in µmol/L, mean (SD)	79.6 (14.2)	78.4 (15.9)

Figure 1. Flow chart of patient inclusion



Sample Size

Sample size calculation was based on the McNemar's test with an expected clinically significant improvement in nocturnal sialorrhea severity in 15% of the placebo intervention and 45% in the treatment group during the double-blind phase, according to previous studies on glycopyrrolate in sialorrhea other than CIS, using an alpha of 0.05 with 80% power. Taking into account 34% discordant pairs with positive efficacy results of glycopyrrolate – negative efficacy results of placebo and 4% discordant pairs with negative efficacy results of glycopyrrolate – positive efficacy results placebo, a total of 32 participants had to finish the double-blind phase, involving the intake of one mg/day glycopyrrolate oral solution or placebo oral solution following six days.

Table 3. Efficacy

	Placebo (N=32)	1 mg (N=32)	2 mg (N=23)
Proportion of patients with a clinical significant improvement based on PGI-I score, n (%)	2 (6.3%)	6 (18.8%)	10 (43.5%)*
PGI-I score, median (IQR)	4 (1)	3 (1)	3 (1)*
PGI-S score, median (IQR)	3 (1)	3 (1)	2 (1)*
NHRS score, median (IQR)	2.5 (2)	2 (3)	1 (1)*
MSQ score, median (IQR)	5.5 (2)	6 (1)	6 (3)
Proportion of patients willing to continue treatment (%)	6 (18.8%)	19 (59.4%)*	16 (69.6%)*
Patient preference during double-blinded phase** (N=32)	2 (6.3%)	15 (46.9%)*	-
Patient preference during open label phase** (N=23)	0 (0.0%)	5 (21.7%)	13 (56.5%)*

* P<0.05 compared to placebo as reference
** Participants who haven't indicated a patient preference intervention are not shown

Data analysis

The proportions of participants with a clinical improvement according PGI-I were compared with the McNemar's test. The PGI-I, PGI-S, NHRS and MSQ scores between the interventions were compared by Wilcoxon matched-pairs signed rank tests.
Although not expected because of the wash-out period based on the elimination half-life of glycopyrrolate, potential carryover effects were evaluated by comparing the efficacy outcome parameters at the end of wash-out with the baseline efficacy outcome parameters using the Wilcoxon matched-pairs signed rank test. All data analyses were performed using SPSS for Windows version 21.0 (IBM, Chicago, IL, USA). An alpha level of 0.05 was considered statistically significant for all performed statistical tests.

Table 4. Adverse events

	Baseline (N=32) Frequency (%)	Placebo (N=32) Frequency (%) of worsening/ improvement*	1 mg (N=32) Frequency (%) of worsening/ improvement*	2 mg (N=23) Frequency (%) of worsening/ improvement*
Cognitive adverse event				
Concentration problems	87.5	0.0/6.3	0.0/6.3	0.0/4.3
Asthenia	84.4	0.0/3.1	0.0/6.3	0.0/4.3
Drowsiness	84.4	0.0/3.1	0.0/6.3	0.0/4.3
Memory impairment	75.0	3.1/3.1	0.0/6.3	0.0/0.0
Accomodation disturbances	25.0	0.0/0.0	0.0/0.0	0.0/0.0
Other central adverse events				
Nervousness	62.5	6.3/6.3	3.1/3.1	4.3/4.3
Prolonged night sleep	78.1	0.0/3.1	0.0/3.1	0.0/0.0
Shortened sleep	18.8	3.1/0.0	6.3/0.0	4.3/0.0
More dreams during night sleep	53.1	3.1/3.1	0.0/9.4	0.0/4.3
Headache	15.6	6.3/3.1	6.3/3.1	0.0/0.0
Dizziness	50.0	0.0/6.3	0.0/3.1	0.0/0.0
Peripheral anticholinergic adverse events				
Xerostomia	37.5	6.3/0.0	6.3/6.3	8.7/4.3
Constipation	50.0	0.0/6.3	0.0/6.3	0.0/4.3
Miction disorders	31.3	0.0/0.0	0.0/0.0	0.0/0.0
Palpitations	25.0	3.1/0.0	3.1/0.0	0.0/0.0
Decreased diaphoresis	15.6	0.0/6.3	0.0/3.1	0.0/0.0
Vaginal dryness	18.2	0.0[a]/0.0[a]	0.0[a]/0.0[a]	0.0[c]/0.0[a]

Table 4. continued Adverse events

	Baseline (N=32) Frequency (%)	Placebo (N=32) Frequency (%) of worsening/ improvement*	1 mg (N=32) Frequency (%) of worsening/ improvement*	2 mg (N=23) Frequency (%) of worsening/ improvement*
Blurred vision	28.1	0.0/0.0	0.0/0.0	0.0/0.0
Skin dryness	18.8	0.0/0.0	0.0/0.0	0.0/0.0
Other adverse event				
Nausea and vomiting	34.4	3.1/3.1	0.0/6.3	0.0/0.0
Diarrhea	25.0	0.0/3.1	0.0/3.1	0.0/0.0
Polyuria and polydipsia	50.0	0.0/0.0	0.0/0.0	0.0/0.0
Orthostatic hypotension	71.9	6.3/0.0	9.4/0.0	0.0/0.0
Diaphoresis	50.0	6.3/0.0	9.4/0.0	4.3/0.0
Skin rash	18.8	0.0/0.0	0.0/0.0	0.0/0.0
Pruritis	15.6	0.0/0.0	0.0/0.0	0.0/0.0
Photosensitivity	12.5	0.0/3.1	3.1/3.1	0.0/3.1
Erectile dysfunction	23.8	0.0[b]/6.3[b]	4.8[b]/0.0[b]	0.0[d]/0.0[d]
Impaired taste	6.3	3.1/3.1	0.0/3.1	0.0/0.0
Flushing	15.6	0.0/0.0	0.0/0.0	0.0/0.0

* compared to baseline
[a] variable only applicable to women (N=11)
[b] variable only applicable to men (N=21)
[c] variable only applicable to women (N=10)
[d] variable only applicable to men (N=13)

Results

A total of 32 participants were included and finished the double-blind phase (see Table 2). Nine participants did not enter the open label phase due to meeting the exclusion criteria for the open label phase including (worsening) constipation and abrupt smoking cessation (Figure 1). Indications for clozapine therapy were schizophrenia, schizoaffective disorder, psychotic disorder and bipolar I disorder (Table 2). The median duration of clozapine therapy at baseline was 83 months.

During the double-blind phase no carry over effects (wash out week one *vs* baseline) were seen in all efficacy outcome parameters including PGI-I, PGI-S, NHRS and MSQ, while during the open label phase (wash out week two *vs* baseline) a significant mean difference in NHRS score was observed (mean difference=-0.43 [-0.83;-0.44], p=0.038)).

Two patients (6.3%) experienced a clinically relevant improvement on placebo, while six patients (18.8%) on glycopyrrolate one mg according to PGI-I (RR=3.0 [0.65;13.76], p=0.289). Neither of the secondary outcome parameters (PGI-S, NHRS, MSQ) did significantly differ.

A statistically significant higher proportion of patients reported a clinical improvement on PGI-I during the open label phase on glycopyrrolate two mg compared to placebo: 43.5% *vs* 6.3%; RR=6.96 [1.23; 20.36], p=0.039 (Table 3).

Significant lower scores on PGI-I (mean difference=-1.04 [-1.52;-0.57] (p=0.001), PGI-S (mean difference=-1.26 [-1.83;-0.70] (p=0.001)) and NHRS (mean difference=-1.13 [-1.61; -0.65] (p=0.001)) were observed in glycopyrrolate two mg compared to placebo.

Compared to placebo, a significantly higher proportion of participants were willing to continue with glycopyrrolate one mg once daily as well as two mg once daily (Table 2).

When comparing glycopyrrolate two mg with one mg once daily, significant lower PGI-I (Mean difference=-0.70 [-1.18;-0.17] (p=0.017)), PGI-S (mean difference=-0.96 [-1.43;-0.52] (p=0.002)) and NHRS (mean difference=-0.87 [-1.26;-0.43] (p=0.004)) scores were found in the two mg dosage.

Adverse events of glycopyrrolate, in terms of worsening of baseline events, were mild/moderate and included diaphoresis (4.3-9.4%), orthostatic hypotension (0-9.4%), xerostomia (6.3-8.7%), erectile dysfunction (0-4.8%), shortened sleep (4.3-6.3%), headache (0-6.3%), nervousness (3.1-4.3%), palpitations (0-3.1%) and photosensitivity (0-3.1%). No worsening of cognitive adverse events was observed in the glycopyrrolate intervention groups (Table 4).

Discussion

Principal findings

The results of our study suggest that glycopyrrolate is effective in reducing clozapine induced nocturnal sialorrhea in psychiatric patients if glycopyrrolate is dosed high enough. During intervention with glycopyrrolate two mg clinical improvement of CIS was found seven times more frequent than with placebo. This is in line with a significant decrease in severity of symptoms (NHRS) and impact (PGI-S). Distinctively, the MSQ score did not differ significantly after glycopyrrolate intervention. This could be explained by an already high rate of satisfaction toward clozapine therapy at baseline (mean MSQ baseline score=5.31 (data not shown)).

Glycopyrrolate one mg is probably more effective than placebo – but less effective than two mg – in the treatment of CIS (RR=3 [p=0.289]), presumably due to the low sample size of this study. Moreover, a significant proportion of participants wished to continue using glycopyrrolate one mg in comparison to placebo.

Glycopyrrolate two mg compared to placebo once daily appeared to be to more effective in improving CIS based on decreases in all efficacy parameters except for MSQ. This indicates a dose related effect of glycopyrrolate. Hence, glycopyrrolate one mg tends to be more effective than placebo and glycopyrrolate two mg in its turn tends to be more effective than one mg in treating CIS than placebo.

However, a significant carryover effect was found on the NHRS during the open-label phase (wash-out week two *vs* baseline), indicating a potential overestimation of lower NHRS scores in glycopyrrolate two mg.

Dosing glycopyrrolate two mg once daily did not result in an increase in adverse events compared to placebo and therefore appears to be a tolerable and safe dose in treating CIS.

As expected, no worsening of cognitive adverse events was observed during the interventions. This is in accordance with the beneficial pharmacological property of glycopyrrolate in not being able to cross the blood brain barrier [15]. Reported adverse events during glycopyrrolate intervention were mild and transient. Most of these adverse events were pharmacologically explained as they were related to the anticholinergic effects of glycopyrrolate.

Implications of these findings with reference to other studies

To date, no drug is registered for the treatment of CIS, although pharmacotherapeutic agents have been studied in various studies and are used in daily practice [9,12].

The use of glycopyrrolate in CIS has been evaluated in a small study and in a number

of case reports. In a randomized double-blind, crossover study glycopyrrolate was compared to biperiden. Glycopyrrolate was given in a dose of 1 mg twice daily and seemed to be an effective agent displaying less impact on cognitive function [14]. Furthermore, in a case study 3 patients suffering from CIS were treated with glycopyrrolate (4–8mg) and showed improvement of CIS. Glycopyrrolate was generally well tolerated [17]. Moreover, in a randomized clinical trial involving patients suffering from Parkinson's Disease associated sialorrhea, glycopyrrolate one mg three times daily has also been shown to be effective [20].
Despite these findings supporting these potential beneficial features of glycopyrrolate in sialorrhea, no randomized clinical trial has ever been conducted to determine the effects of glycopyrrolate compared to placebo on CIS specifically.

