Appendix 1 to: Henning D, Lüno M, Jiang C, et al. Gut–brain axis volatile organic compounds derived from breath distinguish between schizophrenia and major depressive disorder. *J Psychiatry Neurosci* 2023. doi: 10.1503/jpn.220139.

Title: Gut-brain axis volatile organic compounds derived in breath separate schizophrenia and major depressive disorder

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Supplemental Text

Supplemental Methods

Breath gas analysis

To observe the VOCs contained in the subjects' breath gas, Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) was used as a diagnostic tool. PTR-MS uses pre-generated hydronium ions (H_3O^+) to transfer their protons to the desired trace component in the examined gas sample as they react with one another in a designed reaction drift tube [1]. The proton transfer yields ionized products which are subsequently mass analyzed via mass spectrometry using a quadrupole mass analyzer which separates the ions based on their mass to charge ratio (m/z) and transfers them onto a detector for registration [1].

Similar to other established breath gas analysis tests such as the breathalyzer, which deduces a subject's blood alcohol level via the ethanol present in their breath gas and is commonly applied by law enforcement as well as in the medical field, PTR-MS also employs a non-invasive, apparatus-based technique. Subjects' breath samples were collected via Tedlar® bags, which are specific sample devices to collect VOCs and permanent gases. Breath gas probes were taken at awakening, after 30 min, and after 60 min. Since breath gas analysis of some VOCs such as isoprene is dependent on respiratory physiology, a standard procedure was used [1]. All subjects sat quietly for one minute. Then after two minutes of a controlled paced breath rhythm (with a normal RR of 10–12/min), they switched to a spontaneous rhythm and then started breath sampling as recommended by recent methodological work [1].

The samples could then easily be transferred from the Tedlar bags to the sample inlet of the mass spectrometer via a lockable valve installed in the bag through which the gas samples were also collected from the subjects. Upon collection, the breath samples would have a maximum storage life of 10 hours, which meant that they would have to be analyzed on the same day they were collected [2]. All samples were therefore analyzed in the morning right after collection so no breath sample would be stored longer than 4 hours. In order to prevent any spoiling of the samples, all subjects were asked to refrain from any form of extended physical

activity including brushing their teeth, working out, eating and drinking anything but water over the 1-hour timespan in which the samples were collected. After the initial baseline inspection, a two-week follow-up appointment was arranged in which the sampling process was repeated.

Selection of VOCs for analysis

While the overall constitution and functional interaction of the human exhalome are still in the process of being deciphered, several key metabolites have already been identified as part of exhaled breath including isoprene, methanol, acetone, urea and free fatty acids [3, 4]. Most of these compounds, however, have yet to be linked to psychiatric diseases or cerebral metabolism in general. Based on previous studies examining breath gas, 35 VOCs corresponding to known organic compounds were preselected to be analyzed [5]. This included several known VOCs such as isoprene, acetone, 2-pentanone, acetic acid and ethanol, all of which have been previously investigated in real time measurements by PTR-MS (see supplemental table 1). Known exogenous compounds, especially those associated with smoking such as benzene, styrene, toluene and acetonitrile, were excluded from further analysis [6].

Recent literature with identified VOCs from human breath gas analyses was used to preselect the VOCs analyzed in this study [3, 4, 7-19].

Supplemental Discussion

M/z 88 has been linked to several isotopes such as N-ethyl-acetamide, N-methyl-propanamide, pentanamine and N,N-dimethyl-acetamide [20]. Most notably, N,N-dimethyl-acetamide has been described by previous studies as a pollutant from the Tedlar bag material [21]and thus a reduction of this mass could point to a shorter time between breath sampling and measurement in patients. This would, however, not explain why the other masses were reduced in patients as well and why differential effects are seen in patients with schizophrenia. Generally, a prolonged storage time in the bags should amount to a higher loss of substance, although the sample concentration is usually very stable over the first 10 hours [2].

Moreover, the fact that a standardized procedure was used and no difficulties were reported by any participant makes this unlikely. It can therefore not be ruled out that m/z 88 does underline a more specific pulmonary function as it has, for example, been found to be elevated in empyema patients [22].

