ORIGINAL RESEARCH ARTICLE

Unbound Fraction of Clozapine Significantly Decreases with Elevated Plasma Concentrations of the Inflammatory Acute-Phase Protein Alpha-1-Acid Glycoprotein

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Abstract

Background During inflammation, elevated total (unbound plus protein-bound) clozapine plasma concentrations have been observed. Elevated alpha-1-acid glycoprotein concentrations during inflammation are suggested to cause increased plasma clozapine-alpha-1-acid glycoprotein binding, resulting in elevated total clozapine plasma concentrations without significant changes in unbound concentrations. Here, we investigated the association between alpha-1-acid glycoprotein plasma concentrations and clozapine unbound fraction.

Methods First, 25 and 60 μL of alpha-1-acid glycoprotein solution (20 mg/mL) were added to plasma samples ($n = 3$) of clozapine users (spiking experiment). Second, the association between alpha-1-acid glycoprotein plasma concentration and clozapine unbound fraction was assessed in patient samples (patient study). Samples were determined by liquid chromatography-tandem mass spectrometry. Data were analyzed with a paired t test (spiking experiment) and an unpaired t test (patient study).

Results The spiking experiment showed significantly lower mean unbound fractions following 25- and 60-μL alpha-1-acid glycoprotein 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 with normal alpha-1-acid glycoprotein ($n = 20$) samples [525 μg/L vs. 479 μg/L, mean difference = 47 μg/L (95% confidence interval −217 to 310), $p = 0.72$]. Elevated alpha-1-acid glycoprotein samples had a 26% lower mean unbound fraction compared with normal samples [1.22% vs. 1.65%, mean difference = −0.43% (95% confidence interval −0.816 to −0.0443), $p = 0.03$].

Conclusions Both the spiking experiment and patient study showed a significant association between elevated alpha-1-acid glycoprotein plasma concentrations and a lower clozapine unbound fraction. Future studies should include clinical data to examine whether this association is clinically relevant, suggesting any clozapine dose adjustments.

1 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 owing to variability in metabolism, drug–drug as well as food–drug interactions. Therapeutic drug monitoring is therefore considered useful and involves measurement

Key Points

In contrast to many drugs, clozapine is highly bound to the acute phase protein alpha-1-acid glycoprotein (AGP) instead of serum albumin. During inflammation, a substantial rise of the total (unbound plus protein-bound) clozapine plasma concentration into the toxic range has been observed. It has been suggested that elevated 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.

The results of our 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.

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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, owing to drug accumulation in plasma as a result of increased binding to systemic AGP. However, the unbound (and pharmacological active) concentration 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 the absence of toxic side effects in patients with inflammation. The other proposed mechanism is the production of interleukin-6, which is transiently increased during inflammation. Interleukin-6 is claimed to inhibit the metabolism of clozapine by downregulation of the expression of cytochrome P450 1A2 and cytochrome P450 3A4 [13], potentially leading to elevated total clozapine plasma concentrations. In this hypothesis, the 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 the 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. To our knowledge, no studies have yet examined the relationship between AGP plasma concentration and the clozapine unbound fraction. The objective of the current study was to assess this association.

2 Methods

2.1 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 visits, 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 visits, 23,000 day-care hospitalizations, and 380,000 outpatient visits. The Institutional Review Board of the University Medical Center Utrecht determined that the study was not subject to the Medical Research Involving Human Subjects Act, and the University Medical Center Utrecht Biobank approved the 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, St Louis, MO, USA) stock solution, simulating a final AGP concentration nearly 1.5- to 2.3-fold higher than the upper limit of the normal AGP concentration range. In this regard, the amounts of AGP in the spiking experiments reflect the in-vivo amount because AGP plasma concentrations could be up to 2- to 5-fold higher in acute-phase reactions. [21]

The AGP stock solution of 20 mg/mL was prepared in phosphate-buffered saline buffer. Aliquots were then incubated for 1 hour at room temperature to establish an equilibrium. The total and unbound clozapine concentrations were determined in the pre- 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 therapeutic drug monitoring 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 the 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 an unbound plasma concentration, aliquot samples were stored as ultra-filtrate at −20 °C after the protein filtering step, as described below.

2.2 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 University Medical Center 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 liquid chromatography mobile phases consisted of mass spectrometry grade water mixed with formic acid (0.1%) and acetonitrile mixed with formic acid 0.1%, both ordered at Sigma-Aldrich. The LC-MS/MS system included a Thermofisher Hypersil GOLD PFP 50×2.1 mm column (Waltham, MA, USA) pressurized by a Surveyor MS Plus Pump (Waltham). Signal was detected with a Surveyor Autosampler plus (Waltham) and a Thermofisher TSQ Quantum access mass spectrometer with a Heated Electrospray Ionization Probe (Waltham). Data gathering and system control were performed by Xcalibur software (high-performance liquid chromatography) and Thermo Scientific Trace Finder software (mass spectrometry). Using Merck Millipore centrifree YM-30 cat4104 Amicon filters (Darmstadt, Germany), ultra-centrifugation took place at 2500 rpm ($559 \times g$) 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 to 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 European Medicines Agency guideline on bioanalytical method validation [22]. Clozapine unbound fraction was calculated by dividing unbound plasma concentration by the total plasma concentration.