Limitations

A limitation of the study is the glycopyrrolate dose. Dose selection is important for obtaining the optimal balance between efficacy and safety. Based on previous studies and case reports involving the use of glycopyrrolate against sialorrhea, we focused on patient safety by choosing a fairly low/normal dose of glycopyrrolate. The one mg dose showed a numerically lower PGI-I, but this was not statistically significant, probably due to power problems or due to the fairly low dose of glycopyrrolate. Nevertheless, at the end of the double-blind phase significantly more participants wished to continue with glycopyrrolate one mg compared to placebo. It may be clinically advisable to start treatment of CIS with one mg glycopyrrolate before bedtime and double the dose in non-responders, since this dose was not only more efficacious than placebo but also than one mg glycopyrrolate. Future studies should focus on intervention of glycopyrrolate with higher doses of glycopyrrolate in terms of optimizing efficacy.
Furthermore, another limitation of the study could be the lack of objectively quantifiable outcome parameters for sialorrhea. Although our outcome rating scales were all validated, these were not outcome parameters that are fully objective. Because of the setting of the study, it was practically not possible to quantify sialorrhea, for example by measuring the amount of saliva secretion.
Our study evaluated the effects of glycopyrrolate on nocturnal sialorrhea. Although occurrence of CIS is prominent during the night, many patients also suffer from CIS during the daytime. Future studies should evaluate what dose regimen of glycopyrrolate would be optimal to treat sialorrhea during the night and the daytime.

Conclusions

Our results suggest that oral glycopyrrolate two mg but not one mg once daily is an significant effective anticholinergic agent in the treatment of CIS that could diminish the impact of severity of symptoms in psychiatric patients. Glycopyrrolate did not show cognitive adverse events and was shown a well-tolerated drug. Future studies should evaluate the optimum dose of glycopyrrolate in treating CIS.

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References

1 Chakos M, Lieberman J, Hoffman E, Bradford D, Sheitman B. Effectiveness of second-generation antipsychotics in patients with treatment-resistant schizophrenia: a review and meta-analysis of randomized trials. Am J Psychiatry 2001; 158: 518–526.

2 Kane JM, Honigfeld G, Singer J, Meltzer HY, Al E. Clozapine for the treatment-resistant schizophrenic: Results of a US multicenter trial. Psychopharmacology 1989; 99: S60–S63.

3 Grohmann R, Rüther E, Sassim N, Schmidt LG. Adverse effects of clozapine. Psychopharmacology 1989; 99 Suppl: S101-4.

4 Lahdelma L, Appelberg B. Clozapine-Induced Agranulocytosis in Finland, 1982–2007. J Clin Psychiatry 2012; 73: 837–842.

5 Lieberman J a, Safferman a Z. Clinical profile of clozapine: adverse reactions and agranulocytosis. Psychiatr Q 1992; 63: 51–70.

6 Praharaj SK, Arora M, Gandotra S. Clozapine-induced sialorrhea: pathophysiology and management strategies. Psychopharmacology 2006; 185: 265–73.

7 Abdelmawla, Nasser; Ahmed MI. Clozapine and risk of pneumonia. Br J Psychiatry 2009; 194: 467.

8 Robinson D, Fenn H, Yesavage J. Possible association of parotitis with clozapine. Am J Psychiatry 1995; 152: 297–8.

9 Sockalingam S, Shammi C, Remington G. Clozapine-induced hypersalivation : A review of treatment strategies. Can J psychiatry; 52: 377–384.

10 Zorn SH, Jones SB, Ward KM, Liston DR. Clozapine is a potent and selective muscarinic M4 receptor agonist. Eur J Pharmacol 1994; 269: R1–R2.

11 Rabinowitz T, Frankenburg FR, Centorrino F, Kando J. The effect of clozapine on saliva flow rate: a pilot study. Biol Psychiatry 1996; 40: 1132–4.

12 Gaftanyuk O. . RLT. Scolpolamine Patch for Clozapine-Induced Sialorrhea. Psychiatr Serv 2004; 55: 2004.

13 Lechevallier-Michel N, Molimard M, Dartigues J-F, Fabrigoule C, Fourrier-Réglat A. Drugs with anticholinergic properties and cognitive performance in the elderly: results from the PAQUID Study. Br J Clin Pharmacol 2005; 59: 143–151.

14 Liang C-S, Ho P-S, Shen L-J, Lee W-K, Yang F-W, Chiang K-T. Comparison of the efficacy and impact on cognition of glycopyrrolate and biperiden for clozapine-induced sialorrhea in schizophrenic patients: a randomized, double-blind, crossover study. Schizophr Res 2010; 119: 138–44.

15 Paton WDM, Zaimis E. The methonium. Pharmacol Rev 1952; 4: 219–53.

16 Blissit KT, Tillery E, Latham C, Pacheco-Perez J. Glycopyrrolate for treatment of clozapine-induced sialorrhea in adults. Am J Heal Pharm 2014; 71: 1282–1287.

17 Robb AS, Lee RH, Cooper EB, Siedel J V, Nusrat N. Glycopyrrolate for treatment of clozapine-induced sialorrhea in three adolescents. J Child Adolesc Psychopharmacol 2008; 18: 99–107.

18 Duggal HS. Glycopyrrolate for clozapine-induced sialorrhea. Prog Neuropsychopharmacol Biol Psychiatry 2007; 31: 1546–7.

19 Evatt ML. Oral glycopyrrolate for the treatment of chronic severe drooling caused by neurological disorders in children. Neuropsychiatr Dis Treat 2011; 7: 543–7.

20 Arbouw MEL, Movig KLL, Koopmann M, Poels PJE, Guchelaar H-J, Egberts TCG *et al.* Glycopyrrolate for sialorrhea in Parkinson disease: a randomized, double-blind, crossover trial. Neurology 2010; 74: 1203–7.

21 Mier RJ, Bachrach SJ, Lakin RC, Barker T, Childs J, Moran M. Treatment of Sialorrhea With Glycopyrrolate. Arch Pediatr Adolesc Med 2000; 154: 1214.

22 Bird a. M, Smith TL, Walton a. E. Current Treatment Strategies for Clozapine-Induced Sialorrhea. Ann Pharmacother 2011; 45: 667–675.

3.3

Clozapine-induced hypersalivation: the association between quantification, perceived burden and treatment satisfaction reported by patients

Wai Hong Man, Jantine CA Colen - de Koning, Peter FJ Schulte, Wiepke Cahn, Ingrid M van Haelst, Eibert R Heerdink, Ingeborg Wilting

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We read with great interest the recent Therapeutic Advances in Psychopharmacology article by Maher and colleagues entitled "Clozapine-induced hypersalivation: an estimate of prevalence, severity and impact on quality of life" [1]. The authors evaluated the prevalence of CIH in a population of psychiatric patients in a specialized clozapine clinic. In addition they assessed and quantified the severity of CIH – using the Nocturnal Hypersalivation Rating Scale (NHRS) and the Drooling Severity and Frequency Scale (DSFS) – and its impact on global functioning. They reported that clozapine-induced hypersalivation (CIH) was the most prevalent adverse effect negatively impacting the quality of life in patients treated with clozapine.

The NHRS is a validated five-item scale used to determine patient-reported hypersalivation. Owing to their subjective nature, individual differences exist in patient-reported treatment satisfaction and perceived burden of hypersalivation. With regard to the extent to which changes in NHRS score affect patients' perceived burden and treatment satisfaction, we here report an association between NHRS and, firstly, the Patient Global Impression of Severity (PGI-S) five-point scale (regarding CIH specifically) – which has similar anchors for scoring as the Clinical Global Impression-Severity scale – and, secondly, the Medication Satisfaction Questionnaire (MSQ) seven-point scale among patients experiencing CIH before and after treatment with the anticholinergic agent glycopyrrolate.

We included 32 psychiatric patients experiencing nocturnal CIH in a double-blind crossover study (EudraCT number: 2012-002299-15) investigating the association between NHRS score, perceived burden of CIH (PGI-S) and participants' satisfaction (MSQ) with clozapine treatment, comparing before intervention (baseline) and after intervention with glycopyrrolate 1 mg and 2 mg [2]. Thus, further to the study of Maher et al, we investigated the effect of treatment of CIH on severity and perceived burden within patients.

All participants received glycopyrrolate 1 mg QD (N=32) for six consecutive days. This treatment was followed by a washout week and eventually by an optional open-label treatment with glycopyrrolate 2 mg QD (N=23) for six consecutive days. Only patients who were willing to continue and who met the eligibility criteria participated in the open-label treatment. We calculated Spearman rank correlation coefficients to assess the association between the different patient-reported outcome scales.

A decrease in NHRS score after intervention with glycopyrrolate 1 mg QD was significantly associated with a decrease in PGI-S score (r_s (30)=0.747, P<0.001), but with a non-significant change in MSQ score (r_s (30)=-0.165, P=0.366). A decrease in NHRS

Table 1. Patient-reported outcome parameters and associations

	Baseline (N=32)	1 mg (N=32)	Δ1 mg - baseline (N=32)	2 mg (N=23)	Δ2 mg - baseline (N=23)
NHRS, median (IQR)	3 (2)	2 (3)	0 (1)	1 (1)	-1 (1)
PGI-S median (IQR)	3 (2)	3 (1)	0 (2)	2 (1)	-1 (1)
MSQ median (IQR)	5 (2)	6 (1)	0 (0)	6 (3)	0 (1)
NHRS *vs* PGI-S, r_s (*P* value)	0.347 (0.051)	0.616 (<0.001)	0.747 (<0,001)	0.687 (<0.001)	0.264 (0.224)
NHRS *vs* MSQ, r_s (*P* value)	0.179 (0.326)	0.268 (0.139)	-0.165 (0.366)	0.223 (0.306)	0.052 (0.814)

NHRS, Nocturnal Hypersalivation Rating Scale; PGI-S, Patient Global Impression of Severity; MSQ, Minnesota Satisfaction Questionnaire; r_s, Spearman Rank-order Coefficient; IQR, interquartile range

score after intervention with glycopyrrolate 2 mg QD was not significantly associated with a change in either PGI-S (r_s (21)=0.264, P=0.224) or MSQ (r_s (21)=0.052, P=0.814) score. This indicates that clozapine users perceived a decreased burden of CIH, reflected by a decrease in PGI-S, when the NHRS score decreased. However, further decrease in NHRS score did not lead to a coherent decrease in PGI-S, as observed after intervention with glycopyrrolate 2 mg. Satisfaction towards clozapine therapy (MSQ) was not influenced by any intervention.

This could at least partly be explained by the patient selection in our study, as individuals who were already receiving clozapine treatment were very satisfied with clozapine therapy at baseline, despite experiencing CIH (Table 1). This is consistent with findings from a study in which clozapine users reported benefit of treatment and had the intention to continue taking clozapine, despite the presence of CIH [3]. Thus, high satisfaction towards treatment and improved quality of life are likely to promote adherence to clozapine, thereby improving long-term treatment outcomes. Also, subjective experiences of clozapine treatment reported by patients are found to be a useful component of outcome measures [3]. In addition, owing to the selection criteria for the optional open-label treatment (glycopyrrolate 2 mg), the outcomes of this intervention in our study may have been influenced by selection bias.

Overall, given these findings, we suggest that patient-reported quantification of hypersalivation should be placed into perspective with patients' perceived burden of hypersalivation and treatment satisfaction. That is, not all patients tend to respond in the same manner regarding the perception of disease burden and treatment satisfaction in the context of decreased NHRS scores.

In addition, an interesting question arises with regard to whether MSQ score is significantly affected by a decrease in NHRS score specifically in clozapine-naïve patients who are starting clozapine therapy, as these individuals are not yet used to the drug. In this respect, routine use of patients' perceived burden and treatment satisfaction scales in the evaluation of hypersalivation in clinical practice, as suggested by Maher *et al.* [1], would provide insights into this topic.

References

1 Maher S, Cunningham A, Callaghan NO, Byrne F, Donald CM, Mcinerney S *et al.* Clozapine-induced hypersalivation : an estimate of prevalence , severity and impact on quality of life. Ther Adv Psychopharmacol 2016; 6: 178–184.

2 Man WH, Colen-de Koning JCA, Schulte PFJ, Cahn W, van Haelst IMM, Doodeman HJ *et al.* The Effect of Glycopyrrolate on Nocturnal Sialorrhea in Patients Using Clozapine: A Randomized, Crossover, Double-Blind, Placebo-Controlled Trial. J Clin Psychopharmacol 2017; 37: 155–161.