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The second marker related to schizophrenia is m/z 74. Mass m/z 74 can be related to protonated methylguanidine, a guanidine compound, and can be synthesized from creatine and creatinine [23]. Although it might reflect creatine metabolism, methylguanidine was also found to be highly dependent on the diet, e.g. boiled beef and fish [24]. M/z 74 might also correspond to N-butylamine, which naturally occurs in some fruits, but is also used as a key ingredient in a variety of industrial and food products and even in the production of pharmaceuticals [25]. Despite its industrial abundance, the exact metabolism of N-butylamine remains relatively unknown. In this investigation we found a reduction of m/z 74 levels in breath samples of patients with schizophrenia, making it unlikely to be a byproduct of medication, as it should then be increased. Thus, dietary habits are more likely relevant for this reduction.

Supplemental Table 1 Preselected VOCs for analysis

NH_3	Ammonia	18
CH_2CO	Ketene	31
O2	Oxygen	32
H_2S	Hydrogen Sulfide	35
H2O	Water	19
C_2H_3N	Acetonitrile	42
CO2	Carbon Dioxide	45
C2H4O	Acetaldehyde	45
CH_2O_2	Formic Acid	47
C2H5OH	Ethanol	47
C3H4O	Acrolein	57
$(CH_3)_2CO$	Acetone	59

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N(CH3)3	Trimethylamine	60
CH_3COOH	Acetic Acid	61
C3H8O	Isopropanol	61
CH_3CH_2SH	Ethanethiol	63
C_5H_6	Cyclopentadien	67
C4H4O	Furan	69
C5H8	Isoprene	69
C4H6O	Crotonaldehyde	71
C5H10	Penten	71
C4H8O	Butanone	73
$CH_3(CH_2)_3NH_2$	N-Butylamine	74
C6H6	Benzol	79
C4H6O2	y-Butyrolactone	87
C5H10O	2- Pentanone	87
C4H8S	Tetrahydrothiophen	89
C4H8O2	Ethylacetate	89
$CH_3CH_2CH_2CO_2H$	Butyric Acid	90
C4H10S	2- Butanethiol	91
C7H8	Toluol	93

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C6H6O	Phenol	95
C5H8O2	Acetylacetone	101
C6H12O	Hexanal	101
C10H16	Adamantan	137

