1   **Integrative genomic strategies applied to a lymphoblast cell line model reveal specific**

2   **transcriptomic signatures associated with clozapine response**

3

4   Short title: *in vitro* genomic analyses of clozapine response.

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Abstract

Clozapine is an important antipsychotic drug. However, its use is often accompanied by metabolic adverse effects and, in rare instances, agranulocytosis. The molecular mechanisms underlying these adverse events are unclear. To gain more insights into the response to clozapine at the molecular level, we exposed lymphoblastoid cell lines (LCLs) to increasing concentrations of clozapine and measured genome-wide gene expression and DNA methylation profiles. We observed robust and significant changes in gene expression levels due to clozapine (n = 463 genes at FDR < 0.05) affecting cholesterol and cell cycle pathways. At the level of DNA methylation, we find significant changes upstream of the LDL receptor, in addition to global enrichments of regulatory, immune and developmental pathways. By integrating these data with human tissue gene expression levels obtained from the Genotype-Tissue Expression project (GTEx), we identified specific tissues, including liver and several tissues involved in immune, endocrine and metabolic functions, that clozapine treatment may disproportionately affect. Notably, differentially expressed genes were not enriched for genome-wide disease risk of schizophrenia or for known psychotropic drug targets. However, we did observe a nominally significant association of genetic signals related to total cholesterol and low-density lipoprotein levels. Together, these results shed light on the biological mechanisms through which clozapine functions. The observed associations with cholesterol pathways, its genetic architecture and specific tissue effects may be indicative of the metabolic adverse effects observed in clozapine users. LCLs may thus serve as a useful tool to study these molecular mechanisms further.

Introduction

Antipsychotic drugs (APs) play an important role in the treatment of psychotic disorders such as schizophrenia (SCZ). Clozapine is one of the most effective antipsychotic drugs (AP)(Kane et al., 1988; Leucht et al., 2013; Taylor, 2017). However, the decision to prescribe clozapine is complicated by its potential to induce severe adverse effects(Leucht et al., 2013). The most severe adverse effect, with a prevalence of <1%, is clozapine-induced agranulocytosis, a dramatic reduction of white blood cells(Andersohn et al., 2007). More common adverse effects include weight gain, dyslipidemia and type 2 diabetes. These adverse metabolic effects are, in addition to the chance of developing agranulocytosis, the primary reasons for patient noncompliance and discontinuation of treatment(Cohen, 2014; Weiden et al., 2004).

The biological mechanisms underpinning the effect of clozapine, as well as its adverse effects, remain elusive. A twin study estimated that the heritability of APs-induced weight gain is approximately 60%(Gebhardt et al., 2010), suggesting a substantial role for genetic factors. Candidate genes studies of clozapine-induced adverse effects have yielded ambiguous results and lack consistent replication (reviewed by(Chowdhury et al., 2011; Lett et al., 2012; Müller et al., 2013; Roerig et al., 2011; Yan et al., 2013)). Two genome-wide association studies (GWAS) investigating antipsychotic-induced metabolic adverse effects have yielded inconclusive findings, primarily due to insufficient sample sizes and the potential polygenic nature of this trait(Adkins et al., 2011; Malhotra et al., 2012).

Intermediate molecular phenotypes, such as gene expression studies in specific cell lines or tissues, may improve our understanding of the molecular function of clozapine. Previous studies have found that atypical antipsychotic drugs may induce cholesterol metabolism through transcription factors such as sterol regulatory element binding proteins (*SREBP1* and *2*)(Ferno et al., 2011), suggesting that drug-induced cholesterol metabolism is related to these adverse

80 metabolic effects. However, such findings were not consistently replicated when profiling whole

81 blood(Ferno et al., 2011; Harrison et al., 2016; Vik-Mo et al., 2008). It is possible that changes in

82 DNA methylation could mediate changes in gene expression, such as the AP-induced

83 hypomethylation of the *FAR2* gene leading to insulin resistance(Burghardt et al., 2016). However,

84 such studies are currently limited and those performed provide inconsistent results(Burghardt et

85 al., 2016; Houtepen et al., 2016; Kinoshita et al., 2017; Melas et al., 2012; Ota et al., 2014; Rukova

86 et al., 2014; Stapel et al., 2017; Swathy et al., 2017; Swathy and Banerjee, 2017).

87

88 A major obstacle towards understanding clozapine-induced metabolic effects is that clozapine

89 therapy is a relatively rare (~6%) treatment plan in patients diagnosed with

90 schizophrenia(Burghardt et al., 2016; Stroup et al., 2016), of which then only 1% develop CIA.

91 Such factors challenge our ability to adequately sample a large and controlled prospective cohort

92 of patients thereby limiting progress in understanding both metabolic and hematological adverse

93 effects. To augment the lack of available *in vivo* data, we implemented an *in vitro* lymphoblast cell

94 line (LCL) model to study the effects of drug exposure at the molecular level. Cell-based models

95 have been successfully employed for pharmacogenomic studies, including LCL models to study

96 clozapine function(de With et al., 2015; Morag et al., 2010; Welsh et al., 2009; Wen et al., 2012).