3 Waserman J, Criollo M. Subjective experiences of clozapine treatment by patients with chronic schizophrenia. Psychiatr Serv 2000; 51: 666–8.

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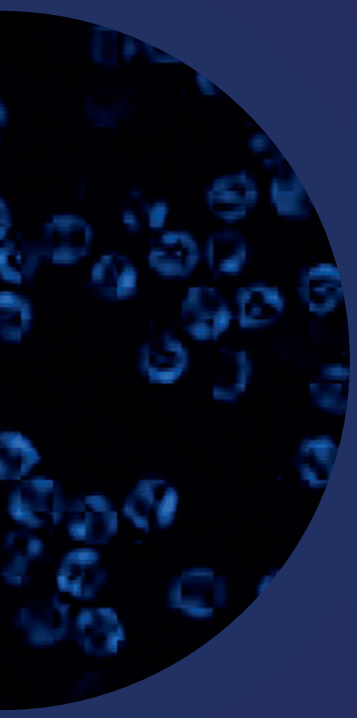
General discussion



General discussion

Clozapine has a unique position within the antipsychotics treatment armamentarium because of its well proven efficacy in refractory schizophrenia on the one hand, and its distinct spectrum of adverse drug reactions (ADRs) including agranulocytosis, myocarditis and sialorrhea [1]. Clozapine is reserved as the last resort pharmacological treatment due to the fear of its potentially life threatening side effects, thereby possibly depriving patients of necessary treatment [2]. Prescribing is a cyclic process involving initiation, monitoring, evaluation and – if necessary – adjustment of pharmacotherapy [3]. Monitoring patients using clozapine is essential to optimize the benefit-harm balance for the individual patient. Medication adherence represents a big challenge in clozapine users [4–6]. Therapeutic drug monitoring of both the parent and the daughter compound provides an indication of clozapine intake only over the past few days given the short elimination half-life of these substances [7]. A (bio)marker that validly corresponds to medication adherence over a longer time-period – comparable to HbA1c measurement in diabetes mellitus patients – would be of great value. In this regard, neutrophil fluorescence that was serendipitously and specifically observed in patients using clozapine at fluorescence channel FL3 could be of interest. FL3-fluorescence is a flow-cytometric parameter that measures nucleated red blood cells and white blood cell viability by propidium iodide (PI) staining. In this thesis we evaluated FL3-fluorescence for the purpose of obtaining information regarding long-term adherence to clozapine therapy.

Monitoring of the clozapine plasma concentrations followed by clinical interpretation and – if necessary – dose adjustments, can also contribute positively to the benefit-harm balance because of the relation between the height of the plasma concentration and the likelihood of the desirable and undesirable response [8–11]. Periodic measurement of the total clozapine plasma concentration is therefore advised in clinical guidelines [12]. However, special patient circumstances including inflammation can affect the total plasma clozapine concentration and its correct interpretation [13,14]. Current guidelines advise dose reduction in case of inflammation [12], because case reports described an increase in total clozapine plasma concentrations, although usually without clinical symptoms that fit to these increased concentrations [13,14]. Some studies have suggested that the free (unbound, active) clozapine plasma concentrations are not altered during inflammation, despite increases in the total clozapine plasma concentration [13,14]. If this is true, then promptly lowering the clozapine dose results in lowering the active, effective plasma concentration, thereby increasing the risk of relapse. In this thesis we evaluated the association between AGP – a protein that is elevated during the acute phase of inflammation – and the clozapine unbound fraction.

Clozapine has a unique side effect profile. These side effects are also important in evaluating the benefit-harm balance in the individual patient. Side effects can negatively affect drug adherence, when the patient is not willing to tolerate the side effects,

and thus have a negative effect on patient outcomes [15,16]. It is therefore important to regularly monitor for side effects that are invalidating from the perspective of the patient in the evaluation of the prescribed drug regimen and to – if necessary – adjust the treatment. Shifting focus to patients' perspectives regarding side effects could strengthen shared decision making, the clinician-patient alliance, improve adherence and thus patient outcomes [17]. In this thesis we focused on sialorrhea as a side effect. Earlier studies have shown that patients consider clozapine induced sialorrhea more relevant than healthcare providers [18].

The aim of this thesis was to provide insight in optimization of monitoring of patients treated with clozapine and to translate these insights into clinical recommendations by, first, examining and elaborating application of FL3-fluorescence as a long-term adherence monitoring biomarker, second, assessing the association between plasma concentration of the acute phase protein alpha-1-acid glycoprotein (AGP) and clozapine unbound fraction and third, studying reporting frequency and treatment of sialorrhea as an example of a non-life threatening ADR but majorly affecting the quality of life in patients using clozapine.

We performed a retrospective observational study that confirmed an earlier serendipitous observation showing neutrophil fluorescence – FL3-fluorescence – to be elevated in clozapine users [19]. FL3-fluorescence was thereby shown to be a potential long-term medication adherence biomarker (*Chapter 2.1*). Explorations on the origin of this biomarker revealed that the neutrophil fluorescence is reflected by a 14kDa soluble protein present in azurophilic granules of neutrophils (*Chapter 2.2*). We developed and evaluated a PK/PD model and nomogram relating FL3-fluorescence as a biomarker providing information about clozapine exposure/adherence over a longer period of time than clozapine plasma concentrations (*Chapter 2.3*). In line with earlier proposed theories we found that elevated AGP plasma concentrations were significantly associated with a lower clozapine unbound fraction. This is most likely explained by increased systemic availability of AGP as a result of inflammation (*Chapter 2.4*). We found the bothersome ADR sialorrhea to be reported almost four times more often with clozapine use than with other antipsychotics. In addition, it was reported eight times more often by patients than by health-care professionals (*Chapter 3.1*). Management of sialorrhea with the anticholinergic agent glycopyrrolate, dosed at 2 mg a day, was found effective and well tolerated in clozapine users, as demonstrated by the results of our placebo-controlled randomized clinical trial (RCT) (*Chapter 3.2*). In monitoring sialorrhea, patient-reported quantification of sialorrhea should be placed into perspective with patients' perceived burden and treatment satisfaction (*Chapter 3.3*).

In this general discussion the following three topics will be put into a broader perspective:

- FL3 fluorescence as a biomarker for long-term adherence to clozapine
- Monitoring unbound clozapine concentrations during inflammation
- Monitoring of clozapine-induced sialorrhea as a clinical biomarker

FL3-fluorescence as a biomarker for long-term adherence to clozapine

A promising adherence biomarker to be used in clinical practice

A biomarker has been defined as a biological characteristic that is objectively measured and evaluated as an indicator of normal biological or pathological processes, or a response to a therapeutic intervention including the assessment of medication adherence [20]. In treatment with clozapine, such a biomarker is desirable because non-adherence to is a major issue in the treatment of schizophrenia in general [21]. The overall prevalence of non-adherence to antipsychotics is estimated at 40% to 50% [22]. In *Chapter 2* we have studied application of FL3-fluorescence as a long-term adherence marker for clozapine use. FL3-fluorescence is the terminology used in this thesis to describe neutrophil autofluorescence in blood of patients using clozapine detected at FL3 (red channel) in flowcytometry.

For biomarkers to be valuable for use in clinical practice, these should meet the following criteria [23,24]:

- Specific
- Sensitive
- Predictive
- Robust
- Non-invasive
- Cost-effective

Chapter 2.1 showed that enhanced FL3-fluorescence is strongly associated with use of clozapine according to the ROC with a cut-off value of the mean plus three times the standard deviation, rendering both high specificity (0.99) and sensitivity (0.94). A high specificity is essential, as false positive results should be avoided for the intended use. However, it should be noted that we did not examine this association specifically in a population of psychiatric patients. It was also demonstrated that the signal remained enhanced for a relative long time after clozapine intake, indicating FL3-fluorescence to be an appropriate adherence marker over a longer time-period. In addition, in *Chapter 2.3* we evaluated our developed model and nomogram and found median absolute prediction errors and the IQR (%) to be relatively low. Robustness is the ability of the FL3 assay to provide consistent results using independent samples. This has not been

extensively studied in this thesis. Moreover, although FL3 measurements are invasive, patients don't require additional blood samples or should not follow special instructions regarding blood sampling, when determining FL3-fluorescence together with a routine complete blood count (CBC) measurement coincidentally. That is, FL3-fluorescence measurement of neutrophil granulocytes is performed by the Abbott Cell-Dyn Sapphire analyser as part of the routine CBC measurement. In this regard, clinicians are basically able to evaluate FL3 measurement results together with the results of the CBC, if determined using a Sapphire analyzer, during the upcoming consultation with the patient using clozapine. Although a cost-effectiveness analysis has not been performed, FL3 measurement is expected to be cost-effective, since the measurement is paired with low costs and poor medication adherence would lead to high costs.

Given the information above, FL3-fluorescence seems to be a biomarker with high potential to be used in clinical practice, since it fulfills several criteria stated above. Although FL3-fluorescence as studied in our current settings can be a promising marker, there are a few limitations that need to be addressed. A limitation for implementation is the availability of hospitals equipped with the specific type of analyzer able to report FL3-fluorescence. As mentioned above, we measured FL3-fluorescence using the Abbott Cell-Dyn Sapphire, which can be considered a method to detect autofluorescence of neutrophils in patients using clozapine. Same autofluorescence can be detected on other fluorescence-activated cell sorting (FACS) devices if excitation and emission wavelengths, similar to the FL3 channel on the Sapphire, are used. Therefore, standardization of these emission and excitation settings is crucial to reproduce FL3-fluorescence. In addition, as described in *Chapter 2.2*, stronger autofluorescence signals were detected at FL8 to FL10 fluorescence channels. Neutrophil autofluorescence should therefore be detected at these channels if available when using FACS devices other than the Sapphire analyzer.

Another limitation is the requirement of steady state concentrations of clozapine for adequate interpretation. The nomogram, when validated, can only be used in patients on stable clozapine dose in steady state over the examined time-period. As CBC measurements take place weekly during the first 18 weeks after initiation of clozapine therapy [25,26], it is during this period that steady state plasma concentrations of clozapine have not been reached yet. Thus, interpretation of FL3 measurements based upon our nomogram during this period is not valid until a steady dose is reached within the 18 weeks. Besides, it is often unnecessary or irrelevant to evaluate clozapine intake over the past 100 days from that moment. Therefore, evaluation of medication adherence based on FL3-fluorescence is suggested to be of value when patient is on a stable clozapine dose for at least three months and thus the marker is not recommended for patients in the initiation phase of clozapine therapy.

Biomarkers with a relative long half-life in the assessment of adherence have been studied before, next to the treatment of interest. For example; very low-dose phenobarbital in adherence on reducing doses of methadone [27]; bromide in hypertension [28]

and methotrexate polyglutamates in juvenile idiopathic arthritis and juvenile dermatomyositis [29]. As opposed to FL3-fluorescence use of mentioned markers induces unnecessary exposure to exogenous substances and is more time-, cost- and work-consuming, hampering use in clinical practice.

FL3-fluorescence is solely practical when translated into an intuitive outcome parameter

Insight in adherence with clozapine treatment is essential to allow the clinician to optimally support the patient with taking the drug. In this regard, FL3-fluorescence can be useful in clinical practice, only if this biomarker is translated into an intuitive outcome parameter. In *Chapter 2.3* a conceptual nomogram was developed to estimate the long-term adherence to clozapine over the past 100 days based upon FL3 measurement value in combination with prescribed clozapine dose. The extent of long-term adherence is expressed as proportion (%) daily doses taken over 100 days, in this thesis termed adherence rate. This outcome parameter, rather than the FL3-fluorescence result, is more intuitive and unambiguous, allowing clinicians to directly interpret this result without in-depth pharmacology knowledge needed. Therefore, measurement of FL3-fluorescence should be routinely translated into adherence rate with prediction intervals and reported as such, based on the nomogram. With their knowledge of pharmacology, pharmacists can play a role in this translation. Similar to the translation of a CYP genotype into an expression profile and metabolic capacity, pharmacist should guide the clinicians how to interpret the result. Additionally, pharmacists could detect any potential non-adherence to clozapine therapy using dispensing gaps of clozapine in their dispensing systems and consequently suggest determining an FL3-measurement proactively.