2.3 Measurement of Alpha-1-Acid Glycoprotein Plasma Concentrations

Alpha-1-acid glycoprotein plasma concentrations were determined using an immunoturbidimetric assay according to the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany) at the Clinical Laboratory of Chemistry and Hematology at Leiden University Medical Center. 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.

2.4 Data Analysis

Differences in mean unbound fraction between the pre- and spiked samples calculated from the spiking experiment were analyzed using a paired t test [Graphpad Prism 7.02 (La Jolla, CA, 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). These results were plotted in scatter graphs and box-and-whisker plots using Graphpad Prism 7.02. An alpha level of 0.05 was considered statistically significant for all performed statistical tests.

3 Results

3.1 Spiking Experiment

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

3.2 Patient Study

A total of 26 remnant samples from patients using clozapine were collected and included in the study (Table 1). Study samples with elevated AGP concentrations ($n = 6$) showed a 25% significantly lower mean unbound fraction [1.22% vs. 1.65%, mean difference $= -0.43\%$ (95% confidence interval -0.816 to -0.0443), $p = 0.03$] in comparison to samples with normal AGP concentrations ($n = 20$). Both clozapine unbound concentrations and total clozapine plasma concentrations did not significantly differ between study samples with elevated AGP and those with normal AGP concentrations [7.2 ng/mL vs. 6.3 ng/mL (mean difference $= 0.9$ ng/mL, 95% confidence interval -4.45 to 2.54, $p = 0.590$) and 525 ng/mL vs. 479 ng/mL (mean difference $= 47$ ng/mL, 95% confidence interval -217 to 310, $p = 0.72$), respectively] (Fig. 2).

4 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

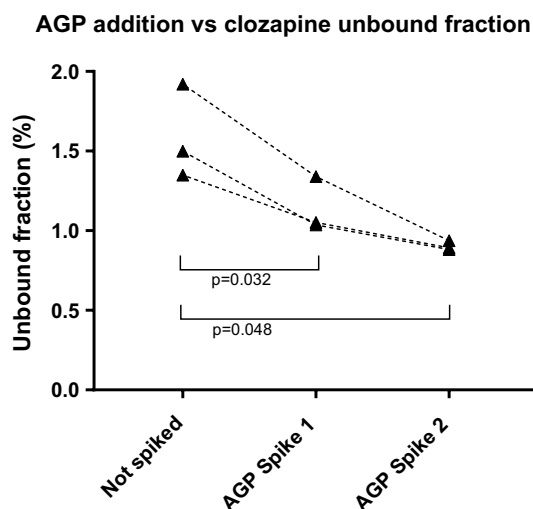**AGP addition vs clozapine unbound fraction**

Fig. 1 Relation between alpha-1-acid glycoprotein (AGP) addition and clozapine unbound fraction. Spiking experiment displaying clozapine unbound fraction 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

(a) Normal and elevated AGP concentration vs unbound concentration of clozapine

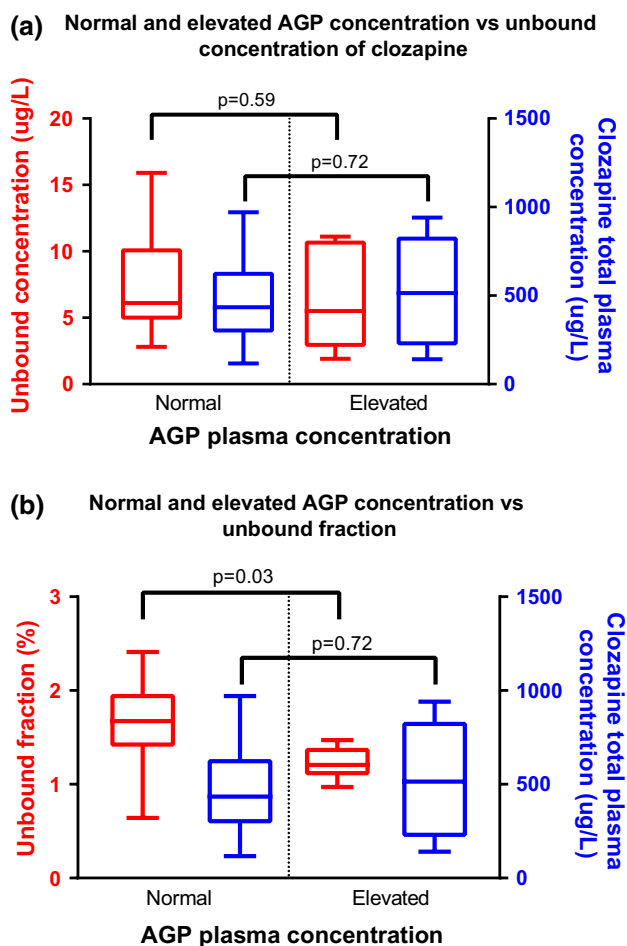**(b)** Normal and elevated AGP concentration vs unbound fraction

