

## **Sex- and lifestyle-related factors are associated with altered hepatic CYP protein levels in people diagnosed with mental disorders**

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### **List of abbreviations:**

ACN (acetonitrile), BMI (body mass index), BSA (bovine serum albumin) CYP (cytochrome P450), Eqn (equation), FA (formic acid), HLM (human liver microsomes), LC–MS/MS (liquid chromatography–mass spectrometry), LOD (limit of detection), LLOQ (lower limit of quantification), MPPGL (microsomal protein pr. g liver), pHLM (pooled HLM), peptide peak area ratio (PAR), PMI (postmortem interval), SIL-IS peptides (stable isotopic labelled-internal standard peptides), and UPLC (ultra-performance liquid chromatography).

## Abstract

In this study, we used human postmortem tissue to investigate hepatic protein expression levels of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 by LC–MS/MS in a population of people suffering from mental disorders (n=171). We report hepatic protein levels of these six CYP isoforms in 171 individuals in total, and define a focused population dataset of 116 individuals after excluding 55 samples due to low Microsomal Protein Per Gram of Liver (MPPGL) yield. Postmortem decay was most likely the reason for the low MPPGL yield in the 55 samples. In the focused population, we found women to have significantly higher protein levels of CYP3A4 than men in addition to decreased CYP3A4 protein levels among obese individuals. Furthermore, MPPGL was negatively correlated with body mass index (BMI). An increase in CYP1A2 protein levels was observed among smokers and increased CYP2E1 protein levels were observed among individuals with a history of alcohol abuse. Finally, individuals who received phenobarbital (CYP3A4 inducer) had significantly higher CYP3A4 levels. In conclusion, lifestyle-related factors prevalent among people suffering from mental disorders are associated with altered CYP protein levels which may alter drug metabolism and affect the efficacy of commonly prescribed drugs. Furthermore, this investigation demonstrates that postmortem hepatic tissue can be used to study how lifestyle and effectors affect hepatic CYP-levels in a large cohort of patients.

## **Significance Statement**

Using a large number of postmortem hepatic tissue specimens (n=116) originating from the autopsy of individuals diagnosed with mental disorders, we were able to show that hepatic CYP-levels were affected by alcohol, smoking, BMI, and sex and that MPPGL was affected by BMI. These lifestyle-related changes may alter drug metabolism and affect the efficacy of commonly prescribed drugs. It is a novel approach to use a large postmortem cohort to investigate how lifestyle and effectors affect hepatic CYP-levels.

## Introduction

Individuals with severe mental disorders have a life expectancy approximately 15-20 years lower than that of the general population (Wahlbeck et al., 2011). Aside from the increased frequency of suicide, known risk factors for reduced life expectancy are the increased prevalence of cardiovascular diseases and lifestyle-related factors, such as diabetes, metabolic syndrome, obesity, smoking, and alcohol and drug abuse (Toftdahl et al., 2016).

Lifestyle-related factors as e.g. obesity and smoking have been associated with altered drug elimination (Brill et al., 2012; Kroon 2007; van Rogen et al., 2018). These findings are corroborated by altered hepatic cytochrome P450 (CYP)-expression or CYP-activity of important drug-metabolizing CYP-isoforms. There is substantial evidence that smoking induces CYP1A2 (Schrenk et al., 1998), heavy drinking (ethanol) induces CYP2E1 (Dangi et al., 2021; Lu and Cederbaum 2008; Novak and Woodcroft 2000), CYP3A4 expression and activity varies between sexes (Wolbold et al., 2003; Yang et al., 2012; Parkinson et al., 2004), and high body mass index (BMI) is associated with reduced CYP3A4 activity (Krogstad et al., 2021; Sandvik et al., 2020; Ulvestad et al., 2013). These changes potentially reduce the efficacy of a wide range of drugs used for the treatment of patients suffering from mental disorders and increase the risk of intoxication. These observations are usually made in populations investigating one specific lifestyle factor by estimating CYP activity either by in vitro assays with human liver microsomes (HLM) or in vivo using the metabolism of a probe compound as a measure of CYP activity (Krogstad et al., 2021; Sandvik et al., 2020; Schrenk et al., 1998). Studies investigating how lifestyle-related factors affect the CYP protein expression levels are much less available and typically include few individuals (<30 individuals) (Achour et al., 2014; Ulvestad et al., 2013; Zhang et al., 2016) or use pooled HLMs (Dangi et al., 2021).

Hepatic CYP expression levels can be quantified in fresh hepatic tissue using western blotting (Shimada et al., 1994) or by targeted proteomics techniques (Achour et al., 2014; Langenfeld et al., 2009; Zhang et al., 2016). Previous studies have shown that quantification of CYP-enzymes can be performed in postmortem hepatic tissue by LC–MS/MS measurement of CYP isoform-specific peptides. Depending on the postmortem interval and temperature, CYP enzymes will degrade over time, but in many cases, meaningful quantification is possible and will reflect antemortem levels (Hansen et al., 2019; Pedersen et al., 2021).

This study aimed to investigate the correlation between hepatic CYP-expression levels and sex and lifestyle-related factors in a subpopulation of the SURVIVE cohort, which is an autopsy-based study of a large number of individuals suffering from mental disorders (n=500) (Banner et al., 2018). By modifying a previously developed LC–MS/MS-based method (Hansen et al., 2019) quantification of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 was performed in postmortem human hepatic tissue and correlated to individual information on alcohol abuse, smoking habits, obesity, and sex.

## Materials and Methods

### Reagents and solutions

Reagents were purchased from Sigma–Aldrich (St. Louis, MO, US) unless otherwise stated. Methyl methanethiosulfonate (MMTS) was dissolved in 2-propanol at a concentration of 80 mM, and tris(2-carboxyethyl)phosphine (TCEP) was prepared as 35 mM in 1.5 M ammonium bicarbonate (Ambic). The Bradford protein assay for Microsomal Protein Per Gram of Liver (MPPGL) determination was from Bio-Rad (Hercules, CA, US), and TPCK-treated trypsin for protein digestion was from AB-SCIEX (Washington, DC). Ultrapure water (type 1) was obtained from a Milli-Q IQ 7000 apparatus (Millipore, Bedford, MA, US).

### Characteristics of the population

The population was part of a Danish autopsy-based study called the SURVIVE project (Banner et al., 2018). The individuals included in the project (n=500) were diagnosed or had indications of diagnosis within the section ICD-10 F20-F29 and F30-F39 ((WHO) 1993). Only 250 of the 500 individuals were selected for this study due to workload considerations; 50% of these were intoxication cases caused by CYP-metabolized drugs, and 50% were nonintoxications. Seventy-nine cases were excluded due to visible degradation of the hepatic tissue, resulting in 171 cases included in this study. The project was approved by the National Committee on Health Research Ethics (registration number 1305373), and the deceased individuals were only included in the study if informed consent was given by the next of kin. A full toxicological analysis was performed for all individuals (Reuss et al., 2021), and additional information regarding alcohol and smoking history was based on statements (police/next-of-kin/general practitioner) collected from the autopsy report. PMI (postmortem interval) information was available in approximately half of the cases and was used to

describe the estimated time from death to autopsy. PMI was estimated as either (days from date of death to date of autopsy) or (days from date of last sign of life to date of find)/2+(days from date of find to date of autopsy), depending on the information available.

### **Preparation of human liver microsomes**

Hepatic tissue was collected from 171 individuals during forensic autopsies as part of the SURVIVE project (Banner et al., 2018). The liver samples were stored at -80 °C until further use. HLM was isolated as previously described (Pedersen et al., 2021). In brief, 500 mg of hepatic tissue was minced with a scalpel and homogenized together with cold homogenization buffer (0.1 M potassium phosphate buffer with 0.125 M potassium chloride and 1 mM EDTA, pH 7.5) in a glass homogenizer (Potter-Elvehjem type glass homogenizer, VWR) using a motor-driven (RW16 motor, IKA) Teflon pestle. The homogenate was centrifuged at  $9000 \times g$  for 20 min, and the supernatant (S9 fraction) was transferred to centrifugation tubes (Beckman Coulter). The S9 fraction was further centrifuged at  $100,000 \times g$  for 75 min at 4 °C using an Optima L-90K ultracentrifuge (Beckman Coulter, Inc.), after which the pellet was carefully dissolved in storage buffer (50 mM phosphate buffer with 250 mM sucrose, pH 7.5) using a glass pipette. The yield of HLM protein (MPPGL) was determined by the Bradford protein assay using a bovine serum albumin (BSA) standard curve. MPPGL was not corrected for process loss (all values reported as uncorrected results).

### **Peptide selection**

Tryptic peptides suitable for LC–MS/MS quantification based on the AQUA strategy (Gerber et al., 2003) were selected based on the literature or in silico digestion. CYP1A2:

YLPNPALQR (Prasad et al., 2018), 2C9: GIFPLAER (Kawakami et al., 2011), 2C19: GHFPLAER (Achour et al., 2014), CYP2D6: SQGVFLAR, CYP2E1: FITLVPSNLPHEATR (Gröer et al., 2014) and CYP3A4 LSLGGLLQPEKPVVLK (Russell et al., 2013). The peptides were purchased as synthetic peptides (SpikeTides™, JPT) and the collision energy was optimized by direct infusion into the mass spectrometer. MRM transitions were evaluated in terms of signal intensity and linearity and the two best performing transitions were selected for each peptide (Supplementary Table S1). Stable isotope-labelled internal standard peptides (SIL-IS peptides) containing C-terminally isotopically labelled Arg ( $^{13}\text{C}_6^{15}\text{N}_4$ ) or Lys ( $^{13}\text{C}_6^{15}\text{N}_2$ ) were purchased in purified forms and in specified quantities (SpikeTides™ TQL, JPT). The SIL-IS peptides contained a tag used for quantification by the manufacturer (JPT quant-tag), which was enzymatically cleaved off during sample preparation (trypsin digestion).

