Alterations of primary fatty acid amides in serum of patients with severe mental illness

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1. ABSTRACT

Cannabis consumption is a well known risk factor for the onset of schizophrenia and evidence accumulates that the endocannabinoid system may play a central role in the disease etiology. Using a clinical bioinformatics approach, we have previously found primary fatty acid amides, which are linked to the endocannabinoid system, to be elevated in drug naive schizophrenia and affective disorder. Here, we provide a detailed description of these findings and expand the investigation by analyzing serum from 74 patients after short term treatment with antipsychotic medication using a liquid chromatography-mass spectrometry (LC-MS) metabolomics approach. We show that primary fatty acid amide (pFAA) levels normalize after treatment with typical but not after treatment with atypical antipsychotic medication. Also, the comparison of pFAA levels in schizophrenia patients to those of sleep deprived healthy volunteers suggests that pFAA abnormalities were not related to changes in the sleep architecture of patients with mental illness. Our findings support the involvement of the endocannabinoid system in the pathology of schizophrenia.

2. INTRODUCTION

Within the last decades, considerable progress has been made in the search for factors contributing to the development of schizophrenia. The endocannabinoid system has become a focus of recent investigations due to its role in modulating both central and peripheral nervous system function (1). Δ^9- tetrahydrocannabinol (THC), an active ingredient in cannabis, acts on the two known endocannabinoid receptors CB1 and CB2. Excessive consumption of cannabis can precipitate a schizophrenia-like state with hallucinations, delusions and emotional lability (2-4) and heavy cannabis use has been found to be associated with a threefold increased risk to develop schizophrenia (5). The current view is that cannabis may trigger episodes of mental illness, rather than being a primary cause. Interestingly, endocannabinoid receptor expression in the human brain overlaps with brain regions reported to be involved in the schizophrenia pathology (6, 7) and various neuronal functions modulated by the endocannabinoid system have been reported to be altered in schizophrenia (8).

Primary fatty acid amides in schizophrenia

Table 1. Demographic details of subjects

	Healthy Volunteer (HV) (n=59)	Drug Naive (PS) Schizophrenia (n=70)	Schizophrenia with "typical" antipsychotics treated (D2)(n=40)	Schizophrenia with "atypical" antipsychotics treated (D2/5HT) (n=34)	Affective Disorder (n=37)	Sleep deprived healthy volunteers (n = 8)
Age[1] (yrs)	27.5 ± 5.9	29.3 ± 10.0	32.3 ± 10.9	30.5 ± 11.9	41.9 ± 18.1	25.8 ± 2.7
Sex (m/f)	33/26	46/24	28/12	23/11	21/16	2/6

[1] values are shown as mean $\pm$ sd

The best understood endocannabinoids are anandamide and 2-arachidonyl glycerol, both of which bind to CB1 and CB2 endocannabinoid receptors. However, other molecules have also been linked to the endocannabinoid system. These include related primary fatty acid amides (pFAAs) such as oleamide. Although pFAAs show little or no affinity to CB1 and CB2 receptors (9-11), their functional link to the endocannabinoid system is based on multiple lines of evidence. Oleamide, the primary amide of oleic acid, for example has been shown to induce clinical symptoms similar to those induced by cannabinoids, in particular the triad of a reduction in body temperature, hypolocomotion and a reduction in pain perception (12). Further, oleamide is involved in the modulation of memory (12), immune functions (13) and accumulates in the CSF of sleep deprived cats (14). Primary fatty acid amides and endocannabinoids are degraded by the same enzyme fatty acid amide hydrolase (FAAH) (15, 16). Therefore, it has been hypothesised that fatty acid amides compete with endocannabinoids at the active binding site of FAAH, thereby increasing the concentration of endocannabinoids by preventing their degradation (17).

Previously, it has been shown that endocannabinoid levels were elevated in cerebrospinal fluid (CSF) of schizophrenia patients (18, 19). We have shown that the pFAA system is elevated in a subgroup of schizophrenia and affective disorder patients (20). Here, we expand this investigation by analysing patients after short term antipsychotic treatment and a comparison with pFAA levels in sleep deprived subjects. With this in mind, we used liquid chromatatography-time-of-flight-mass spectrometry (LC-TOF-MS) to profile serum pFAAs of subjects with schizophrenia, affective disorder and sleep deprivation.