Issues that need to be resolved before FL3-fluorescence may enter into clinical practice

We propose the following directions for future research. First, we suggest that our conceptual nomogram should be validated prospectively in a psychiatric setting with external data from Medication Event Monitoring Systems (MEMS) or drug formulations with an ingestible event marker (IEM) sensor as indicators for 'true' intake in clinical practice, paired with TDM data. This could be done by comparing adherence rates derived from either IEM or MEMS over a longer period of time next to nomogram-based adherence (*Chapter 2.3*) using well documented FL3-measurements and clozapine plasma concentration measurements. Second, future studies should bear in mind that FL3-fluorescence was detected at the FL3 fluorescence channel on the Sapphire analyzer. As described in *Chapter 2.2*, clozapine associated neutrophil fluorescence has been studied with detection channels other than FL3, resulting in even stronger signals. This underlines the need for standardization, as neutrophil autofluorescence seemed to be a basic phenomenon seen in clozapine users within the range

of certain exciting and emission wavelengths. Future studies should therefore aim to standardize these settings to further explore the clinical validity of the nomogram. If not possible to determine neutrophil autofluorescence under the same settings as the FL3 channel, a new model should be developed and evaluated to obtain a nomogram based on the alternative concerning autofluorescence signal. Third, a cost-effectiveness study should be performed to evaluate the role of FL3-fluorescence in clinical practice. In addition, robustness of FL3 measurements should also be examined to see if minor perturbations in conditions would affect FL3 results.

Monitoring unbound clozapine concentrations during inflammation

Measuring clozapine unbound plasma concentrations: implications for implementation in routine practice

While the unbound concentration is responsible for both efficacy and toxicity, therapeutic drug monitoring of clozapine usually involves the determination of total plasma concentrations, because quantitation of total plasma concentrations is a less expensive, more convenient and quicker measurement than measuring unbound plasma concentration [30]. Total clozapine plasma concentrations are assumed to directly correspond to unbound concentrations. However, specific conditions could affect unbound fraction thereby disturbing the association between total and unbound plasma concentration [31]. In *Chapter 2.4* a significantly lower clozapine unbound fraction was observed in case of elevated alpha-1-acid glycoprotein (AGP) plasma concentrations. AGP is an acute phase protein that is elevated during inflammation. In general, altered plasma protein binding affects the pharmacokinetics of certain drugs. For drugs 1) with high protein binding, 2) with high intrinsic clearance (Cl_{int}), 3) which dosing is not titrated to effect and 4) with a narrow therapeutic index, this may result in clinically relevant changes [32].

- Ad 1. Drugs with high protein binding such as phenytoin (95% to albumin) and clozapine (95% to AGP) are highly affected by altered unbound fraction, for instance a change in bound percentage from 95% to 90%, would result in a doubling of unbound concentration (from 5% to 10%) [32].
- Ad 2. Drugs with low extraction ratios and thereby low Cl_{int} are not substantially affected by changes in organ perfusion and drug metabolism or clearance. Otherwise, alterations of Fu in drugs with a high Cl_{int} such as propranolol and nifedipine could result in clinically relevant increases in clearance [32]. Since more unbound drug is systemically available, increased clearance and more extensive distribution could take place. Clozapine can be considered as a low clearance drug with an extraction ratio of 0.2.

- Ad 3. In drugs where the pharmacological effect is difficult to measure (antibiotics or antipsychotics), alterations in unbound fraction do not instantly affect clinical outcome, potentially increasing the risk of underexposure.
- Ad 4. Due to alteration of unbound concentrations drugs with a narrow therapeutic index such as theophylline and clozapine, risk of toxicity is increased.

Given the information above, standard determination of protein-unbound plasma concentrations instead of complementary to total plasma concentrations is preferable for drugs that are high protein bound with a high intrinsic clearance, a narrow therapeutic index and where dosing is not titrated to effect. Clozapine, with the exceptions of the second feature, meets with all features. As for the second feature with clozapine being a low clearance drug, it is expected that unbound concentrations are not dramatically affected by changes in systemic AGP. Therefore, in case systemic AGP is changed, monitoring of clozapine unbound concentrations is recommended over total clozapine plasma concentrations to confirm the unaffected unbound concentrations.

How to deal with monitoring and dosing of clozapine in patients with inflammation?

Common practice is to lower clozapine dosing in case of inflammation, because of increased total clozapine plasma concentration [12]. However, as we have argued above, elevated alpha-1-acid glycoprotein (AGP) concentrations during inflammation causes increased clozapine - plasma AGP binding, resulting in elevated total clozapine plasma concentration, but not unbound concentration. In *Chapter 2.4*, we reported a decreased unbound fraction in case of elevated AGP concentrations.

Although we have demonstrated that alterations of unbound fraction is mechanistically related to plasma AGP, elevated AGP as seen during inflammation serves as a trigger for more intense monitoring of unbound concentrations rather than a trigger indicating prompt dose reduction of clozapine. Routinely monitoring of clozapine unbound concentrations should therefore commenced in practice. More specifically, in case of a suspected inflammation and thus altered AGP protein binding, clinicians should send the blood sample to a laboratory that is able to determine unbound fractions of clozapine as this is not a complicated measurement with current analytical techniques. Since it is difficult to follow the exact time-period of the transient clozapine redistribution owing to altered AGP we suggest the consideration to just hold on to the maintenance dose initially, accompanied by monitoring of clozapine unbound plasma concentrations. Yet, for clinicians, it can feel unnatural and counterintuitive to handle according to the paradox of maintaining the drug dose while an acute rise of total plasma concentration into toxic ranges is observed. Again, routinely monitoring of unbound concentrations would take away any constraints to do so.

Recommendations for future research regarding monitoring unbound concentrations

There are several recommendations for future research regarding the monitoring of unbound concentrations of clozapine. First, clozapine, being an alkaline drug, is highly bound to AGP [33]. In this respect, determinants of altered systemic AGP concentrations in patients using clozapine are an area of interest. Previous studies have consistently shown alterations in protein binding and unbound concentrations of several drugs (ceftriaxone, cefazolin, flucloxacillin) in specific pathological conditions or patient groups including the critically ill, patients with cancer, trauma, infections and other inflammatory reactions as AGP increases of three- to five-fold have been observed [32]. Moreover, the type of inflammation (e.g. pneumonia, fever or urethritis) should be documented, because it has not been established yet to what extent AGP is affected in each type of inflammation.

Second, clinical outcome measures should be included in future research, since solely a conceptual mechanistic explanation is provided in *Chapter 2.4*. Clinical outcome measurements could include the observation or follow-up of ADRs that are directly dependent on clozapine exposure such as tachycardia, sedation, seizures and hypotension [34,35]. This would then explain the discrepancy between the acute rise of total clozapine concentrations and absence of clinical toxicity.

Third, the therapeutic reference values of unbound plasma concentrations should be established. Unfortunately, data have been produced without sufficient control of known parameters influencing the equilibrium between protein-bound and unbound plasma concentrations [31], complicating establishment of a target range for unbound concentrations. The unbound fraction can be used to estimate this range by multiplying the reference range of total plasma concentrations to the percentage fraction. Crucial for this method of establishment is the certainty to which this fraction is determined in patients without inflammation, since this represents the unbound fraction under physiologically settings.

Fourth, the unbound measurement assay should be validated and be able to detect plasma concentrations that are low enough. Determination with LC/MS-MS could offer a solution owing to the high sensitivity of this assay. Unfortunately, not all clinical laboratories are equipped with the tools needed to prepare and detect samples. It is expected that determination would mainly take place at specialized laboratories.

Monitoring of clozapine-induced sialorrhea as a clinical biomarker

Outcome measures in monitoring patients with clozapine induced sialorrhea: what does really matter?

In this thesis we have studied monitoring of sialorrhea, an invalidating ADR in clozapine therapy. Sialorrhea is an ADR that is both frequently observed (estimated

prevalence of 30-90% [36]) and difficult to treat in patients using clozapine [37–39]. Most patients exhibit sialorrhea only during sleep [36,39–42]. It reduces quality of life and can lead to complications including parotitis, mucositis, sleep disorders and aspiration pneumonia [12,15,38,43,44]. Moreover, sialorrhea can be socially incapacitating [39] and can result in poor medication adherence or even patient-initiated discontinuation of clozapine [45], potentially leading to severe psychiatric deterioration. It also remains a challenge to objectively quantify the severity of sialorrhea in daily practice. Several studies have suggested methods to objectively determine salivary flow in general, but none of them, however, seems to be easily implemented in practice nor standardized. These methods require a clinical experimental setting to accurately carry out and include the use of radioactive isotopes [46], suction bags used as collection units for saliva, cup-like collection devices [47] and absorbent cotton dental rolls [48] (Table 1). In contrast, subjective outcome measures including severity of complaints of nocturnal sialorrhea can be more easily acquired in practice by using validated psychometric instruments such as the Patient Global Impression of improvement (PGI-I) questionnaire, Nocturnal Hypersalivation Rating Scale (NHRS) and Medication Satisfaction Questionnaire (MSQ), as demonstrated in *Chapter 3.2*.

More important is the question whether objective monitoring of sialorrhea in daily practice is necessary to initiate treatment of sialorrhea. From a clinical perspective sialorrhea is not a life-threatening ADR requiring additional interventions. Yet, from a patients' perspective what matters is whether sialorrhea intervenes with sleep, causes waking up with a wet pillow or disturbs day-time activities thereby negatively impacting quality of life and potentially impairing medication adherence. Questionnaires based subjective assessment of sialorrhea tackle these aspects since subjective experience from patients' perspectives are the relevant outcome measures that matter in evaluating this ADR. The approach to act upon patient reported severity can be both convenient and practical. As demonstrated in *Chapter 3.3* the patient-reported quantification of sialorrhea does not necessarily correspond with patients' perceived burden and treatment satisfaction. Impact of severity should therefore be evaluated on individual base. Thus, even though objective outcome measures would be available in clinical practice, these would still need to be put into patients' perspectives. Again, this underlines the importance of taking a more patient-centered approach, thereby incorporating the patients' perspective in the clinical evaluation. Therefore, routinely use of patients' perceived burden and treatment satisfaction scales in the evaluation of sialorrhea in clinical practice is of great importance. Since patient-clinician visits are time-limited filling in the questionnaires and scales regarding sialorrhea through an eHealth portal prior to the visit would be a time-efficient possibility. This is in line with the upcoming rise of digital healthcare [49].

Table 1. Tools to measure sialorrhea

	Quantitative	Qualitative
Objective	Radioactive isotopes Suction bags used as collection units Cup-like collection devices Absorbent cotton dental rolls	Quality of Life Scale (QoLS) Medication Satisfaction Questionnaire (MSQ) Pittsburgh Sleep Quality Index (PSQI)
Subjective	Nocturnal Hypersalivation Rating Scale (NHRS) Drooling rating scale (DRS) Drooling Severity and Frequency Scale (DSFS)	Patient Global Impression of Severity (PGI-S) questionnaire Patient Global Impression of Improvement (PGI-I) questionnaire Visual Analogue Scale (VAS)

Monitoring sialorrhea in clozapine therapy: need for acknowledgement and patient-centered approach

In *Chapter 3.1* we evaluated clozapine induced sialorrhea (CIS) reporting patterns using VigiBase, an international database for ADRs, and demonstrated a discrepancy in sialorrhea reporting rates among patients and health-care professionals. The reporting rate of sialorrhea was found ten times higher in patients in comparison to health care professionals. It either suggests that clinicians consider reporting clozapine-related ADRs other than sialorrhea to be of more importance or that they are unaware of CIS. Health-care professionals should pay more attention to this non-threatening, but bothersome ADR, because acknowledging and addressing CIS could contribute to guiding patients in their clozapine therapy. This can lead to improved medication adherence and therefore lowering risks of psychiatric deterioration. Furthermore, it enables to evaluate aspects of the intended therapy that directly matters to patient, offering patients to be part of the clinical decision-making process.