### Trypsin digestion

The isolated HLMs and added SIL-IS peptides were subjected to enzyme digestion following a previously described protocol, with minor deviations (Hansen et al., 2019). In brief, all HLM preparations were diluted in microsomal storage buffer to a working solution of 5 µg protein/µL. In Eppendorf LoBind tubes, 10 µL of diluted HLM protein (50 µg) was added to 10 µL of H<sub>2</sub>O and 5 µL of IS-peptides (200 fmol CYP1A2/µL, 100 CYP2C19 fmol/µL, 500 CYP2C9 fmol/µL, 150 CYP2D6 fmol/µL, 500 CYP2E1 fmol/µL, and 500 CYP3A4 fmol/µL) dissolved in 0.1 M AmBic with 20% acetonitrile (ACN). Then, 5 µL of a 12% sodium deoxycholate (SDC) solution was added to the mix and incubated at 80 °C for 10 min with vigorous rotational shaking. The denatured mix was cooled to 60 °C, and 5 µL of 35

mM TCEP was added, followed by incubation for 20 min at 60 °C. The mix was then cooled to room temperature and alkylated by incubation with 5 µL of 80 mM MMTS for 20 min. The solution was diluted with 70 µL of 25 mM AmBic, and 10 µL of trypsin mix (0.033 µg trypsin/µL) prepared in 25 mM AmBic with 2 mM CaCl<sub>2</sub> was added. The digestion proceeded for 4 h at 37 °C, after which the SDC was precipitated by the addition of formic acid (FA) to a final concentration of 0.3%. The solution was vigorously vortexed followed by centrifugation at 15,000 × g for 2 min, after which the supernatant was moved to a glass vial and analysed by LC–MS/MS.

### Estimation of CYP levels

The digested peptides were analysed using UPLC (ultra-performance liquid chromatography) (Waters Acquity) coupled to a triple quadrupole mass spectrometer (Waters Xevo TQ-S) by adding the MRM-transition described in Table S1 to a previously described LC–MS/MS method (Hansen et al., 2019). The targeted LC–MS/MS method consisted of monitoring the selected unique signature peptides along with SIL-IS peptides containing either stable or isotopically labelled Arg (\***R** = R+10 Da) or Lys (\***K** = K+8 Da). Samples were analysed in duplicate, and the mean of the duplicates was reported. See supplementary data Table S1 for information regarding peptide sequences, m/z of the MRM transitions, cone voltage, and optimized collision energy for the signature peptides and SIL-IS used in this study.

$$\text{pmol}_{\text{signature peptide}} = \frac{\text{Area signature peptide}}{\text{Area SIL-IS peptide}} \times \text{pmol}_{\text{SIL-IS peptide}} \quad \text{Eqn. 1}$$

$$[\text{CYP}] = \frac{\text{pmol}_{\text{signature peptide}}}{\text{mg HLM protein}} \quad \text{Eqn. 2}$$

CYP concentrations were determined based on the peptide peak area ratio (PAR) response by AQUA (Gerber et al., 2003) (Eqn. 1); the CYP levels are normalized for HLM and reported as pmol CYP/mg HLM (Eqn. 2).

### Method validation

An ultra HLM pool (n=150, lot#38294) referred to as pooled HLM (pHLM) was purchased from BD Biosciences (New Jersey, US) and used for method validation.

To determine the precision of the method, the pHLM was analysed as four technical replicates on seven different days. The precision was calculated for within- and between-run variation using a one-way ANOVA approach, and CV < 20% was accepted. The limit of detection (LOD) and lower limit of quantification (LLOQ) of the method were determined based on the SIL-IS-peptide because no blank matrix exists. HLM was spiked with low concentrations of SIL-IS-peptides CYP1A2 (50 fmol), CYP2C19 (50 fmol), CYP2C9 (125 fmol), CYP2D6 (75 fmol), CYP2E1 (125 fmol), and CYP3A4 (125 fmol) and was subjected to enzyme digestion performed as three reactions per day on three different days (a total of nine reactions) with a CV < 20%. The LOD was estimated as three times the standard deviation (SD) of the reactions, and the LLOQ was estimated as ten times the SD. The peak areas were converted into fmol by one-point calibration using the average SIL-IS-peptide peak area from five samples. The trueness of the method at the peptide level was investigated for all CYP isoforms by comparing the closeness of agreement between duplicate unspiked HLM samples and duplicate HLM samples spiked with unlabelled synthetic target peptides (SpikeTides™ TQ, JPT). Trueness was reported as the mean of two peptide levels, CYP1A2 (400/800 fmol), CYP2C19 (100/200 fmol), CYP2C9 (1000/2000 fmol), CYP2D6 (150/300 fmol), CYP2E1(1000/2000 fmol), and CYP3A4 (1000/2000 fmol) per 50 µg HLM. The trueness of the method for the protein level was evaluated for CYP2C9, CYP2C19, CYP2D6,

and CYP3A4 using commercially available isolated quantified recombinant CYP protein at 10 nmol/mL purchased from Cypex (Dundee, Scotland, UK). Purified CYP proteins (150/300 fmol CYP2C19, 1000/2000 fmol CYP2C9, 150/300 fmol CYP2D6, and 1000/2000 fmol CYP3A4) were spiked into duplicate HLM samples and compared to duplicate unspiked HLM samples. Trueness was reported as a mean of the two spike levels and calculated using Eqn. 3.

$$\text{Trueness (\%)} = \frac{[\text{spiked samples}] - [\text{unspiked samples}]}{\text{spike concentration}} \times 100 \quad \text{Eqn. 3}$$

### Statistical analysis

The CYP protein levels were not normally distributed for any of the investigated CYP isoforms; therefore, Wilcoxon rank sum tests were performed for analysis of two variables, and Kruskal–Wallis tests with pairwise Wilcoxon rank sum post hoc tests were performed for analysis of multiple variables using R (R Core Team 2021). Significance levels were reported as \*(<0.05), \*\*(<0.01), and \*\*\* (<0.0001) or exact p-values.

## Results

The performance of the LC–MS/MS proteomics method for estimating CYP expression in terms of LOD/LLOQ, ULOQ, precision, and trueness validated in pHLM is presented in Table 1.

Microsomal yield (MPPGL) and CYP protein level estimations for CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 are presented in Table 2. All samples with MPPGL < 4.0 mg/g liver (55 samples) were excluded in an attempt to remove the most degraded samples from our population. The use of a MPPGL cut-off value to exclude degraded samples was motivated by the unexpected association between increasing mean CYP protein levels (pmol CYP/mg HLM) at increasing MPPGL cut-off values which is presented in Figure 2. The population with a MPPGL above the cut-off value of 4.0 mg/g was annotated as the ‘focused population’ and consisted of 116 samples. The mean MPPGL of the focused population was 6.1 mg/g. For CYP2C19, two samples were lower than the LLOQ, and for CYP2D6, 15 samples were lower than the LLOQ, of which eight samples were lower than the LOD. Due to large variation between duplicates, thirteen CYP3A4 measurements were excluded. The removal of samples with MPPGL < 4.0 mg/g increased the population means of the CYP protein levels by 20-25% in the focused population compared to the total population (Table 1). The distribution and mean CYP protein levels of the focused population are displayed in Figure 1, along with pHLM measurements.

The characteristics of the focused population are shown in Table 3. We observed a significant difference between men and women for CYP3A4 protein levels in the focused population, with women having almost twice as high a median level of CYP3A4 compared to men (Figure 3).

We observed a statistically significant difference between obese individuals and individuals with normal BMI (Figure 4). The obese individuals exhibited less than half of the median hepatic CYP3A4 levels compared to the other BMI categories. There was no statistically significant difference between BMI groups for any other measured CYP isoforms (data not shown). However, we observed a trend of low MPPGL among individuals with high BMI (Figure S1). The distribution of MPPGL for all samples and the focused population is presented by sex in Figure S2 and S3, and the relation between liver weight and BMI in the focused population in Figure S4.

The median CYP3A4 was 4-fold higher among individuals positive for phenobarbital (n=5) in the toxicology analysis (Figure 5), and this difference was statistically significant.

The median CYP2E1 protein levels among individuals with no history or unknown history of alcohol abuse were 23.5 and 23.4 CYP2E1 pmol/mg HLM, respectively, whereas the individuals with a history of alcohol abuse had a 30% higher median level of 33.6 pmol/mg HLM (Figure 6). Additionally, we observed a 2-fold higher median CYP1A2 protein level among individuals with a history of smoking than among individuals with an unknown smoking history (Figure 6).

## Discussion

In this study, we investigated hepatic CYP protein levels by a validated LC–MS/MS proteomics method in a large postmortem population and correlated the data to information about sex- and lifestyle-related factors. By correlating the history of smoking with CYP protein levels, we found a 2-fold higher CYP1A2 level among smokers than among individuals with an unknown smoking history (Figure 6). Although the exact induction degree of CYP1A2 due to smoking is biased by possible smokers in the unknown smoking history group, to the best of our knowledge, this is the first study to show that smoking induces an increase in hepatic CYP1A2 at the protein level in humans. Huang et al. found that it is a combination of the CYP1A2\*1F/\*1F genotype and smoking that induces CYP1A2 activity (Huang et al., 2016) this an uninvestigated covariate in this study, and this could bias the found effect of smoking on CYP1A2 induction.

Our data showed a 30% increase in CYP2E1 protein levels in individuals reported to have a history of alcohol abuse compared to individuals with no history or unknown history of alcohol abuse (Figure 6). Similar findings have previously been reported in other studies (Lu and Cederbaum 2008; Novak and Woodcroft 2000). This increase in CYP2E1 protein levels is lower than that reported by Dangi and colleagues, who found a 2-3.4-fold difference when investigating CYP2E1 (quantified by LC–MS/MS) in pooled HLMs from normal donors and heavy alcohol consumers (Dangi et al., 2021). Considering that we do not know the exact alcohol abuse level of our population, the lower fold-difference can be explained by a more moderate alcohol abuse level in our population. The 75<sup>th</sup>-percentile CYP2E1 level (corresponding to the more heavy alcohol abusers) in our population was 59.5 pmol/mg HLM, a 2.5-fold difference from the nonabusers. Other proteomics studies have failed to detect a correlation between CYP protein levels and smoking or alcohol consumption;

however, the number of affected individuals was much lower (Achour et al., 2014; Zhang et al., 2016).