3. MATERIAL AND METHODS

3.1. Clinical samples

The Ethical committee of the Medical Faculty of the University of Cologne reviewed and approved the protocol of this study and the procedures for sample collection and analysis. All study participants gave their written informed consent. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Serum samples were collected from 248 individuals, comprising 70 drug-naive patients diagnosed with first episode paranoid schizophrenia due to duration of illness (DSM-IV 295.30), 74 acute paranoid schizophrenics treated with antipsychotic medication, 37 affective disorder patients and 59 demographically matched healthy volunteers with no family history of schizophrenia or other detectable psychiatric, neurological or medical

history and 8 healthy volunteers who were sleep deprived for 24h (Table 1). Antipsychotic medication was either 'typical' (D_2-antagonistic) or 'atypical' ($5HT_{2A}/D2$ antagonistic).

### 3.2. Preparation of serum samples

Acetonitrile (120 µL, Sigma Aldrich) was added to a 30 µL aliquot of each serum sample in 1.5 mL Eppendorf tubes to protein precipitate the samples and left to stand for 15 minutes. The tubes were then vortexed and centrifuged at 13000rpm for 15 min at room temperature in a Sanyo Micro Centaur centrifuge. The supernatant was transferred into Total Recovery vials (Waters, screw 12x32mm & cap with PTFSE/ Silicone septa) and evaporated to complete dryness in a Genevac EZ-2. The pellet was re-suspended in 100 µL of 95:5 H_2O:Acetonitrile and stored at -80°C.

3.3. Liquid Chromatography

5 µL aliquots of the reconstituted serum samples were analysed on a Waters ACQUITY™ Ultra Performance Liquid Chromatography system using a (2.1 x 100) mm ACQUITY BEH C18 column (1.7 µm particle size), maintained at 40°C, at a flow rate of 0.6 mL/min. All samples were eluted using a gradient of 0-20%B from 0 to 4 min, followed by 20-95%B from 4 to 9 min. The column was then washed with 100% B until 14 minutes before returning to the starting conditions (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile). After acquiring the raw data of all randomized samples, the run was repeated.