97 Here, we exposed LCLs to increasing doses of clozapine and collected both expression and

98 methylation profiles. Through integrative genomic analyses, we aim to extrapolate *in vitro*

99 molecular signatures of clozapine towards relevance of *in vivo* function and study clozapine

100 response without the need to assemble a large cohort of patients. We identified significant

101 changes in transcriptomic signatures associated with cholesterol and cell cycle pathways. By then

102 integrating these molecular profiles with genome-wide association study (GWAS) summary

103 statistics of different traits and diseases, we show that clozapine-associated genes also overlap

104 with genetic signals related to total cholesterol and low-density lipoprotein (LDL) levels but not

105   schizophrenia genetic risk. Clozapine-associated genes are furthermore related to specific human

106   tissues, such as liver and those involved in immune, endocrine and metabolic functioning.

107

108   **Material and Methods**

109   *Lymphoblast cell lines*

110   We used lymphoblast cell lines (LCLs) from four unrelated samples, all part of the collection of

111   Utah residents of Northern and Western European ancestry (HapMap CEPH/CEU phase

112   1)(Consortium and †The International HapMap Consortium, 2003). We obtained LCLs from the

113   Coriell Institute for Medical Research (Camden, NJ, USA) and maintained the cell lines as

114   previously described(Consortium and †The International HapMap Consortium, 2003; de With et

115   al., 2015). To study methylation changes after exposure to clozapine, we performed a separate

116   experiment using six LCLs (HapMap, CEPH/CEU phase 1) consisting of two parent-offspring

117   trios.

118   *In vitro experimental design and clozapine exposure*

119   We exposed LCLs to different clozapine concentrations, with clinical concentration set at

120   2μM(Baumann et al., 2004). Clozapine, purchased from Sigma Aldrich, was dissolved in culture

121   medium with dimethyl sulfoxide (DMSO), with a maximum concentration of 0.1%. Cell lines were

122   exposed for 24 hours to clinical concentration (Supplementary Methods), 10x, 50x and 100x

123   clinical concentration (20μM-100 μM-200 μM clozapine) and vehicle (DMSO); each concentration

124   was measured in 4 cell lines, after which RNA was obtained for gene expression analysis

125   (Supplemental Figure 1A). To study DNA methylation changes in response to clozapine, we

126   performed an experiment similar to the gene expression study; LCLs were exposed to different

127   concentrations of clozapine (vehicle (DMSO), 1x, 20x, 40x and 60 times clinical concentration)

128   and exposure times were 24h and 96h (Supplemental Figure 1B). We measured cell viability using

129   the TC10 automated cell counter.

130   *Sample processing and data collection*

131   After desired exposure time, we lysed cells and performed RNA and DNA collections using

132   column-based extraction methods from Qiagen according to manufacturer's instructions

133   (Supplementary Methods). Gene expression profiling was carried out using Illumina® HumanHT-

134   12 v4 Expression BeadChip technology. DNA methylation assays were performed with Illumina®

135   Infinium HumanMethylation450 Beadchip arrays.

136   *Data preprocessing and normalization*

137   We processed raw gene expression values using the "Lumi" R-package(Du et al., 2008). We log2

138   transformed and quantile normalized the raw data, keeping only expressed gene transcripts

139   (detection p < 0.01) for further analysis (22,926 probes). We processed DNA methylation values

140   using the "WateRmelon" Bioconductor package(Pidsley et al., 2013), removing probes that were

141   known to cross-hybridize, probes containing SNPs in target CpG regions, probes with detection

142   p-value greater than 0.01 in 5% of samples, and probes with beadcounts > 3 (n=86,068 probes

143   in total)(Chen et al., 2013; Price et al., 2013). We normalized the data using the *dasen* function

144   and computed β-values, defined as the ratio of the methylated probe intensity and the overall

145   intensity (sum of methylated and unmethylated probe intensities), to measure methylation levels.

146   To limit the effect of heteroskedasticity, we included only variable probes with β-values between

147   0.2 – 0.8 in our analyses (165,014 probes)(Du et al., 2010).