For CYP3A4, we found a significantly higher median level of CYP3A4 protein among women compared to men (Figure 3) in the focused population. This difference is similar to the 2-fold increase in protein expression, which has been reported using western blotting (Wolbold et al., 2003), in line with a 2-fold higher CYP3A4 enzyme activity in female cryopreserved hepatocytes (Parkinson et al., 2004), and comparable to results of gene expression studies (Yang et al., 2012). Furthermore, we found 4-fold higher median CYP3A4 protein levels among five cases positive for phenobarbital, a known inducer for CYP3A4 (Figure 5).

Other studies have reported a correlation between CYP3A4 activity and body weight (Krogstad et al., 2021) or BMI (Sandvik et al., 2020) or CYP3A4 expression and BMI (Ulvestad et al., 2013). When categorizing BMI, we observed a statistically significant difference in CYP3A4 levels between individuals with normal BMI and obese individuals (Figure 4). The other investigated CYP isoforms did not exhibit significant correlations with BMI. Furthermore, we observed a negative correlation between BMI and MPPGL (Figure S1), which may be a result of higher fat content in the tissue among individuals with high BMI (Fan et al., 2018).

The expression level of CYP3A4 was twofold higher in females, but BMI/CYP3A4 data were not separated by sex, which could be a source of bias for the found correlation because the BMI distribution of women was lower than that of men in the focused population (Table 2). The studies by Krogstad et al. 2021, Sandvik et al. 2020 and Ulvestad et al. 2013 also included a mix of males and females. When separating the data on sex (female n=43, male n=70), the median CYP levels for normal/obese patients were 33.0/13.2 pmol/mg HLM for

females and 14.8/7.0 pmol/mg HLM for males, but these differences were not statistically significant. The trend of BMI impacting the CYP3A4 hepatic content is still present, but further studies should remove sex as a source of bias when investigating the BMI/CYP3A4 correlation.

A limitation of the method used in this study is that the trueness estimations are not close to 100% for the quantification of some of the CYP protein forms. The method validation (Table 1) established acceptable results for limits of quantification, precision (within-run and intermediate/total precision <17.6%) and trueness when evaluated on the levels of synthetic peptides spiked into the correct matrix (<26% deviation). However, when quantifying whole purified CYP proteins spiked into the correct matrix we reached acceptable trueness only for some isoforms (e.g. CYP2D6, <22% deviation) whereas we underestimated other isoforms (e.g. CYP3A4 and CYP2C19, <59% and <44% deviation). We ascribe this to incomplete trypsin cleavage and release of signature peptides in the context of a whole protein since the trueness estimates from experiments with cleavable synthetic peptides were better.

Another major limitation of this study is the likely presence of postmortem degradation in our dataset. The postmortem stability of CYP1A2 and CYP3A4 was previously investigated in a postmortem population (Hansen et al., 2019), and using a porcine animal model (Pedersen et al., 2021). Both studies found PMI and temperature to be very important for the protein stability of the CYP isoforms, and low MPPGL has been associated with postmortem degradation (Hansen et al., 2019; Pedersen et al., 2021). In the study by Pedersen et al. 2021, both MPPGL and the concentration of CYP isoforms (pmol/mg microsomal protein) decreased as the liver was putrefied. In the current investigated population, there was a clear positive correlation between MPPGL (using a rising MPPGL cut-off level) and CYP levels (Figure 2), which is in contrast to results by Zhang et al. 2015 (Zhang et al., 2015) who found no association between MPPGL content and CYP activities. The focused population had a

mean uncorrected MPPGL of 6.1 mg/g (Table 2), which is lower than the 15 mg/g reported by Vasilogianni et al. 2021. We have previously shown that the protocol used for liver microsome purification (Pedersen et al. 2021) was performed with high repeatability and satisfying uncorrected yields. Therefore, we believe that the most plausible reason for the low MPPGL values and the association between MPPGL and CYP levels is postmortem degradation. We introduced an MPPGL cut-off value to remove the most degraded samples from the population. The choice of an MPPGL cut-off value of 4.0 mg/g is based on Figure 2, as an compromise between excluding the most degraded samples but still maintaining a large sample size. In our attempt to exclude degraded samples we may have excluded nondegraded samples with naturally low levels of MPPGL from our population, and we may still have moderately degraded samples in our population; however, this was a transparent way of improving the overall quality of the population. It should be noted that the aforementioned correlations also were significant in the total population. No correlation between PMI and CYP-levels was found (data not shown).

The estimated CYP levels in the pHLM were within or close to published literature values (Table 1) for all CYP isoforms, indicating that the developed method performs on par with previously published methods.

Comparing the mean value of the different CYP isoforms for the focused population with the pHLM is another way to assess the impact of postmortem degradation. The mean values of CYP2C9, CYP2D6, CYP2E1, and CYP3A4 in the focused population were 33-45% lower than those in the pHLM. Postmortem degradation could be a possible explanation for this observation. The limitation of this approach is of course the difference in the populations behind the pHLM compared to this study, where differences in sex and lifestyle factors and genetics may explain the lower found levels.

Due to the large natural interindividual variation of up to 30-100-fold in the expression of CYP isoforms (Tracy et al., 2016) a large number of individuals is needed to obtain sufficient power to show a statistically significant difference for a given effector. Some studies of CYP-effectors are clearly underpowered, but by using postmortem material, we increased access to human hepatic specimens and were able to find several correlations. By investigating postmortem hepatic tissue from a population with mental disorders, we found that smoking was correlated with induced CYP1A2 levels, excessive alcohol consumption correlated with induced CYP2E1 expression, and increasing BMI correlated with reduced hepatic CYP3A4 expression and a trend of reduced hepatic MPPGL content. The limitation of the postmortem approach is the likely postmortem degradation of samples. Even though there are signs of postmortem degradation in the focused population, the power of a large number of individuals made us able to find the aforementioned correlations verifying the usefulness of postmortem tissue for studying CYP expression levels in large cohorts of individuals. In the investigated population with mental disorders, there was a large proportion of smokers, individuals with excessive alcohol consumption, and obesity, and these lifestyle factors resulted in altered hepatic CYP levels. Considering that numerous commonly prescribed drugs are metabolized by CYP1A2 and CYP3A4 (Williams et al. 2004), the altered CYP levels in a population of people suffering from mental disorders results in affected drug metabolism, thus lowering the efficacy of standard doses in this population. In conclusion, these findings emphasize the need for a personalized medicine approach in a population of people suffering from mental disorders.

### **Data Availability Statement**

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

### **Authorship Contributions**

Participated in research design: Pedersen, Hansen, Hasselstrøm, Banner, Jornil

Conducted experiments: Pedersen, Hansen

Performed data analysis: Pedersen, Hansen, Hasselstrøm, Jornil

Wrote or contributed to the writing of the manuscript: Pedersen, Hansen, Hasselstrøm, Banner, Jornil

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## **Footnotes**

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## **Conflict-of-interest**

No author has an actual or perceived conflict of interest with the contents of this article.

## Figure Legends

Figure 1: Distribution of CYP protein levels in the focused postmortem population. Left: Highly abundant CYP isoforms CYP1A2, CYP2C9, CYP2E1, and CYP3A4. Right: Lowly abundant CYP isoforms CYP2C19 and CYP2D6. Sample sizes, n=101-116. The horizontal bar represents the mean of the isoform. The black dots represent CYP protein level estimations of the commercially available pHLM (n=150)

Figure 2: Association between CYP levels and MPPGL cut-off. One data point represents the mean CYP level for the population at a given MPPGL cut-off. n=171(all data), n=28 (MPPGL cut-off=7 mg/g). Population size for CYP3A4 was from ‘all data’ to MPPGL cutoff=7 mg/g n=143, 128, 115, 103, 73, 45, 24.

Figure 3: Correlation between CYP3A4 protein levels and sex. The horizontal bar represents the median of the group. Wilcoxon rank sum test.

Figure 4: Association between BMI and CYP3A4 protein levels. The horizontal bar represents the median of the group. Kruskal–Wallies test CYP3A4 p= 0.009. Pairwise Wilcoxon rank sum test (Normal vs. Obese, p=0.0029)

Figure 5: CYP3A4 induction by phenobarbital. CYP3A4 protein levels among phenobarbital positive cases (n=5, three men, two women). Wilcoxon rank sum tests were performed. The horizontal bar represents the median of the population

Figure 6: CYP induction is associated with alcohol abuse (left) and smoking (right). The horizontal bar represents the median of the group. Yes: known history of alcohol/smoking, No: no history of alcohol. Unknown: Unknown history. Left: Kruskal–Wallies test  $p=0.01$ . Pairwise Wilcoxon rank sum test (yes vs. no  $p=0.017$ , yes vs. unknown  $p=0.017$ ). Right: Wilcoxon rank sum test,  $p=0.019$

## Tables

Table 1: Method validation results of the investigated CYP isoforms

<b>CYP</b>	<b>1A2</b>	<b>2C19</b>	<b>2C9</b>	<b>2D6</b>	<b>2E1</b>	<b>3A4</b>
pHLM (pmol/mg HLM)	24.2	5.4	75.3	13.5	63.6	38.8
Within-run precision (%)	10.7	8.5	6.2	8.4	9.0	15.6
Intermediate/total precision (%)	12.9	11.9	6.9	12.2	9.3	17.6
LOD (pmol/mg HLM)	0.23	0.17	0.34	0.38	0.64	0.33
LLOQ (pmol/mg HLM)	0.75	0.58	1.13	1.3	2.12	1.12
ULOQ (pmol/mg HLM)	180	83	390	100	325	400
Trueness (%) (synthetic peptides)						
Low level	118	123	104	78	92	95
High level	126	122	120	97	82	95
Trueness (%) (protein)						
Low level	-	56	69	78	-	41
High level	-	58	88	97	-	62

pHLM (pooled HLM), LOD limit of detection, LLOQ lower limit of quantification, ULOQ upper limit of quantification

Table 2: MPPGL and CYP levels of the total and the focused postmortem populations.