Pharmacotherapeutic management of clozapine induced sialorrhea

Monitoring patients using clozapine involves acting upon observations regarding ADRs as well. Treating sialorrhea is challenging and consists of pharmacotherapeutic management, including off-label treatment with anticholinergic agents such as oral intake of glycopyrrolate, application of scopolamine transdermal patches or sublingual application of ophthalmic atropine drops [12,38]. *Chapter 3.2* showed that glycopyrrolate 2 mg a day was an effective and tolerable treatment of CIS. Within the anticholinergic agents, glycopyrrolate is distinctive because of its minimal blood-brain barrier passage [12], thereby lacking central cognitive adverse effects. M_4 agonism of clozapine is suggested to be related to sialorrhea and glycopyrrolate [38,39,43,50]. In contrast to atropine and scopolamine, glycopyrrolate has particularly strong M_4 antagonistic effects. Hence, the efficacy of glycopyrrolate can be explained in terms of this receptor. However, inherent to the drug class effects of anticholinergic agents, glycopyrrolate

like atropine and scopolamine contributes to the anticholinergic burden in patients using clozapine. This is considered of high relevance because clozapine itself is a strong antagonist of all muscarinic receptor subtypes except for the muscarinic M_4 subtype. Therefore, it is important to monitor anticholinergic symptoms including obstipation, urinary retention, increased heart rate and blurred vision. Though, according to our study in *Chapter 3.2* glycopyrrolate dosed at 2 mg a day was found a tolerable agent with absence of cognitive symptoms. In case of nocturnal CIS, clinicians can start treatment with glycopyrrolate at a dose of 1 mg each day before bedtime and elevate the dose to 2 mg each day guided by clinical response and anticholinergic burden. If sialorrhea also occurs during the day, glycopyrrolate can be dosed 1 mg three times a day.
Efficacy of treatment can be monitored by instructing patients to keep a diary with questionnaires regarding their experiences with sialorrhea. In this way sialorrhea can be adequately monitored as a clinical biomarker.

Implications for clinical practice

This thesis leads to the following implications for clinical practice:

- Our conceptual nomogram is a first step for health-care professionals to obtain indicative information regarding long-term clozapine adherence. This can be used in addition to short-term adherence information as obtained by TDM.
- Clinicians and pharmacists should be given the relevant education and training to interpret long-term adherence to clozapine based on FL3-fluorescence.
- Monitoring of clozapine unbound plasma concentrations should become a routine in practice and should be performed more frequently during inflammation.
- In patients with inflammation-induced elevated total clozapine concentrations, clozapine dose should be adjusted only based on protein-unbound concentrations, instead of on total plasma concentrations only.
- Patient reported quantification of sialorrhea should be placed into perspective with patients' perceived burden of hypersalivation and treatment satisfaction. In addition, patients using clozapine should keep a diary regarding their experiences with sialorrhea.
- Health-care professionals could benefit from incorporating sialorrhea as a relevant topic of discussion into the shared decision process with patients when initiating and monitoring clozapine therapy.
- Treatment of clozapine-induced sialorrhea could involve glycopyrrolate 2 mg/day since it has been shown to be an effective and tolerable anticholinergic agent.

Conclusions

Clozapine is a remarkable, yet valuable antipsychotic agent within the antipsychotics treatment armamentarium. Adequate monitoring during the prescribing process of clozapine contributes to optimizing the benefit-harm balance for the individual patient. FL3-fluorescence has shown to be a potential biomarker for adherence to clozapine therapy over a longer time-period. Furthermore, clozapine unbound fraction was shown to be lower during inflammation based on elevated AGP-clozapine binding in plasma consequently influencing correct interpretation of clozapine serum levels. Glycopyrrolate 2 mg has proven to be an effective and tolerable anticholinergic agent in the treatment of sialorrhea in patients using clozapine. We have moved a step closer to optimizing treatment with clozapine by translating these insights into clinical recommendations and contributing to rational, effective and safe use of clozapine.

References

1 Kane J, Honigfeld G, Singer J, Meltzer H. Clozapine for the treatment-resistant schizophrenic. A double-blind comparison with chlorpromazine. Arch Gen Psychiatry 1988; 45: 789–96.

2 Grohmann R, Rüther E, Sassim N, Schmidt LG. Adverse effects of clozapine. Psychopharmacology 1989; 99 Suppl: S101-4.

3 De-Vries T, Henning R, Hogerzeil H, Fresle D. Guide to Good Prescribing: a practical manual. WHO 1994; 142.

4 Brown MT, Bussell JK. Medication adherence: WHO cares? Mayo Clin Proc 2011; 86: 304–314.

5 Lam WY, Fresco P. Medication Adherence Measures: An Overview. Biomed Res Int 2015; 2015: 217047.

6 Wongvipat N. Adherence matters. Therapeutic drug monitoring (TDM) may detect drug failure. Posit Living 1999; 8: 16, 18–9.

7 Flanagan RJ, Spencer EP, Morgan PE, Barnes TRE, Dunk L. Suspected clozapine poisoning in the UK/Eire, 1992-2003. Forensic Sci Int 2005; 155: 91–9.

8 Meltzer HY. Clozapine: balancing safety with superior antipsychotic efficacy. Clin Schizophr Relat Psychoses 2012; 6: 134–44.

9 Remington G, Agid O, Foussias G, Ferguson L, McDonald K, Powell V. Clozapine and therapeutic drug monitoring: is there sufficient evidence for an upper threshold? Psychopharmacology 2013; 225: 505–18.

10 Mitchell PB. Therapeutic drug monitoring of psychotropic medications. Br J Clin Pharmacol 2000; 49: 303–12.

11 Hiemke C, Dragicevic A, Gründer G, Hätter S, Sachse J, Vernaleken I *et al.* Therapeutic monitoring of new antipsychotic drugs. Ther Drug Monit 2004; 26: 156–60.

12 Schulte P, Bakker B, Bogers J, Cohen D, van Dijk D. Guideline for the use of clozapine by the Netherlands Clozapine Collaboration Group, version 05-02-2013. www.clozapin-epluswerkgroep.nl.

13 Leung JG, Nelson S, Takala CR, Gören JL. Infection and inflammation leading to clozapine toxicity and intensive care: a case series. Ann Pharmacother 2014; 48: 801–5.

14 Espnes KA, Heimdal KO, Spigset O. A puzzling case of increased serum clozapine levels in a patient with inflammation and infection. Ther Drug Monit 2012; 34: 489–92.

15 Taylor DM, Douglas-Hall P, Olofmjana B, Whiskey E, Thomas A. Reasons for discontinuing clozapine: Matched, case-control comparison with risperidone long-acting injection. Br J Psychiatry 2009; 194: 165–167.

16 Higashi K, Medic G, Littlewood KJ, Diez T, Granström O, de Hert M. Medication adherence in schizophrenia: Factors influencing adherence and consequences of nonadherence, a systematic literature review. Ther Adv Psychopharmacol 2013; 3: 200–218.

17 Kane JM, Kishimoto T, Correll CU. Non-adherence to medication in patients with psychotic disorders: epidemiology, contributing factors and management strategies. World Psychiatry 2013; 12: 216–26.

18 Qurashi I, Stephenson P, Chu S, Duffy C, Husain N, Chaudhry I. An evaluation of subjective experiences, effects and overall satisfaction with clozapine treatment in a UK forensic service. Ther Adv Psychopharmacol 2015; 5: 146–50.

19 Hoffmann J, Lillholm A. Neutrophil fluorescence in patients using clozapine. Ned Tijdschr Klin Chem 2000; 25: 108.

20 Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001; 69: 89–95.

21 Keith SJ, Kane JM. Partial compliance and patient consequences in schizophrenia: our patients can do better. J Clin Psychiatry 2003; 64: 1308–15.

22 Lacro JP, Dunn LB, Dolder CR, Leckband SG, Jeste D V. Prevalence of and risk factors for medication nonadherence in patients with schizophrenia: a comprehensive review of recent literature. J Clin Psychiatry 2002; 63: 892–909.

23 Whomsley P. Biomarkers: Basic Principles and Applications. Eur J Hosp Pharm Sci Pract 2010; 16: 16–18.

24 Makowski G. Advances in Clinical Chemistry. Gulf Professional Publishing 2015.

25 Lieberman JA, Safferman AZ. Clinical profile of clozapine: Adverse reactions and agranulocytosis. Psychiatr Q 1992; 63: 51–70.

26 Kar N, Barreto S, Chandavarkar R. Clozapine monitoring in clinical practice: Beyond the mandatory requirement. Clin Psychopharmacol Neurosci 2016; 14: 323–329.

27 Wolff K, Hay AA, Raistrick D, Feely M. Use of 'very low-dose phenobarbital' to investigate compliance in patients on reducing doses of methadone (detoxification). J Subst Abuse Treat 1993; 10: 453–458.

28 Braam RL, Van Uum SHM, Lenders JWM, Thien T. Bromide as marker for drug adherence in hypertensive patients. Br J Clin Pharmacol 2008; 65: 733–736.

29 Hawwa AF, AlBawab A, Rooney M, Wedderburn LR, Beresford MW, McElnay JC *et al.* Methotrexate polyglutamates as a potential marker of adherence to long-term therapy in children with juvenile idiopathic arthritis and juvenile dermatomyositis: an observational, cross-sectional study. Arthritis Res Ther 2015; 17: 295.

30 Farooq S, Naeem F. Tackling nonadherence in psychiatric disorders: current opinion. Neuropsychiatr Dis Treat 2014; 10: 1069.

31 Nilsson LB. The bioanalytical challenge of determining unbound concentration and protein binding for drugs. Bioanalysis 2013; 5: 3033–50.

32 Roberts JA, Pea F, Lipman J. The clinical relevance of plasma protein binding changes. Clin Pharmacokinet 2013; 52: 1–8.

33 Flanagan RJ, Yusufi B, Barnes TRE. Comparability of whole-blood and plasma clozapine and norclozapine concentrations. Br J Clin Pharmacol 2003; 56: 135–138.

34 Devinsky O, Honigfeld G, Patin J. Clozapine-related seizures. Neurology 1991; 41: 369–71.

35 Khan AY, Preskorn SH. Examining concentration-dependent toxicity of clozapine: role of therapeutic drug monitoring. J Psychiatr Pract 2005; 11: 289–301.

36 Bird AM, Smith TL, Walton AE. Current Treatment Strategies for Clozapine-Induced Sialorrhea. Ann Pharmacother 2011; 45: 667–675.

37 Lakraj A, Moghimi N, Jabbari B. Sialorrhea: Anatomy, Pathophysiology and Treatment with Emphasis on the Role of Botulinum Toxins. Toxins 2013; 5: 1010–1031.

38 Sockalingam S, Shammi C, Remington G. Clozapine-induced hypersalivation : A review of treatment strategies. Can J psychiatry; 52: 377–384.

39 Praharaj SK, Arora M, Gandotra S. Clozapine-induced sialorrhea: pathophysiology and management strategies. Psychopharmacology 2006; 185: 265–73.

40 Grohmann R, Rüther E, Sassim N, Schmidt LG. Adverse effects of clozapine. Psychopharmacology 1989; 99 Suppl: S101-4.

41 Maher S, Cunningham A, Callaghan NO, Byrne F, Donald CM, Mcinerney S *et al.* Clozapine-induced hypersalivation : an estimate of prevalence , severity and impact on quality of life. Ther Adv Psychopharmacol 2016; 6: 178–184.

42 Waserman J, Criollo M. Subjective experiences of clozapine treatment by patients with chronic schizophrenia. Psychiatr Serv 2000; 51: 666–8.

43 Miranda-Rius J, Brunet-Llobet L, Lahor-Soler E, Farré M. Salivary secretory disorders, inducing drugs, and clinical management. Int J Med Sci 2015; 12: 811–824.

44 Van Os J. A salience dysregulation syndrome. Br J Psychiatry 2009; 194: 101–103.

45 Krivoy A, Malka L, Fischel T, Weizman A. Predictors of clozapine discontinuation in patients with schizophrenia. 2008; 26: 311–315.