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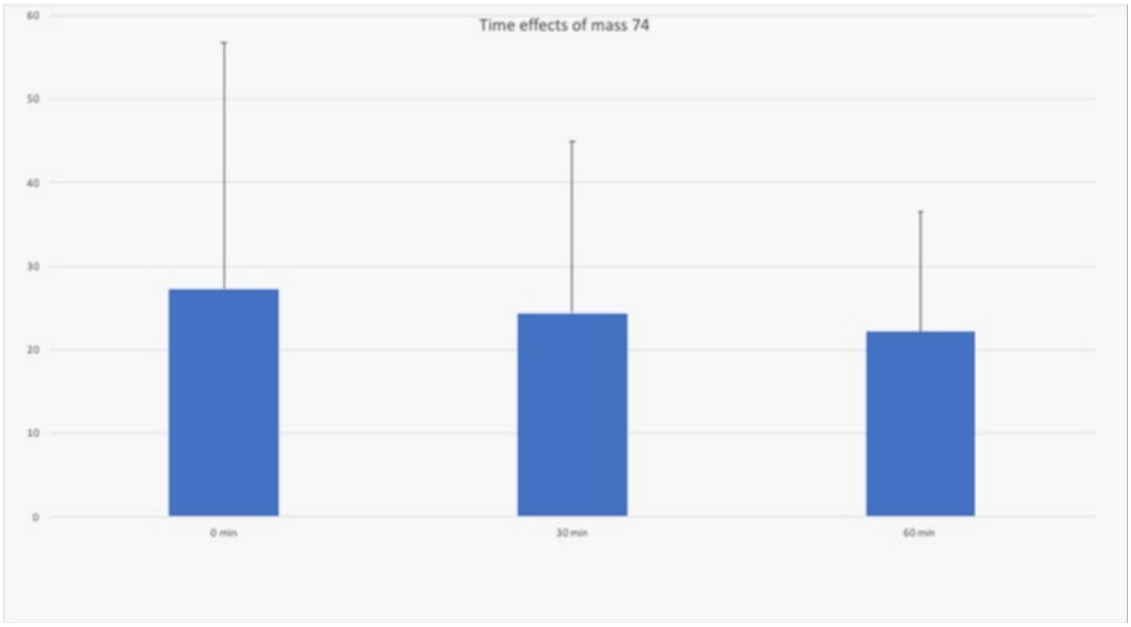
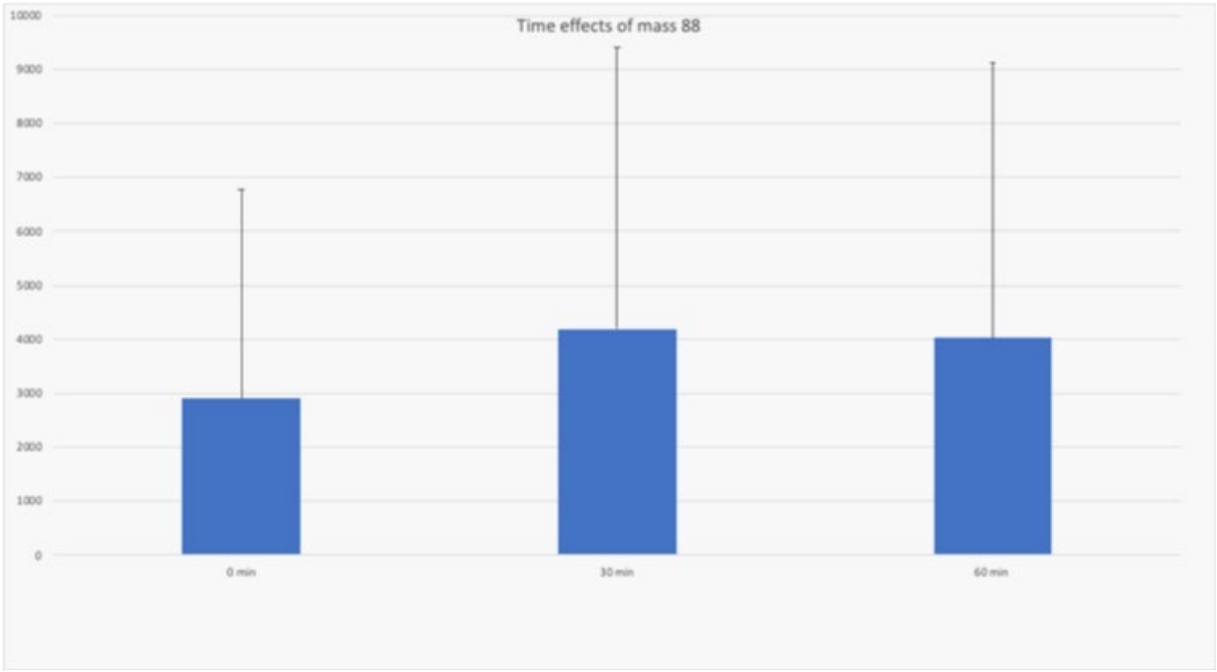
Supplemental Figure Time effects of VOCs for the baseline, 30 minutes and 60 minutes measurement after awakening: A: m/z 74, B: m/z88, C: m/z 90, D: m/z 60, E: m/z 69