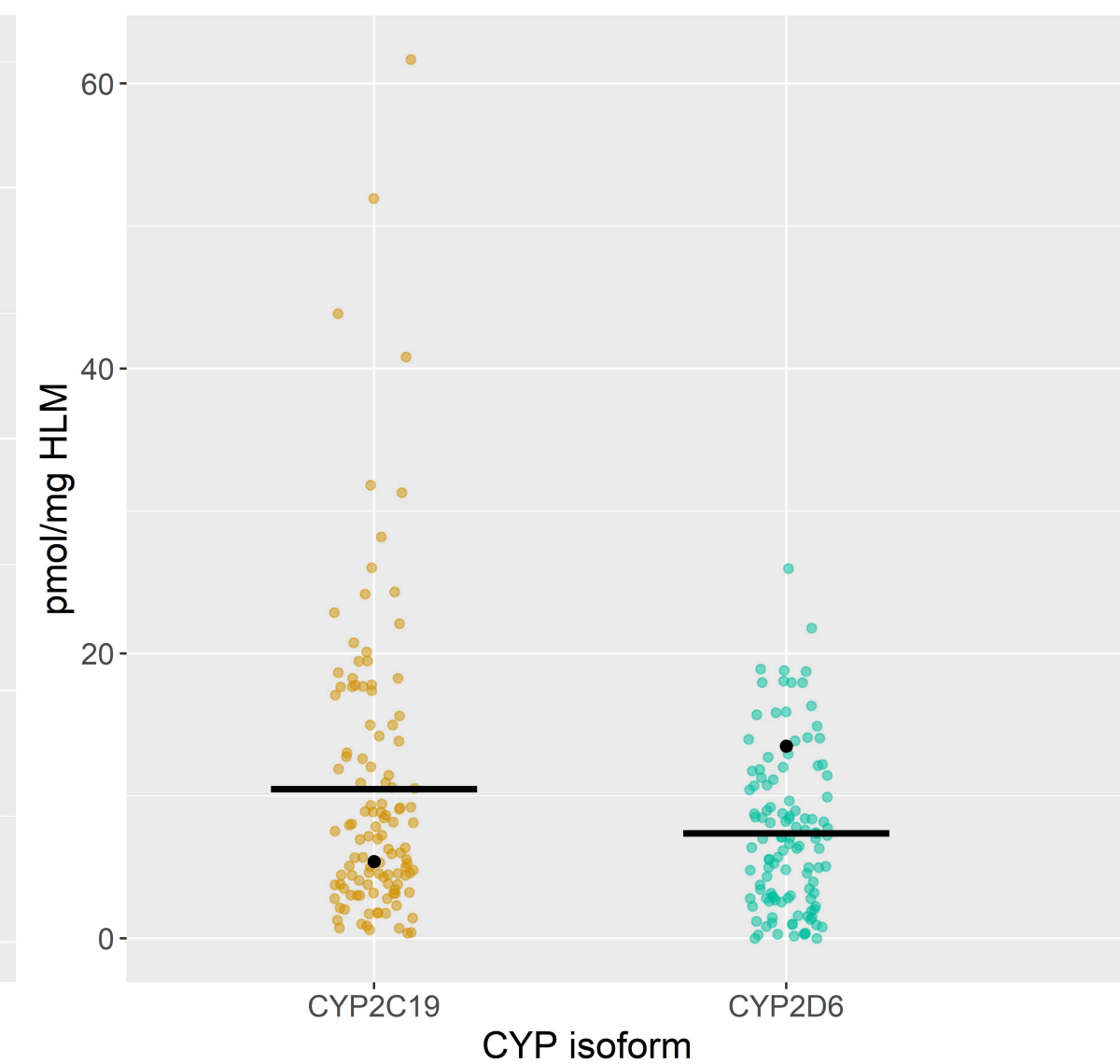
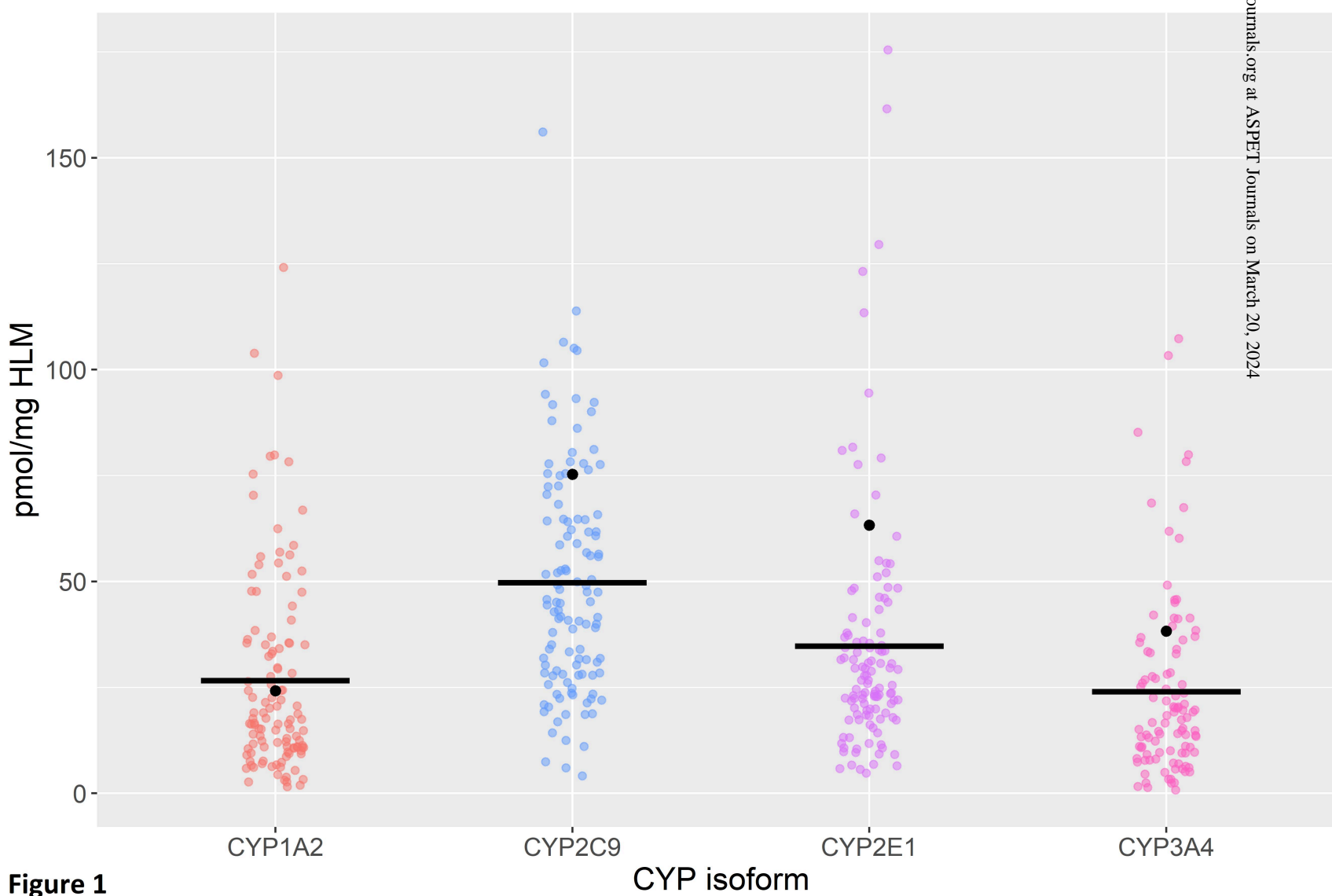
	<b>Total postmortem population</b>	<b>Focused postmortem population (MPPGL cut-off = 4.0 mg/g)</b>	<b>pHLM ±SD</b>	<b>Literature values range*</b>
<b>n</b>	171	116	150	
<b>Mean PMI (days) †</b>	5.2 (1-20) (n=77)	4.7 (1-10) (n=54)		
<b>MPPGL (mg/g) mean (range)</b>	5.0 (1.2-11.8)	6.1 (4.0-11.8)		15.8 (8.8-22.8)
<b>CYP protein levels (pmol/mg HLM)</b>				
<b>CYP1A2</b>	21.9 (97% CV)	26.6 (88% CV)	24.2±3.1	12.8-29.4
<b>CYP2C9</b>	40.3 (71% CV)	49.7 (54% CV)	75.3±5.1	37.3-80.2
<b>CYP2C19</b>	8.0 (118%CV)	10.5 (101% CV)	5.4±0.62	2.18-5.31
<b>CYP2D6</b>	6.2 (91% CV)	7.4 (76% CV)	13.5±1.6	9.3-17.2
<b>CYP2E1</b>	28.9 (97% CV)	34.7 (91% CV)	63.6±5.9	51.3-66.1
<b>CYP3A4</b>	19.0 (105% CV)	24.0 (91% CV)	38.8±6.0	32.6-68.1