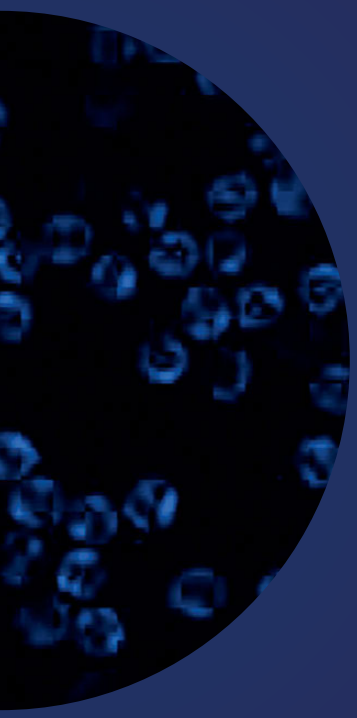
46 Ekedahl C, Hallén O. Quantitative measurement of drooling. Acta Otolaryngol 1973; 75: 464–9.

47 Sochaniwskyj AE. Drool quantification: noninvasive technique. Arch Phys Med Rehabil 1982; 63: 605–7.

48 Reid SM, Johnson HM, Reddihough DS. The Drooling Impact Scale: a measure of the impact of drooling in children with developmental disabilities. Dev Med Child Neurol 2010; 52: e23-8.

49 Biagianti B, Hidalgo-Mazzei D, Meyer N. Developing digital interventions for people living with serious mental illness: Perspectives from three mHealth studies. Evid Based Ment Health 2017; 20: 98–101.

50 Davydov L, Botts SR. Clozapine-induced hypersalivation. Ann Pharmacother 2000; 34: 662–5.



Summary

Summary

Clozapine was introduced into clinical practice in 1972 and subsequently withdrawn in 1975 due to reports of potentially fatal agranulocytosis. Clozapine was reintroduced, accompanied by strict monitoring requirements in 1989 due to the effort of clinicians. Clozapine is regarded a unique antipsychotic agent because of its anti-aggressive, antisuicidal features and efficacy in refractory schizophrenia. Next to these clinical advantages, clozapine is also known for various adverse drug reactions (ADRs) including sialorrhea (30-80%), agranulocytosis (0.8%) and myocarditis (1%). Monitoring by means of laboratory monitoring, including determination of total blood count and therapeutic drug monitoring (TDM), and monitoring of clinical adverse effects is essential for optimization of the benefit-risk balance. Monitoring involves continuous observation, analysis of measurements and act upon these observations. In this thesis three aspects of monitoring in clozapine therapy were studied:

- The applicability of FL3-fluorescence as a biomarker to monitor long term adherence to clozapine therapy;
- The effects of AGP concentration during inflammation on the unbound fraction of clozapine plasma concentrations in patients, contributing to better interpretation of clozapine TDM results;
- Optimization of recognition and treatment of sialorrhea as an ADRs in patients using clozapine.

Since non-adherence is a major problem in schizophrenia (overall prevalence between 40% and 50%), potentially leading to psychiatric deterioration, monitoring of medication adherence to clozapine therapy is useful. TDM is used for both optimization in dosing as well as confirming adherence. However, it only provides information of its intake for a few days prior to blood sampling at most. Elevated neutrophil fluorescence – termed FL3-fluoresence – was serendipitously observed in patients using clozapine and, in this regard, a potential interesting target as an adherence biomarker to clozapine therapy.

Monitoring results need, however, to be put into both patient and clinical context. Studies have, for example, shown that adequate interpretation of TDM of clozapine can be affected by conditions including inflammation. Inflammation influences the balance between bound and unbound clozapine in serum and lead to elevated total (protein bound plus unbound) clozapine concentrations. This may be the result of an increase of the systemic acute phase protein 'alpha-1-acid glycoprotein' (AGP) following inflammation.

Monitoring and management of bothersome adverse drug reactions (ADRs) including sialorrhea can improve adherence by ameliorating the benefit-risk balance of clozapine from patient perspective. Studies have shown differences in perspectives on prevention and management of sialorrhea among patients and health-care providers.

Chapter 2 focusses on use of laboratory biomarkers that may be beneficial and worthwhile to monitor in patients using clozapine in clinical practice with the aim of improving safe use.
Non-adherence to medication is a major issue in the treatment of schizophrenia (overall prevalence between 40% and 50%), potentially leading to psychiatric deterioration.
Reliable monitoring tools to evaluate clozapine adherence over a short and long period of time are clearly warranted. In this respect we believed that the serendipitous observation of fluorescent neutrophils in users of clozapine called FL3-fluorescence could be of high interest. This association between clozapine use and FL3-fluorescence in a Dutch patient population was studied retrospectively in a cross-sectional setting and the association was confirmed in *Chapter 2.1*. FL3-fluorescence was found significantly higher in clozapine users (N=124) compared to non-users (N=38,266) (U=240,179, P<0.001) and was found to be increased with increasing clozapine dose. These results provided initial information on the concept of neutrophil fluorescence serving as a potential long-term adherence marker for clozapine use.
To understand the underlying mechanism and to explore neutrophil fluorescence in clozapine users in further detail, the subcellular localization and source of neutrophil fluorescence was extensively studied in *Chapter 2.2*. Explorations on the origin of this clozapine associated neutrophil fluorescence revealed that the neutrophil fluorescence was reflected by a 14kDa soluble protein present in azurophilic granules of neutrophils. This protein could be an auto-fluorescent protein already present in the cell and upregulated by clozapine or a protein altered by clozapine to express fluorescence.
From this point of view in *Chapter 2.3*, a PK/PD model relating FL3-fluorescence to clozapine exposure was developed and evaluated using data from 27 patients initiating clozapine. The model was best described with an Emax model and displayed a half-life of approximately 230 hours, indicating FL3-fluorescence to be suitable as a biomarker providing information over a long period. Consequently, with this PK/PD model a conceptual nomogram for estimation of long-term adherence based upon FL3-fluorescence values and clozapine dose was developed. Evaluation showed a significant nonlinear relationship between clozapine dose and FL3-fluorescence evidenced by the Emax model. With the developed model and nomogram, clinicians are offered information regarding long-term adherence based on prescribed clozapine dose and FL3-fluorescence. Together with TDM the use of the nomogram could depict any possible non-adherence both short and long-term.
TDM of clozapine involves measurement of total (unbound plus protein-bound) clozapine plasma concentrations, while the unbound plasma concentrations are responsible for the clinical efficacy and toxicity. It is established that a rise in total clozapine plasma concentrations into toxic ranges is seen during inflammation. The observation that this rarely results in clinical toxicity, suggests a discrepancy between measured total clozapine plasma concentrations and clinical presentation in case of inflammation. In *Chapter 2.4*, the effects of inflammation on both unbound and total

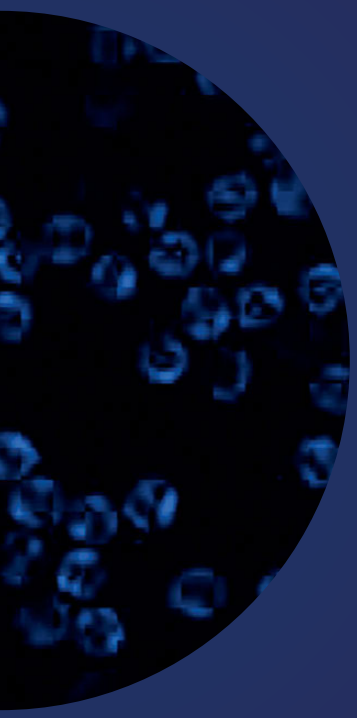
clozapine plasma concentrations were studied by conducting a spiking experiment and a patient study. Both spiking experiment and patient study showed a significant association between elevated AGP plasma concentrations and a lower clozapine unbound fraction. The spiking experiment showed significantly lower mean unbound fractions following 25 and 60 µL AGP spikes (relative reductions of 28.3%, p=0.032 and 43.4%, p=0.048). In the patient study, total clozapine plasma concentrations were 10% higher in elevated (n=6) compared to normal AGP (n=20) samples (525 µg/L *vs* 479 µg/L, mean difference=47 µg/L [95% CI: -217, 310], p=0.72). Elevated AGP samples had a 26% lower mean unbound fraction compared to normal samples (1.22% *vs* 1.65%, mean difference=-0.43% [95%CI: -0.816; -0.0443], p=0.03). Linear regression analysis showed a significant correlation between AGP concentration and unbound fraction (r^2 =0.214, p=0.017). Monitoring of unbound clozapine plasma concentration rather than total clozapine plasma concentrations was suggested to be more suitable during inflammation.

In *Chapter 3* the safety aspect of clozapine therapy was assessed by focusing on one of the ADRs carrying high risk for nonadherence due to its high impact on well-being from patient's perspective; clozapine-induced sialorrhea.
In *Chapter 3.1*, ADR reporting patterns related to drug-induced sialorrhea were compared between clozapine (N=243,564) and other antipsychotic agents (N=925,690) using WHO pharmacovigilance data from VigiBase. Sialorrhea was reported almost four times more often with clozapine use than with use of other antipsychotics (ROR=3.60; 95% CI 3.41 – 3.79) and was reported eight times more often by patients (ROR=19.8; 95% CI 15.1 – 25.9) than by health-care professionals (ROR=2.44; 95% CI 2.27 – 2.63). The high sialorrhea reporting rate might seem to relate to muscarinic M_4 and dopamine D_1 receptor agonism. Since invalidating ADRs could negatively affect medication adherence, these findings indicate the importance for health-care professionals to incorporate sialorrhea into the shared decision process when considering initiation and management of clozapine therapy.
In *Chapter 3.2*, the efficacy and safety of glycopyrrolate was assessed for the treatment of clozapine-induced sialorrhea (CIS) in 32 patients. The proportion of patients with a clinical improvement according to the Patient Global Impression of Improvement (PGI-I) scale did not significantly differ between glycopyrrolate one mg and placebo (18.8% versus 6.3%, p=0.289), but glycopyrrolate two mg once daily *vs* placebo did (43.5% versus 6.3%, p=0.039). Glycopyrrolate two mg but not one mg once daily was shown to be a significantly effective anticholinergic agent in the absence of cognitive adverse events.
In *Chapter 3.3*, the extent to which changes in Nocturnal Hypersalivation Rating Scale (NHRS) scores affect patients' perceived burden and treatment satisfaction are studied and assessed. Patient differed regarding the perception of disease burden and treatment satisfaction in the context of decreased NHRS scores. Patient-reported

quantification of sialorrhea should be placed into perspective with patients' perceived burden of sialorrhea and treatment satisfaction.

In the final chapter (*Chapter 4*), the implications and results from the different studies are placed in a broader perspective. Three topics are discussed: FL3-fluorescence as a biomarker for long-term adherence to clozapine; monitoring unbound clozapine concentrations during inflammation; and monitoring of clozapine-induced sialorrhea as a clinical biomarker. With the research presented in this thesis we have moved a step closer to optimizing treatment with clozapine contributing to rational, effective and safe use of clozapine and has led to the following recommendations:

- FL3-fluorescence has shown to be a potential biomarker for long-term clozapine use. Our conceptual nomogram involving FL3-fluorescence is a first step for health-care professionals to obtain indicative information regarding long-term clozapine adherence;
- In patients with inflammation-induced elevated total clozapine concentrations, clozapine dose should be adjusted only based on protein-unbound concentrations, instead of on total plasma concentrations only;
- Health-care professionals could benefit from incorporating sialorrhea as a relevant topic of discussion into the shared decision process with patients when initiating and monitoring clozapine therapy;
- Treatment of clozapine-induced sialorrhea could involve glycopyrrolate 2mg/day since it has been shown to be an effective and tolerable anticholinergic agent;
- Patient reported quantification of sialorrhea should be placed into perspective with patients' perceived burden of hypersalivation and treatment satisfaction.