148

149   *Statistical analyses*

150   To detect clozapine-induced molecular changes, for each probe, we tested for association

151   between gene expression (1) or DNA methylation levels (2) with increasing clozapine

152   concentrations using the following linear models implemented in R using the "Limma"

153   package(Smyth, n.d.):

154

155 $$Y_{g,i} = \beta0_{g,i} + \beta1_{g,i}Cloz + \beta2_{g,i}RIN + \varepsilon_{g,i} \quad (1)$$

156 $$Y_{m,i} = \beta0_{m,i} + \beta1_{m,i}Cloz + \varepsilon_{m,i} \quad\quad (2)$$

157

158 where Y is the normalized gene expression (g) or DNA methylation levels (m) for an individual

159 probe, b0 the intercept, b1 the effect of clozapine concentration, b2 the effect of RIN, and e the

160 residual variation of the model. We ran each model for each probe per individual i and

161 subsequently performed a meta-analysis across all individuals by combining p-values using

162 Stouffer's method with directionality of the effect sizes taken into account. We included RNA-

163 integrity number (RIN) as a covariate in the gene expression model and applied a Bonferroni

164 correction to correct for multiple testing, resulting in a significance threshold of $p < 2.18 \times 10^{-6}$ for

165 the gene expression analysis (n = 22,926 probes) and $p < 3.03 \times 10^{-7}$ for the DNA methylation

166 analysis (n = 165,014 probes).

167 *Gene ontology analysis*

168 We performed functional gene ontology analysis using DAVID (Database for Annotation,

169 Visualization and Integrated Discovery, version 6.8, interrogated February 2018)(Huang et al.,

170 2009a, 2009b), with default settings (Supplementary Methods).

171 *Functional enrichment analysis of DNA methylation data*

172 We used Genomic Regions Enrichment of Annotations Tools (GREAT, v3.0) to predict the

173 biological function of the top methylation probes associated with clozapine exposure. GREAT

174 links both proximal and distal genomic CpG sites with their putative target genes and implements

175 both a gene-based test and a region-based test using the hypergeometric and binomial test,

176 respectively, which allows us to assess enrichment of genomic regions in biological annotations

177 of pathway databases (Supplementary Methods)(McLean et al., 2010). The statistical outputs of

178 GREAT for both gene-based and region-based tests were subsequently adjusted for multiple

179 testing using Bonferroni correction.

6

180 *Weighted gene co-expression network analysis*

181 We performed a gene expression network analysis using weighted gene co-expression network

182 analysis (WGCNA) in R. Briefly, WCGNA identifies distinct modules using the shared variation in

183 gene expression based on pairwise correlation. To account for the biases related to differing

184 probe numbers between genes assayed on the array, we provided as input the mean probe

185 expression of genes residing within nominally significant differentially expressed genes (p<0.05),

186 considering 5,708 probes within 4,897 genes(Horvath, 2011; Langfelder and Horvath, 2008;

187 Zhang and Horvath, 2005). To assign biological function to each WCGNA module, we performed

188 gene ontology analysis using DAVID we performed.

189 *Additional DNA methylation analyses*

190 We ran a candidate gene study for CpG sites in close proximity of genes with evidence of

191 clozapine-induced differential gene expression. Methylation probes within the gene body, in the

192 3' and 5' untranslated regions and up to 1,500 nucleotides upstream of the transcription start site

193 of the 463 'top genes' (p<0.05) were selected for a post-hoc analysis (n = 1,004). These results

194 can be found in Table 1 of the Supplemental Material.

195 *GTEx cross tissue analysis*

196 To translate the *in vitro* effects of clozapine to *in vivo* human biology, we investigated how

197 clozapine genes behave across 22 human tissues represented in the GTEx data set. We

198 downloaded gene level quantifications (version 6, date: April 24, 2019) from the GTEx Project

199 web portal(Melé et al., 2015) and transformed gene expression values using a log2 transformation

200 (1 + RPKM value). We then tested 1) if clozapine-associated genes have higher or lower average

201 expression within each tissue and 2) if between tissue distance is different for clozapine-

202 associated genes compared to the expected based on chance. To calculate between tissue

203 distance, we used the top half most variable genes across GTEx samples that were also

204 significantly detected in our *in vitro* assay (n=7,025). We then performed multidimensional scaling

205   using the isoMDS() function in the MASS R package (v7.3)(Melé et al., 2015; Venables and

206   Ripley, 2002) and calculated between tissue Euclidean distances using the dist() function in R

207   (v.3.3.3) and the median gene expression values for each tissue for clozapine associated genes

208   (N=463). To established whether clozapine-associated genes significantly deviated from the

209   expected, we established a null distribution by repeating this procedure using the Euclidean

210   distance for samplings of similarly-sized sets of genes. Within each sampling, genes were

211   sampled with a probability that matched the distribution of average gene expression of clozapine-

212   associated genes in our LCL experiment. This was done to account for differentially expressed

213   genes having higher gene expression levels. Our null distribution therefore better captured the

214   expected effect. Null distributions were built separately for the expected average gene expression

215   within each tissue and for the expected Euclid distance for each tissue pair. P-values were then

216   established by calculating the proportion of samplings that were higher or lower than the statistic

217   of the observed clozapine-associated genes and corrected for multiple testing by Bonferroni

218   correction. Analyses were conducted separately for genes upregulated and downregulated after

219   clozapine exposure.