3.4. Mass spectrometry

The eluent from the analytical column was coupled directly to an orthogonal acceleration time of flight (oa-ToF) mass spectrometer (LCT Premier™, Waters, Milford MA) operating in positive electrospray ionisation (ESI) mode. A sample cone voltage of 35V was used throughout to minimise fragmentation. A capillary voltage of 3.0 kV positive ion was used for the analysis. The source temperature was set at 120°C and the desolvation gas temperature at 350°C with a nitrogen desolvation gas flow of 800 L h^{-1}. The lock mass reference compound used for mass alignment was leucine enkephalin at a concentration of 200 pg/µL. This was introduced through the LockSpray reference probe at a flow rate of 30 µL/min. The instrument was calibrated from an infusion of a sodium formate reference solution. Data from a system suitability test mix (Waters, Milford MA) containing 5 compounds was acquired at the start of the analysis and at regular intervals throughout the run as well as data from a quality control serum sample.
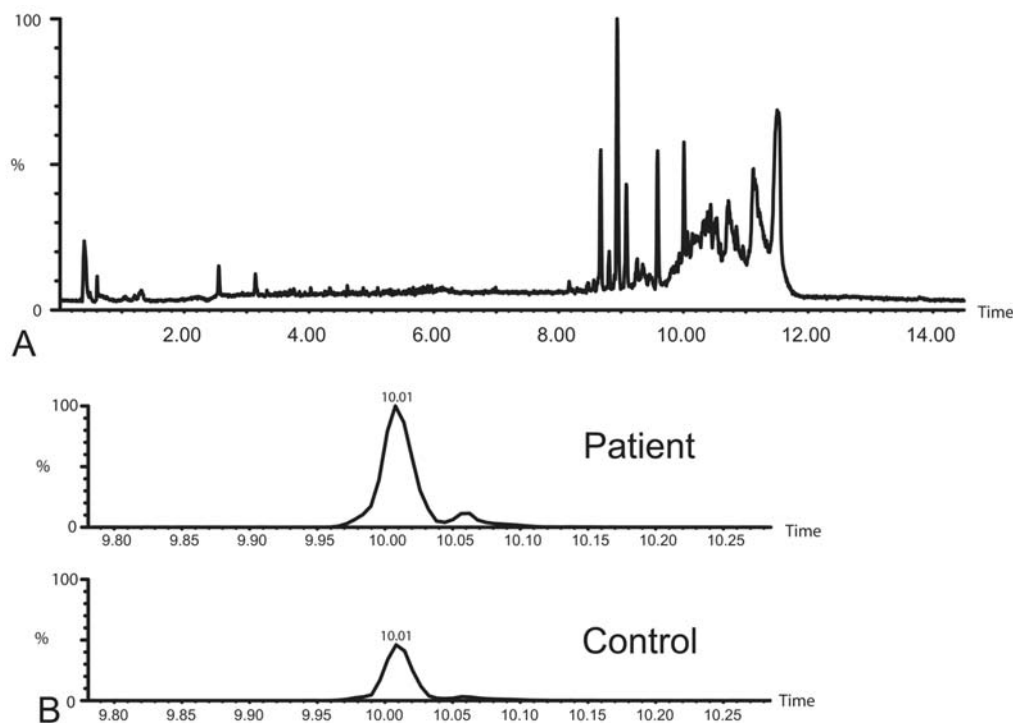
Figure 1. A: Total Ion Current (TIC) chromatogram. B: Extracted Ion Chromatogam (XIC) of oleamide in a typical patient (top panel) and control sample (lower panel). The plot shows intensities relative to those observed in the patient sample (top panel)

The elemental composition of detected compounds of interest were determined using a combination of exact mass measurements and isotopic ratio matching using the elemental composition tool embedded into MassLynx v 4.0 software (Waters). The identification is based on mass error, the double bond equivalent of the suggested formula and the match of the isotopic pattern of the suggested formula with the observed mass spectrum (iFit). The identity of the molecules was confirmed by comparing the retention times of purchased standards as well as intensity changes of the molecules in spiked serum samples.

3.5. Data processing

The data was deconvoluted and aligned using the MarkerLynx application manager within MassLynx. The table of m/z and retention time pairs with associated intensities generated was exported to SIMCA-P multivariate statistical analysis software for further analysis.

3.6. Statistical analysis

The analysis strategy that led to the identification of a network of primary fatty acid amides has been described elsewhere (20). In short, univariate analysis was performed to investigate molecular abnormalities in schizophrenia compared to healthy volunteers. To account for multiple hypothesis testing, the False Discovery Rate was controlled according to Benjamini and Hochberg (21). Metabolite peaks were then encoded in a network and Markov chain clustering was used to identify molecular

networks (20). The molecular network of pFAA described in this study comprised 26 ions which featured a median FDR of 0.0196 (range: 0.0035 – 0.7627).

A multivariate Partial Least Squares (PLS) model based on 26 ions was generated using SIMCA-P+ (version 10.5, Umetrics, Umeá, Sweden). Variables were scaled to have a mean value of 0 and standard deviation of 1. The free software package R (http://cran.r-project.org/) was used for all other analyses.

4. RESULTS

4.1. Characterisation of serum metabolites changes
LC-TOF-MS was used to profile 248 sera from patients with different mental disorders, sleep deprived healthy volunteers and appropriately matched controls. A representative Total Ion Chromatogram (TIC) of a patient serum sample is shown in Figure 1A. The samples were analysed in duplicate by carrying out a second independent replication of the entire experiment after the first one. A clinical bioinformatics approach described previously led to the identification of a network of 26 ions corresponding to 6 different primary fatty acid amides (plus respective adducts) (20). In this study all pFAA levels were found to be elevated in drug naive first onset schizophrenia patients compared to controls (Table 2). Figure 1B shows an extracted ion chromatogram (XIC) for a representative patient and control sample. Findings were reproducible between measurement replicates (data not shown). The molecular formulae of these molecules were determined