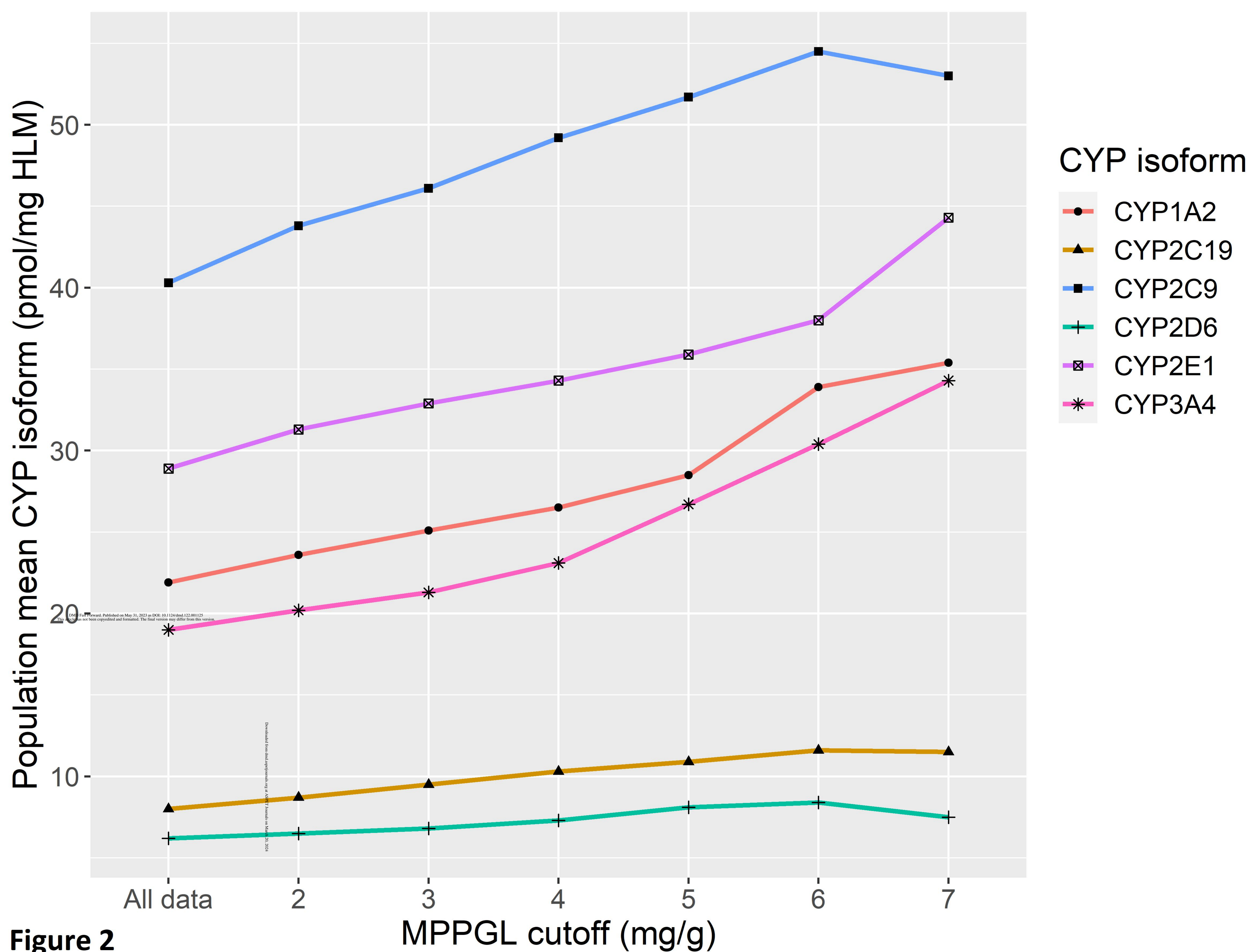
\* (Achour et al., 2014; Gröer et al., 2014; Kawakami et al., 2011; Michaels and Wang 2014; Ohtsuki et al., 2012). MPPGL (uncorrected for process loss) (microsomal protein per g liver): (Vasilogianni et al., 2021). †Information about PMI (postmortem interval) was available in only 77/171 cases for the total population and in 54/116 cases for the focused population. pHLM (pooled HLM).

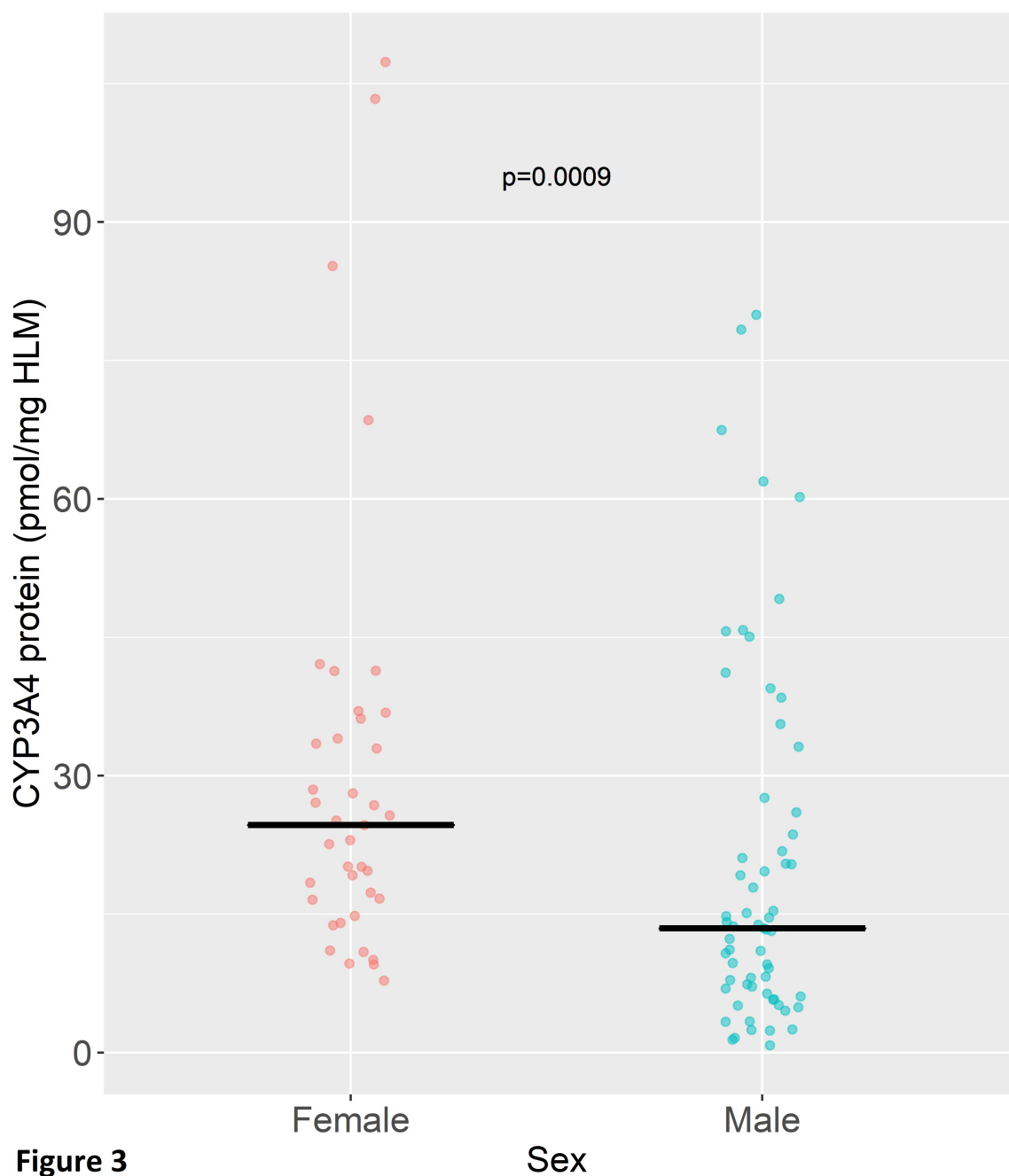
Table 3: Characteristics of the focused postmortem population.

	<b>Men</b>	<b>Women</b>
<b>n =116</b>	72 (62%)	44 (38%)
<b>Age at time of death (years)</b>	46 (18-82)	51.5 (18-85)
<b>Body weight (kg)</b>	81.2 (53-119)	65.5 (38-115)
<b>Liver weight (g)</b>	1,740 (972-3,512)	1,822 (1,076-3,504)
<b>Mean BMI (kg/m<sup>2</sup>)</b>	26.0 (16.6-37.6)	24.3(15.6-50.4)
<b>Underweight</b>	2 (3%)	4 (9%)
<b>Normal</b>	26 (36%)	24 (56%)
<b>Overweight</b>	32 (46%)	9 (21%)
<b>Obese</b>	10 (14%)	6 (14%)
<b>Alcohol history</b>		
<b>Yes</b>	27 (38%)	11 (25%)
<b>No</b>	15 (21%)	8 (18%)
<b>Unknown</b>	30 (42%)	25 (57%)
<b>Smoking</b>		
<b>Yes</b>	18 (25%)	12 (27%)
<b>No</b>	0	1 (2%)
<b>Unknown</b>	54 (75%)	31 (70%)

Data are presented as the mean and range or absolute numbers and percentages. Information about body weight and BMI was unknown for one woman and two men. BMI groups: Underweight (<18.5), normal (18.5-24.9), overweight (25-29.9), and obese (>29.9).