220   *MAGMA gene-set analysis*

221   To investigate if clozapine-associated genes were enriched for schizophrenia or cardiovascular-

222   related genetic association signals, we performed gene-set analysis using MAGMA (multimarker

223   analysis of genomic annotation)(de Leeuw et al., 2015). We tested the following three gene-sets,

224   each with three different significance thresholds:

225   1.   All genes differentially expressed, either up- or downregulated;

226   2.   Upregulated differentially expressed genes;

227   3.   Downregulated differentially expressed genes.

228   We obtained GWAS summary statistics for schizophrenia(Schizophrenia Working Group of the

229   Psychiatric Genomics Consortium, 2014), body mass index(Yengo et al., n.d.), coronary artery

8

230   disease(Nikpay et al., 2015), type 2 diabetes(Scott et al., 2017), total cholesterol, triglycerides,

231   high-density-lipoprotein, and low-density-lipoprotein(Scott et al., 2017; Surakka et al., 2015). For

232   each GWAS, we computed aggregate gene-level test statistics using a 10kb window around the

233   transcription start and end site of each gene; a total of 11,533 genes were available for analysis.

234   We then tested for association between GWAS gene level Z-scores and experimental gene-sets

235   (defined above). We estimated linkage disequilibrium (LD) using the 1000 Genomes European

236   reference panel(1000 Genomes Project Consortium et al., 2015; Scott et al., 2017; Surakka et

237   al., 2015) and ran MAGMA using a two-sided competitive test by accounting for probes tested in

238   our experiment while also correcting for gene size, SNP density, minor allele count and LD.

239   *Antipsychotic drug gene-sets and enrichment of schizophrenia heritability*

240   As a complementary analysis, we investigated whether manually curated antipsychotic drug

241   gene-sets overall are associated with schizophrenia heritability. We performed gene-set analysis

242   on antipsychotic drug targets that were previously reported to be enriched for schizophrenia

243   heritability [55]. Drug target list were curated using drug-gene interaction databases(Roth et al.,

244   2000; Wagner et al., 2016). We identified 50 sets of drugs with ATC (Anatomical Therapeutic

245   Chemical) code N05A, a drug class to which all antipsychotic drugs belong, including clozapine.

246   Using the schizophrenia GWAS as input to MAGMA, we ran gene-set analysis (1) per individual

247   N05A antipsychotic drug gene-set, (2) for all N05A drug target genes combined, and for (3) all

248   N05A drug target genes combined excluding clozapine.

249   *LD Score Regression estimation of heritability and genetic correlations*

250   Pre-computed LD scores from the 1000 Genomes European reference panel provided through

251   the LD Score Regression (LDSR) *github* and GWAS summary statistics file processed and

252   reformatted to s*umstats* format were used as input to (LDSR) to estimate SNP-based heritability

253   (SNP-h^2)(B. K. Bulik-Sullivan et al., 2015). Genetic correlations between traits were estimated

254   using cross-trait LDSR via the *--rg flag(B. Bulik-Sullivan et al., 2015)*.

255   **Results**

256   *Clozapine exposure induces widespread gene expression changes*

257   We exposed lymphoblast cell lines to different concentrations of clozapine and tested for

258   subsequent changes in gene expression levels (Supplemental Figure 1A). In total, we tested

259   22,926 gene expression probes, of which 5,708 showed nominal significance (p<0.05) and 518

260   probes exceeded a Bonferroni-corrected $p < 2.18^*10^{-6}$. These 518 probes consisted of 234 up-

261   regulated probes and 284 down-regulated probes, representing a total of 463 unique genes. The

262   top 10 up-regulated and down-regulated probes are shown in Table 1. See Supplement for the

263   complete list of differentially expressed genes.

Up-regulated genes		Down-regulated genes	
Gene	P-value	Gene	P-value
STMN1 (Stathmin 1)	5.16^*10^{-16}	LSS (Lanosterol synthase)	6.14^*10^{-17}
HIST2H2AC (Histone cluster 2 H2A family member C)	1.98^*10^{-15}	MAL (T-cell differentiation protein)	1.10^*10^{-16}
RPS7 (Ribosomal protein S7)	2.52^*10^{-15}	MIR1974 (MicroRNA 1974)	5.56^*10^{-16}
HIST1H2BJ (Histone cluster 1 H2B family member J)	4.42^*10^{-15}	LDLR (Low density lipoprotein receptor)	3.97^*10^{-14}
HIST2H2AA3 (Histone cluster 1 H2A family member A3)	1.97^*10^{-14}	DHCR7 (7-dehydrocholesterol reductase)	4.26^*10^{-14}
RPS15 (Ribosomal protein S15)	2.17^*10^{-14}	PASK (PAS Domain Kinase)	5.09^*10^{-14}
HIST2H2AA4 (Histone cluster 2 H2A family member A4)	2.78^*10^{-14}	RGS1 (Regular Of G Protein Signaling 1)	2.31^*10^{-13}
HIST1H2AC (Histone cluster 1 H2A family member C)	2.79^*10^{-14}	LYPD6B (LY6/PLAUR Domain Containing 6B)	4.38^*10^{-13}
AURKA (Aurora Kinase A)	5.19^*10^{-14}	TPP1 (Tripeptidyl Peptidase 1)	6.77^*10^{-13}
SGOL1 (Shugoshin 1)	2.09^*10^{-13}	RENBP (Renin Binding Protein)	7.54^*10^{-13}

Table 1. Top 10 up- and downregulated genes after clozapine exposure. Gene symbols are shown with corresponding gene name and p-value of differential gene expression analysis. A full list of genes is in Supplementary Table 1.