Table 2. Summary of pFFA changes in schizophrenia, affective disorder and sleep deprivation

Metabolite	p-value first replicate	FC schizophrenia	FC affective disorder	FC sleep withdrawal
Oleamide[1]	< 0.001[2]	1.20	1.29	1.24
Linoleamide	0.121	1.08	1.11	1.44
Heptadecenoic amide (17:1) [1,3]	< 0.001[2]	1.22	1.29	1.25
Palmitic amide[1,3]	< 0.001[2]	1.30	1.42	1.34
Palmitoleic amide[1,3]	< 0.001[2]	1.20	1.26	1.24
Myristic amide[1,3]	0.013	1.15	1.16	1.15

Each molecule is represented by the sum of all corresponding peaks in the extracted metabolic network. P-values were determined using parametric t-test. [1] Significantly changed in both replicates. [2] $P < 0.05$ using non-parametric Wilcoxon Rank sum tests. The fold change (FC) for each molecule was calculated as the ratio of the average intensity of the respective patient group and the average of the control group and was based on the first replicate of the analysis. [3] Tentative identification.
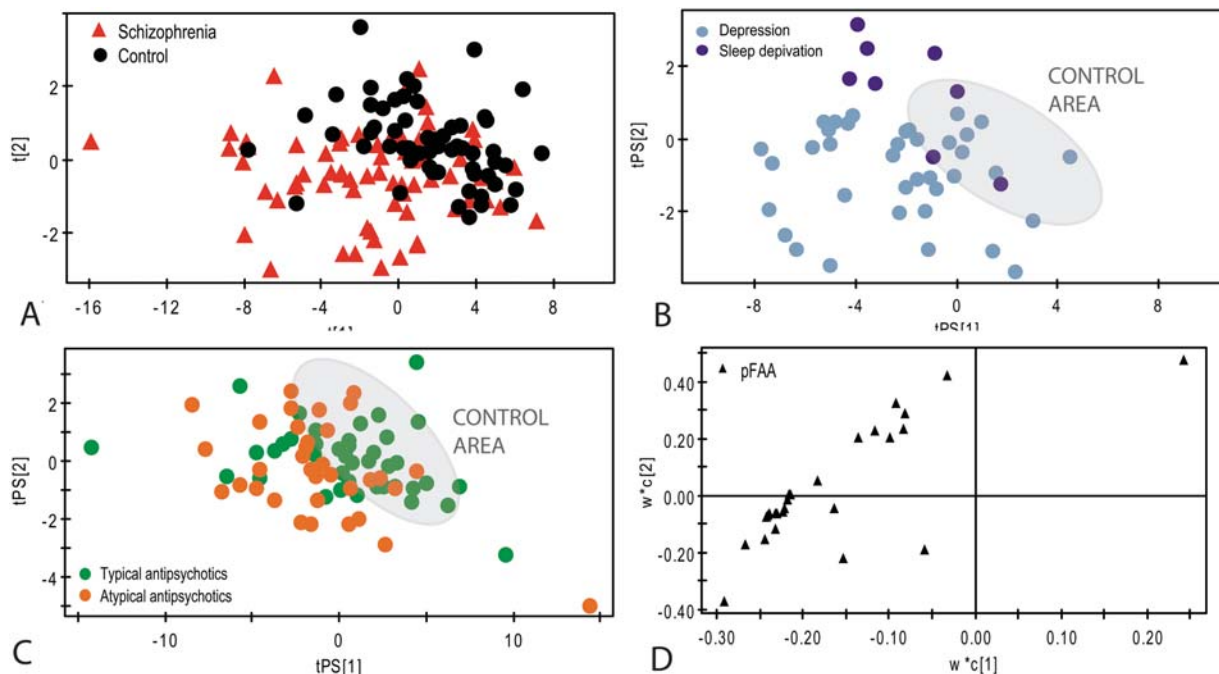
Figure 2. A: PLS scores plot of schizophrenia patients and controls based on mass spectrometric peaks of primary fatty acid amides. B: Predicted scores of affective disorder patients and sleep deprived healthy volunteers based on the PLS mode shown in A. C: Predicted scores of patients treated with typical and atypical antipsychotic medication. The profile normalizes after treatment with typical antipsychotic medication. D: PLS weights plot.

with the elemental composition tool embedded in the MassLynx software (Table 2). We were able to confirm the identification of oleamide and linoleamide using commercially available standards, which were re-run in pure form as well as spiked into serum samples.