Samenvatting

Samenvatting

Clozapine werd voor het eerst in 1972 in de klinische praktijk geïntroduceerd, maar werd in 1975 van de markt gehaald vanwege meldingen over agranulocytose. Op dringend verzoek van artsen werd clozapine in 1989 geherintroduceerd in de praktijk met als voorwaarde dat het gebruik gepaard gaat met strikte monitoring procedures. Clozapine kan worden beschouwd als een uniek antipsychoticum met anti-agressieve, anti-suïcidale eigenschappen en werkzaamheid bij therapieresistente schizofrenie. Naast deze klinische voordelen staat clozapine ook bekend om diverse bijwerkingen zoals speekselvloed (30-80%), agranulocytose (0.8%) en myocarditis (1%).
Het monitoren van laboratoriumwaarden zoals het bepalen van het bloedbeeld en geneesmiddelspiegels en het monitoren van bijwerkingen zijn essentiële handelingen voor het optimaliseren van de verhouding tussen voordelen en risico's van clozapine therapie. De monitoringuitslagen dienen echter in de juiste klinische context van de patiënt te worden geplaatst. Onderzoek heeft laten zien dat interpretatie van clozapine geneesmiddelspiegels beïnvloed kunnen worden door klinische omstandigheden zoals een ontsteking. Mogelijk is dit te verklaren door een stijging van de hoeveelheid acute fase eiwit 'alfa 1-zuur-glycoproteïne' (AGP) in het bloed als gevolg van een ontsteking. Dit zou kunnen leiden tot een verandering in de verhouding tussen eiwit gebonden en ongebonden clozapine in bloedplasma.
Aangezien therapieontrouw een groot en vaak voorkomend probleem is bij patiënten met schizofrenie (met een prevalentie tussen 40% en 50%) en kan leiden tot psychiatrische verslechtering, is het monitoren van de mate van therapietrouw aan clozapine een waardevolle handeling. Verhoogde fluorescentie in neutrofielen – FL3-fluorescentie genaamd- werd per toeval waargenomen bij patiënten die clozapine gebruikten. In dit opzicht is FL3-fluorescentie een mogelijke interessante indicator voor de mate van therapietrouw aan clozapine gebruik.
Het monitoren en behandelen van invaliderende bijwerkingen zoals speekselvloed zou de mate van therapietrouw kunnen verbeteren aangezien vanuit patiëntperspectief de risico's en nadelen die gepaard gaan met clozapine behandeling kunnen worden geminimaliseerd. Onderzoeken hebben laten zien dat er een verschil bestaat in het bewustzijn, preventie en behandeling van speekselvloed tussen patiënten en zorgverleners.
Het doel van dit proefschrift was het verbeteren van clozapine therapie bij patiënten door het monitoren te optimaliseren. Specifieke doelstellingen waren daarbij:

- Het beoordelen van de toepasbaarheid van FL3-fluorescentie als een biomarker voor het monitoren van therapietrouw aan clozapine gebruik over een lange termijn;
- Het bestuderen van de effecten AGP concentraties op eiwit ongebonden fractie van plasma clozapine concentratie bij patiënten;
- Het optimaliseren van erkenning en behandeling van speekselvloed als bijwerking bij patiënten die clozapine gebruiken.

Hoofdstuk 2 richt zich op het gebruik van biomarkers in het bloed die in de klinische praktijk van waarde kunnen zijn in het monitoren van patiënten, die clozapine gebruiken, ten einde het veilige gebruik van dit middel te bevorderen. Therapieontrouw is een groot probleem bij de behandeling van schizofrenie (met een totale prevalentie tussen 40% en 50%) en kan uiteindelijk leiden tot psychiatrische verslechtering. Betrouwbare hulpmiddelen die de mate van therapietrouw aan clozapine over een korte en lange tijdsperiode monitoren zijn benodigd. In dit opzicht lijken de fluorescente neutrofielen – genaamd FL3-fluorescentie – die per toeval bij clozapine gebruikers werden ontdekt van groot belang. Het verband tussen het gebruik van clozapine en FL3-fluorescentie werd in een retrospectieve dwarsdoorsnede-onderzoek bestudeerd bij een Nederlandse patiëntenpopulatie in *Hoofdstuk 2.1*. Het FL3-fluorescentie signaal was significant hoger bij clozapine gebruikers (N=124) in relatie tot patiënten die geen clozapine gebruikten (N=38,266) (U=240,179, P<0.001). Tevens steeg het FL3-fluorescentie signaal naarmate de clozapine dagdosering hoger werd. Deze bevindingen dragen bij aan het concept waarin neutrofiele fluorescentie kan worden toegepast als potentiele indicator voor therapietrouw aan clozapine over een lange tijdsperiode.

Het onderliggend mechanisme van neutrofiele fluorescentie werd verder verkend in *Hoofdstuk 2.2*. Clozapine geassocieerde neutrofiele fluorescentie bleek afkomstig te zijn van een water oplosbaar eiwit ter grootte van 14 kDa, dat aanwezig is in azurofiele granula van neutrofielen. Dit eiwit zou een autofluorescerend eiwit kunnen zijn dat reeds aanwezig is in de cel en door clozapine wordt vermeerderd of een eiwit dat dusdanig door clozapine wordt vervormd dat het fluorescentie vertoont.

Met deze uitgangsbasis is in *Hoofdstuk 2.3*. een PK/PD model ontwikkeld en geëvalueerd dat het verloop van neutrofiele FL3 fluorescentie in de tijd na starten van clozapine beschrijft.

Dit werd gedaan met data van 27 verschillende patiënten die begonnen met clozapine behandeling. FL3-fluorescentie verandering onder invloed van clozapine werd het best beschreven met een effect compartiment model. Tevens bleek de halfwaardetijd van de FL3-fluorescentie verhoging na clozapine gebruik ongeveer 230 uur te zijn, hetgeen aangeeft dat FL3-fluorescentie daadwerkelijk een goede maat kan zijn voor therapietrouw voor de langere termijn. Aan de hand van dit PK/PD model werd vervolgens een conceptueel nomogram ontwikkeld voor het voorspellen van therapietrouw aan clozapine voor de langere termijn op basis van een FL3-fluorescentie waarde en clozapine dosering. Evaluatie van dit nomogram liet een significante niet-lineaire relatie zien tussen clozapine dosering en FL3-fluorescentie signaal. Met ons voorgesteld nomogram kan op basis van de clozapine dosering en FL3-fluorescentie een indicatie worden verkregen wat de mate van therapietrouw is geweest over een langere periode. Dit brengt FL3-fluorescentie weer een stap dichter bij de praktische bruikbaarheid als lange termijn therapietrouw marker.

Het monitoren van clozapine bloedspiegels bestaat in de praktijk uit het bepalen van totale (eiwit ongebonden en gebonden) clozapine plasma concentraties, ondanks

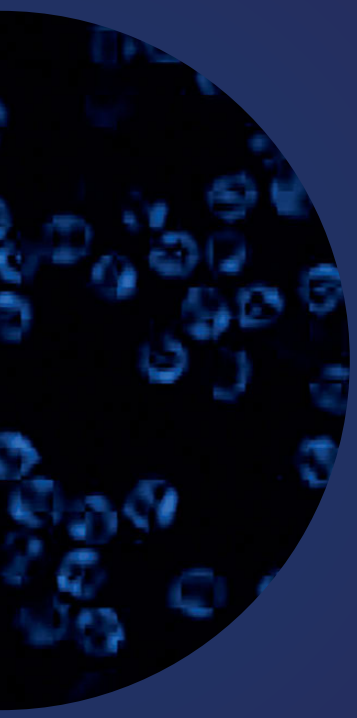
het feit dat de eiwit ongebonden plasma concentratie verantwoordelijk is voor het farmacologisch effect. In de literatuur worden stijgingen in totale clozapine plasma concentraties tot in het toxisch gebied waargenomen bij clozapine patiënten met een ontsteking. Deze observatie resulteert echter zelden tot klinische toxiciteit. Dit suggereert een discrepantie tussen gemeten totale clozapine plasma concentraties en het klinisch beeld bij patiënten met een ontsteking.
In *Hoofdstuk 2.4* zijn de effecten van een ontsteking op de eiwit ongebonden en totale clozapine plasma concentraties onderzocht met een spiking experiment en een patiëntonderzoek. Zowel het spiking experiment als het patiëntonderzoek lieten een significante associatie zien tussen verhoogde AGP plasma concentraties en een verlaagde ongebonden fractie van clozapine. Het spiking experiment liet een significant lagere ongebonden fractie zien na AGP toevoegingen van 25 en 60 µL aan clozapine bloedmonsters (relatieve reductie van 28.3%, p=0.032 en 43.4%, p=0.048). In het patiëntonderzoek waren totale clozapine plasma concentraties 10% hoger bij bloedmonsters met verhoogde AGP (N=6) in relatie tot de monsters met normale AGP (N=20) (525 µg/L *vs* 479 µg/L, gemiddeld verschil=47 µg/L [95% CI: -217, 310], p=0.72). De monsters met verhoogde AGP concentraties hadden een 26% lagere gemiddelde ongebonden fractie vergeleken met de monsters met normaal AGP concentraties (1.22% *vs* 1.65%, gemiddeld verschil=-0.43% [95%CI: -0.816; -0.0443], p=0.03). Lineaire regressie analyse liet een significante correlatie zien tussen de AGP concentratie en de ongebonden fractie van clozapine (r^2=0.214, p=0.017). Het monitoren van ongebonden plasma concentraties in plaats van enkel de totale clozapine plasma concentraties lijkt aanbevolen bij patiënten met een ontsteking.

In *Hoofdstuk 3* komt een van de bijwerkingen van clozapine, die vanwege de ingrijpende ervaringen voor patiënten, een hoog risico op therapieontrouw aan clozapine met zich meedraagt, aan bod; namelijk clozapine geïnduceerde speekselvloed.
In *Hoofdstuk 3.1* werden meldingen van de bijwerking speekselvloed vergeleken tussen clozapine (N=243,564) en andere antipsychotica (N=925,690) aan de hand van data uit VigiBase, een internationale database met meldingen over geneesmiddelbijwerkingen. Speekselvloed werd bijna vier maal zo vaak gerapporteerd bij clozapine gebruik vergeleken met gebruik van andere antipsychotica (ROR=3.60; 95% CI 3.41 – 3.79). Daarnaast werd het acht maal vaker door patiënten gerapporteerd (ROR=19.8; 95% CI 15.1 – 25.9) dan door zorgverleners (ROR=2.44; 95% CI 2.27 – 2.63). Dit algehele hoge meldingspercentage bij clozapine houdt mogelijk verband met diens receptorprofiel; namelijk stimulatie van muscarinerge M_4 en dopamine D_1 receptoren. Aangezien invaliderende bijwerkingen de mate van therapietrouw negatief kunnen beïnvloeden, laten deze bevindingen het belang zien van het bespreken van speekselvloed als relevant gespreksonderwerp in het gedeelde besluitvormingsproces wanneer wordt gestart met clozapine behandeling.

In *Hoofdstuk 3.2* werd de effectiviteit en veiligheid van glycopyrronium voor de behandeling van nachtelijk clozapine-geïnduceerd speekselverlies (CIS) onderzocht bij 32 patiënten middels een placebo gecontroleerd cross-over onderzoek. De proportie patiënten met een klinische verbetering op basis van de Patient Global Impression of Improvement (PGI-I) schaal verschilde niet significant tussen glycopyrronium 1mg/dag en placebo (18.8% versus 6.3%, p=0.289), maar wel tussen glycopyrronium 2mg/dag en placebo (43.5% versus 6.3%, p=0.039). Glycopyrronium 2mg/dag in tegenstelling tot 1mg/dag werd effectief bevonden en liet bovendien geen cognitieve bijwerkingen zien.
In *Hoofdstuk 3.3* werd de invloed van veranderingen in de Nocturnal Hypersalivation Rating Scale (NHRS) scores op de ervaren last van de patiënt en diens tevredenheid over de psychiatrische behandeling bestudeerd. Een daling van de hoeveelheid speekselverlies op basis van de NHRS score resulteerde niet bij iedereen in significante verschillen in de door de patiënt ervaren last en tevredenheid over de behandeling. De hoeveelheid speekselverlies gerapporteerd door patiënten dient om die reden in context te worden geplaatst met de door de patiënt ervaren last aan speekselvloed en diens tevredenheid over de algehele psychiatrische behandeling.