264   Gene ontology enrichment analysis of up-regulated transcripts showed significant functional

265   enrichment for cholesterol metabolism (13 genes, $p = 4.15^*10^{-15}$) and steroid biosynthesis (11

266   genes, $p = 1.01^*10^{-8}$) (Table 2), while analysis of down-regulated transcripts were enriched for cell

267   division processes and related annotations, such as mitosis (44 genes, p = 1.87^*10^{-39}),

268   chromosome (49 genes p = 3.03^*10^{-35}) and nucleosome (16 genes, p = 3.12^*10^{-14}) and other cell

269   cycle pathways (Table 2).

270   *Gene expression network analysis after clozapine exposure*

271   WGCNA network analysis of clozapine-induced differentially expressed genes (N=4,987) yielded

272   15 co-expression modules, ranging in size from n=61 to 1,791 genes. Five gene co-expression

273   modules were altered upon clozapine exposure: M14 (upregulated, 61 genes), M10 (upregulated,

274   155 genes), M9 (upregulated, 158 genes), M3 (upregulated, 579) and M1 (downregulated, 1,791

275   genes), which were nominally significant. The M14 co-expression module was enriched for genes

276   involved in cholesterol metabolism (13 genes, p = 4.8^*10^{-15}), the M1 co-expression module was

277   enriched for genes involved in cell cycle (133 genes, p = 7.3^*10^{-32}) and the M9 and M3 co-

278   expression module was enriched for mitochondrial genes (15 genes, p = 6.7^*10^{-6} and 46 genes p

279   = 3.8^*10^{-7} respectively). The M10 co-expression module was enriched for genes involved in the

280   nucleosome (9 genes, p = 1.4^*10^{-7}).

281   *Minimal changes in DNA methylation at single CpG sites after clozapine exposure*

282   We then performed DNA methylation profiling to assess whether these effects were due to

283   epigenetic changes. For statistical analysis, we applied an analytical approach similar to our gene

284   expression analyses. Targeted analysis on the 1,004 CpG sites near the 463 differentially-

285   expressed genes revealed 3 probes exhibiting significant changes in DNA methylation (p <

286   1.08^*10^{-4}), including a probe upstream of the low-density lipoprotein receptor (LDL-R) gene

287   (cg22971501, p=$4.75*10^{-5}$) after 24h; one probe upstream of the cyclin F (CCNF) gene showed

288   a significant change in DNA methylation after 96h (Table S3). Beyond these examples, global

289   methylation differences were not observed after 24h or 96h at the level of individual probes.

290

291

Functional annotation cluster	Enrichment score	Best P-value	Best fold enrichment	Average P-value	Average fold enrichment	Number of pathways
Upregulated pathways						
Cholesterol/lipid/steroid biosynthesis	8.44	4.15^*10^{-15}	46.92	1.19^*10^{-3}	18.46	19
Cholesterol/Steroid biosynthesis	4.77	1.01^*10^{-8}	68.45	4.66^*10^{-4}	54.32	3
Down-regulated pathways						
Mitosis/cell division	31.26	1.87^*10^{-37}	14.77	1.12^*10^{-21}	10.69	5
Chromosome/centromere	16.47	3.03^*10^{-35}	21.32	7.76^*10^{-8}	15.79	9
Histone/nucleosome	6.93	3.12^*10^{-14}	20.72	1.73^*10^{-3}	9.13	18
Spindle	6.02	4.95^*10^{-12}	12.89	7.45^*10^{-4}	10.13	6
Nucleotide/ATP-binding	5.72	1.46^*10^{-13}	2.71	7.78^*10^{-5}	2.47	5
Microtubule/kinesin	4.60	1.90^*10^{-4}	16.92	4.99^*10^{-2}	8.56	19

Table 2. Main functional annotations associated with clozapine exposure based on gene expression. Output of pathway enrichment analyses using DAVID is shown. The enrichment score is used as the main metric of importance. It is defined as the geometric mean of all enrichment p-values of each annotation term within the group. It is expressed as the minus log of the p-value, an enrichment score of 1.3 is nominally significant. This table shows clusters with an enrichment score > 4.6, corresponding to a p-value < 0.01. Fold enrichment is a measure to express the enrichment of this particular group of genes in comparison with the genes in the human genome. A list of all pathways is available in supplemental information.