CYP3A4 protein (pmol/mg HLM)

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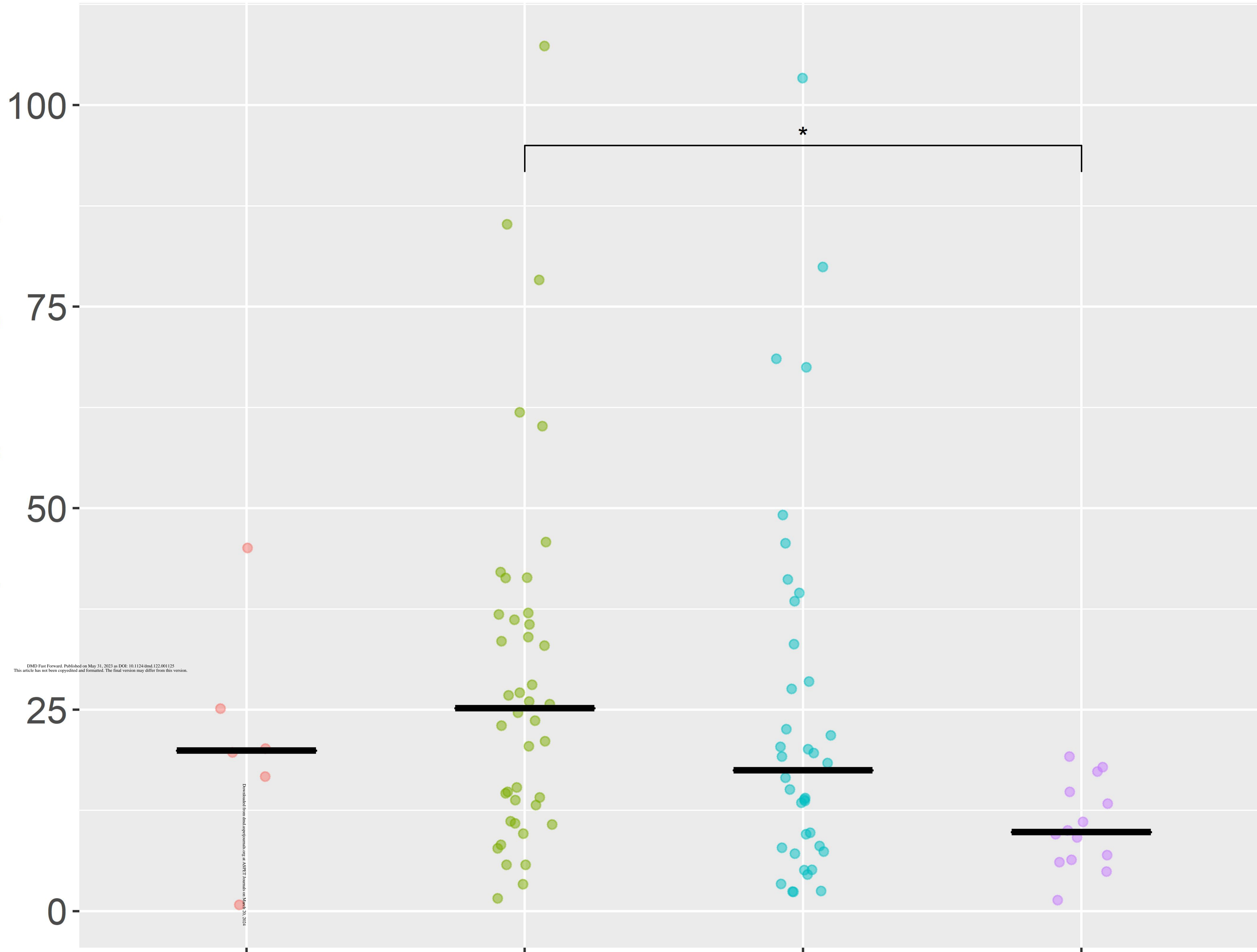
Underweight