In *Hoofdstuk 4* worden de implicaties en resultaten van de verschillende onderzoeken in dit proefschrift in een breder perspectief gebracht. Daarbij komen drie onderwerpen aan bod: FL3-fluorescentie als therapietrouw biomarker voor de langere termijn; het monitoren van eiwit ongebonden clozapine concentraties bij patiënten met een ontsteking; en het monitoren van clozapine-geïnduceerd speekselvloed als klinische biomarker. Met het onderzoek uit dit proefschrift is een nieuwe stap gezet richting het optimaliseren van clozapine behandeling. Dit draagt bij aan een rationeel, effectief en veilig gebruik van clozapine en heeft geleid tot volgende aanbevelingen:

- FL3-fluorescentie is een bewezen potentiele therapietrouw biomarker aan clozapine gebruik voor de lange termijn. Met ons conceptuele nomogram is een eerste stap gemaakt om voor zorgverleners indicatieve informatie over clozapine inname over een langer tijdsperiode te verkrijgen;
- Bij patiënten met verhoogde totaal clozapine concentraties, als gevolg van een ontsteking, wordt aanbevolen om clozapine te doseren op geleide van clozapine ongebonden plasma concentraties in plaats van enkel de totale plasma concentraties;
- Zorgverleners doen er voordeel aan door speekselvloed mee te nemen als een relevant gespreksonderwerp in het gedeelde besluitvormingsproces met patiënten bij het starten en monitoren van de clozapine behandeling;
- Behandeling van clozapine geïnduceerd speekselvloed kan bestaan uit glycopyrronium gedoseerd in 2 mg/dag aangezien het een effectief en verdraagzaam anticholinergisch geneesmiddel is bewezen;
- De hoeveelheid speekselverlies gerapporteerd door patiënten dient in context te worden geplaatst met de door de patiënt ervaren last aan speekselvloed en diens tevredenheid over de algehele psychiatrische behandeling.



Dankwoord

Dankwoord

Na een intensieve doch mooie periode van 6 jaar is het zover! Met het schrijven van dit dankwoord ben ik bij de eindhalte gekomen van mijn wetenschappelijke reis in de vorm van dit promotietraject. Tijdens mijn wetenschappelijke reis hebben veel mensen mij bijgestaan. Allereerst wil ik mijn promotieteam – in mijn ogen de dreamteam- bedanken. Om mee te beginnen gaat mijn dank uit naar mijn promotoren prof. dr. Toine Egberts en prof. dr. Wouter van Solinge.

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Beste Maarten en Albert, het overgrote deel van hoofdstuk 2 had er wellicht zeer anders uitgezien zonder jullie. Het bewijst weer eens dat apothekers op dezelfde golflengte zitten als klinisch chemici (in dit geval 630 ± 30 nm). Ik voel me altijd welkom als ik zo nu en dan op jullie kamer beland om het over FL3 fluorescentie te hebben. We zullen er ooit achter komen wat nou die FL3 fluorescentie precies is! Dank voor de prettige samenwerking. Hanneke den Breeijen, Leon Stijvers en Mark de Groot wil ik bedanken voor hun bijdrage aan het opzetten, managen, extraheren van datasets uit de UPOD database. Het is voor promovendi een geschenk om op die manier ondersteund te worden.
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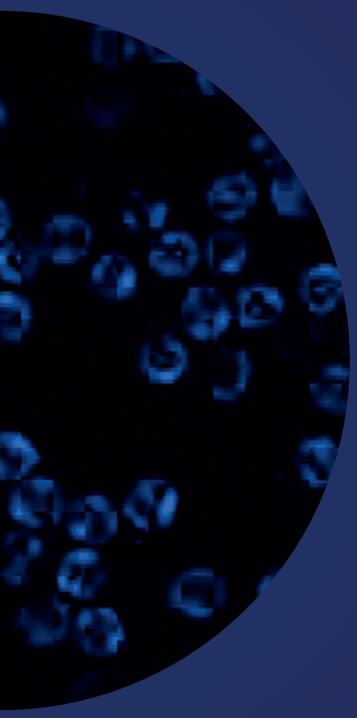


List of co-authors

List of publications

About the author

List of co-authors of manuscripts presented in this thesis

Affiliations at the time the research was conducted
Presented in alphabetical order

Dr. M.J. (Maarten) ten Berg
Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, the Netherlands

Ir. J.H. (Hanneke) den Breeijen
Department of Clinical Pharmacy, University Medical Centre Utrecht, Utrecht, the Netherlands

Prof. dr. W. (Wiepke) Cahn
Department of Psychiatry, University Medical Centre Utrecht, Utrecht, the Netherlands

Drs. J.C.A. (Jantine) Colen – de Koning
Department of Clinical Pharmacy, Medical Centrum Alkmaar, the Netherlands

Drs. H.J. (Jeroen) Doodeman
Department of Clinical Pharmacy, Medical Centrum Alkmaar, the Netherlands

Drs. J.W. (Jan Willem) Douma
Department of Clinical Pharmacy, University Medical Centre Utrecht, Utrecht, the Netherlands

Prof. dr. A.C.G. (Toine) Egberts
Department of Clinical Pharmacy, University Medical Centre Utrecht, Utrecht, the Netherlands
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, the Netherlands

Dr. I.M. (Ingrid) van Haelst
Department of Clinical Pharmacy, Medical Centrum Alkmaar, the Netherlands

Dr. E.R. (Rob) Heerdink
Department of Clinical Pharmacy, University Medical Centre Utrecht, Utrecht, the Netherlands
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, the Netherlands
Research Group Innovation of Pharmaceutical Care, University of Applied Sciences Utrecht, the Netherlands

Dr. G.W.K. (Gerard) Hugenholtz
Department of Hospital Pharmacy, Diakonessenhuis, Utrecht, the Netherlands

Dr. A. (Albert) Huisman
Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, the Netherlands

Prof. dr. A.D.R. (Alwin) Huitema
Department of Clinical Pharmacy, University Medical Centre Utrecht, Utrecht, the Netherlands
Department of Pharmacy & Pharmacology, Netherlands Cancer Institute, the Netherlands

Drs. C.A. (Arnold) Koekman
Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, the Netherlands

Dr. E.M. (Erik) van Maarseveen
Department of Clinical Pharmacy, University Medical Centre Utrecht, Utrecht, the Netherlands

Dr. C. (Coen) Maas
Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, the Netherlands

Dr. R.H.B. (Ronald) Meijboom
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, the Netherlands

Dr. A. (Alejandro) Perez- Pitarch
Pharmacy Department, University Clinical Hospital of Valencia, Spain
Department of Pharmacy and Pharmaceutical Technology, University of Valencia, Spain

Dr. P.F.J. (Peter) Schulte
Mental Health Services North-Holland North, Heerhugowaard, the Netherlands

Prof. dr. W.W. (Wouter) van Solinge
Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, Utrecht, the Netherlands

Dr. P.C. (Patrick) Souverein
Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, the Netherlands

Dr. T. (Tamar) Tak
Department of Respiratory Medicine, Laboratory of Translational Immunology, University Medical Centre Utrecht, the Netherlands

Dr. I. (Ingeborg) Wilting
Department of Clinical Pharmacy, University Medical Centre Utrecht, Utrecht, the Netherlands

Drs. S.A.J. (Jytte) de With
Department of Psychiatry, University Medical Centre Utrecht, Utrecht, the Netherlands

Publications related to this thesis

Man WH, Pérez-Pitarch A, Wilting I, Heerdink ER, van Solinge WW, Egberts ACG, Huitema ADR. Development of a nomogram for the estimation of long-term adherence to clozapine therapy using neutrophil fluorescence. Br J Clin Pharmacol (in press).

Man WH, Colen-de Koning JC, Schulte PF, Cahn W, van Haelst IM, Heerdink ER, Wilting I. Clozapine-induced hypersalivation: the association between quantification, perceived burden and treatment satisfaction reported by patients. Ther Adv Psychopharmacol 2017; 7: 209–210.

Man WH, Colen-de Koning JC, Schulte PF, Cahn W, van Haelst IM, Doodeman HJ, Egberts TC, Heerdink ER, Wilting I. The Effect of Glycopyrrolate on Nocturnal Sialorrhea in Patients Using Clozapine: A Randomized, Crossover, Double-Blind, Placebo-Controlled Trial. J Clin Psychopharmacol 2017; 37: 155-161.

Colen-de Koning JC, Man WH, Wilting I, Schulte PF, Doodeman HJ, Cahn W, Heerdink ER, Egberts TC, van Haelst IM. Effect van glycopyrroniumbromide op nachtelijk clozapine geïnduceerd speekselverlies bij patiënten met een psychiatrische aandoening. PW Wetenschappelijk Platform 2015;9: a1541.

Man WH, de Koning JC, Schulte PF, Wilting I, Heerdink ER. Glycopyrroniumbromide bij clozapine-geïnduceerd speekselverlies. Psyfar 2013; 4: 26-29.

Man WH, Ten Berg M, Wilting I, Huisman A, Cahn W, Douma JW, den Breeijen H, Heerdink ER, Egberts T, van Solinge W. Fluorescence of neutrophil granulocytes as a biomarker for clozapine use. Eur Neuropsychopharmacol 2013; 23: 1408-13.

Publications not related to this thesis

Panizzutti R, Fisher M, Garrett C, Man WH, Sena W, Madeira C, Vinogradov S. Association between increased serum D-serine and cognitive gains induced by intensive cognitive training in schizophrenia. Schizophr Res (in press).

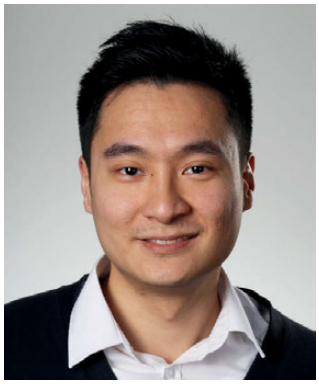
Man WH, Madeira C, Zhou X, Merzenich MM, Panizzutti R. TNF-A Levels throughout the critical period for experience-dependent plasticity in the rat primary auditory cortex. Neurochem Neuropharm Open Access 2015; 1: 105.

van Maarseveen EM, Man WH, Proost J, Neef C, Touw DJ. Chronopharmaco kinetics of once daily dosed aminoglycosides in hospitalized infectious patients. Int J Clin Pharm 2015; 37: 342-7.

Man WH, Hagen EC, Wielders JP, Malingré MM. Verhoogde concentraties metanefrines in urine bij methylfenidaat gebruik kan leiden tot verdenking feochromocytoom. Ned Tijdschr Klin Chem Labgeneesk 2014; 39: 34-6.

van Maarseveen EM, Man WH, Touw DJ, Bouma AW, van Zanten AR. Continuous and intermittent infusion of vancomycin equally effective: review of the literature. Ned Tijdschr Geneeskd 2011; 155: A2667.

About the author



Wai Hong Man was born on 29 June 1987 in Terneuzen, the Netherlands. In 2005 he completed secondary school (gymnasium) at the 'Reynaertcollege Hulst'. He attended his studies in Pharmacy at the University of Utrecht and obtained his Master of Science degree in pharmacy in 2012.
After obtaining his Master's degree he started his professional career with a PhD research project combined with his residency in hospital pharmacy on March 2012 at the Department of Clinical Pharmacy of the University Medical Center Utrecht. During his training in hospital pharmacy he was a member of the Special Interest Group Psychiatry of the NVZA and the Clozapine Plus Werkgroep. He received his degree as a hospital pharmacist in January 2017.
As of October 2017 he holds a position as a hospital pharmacist at the Department of Clinical Pharmacy of Meander Medical Centre at Amersfoort, with clinical psychopharmacology and mental health care as areas of interest. Wai Hong lives together with his fiancé Karen in de Bilt, Utrecht.