4.2. Effects of antipsychotic treatment and cannabis consumption on the extracted metabolic network
74 patients investigated in this study were treated with typical (D_2-antagonistic, n=40) or atypical ($5HT_{2A}$/D2 antagonistic, n=34) antipsychotic medication. A multivariate PLS model discriminating first onset schizophrenia patients from controls was built using the 26 pFAA ions (Figure 2A, representing the training performance of the model; weights shown in Figure 2D). Based on this model and using the same components t[1] and t[2], we predicted the PLS - scores of patients treated with antipsychotic medication and observed that the

metabolic network of pFAAs showed normalization of the profile after short term treatment with typical antipsychotic medication for an average of nine days. This normalization was not or to a far lesser degree visible after treatment with atypical antipsychotics (Figure 2C).

Due to the prevalent consumption of cannabis among schizophrenia patients and especially in the context of endocannabinoid system related metabolites, the effect of cannabis consumption on the profile of the extracted metabolic network was analysed. We found that the degree of cannabis consumption did not have an effect on the alteration of fatty pFAA levels (data not shown).

4.3. Alteration of the extracted metabolic network in affective disorder and sleep deprived healthy volunteers
As described previously, the profile of the metabolic network identified in this study was more

severely altered in the serum of affective disorder patients and PLS models showed an almost complete separation from controls (Figure 2B, using the same PLS components as in Figure 2A). Patients suffering from affective disorder were significantly older than healthy volunteers. We investigated the correlation between pFAA levels (represented by the sum of the respective ions) and age in all individual disease groups as well as controls and found Linoleamide to be negatively correlated with age in affective disorder ($p = 0.027$). Due to the observed pFAA increases in affective disorder, the difference in age compared to controls is unlikely to have affected the results.

pFAA levels of healthy volunteers were increased after one night of sleep deprivation. The PLS scores plot in Figure 1B shows sleep deprived healthy volunteers featured PLS scores different from both first onset schizophrenia and affective disorder. This finding was mainly cause by increased linoleamide levels compared to both schizophrenia patients as well as controls (Table 2).

5. DISCUSSION

We have carried out a mass spectrometry based profiling study involving 248 serum samples using LC-TOF-MS. We employed a clustering procedure and identified a metabolic network of serum pFFAs. These analytes were significantly elevated in drug naive patients with first onset, paranoid schizophrenia (20). As outlined above, pFAAs are thought to compete with endocannabinoids for binding to the active site of their common degrading enzyme fatty acid amide hydrolase (FAAH) resulting in increased levels of endocannabinoids (17). However, knock out mouse studies of FAAH suggest that the biological function of the primary fatty acid amide oleamide is not dependent on, but regulated by the activity of FAAH (11). Therefore, Lichtman *et al.* suggested that oleamide may bind to yet unidentified receptors. Increased concentrations of "the classical" endocannabinoid anandamide have been found previously in the cerebrospinal fluid (CSF) of drug naive, first onset schizophrenia patents but not in serum of the same patients (19). The authors analysed a subset of the same sample collection as investigated in the present study allowing a cross-comparison of respective changes in classical endocannabinoid and pFAA levels. The alteration of pFAAs in the serum of patients with schizophrenia and affective disorder (where classical endocannabinoid levels were found to be normal) may suggest that these molecules play a role in the disease process of both diseases beyond their mere competition for degradation by FAAH. Elevated levels of the endocannabinoid anandamide have also been found in patients in an initial prodromal state of schizophrenia and have been suggested to play a protective role during the early stage of schizophrenia (22). This is further supported by the previously reported negative correlation between CSF anandamide levels and psychotic symptoms (19) as well as negative symptoms in certain schizophrenia subgroups (23).