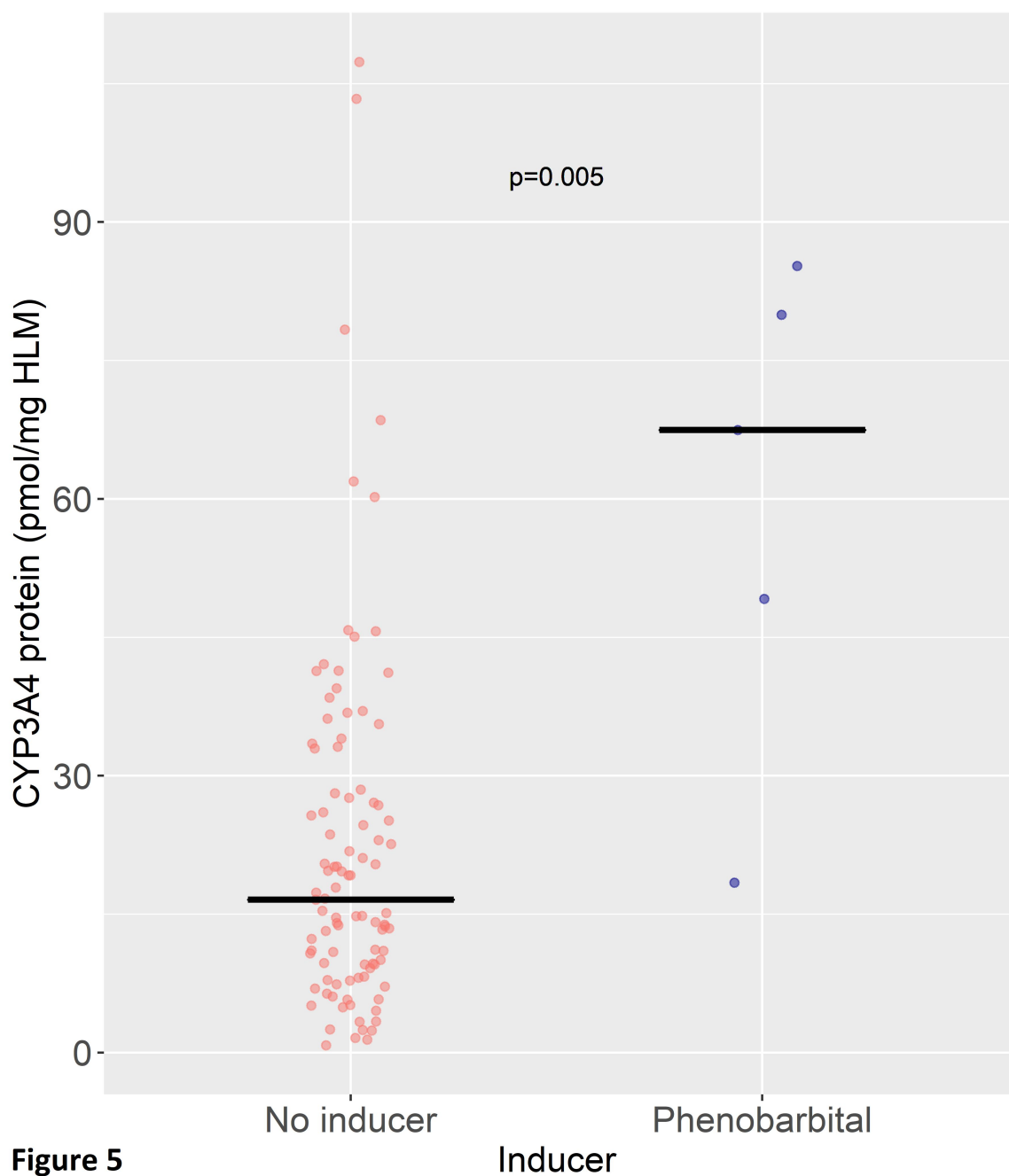
Normal

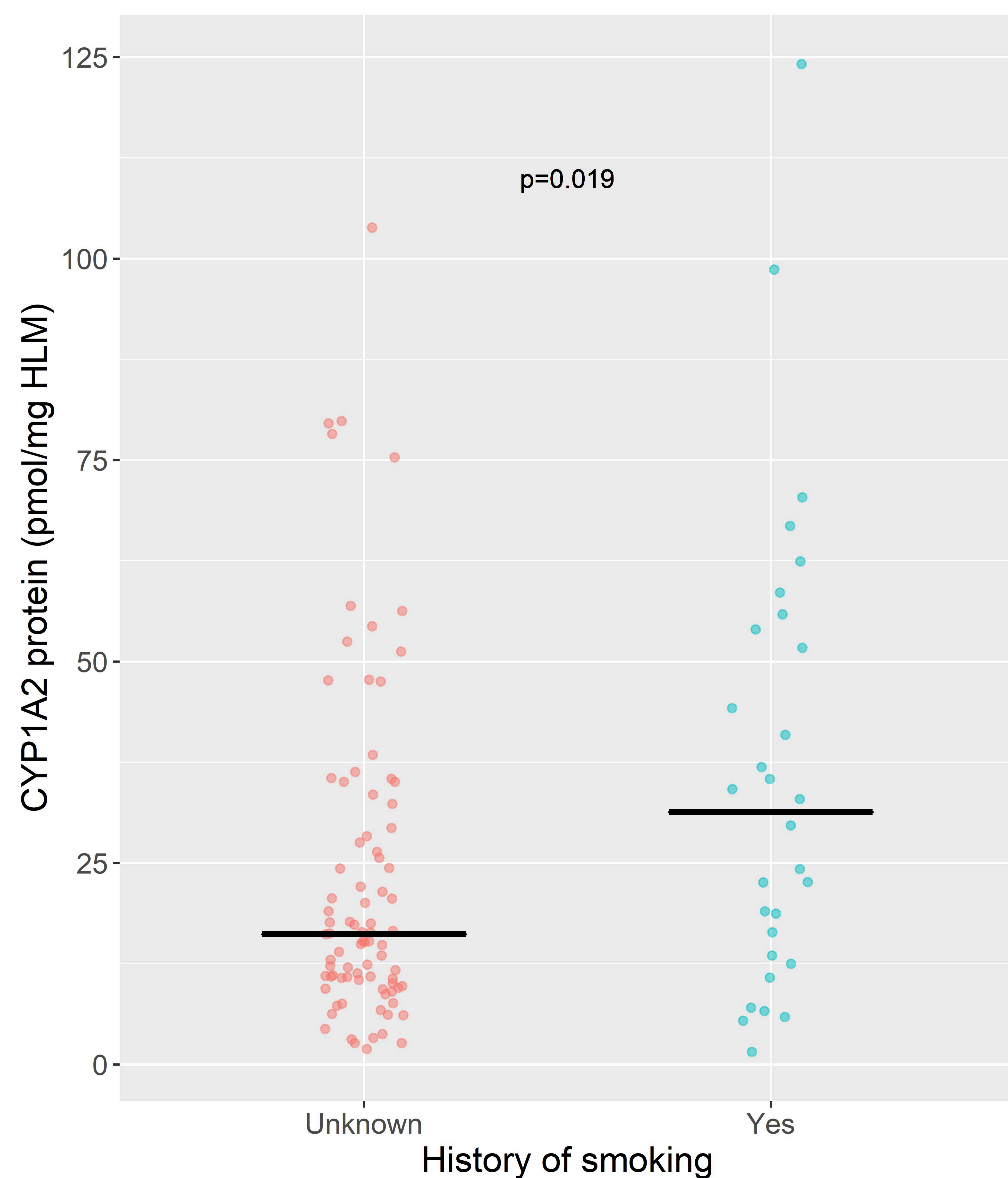
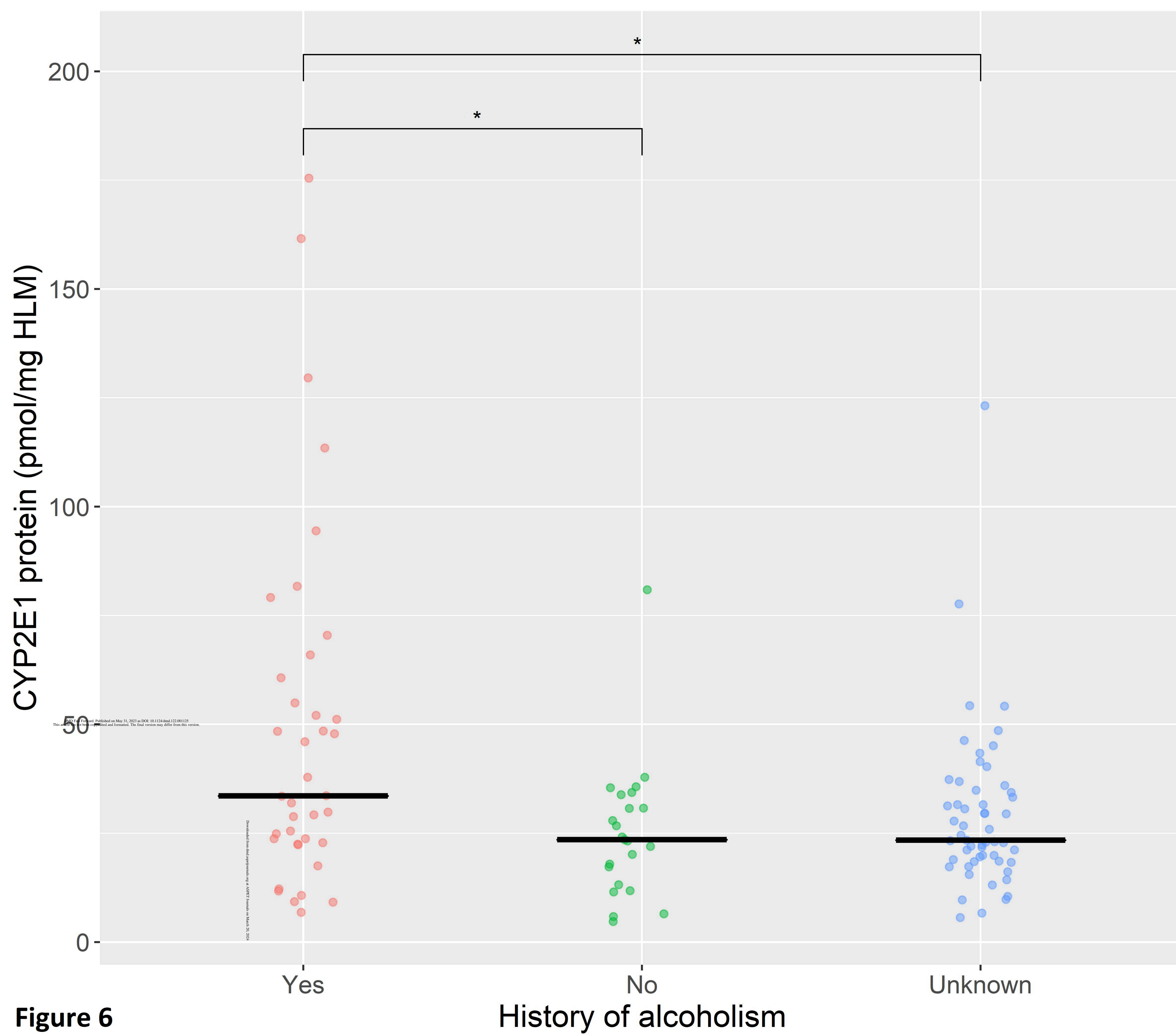
Overweight

Obese

Figure 4







**Figure 6**

**Supplementary data**

**Sex- and lifestyle-related factors are associated with altered hepatic CYP protein levels  
in people diagnosed with mental disorders**

Kata W. Pedersen, Jakob Hansen, Jytte Banner, Jørgen B. Hasselstrøm and Jakob R. Jornil.

Table S1: Transitions for LC–MS/MS quantification. The tryptic peptides were manually tuned by direct infusion, and evaluated in regard to signal intensity and linearity

CYP	Sequence	Precursor ion (m/z)	CE (eV) (quantifier/qualifier)	Product ion (m/z) (quantifier/qualifier)
CYP1A2	DITGALFK	432.7++	21/21	636.4+/535.3+
	DITGALFK*	436.8++	21/21	644.5+/543.4+
2C19	GHFPLAER	463.75++	19/17	195.09+/585.34+
	GHFPLAER*	468.75++	19/17	195.09+/595.34+
2C9	GIFPLAER	451.76++	13/13	366.71+/585.30+
	GIFPLAER*	456.76++	13/13	371.71+/595.34+
CYP2D6	SQGVFLAR	439.25++	13/15	216.1+/662.4+
	SQGVFLAR*	444.25++	13/15	216.1+/672.4+
CYP2E1	FITLVPSNLPHEATR	847.97++	30/30	1121.6+/561.3
	FITLVPSNLPHEATR*	852.97++	30/30	1131.58+/566.29+
CYP3A4	LSLGGLQPEKPVVLK	564.3+++	15/15	745.9+/789.5
	LSLGGLQPEKPVVLK*	567.3+++	15/15	750+/793.5

Collision energy (CE)

**R\***: isotopically labeled arginine ( $^{13}\text{C}_6^{15}\text{N}_4$ , +10 Da)

**K\***: isotopically labeled lysine ( $^{13}\text{C}_6^{15}\text{N}_2$ , +8 Da)

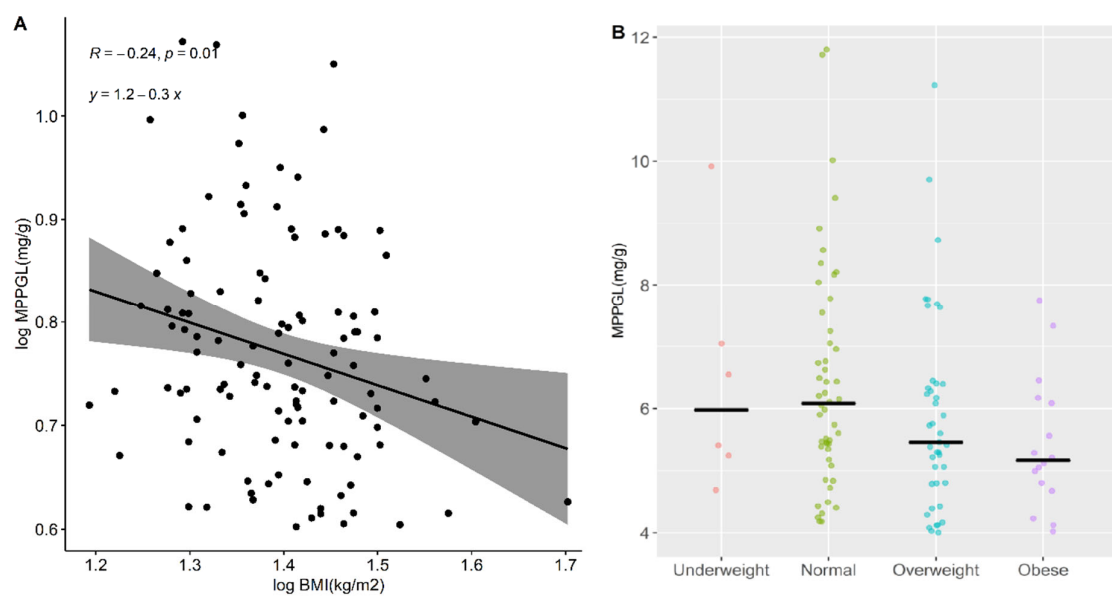


Figure S1: Correlation between MPPGL and BMI in the focused population. A: Pearson's correlation coefficient of log transformed MPPGL and BMI. B: MPPGL and categorized BMI.