293   *DNA methylation is affected at the pathway level*

294   To investigate if our top associated DNAm probes aggregated to changes at the pathway level,

295   we performed pathway enrichment analysis using GREAT, which incorporates functional

296   annotation from various databases to predict cis-regulatory function of genomic regions of

297   interest. When considering probes exhibiting FDR < 10%, we observed enrichment for protein

298   binding and regulation of cellular processes after 24h of clozapine treatment. When considering

299   the top 1000 probes, we also observed enrichment of immune-related functions, such as the

300   Major Histocompatibility Complex (MHC) class II protein complex and Graft-versus-host disease

301   after 24h. We found significant enrichment for estradiol regulation and various embryonic

302   developmental processes (Figure 1 and Table S5) when considering the top 1000 probes after
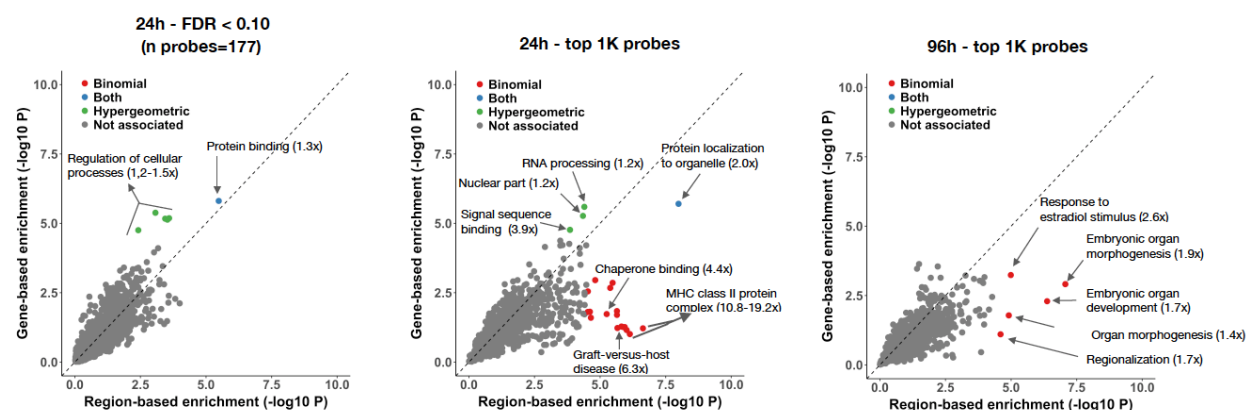
303   96h.

305   **Figure 1. Clozapine-associated DNAm probes concentrate to specific biological annotations.**
306   Genomic Regions Enrichment of Annotations Tool (GREAT) was used to assign biological context to
307   genomic regions associated to clozapine exposure. GREAT uses the annotation of nearby genes and
308   regulatory elements to performed enrichment analysis across known functional databases. The x-axis
309   shows the -log 10 p-value of the region-based analysis (binomial test), while the y-axis shows the
310   enrichment for the gene-based analysis (hypergeometric overlap test). Results are shown for (A) the top
311   probes after 24 hours (FDR < 0.10, n=177), (B) the top 1,000 probes after 24 hours, and (C) the top 1,000
312   probes after 96 hours. Each dot represents an annotation. Significant annotations, after Bonferroni
313   correction, are color-coded according to the test used.

316   *DNA methylation changes are time-dependent*

317   To examine if clozapine affected each time point similarly, we correlated effect sizes at each CpG

318   probe between time points. Across all probes, we found a significant negative correlation between

319   the Z-score of the association between DNA methylation levels and clozapine exposure at 24h

320   and 96h (Pearson r = -0.32, P<2.2x10^{-16}). This correlation was preserved among probes at FDR

321   < 10% at 24h (n=177, Pearson r = -0.38, P=1.3x10^{-7}) but not among the top probes at 96 hours

322   (n=177, Pearson r = -0.12, P = 0.10), highlighting possible time-dependent effects after clozapine
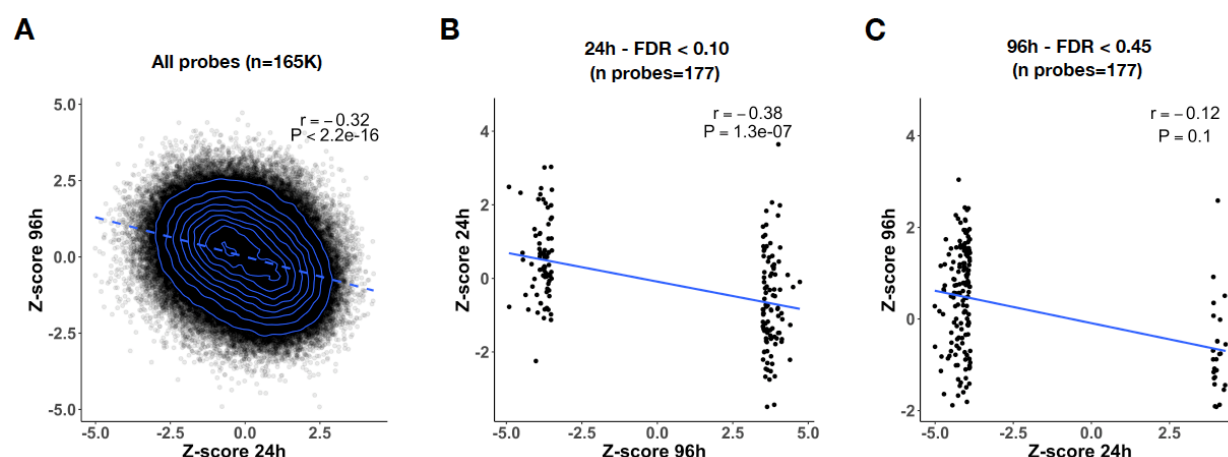
323   exposure (Figure 2).