Fig. 2 Alpha-1-acid glycoprotein (AGP) plasma concentrations vs. clozapine unbound fraction in the patient study. The relation between AGP plasma concentrations and the clozapine unbound fraction. Clozapine unbound concentrations in the patient study are in 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 blue are the clozapine total plasma concentrations, depicted in red are the clozapine unbound fraction (above) and unbound concentrations (below). A significant lowered mean unbound fraction was found in the elevated AGP group in comparison to the normal AGP group, while no significant differences were found in clozapine unbound concentrations. An increase in mean total clozapine plasma concentration was found in the elevated AGP group, but was not statistically significant

by the results of the patient study that demonstrated a lower unbound fraction in the elevated AGP group compared with the normal AGP group, while unbound concentrations were not statistically different. 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 with the larger normal AGP group ($n = 20$). In addition, guided by therapeutic drug monitoring, clinicians could have reduced the clozapine dose anticipating high plasma concentrations owing to inflammation, resulting in lowered total plasma concentrations within the therapeutic window for the total concentration.

In the case of inflammation, high concentrations of AGP can lead to a lowered unbound fraction of clozapine. This is most likely owing 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. These findings constitute a reason for future studies to further examine this association involving clinical assessments of the patient.

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. In a previous study, the clozapine unbound fraction was 5.5% [17]; however, any distinctions in inflammatory/non-inflammatory state were not assessed. Moreover,

the higher fraction that was found in this previous study could be attributed to the assay (high-performance liquid chromatography), which differed from ours. In our study, we used a validated assay involving liquid chromatography-mass spectrometry, which most likely provides results with more accuracy and precision. Last, certain conditions of sample preparation can highly affect the fraction, including the temperature. The major strength of our study is that the results of the spiking experiment and patient study both

Table 1 Results of the patient study

Patient sample	AGP concentration (g/L)	Clozapine unbound concentrations (µg/L)	Total clozapine concentrations (µg/L)	Clozapine unbound fraction (%)
1	3.45	10.7	940	1.14
2	3.27	7.7	797	0.97
3	1.98	3.1	246	1.24
4	1.94	11.1	750	1.47
5	1.24	3.3	278	1.17
6	1.21	1.9	140	1.36
7	1.17	8.4	571	1.47
8	1.12	4.8	252	1.89
9	1.10	5.8	467	1.24
10	1.07	9.1	655	1.39
11	1.04	12.6	823	1.53
12	0.96	4.3	287	1.49
13	0.96	11.1	971	1.14
14	0.95	15.9	942	1.69
15	0.94	5.7	550	1.03
16	0.91	5.0	785	0.64
17	0.90	10.6	534	1.99
18	0.88	6.4	386	1.66
19	0.88	10.9	474	2.30
20	0.84	3.0	150	2.00
21	0.83	7.0	402	1.74
22	0.77	5.4	374	1.44
23	0.75	3.0	136	2.22
24	0.71	2.8	117	2.41
25	0.70	7.3	391	1.86
26	0.61	5.6	304	1.83

AGP alpha-1-acid glycoprotein

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 because 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 because the equilibrium between protein-bound and unbound concentrations is dependent of temperature. [23]. Last, the individual's intra-individual course of clozapine plasma concentrations was not followed up during the inflammatory course. Intraindividual variations are therefore not included in our analysis.

Although we provide here the first evidence for altered clozapine pharmacokinetics as a result of AGP, follow-up in-depth pharmacological and clinical studies are needed. Future studies of larger sample sizes are preferable to further examine the effects of AGP during inflammation on clozapine total and protein unbound plasma concentrations, also taking into account the multiple moments of sampling within the full time-course of inflammation.

5 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 owing to elevated AGP concentrations. Future studies should further explore this association in larger study populations, and include clinical assessments.

Compliance with Ethical Standards

Conflict of interest Wai Hong Man, Ingeborg Wilting, Eibert Heerdink, Gerard Hugenholtz, Tim Bognár, Maarten ten Berg, Wouter van Solinge, Toine Egberts, and Erik van Maarseveen have no conflicts of interest that are directly relevant to the content of this article.

Ethics approval The Institutional Review Board of the University Medical Center Utrecht determined that the study was not subject to the Medical Research Involving Human Subjects Act, and the University Medical Center Utrecht Biobank approved the use of anonymized remnant material for this study.

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