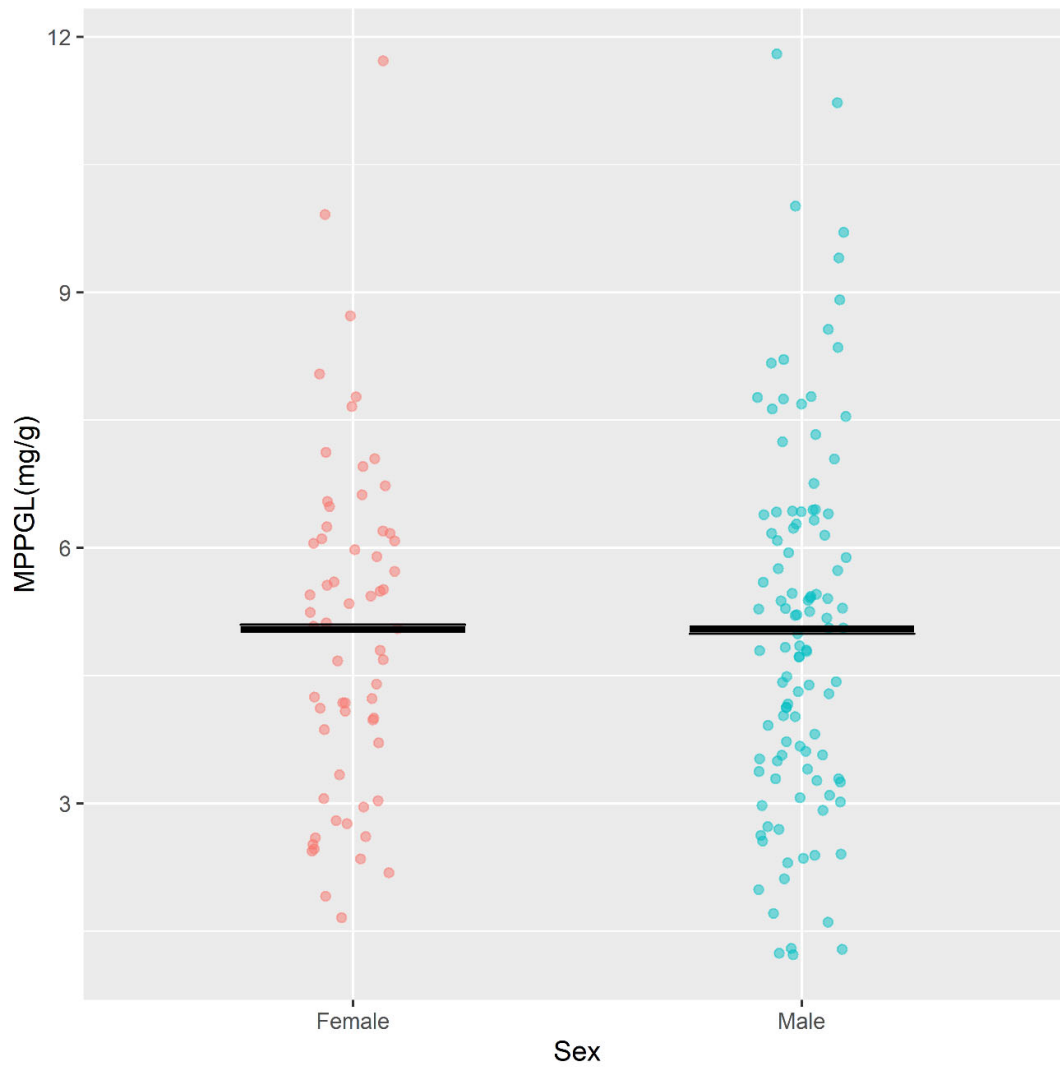


Figure S2: MPPGL distribution for female and male all individuals (n=171). (bold line = mean value, female/male= 5.04/5.05 mg/g, thin line = median value female/male= 5.10/4.99 mg/g), Wilcoxon ranked sum test: p=0.91.

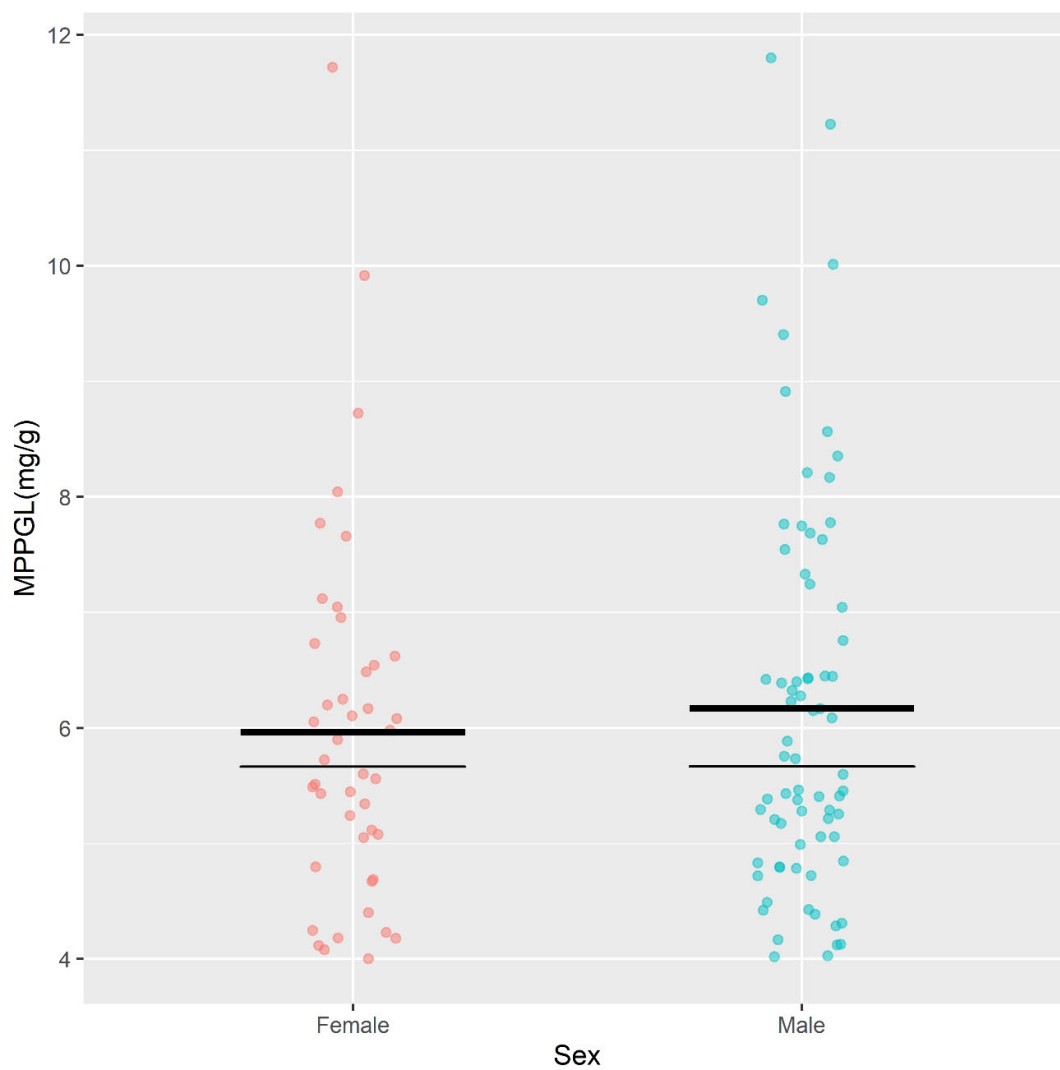


Figure S3: MPPGL distribution for male and female in focused pop(n=116). (bold line = mean value, female/male= 5.96/6.17 mg/g, thin line = median value female/male= 5.66/5.67 mg/g), Wilcoxon ranked sum test:  $p=0.65$ .

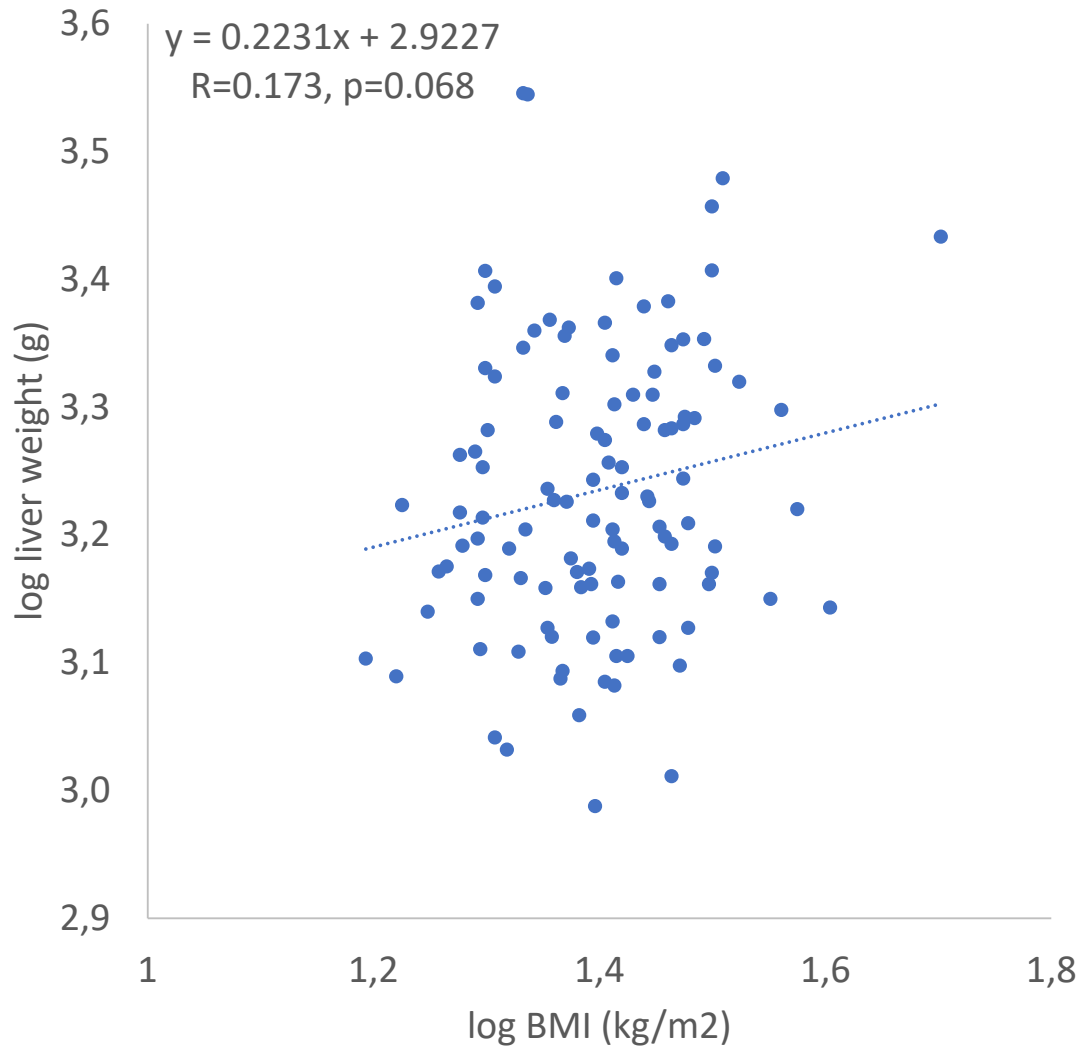


Figure S4: A: Correlation between liver weight and BMI in the focused population. Pearson's correlation coefficient of log transformed MPPGL and BMI.