324

325

Figure 2. Clozapine-induced DNA methylation changes are time-dependent. Correlation analysis was
performed between DNAm probe associations after 24 and 96 hours of clozapine exposure. The Pearson
correlation was computed for (A) all probes tested, (B) the top probes associated after 24hour exposure
(FDR < 0.10, n = 177 probes), and (C) the top 177 probes after 96 hours of drug exposure.

330   *Clozapine transcriptomic profiles highlight multi-tissue effects in GTEx*

331   We then asked whether our *in vitro* derived transcriptomic signatures could be used to help

332   translate the function of clozapine in humans. For this purpose, we used gene expression data

333   from the GTEx Project, including LCLs (n = 22 GTEx tissues, Figure 3A). First, we overlapped

334   preferentially-expressed genes of each GTEx tissue, as previously reported(Melé et al., 2015),

335   with the clozapine-associated genes detected in our assay. Preferentially expressed genes in

336   GTEx-LCLs exhibited the most overlap with the genes identified by our experiment followed by

337   whole blood (Supplementary Figure 2). We then investigated if the mean tissue expression of

338   clozapine-associated genes was significantly different from the mean expression of all the genes

339   tested in our experiment. We found that genes downregulated by clozapine have lower expression

340   in all tissues except testis and LCLs (Figure 3B and Table S6). Downregulated genes are enriched

341   for cell cycle processes and their higher expression in the testis and LCLs likely represent the

342   proliferative nature of these tissues. Genes upregulated by clozapine have significantly higher

343   average expression in liver, muscle, lung, and fibroblasts, and lower average expression in testis

344   tissue. We then asked whether clozapine-associated genes have different between-tissue

345   distances as a proxy to investigate possible functional links with other tissues. Distance is

346   calculated using the Euclidean distance measure. A lower value indicates that two tissues are

347   more similar while a higher distance value indicates that these tissues are more dissimilar. Of the

348   406 tissue pairs, we found that genes upregulated by clozapine have significantly deviating

349   between-tissue distances across 31 pairs of tissues (Figure 3C and Table S7). Spleen tissue

350   stands out with having the most different distance with other tissues (N=20). We also found

351   multiple differences for adipose, lung, and breast tissue pairs. The distance between cervix uteri

352   and ovary tissue, adrenal gland and thyroid tissue, muscle and nerve tissue are significantly more

353   dissimilar as well for upregulated clozapine genes compared to all genes detected in our assay.