5.1. Effects of antipsychotic treatment on the pFAA metabolic network

We found that short term treatment for an average of 9 days with typical antipsychotic medication resulted in a normalization of the altered profile of pFFAs. This normalization was not visible after short term treatment with atypical antipsychotic medication. These findings align well with reports from Giuffrida *et al.* who found that levels of the classical endocannabinoids in schizophrenia CSF normalized after short term treatment with typical, but not atypical antipsychotic medication (19). They hypothesized that this phenomenon is due to the higher affinity of typical antipsychotics to dopamine receptors as activation of dopamine receptors is known to increase anandamide levels (19). The results of the present study may suggest a similar mechanism of action of pFAAs in serum.

5.2. Alteration of the pFAA metabolic network in affective disorder

Primary fatty acid amides were more severely altered in affective disorder as compared to schizophrenia patients. It can not be excluded that drug treatment of these patients had an effect on this alteration. It is well known that sleep deprivation is one of the most frequent symptoms in depression and Irmisch *et al* found significant negative correlations of the serum concentrations of certain fatty acids (amongst others myristic, palmitic, palmitoleic, oleic and linoleic acid) with the degree of sleep disturbance (24). This is interesting as oleic acid and linoleic acid are precursors of the well known sleep inducing molecules oleamide and linoleamide. However, the degree of separation between affective disorder patients and healthy volunteers as well as the normalization of the metabolic profile in schizophrenia patients after treatment with typical antipsychotics argue for a distinct function of primary fatty acid amides in patients with severe mental illness.

To further investigate the effect of sleep alterations on the primary fatty acid amide levels identified in this study, we analysed sera of healthy volunteers who had been sleep deprived for 24 hours prior to blood collection. Fatty acid amide levels were increased after sleep deprivation and, with the exception of linoleamide levels, comparable to those observed in schizophrenia. Further studies and larger sample cohorts will be required to assess the relationship between psychiatric disorders, sleep disturbances and pFAA levels.

Interestingly, Giuffrida *et al* did not observe any changes in endocannabinoid levels in CSF or serum of affective disorder patients (19) suggesting that pFAAs may play a distinct role in schizophrenia and affective disorder, and that the regulation of pFAAs may be independent from the classical endocannabinoids. De Marchi *et al.* reported previously that levels of FAAH mRNA decreased in schizophrenia blood with clinical remission (although the results of this study are based on a small set of 12 schizophrenia samples) (25). Furthermore, Gobbi *et al.* reported that selective inhibition of FAAH had antidepressant-like effects in mice which generated great interest in FAAH as a putative drug target for depression

(26). However, in light of unaltered levels of anandamide in the serum of drug naive, first onset, paranoid schizophrenia as well as affective disorder patients (19), disease-associated changes in FAAH seem unlikely suggesting that the increased levels of pFAAs may be due to alterations upstream in their biosynthesis pathway.

6. CONCLUSION

Serum levels of primary fatty acid amides were found to be significantly elevated in serum of first-onset drug-naive schizophrenia and affective disorder patients. The alterations normalized following short-term treatment with typical (but not atypical) antipsychotic medication. As the mechanism of action of primary fatty acid amides is still poorly understood, we can only speculate as to the function of the respective metabolites in the disease process of schizophrenia and major affective disorder. Further studies are required to elucidate the upstream mechanisms of biosynthesis as well as the down-stream receptor systems of fatty acid amides. The results of this study further strengthen the involvement of the endocannabinoid system in the pathology of schizophrenia.

7. ACKNOWLEDGEMENT

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Abbreviations: FAAH, Fatty Acid Amide Hydrolase; pFFA, primary Fatty Acid Amide; THC: Delta-9 Tetrahydrocannabinol; XIC, extracted ion chromatogram

Key Words: Schizophrenia, Affective Disorder, Primary Fatty Acid Amides, Endocannabinoid System, Blood, Serum, Mass Spectrometry

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