A



B



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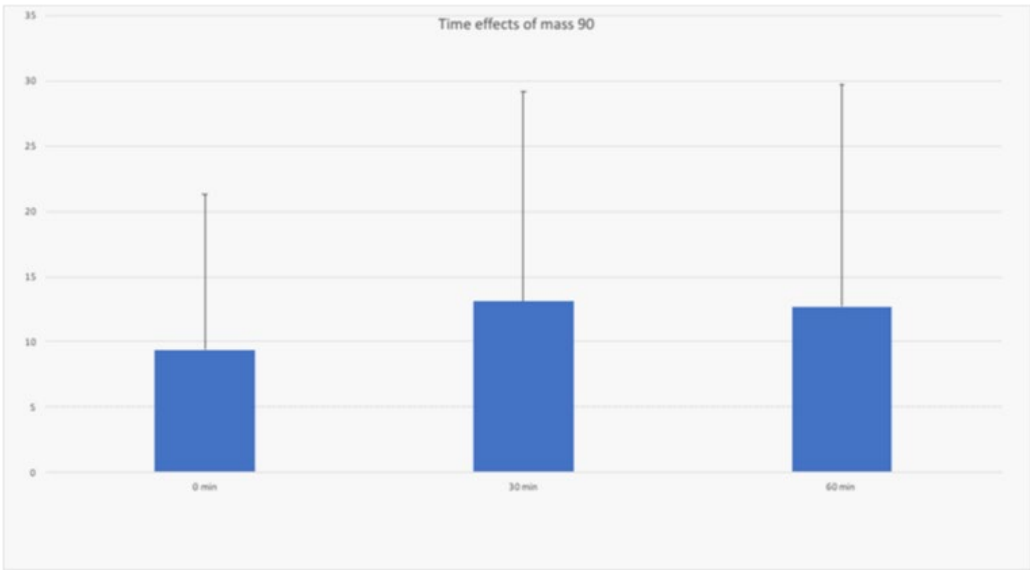
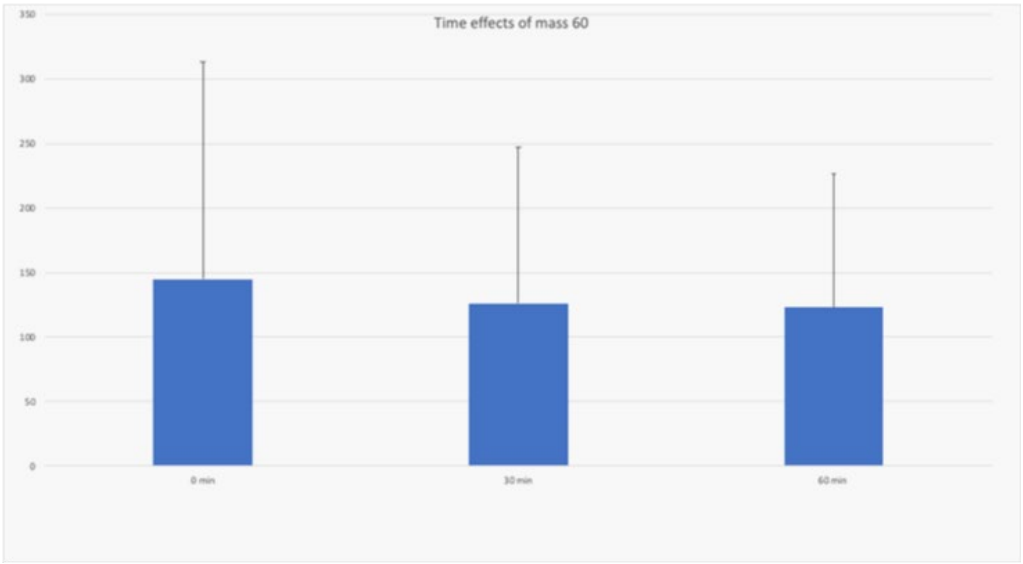
C



D



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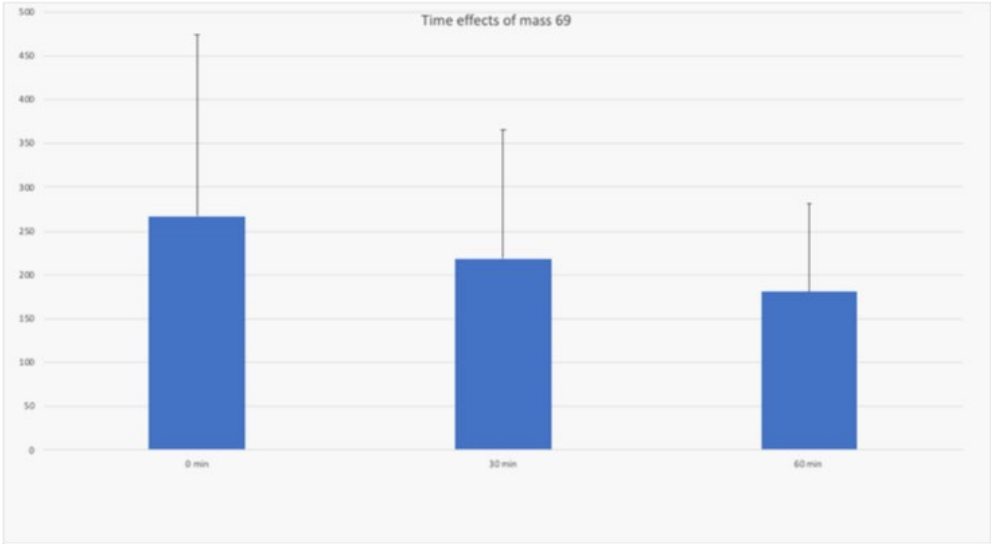
E



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