354   For downregulated clozapine genes, we detected significantly dissimilar distances for 27 tissue-

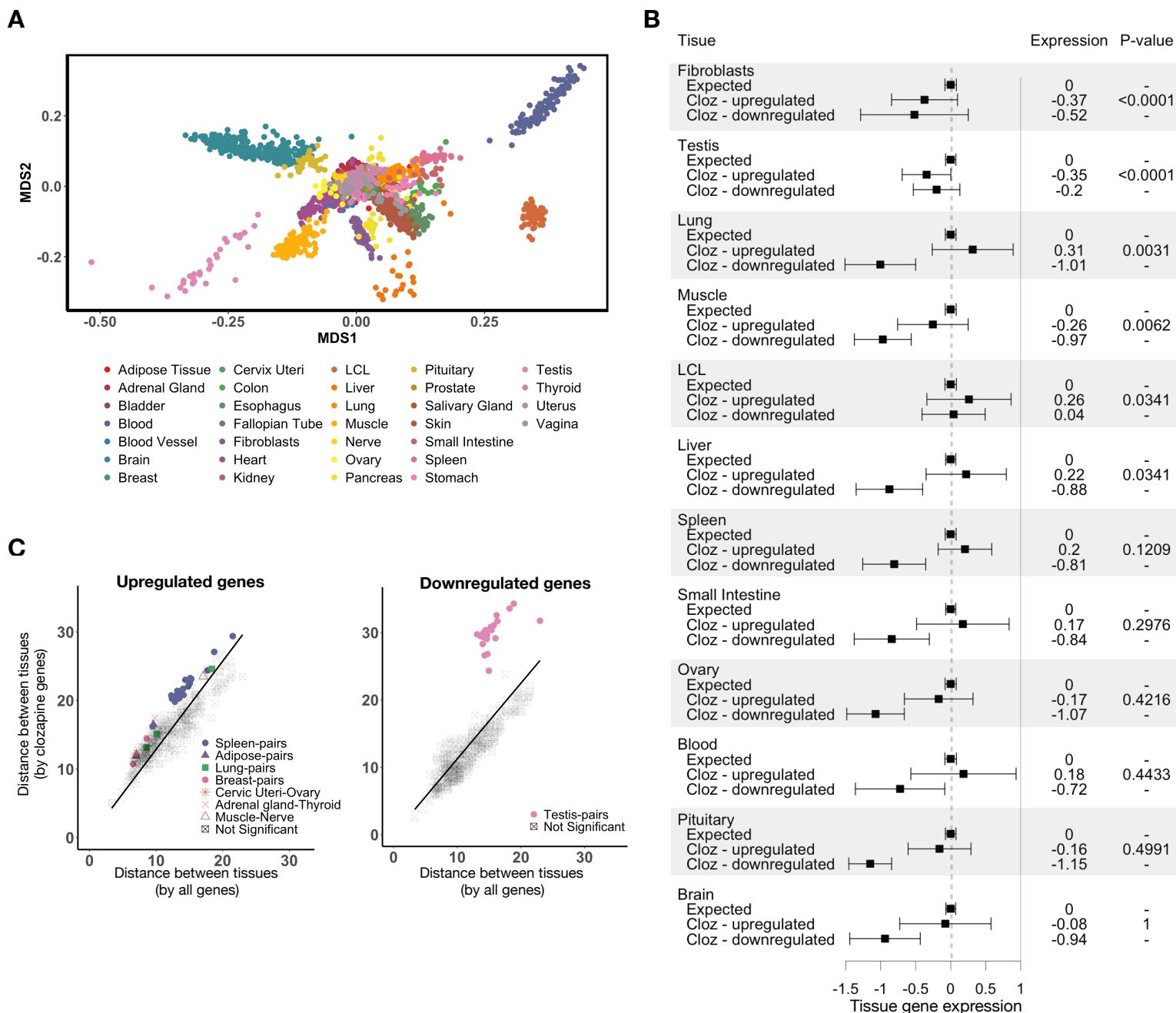355   pairs, all involving testis tissue (Figure 3C).

15

Figure 3. Clozapine-associated genes show tissue-specific expression patterns in GTEx. (A) Using gene expression data from the GTEx project, multidimensional scaling (MDS) was performed to visualize tissues and their relationships in a parsimonious way. The top half most variable genes (n=7,025), that were also significantly detected in our experiment, across a random subset of 2,000 GTEx samples were used as input in the MDS analysis. (B) A forest plot visualizing mean tissue gene expression of clozapine-associated genes across several tissues. Clozapine genes were divided into groups that are up regulated and down regulated after drug exposure. The expected mean gene expression, based on 10,000 weighted samplings, is shown as well (dotted vertical line). Within each tissue, the observed mean tissue gene expression (x-axis) is normalized by subtracting the expected mean gene expression. P-values indicate whether the mean gene expression of clozapine up regulated genes significantly deviate from the expected mean expression within a tissue. P-values are corrected for the number of tissues tested. Downregulated genes were not tested. See Supplementary Tables for results of all tissues (C) Between tissue distance across all GTEx tissue pairs. Each point represents one tissue pair. The y-axis shows the Euclidean distance between tissues computed using only clozapine-associated genes. The x-axis shows the expected mean Euclidean distance across 50,000 weighted samplings. Tissue pairs for which the between tissue distance, based on clozapine genes only, significantly deviates from mean expected distance (Padjusted <0.05), are color-coded. P-values are adjusted for multiple testing by Bonferroni correction (n test = 465 pairs x tests = 930).

356 *Clozapine transcriptome signatures are not enriched for schizophrenia disease risk*

357 Previous studies have found associations between schizophrenia genetic susceptibility and

358 antipsychotic drug targets(Gaspar and Breen, 2017; Skene et al., 2018). To investigate whether

359 clozapine-induced *in vitro* gene expression signatures are also associated with genetic

360 susceptibility of schizophrenia, we conducted gene-set enrichment analysis using all differentially

361 expressed genes (n=463). We did not observe a significant enrichment (p = 0.91). We then

362 considered upregulated genes, and did not observe significant enrichment (p = 0.74), nor for

363 downregulated genes (p = 0.64). Gene sets defined according to <1% and <5% FDR did not

364 change these findings (Table S8).

365

366 To further understand these findings, we investigated whether SCZ genetic susceptibility

367 aggregates to antipsychotic drug target genes with and without clozapine targets. As SCZ genetic

368 risk has been associated with antipsychotic drug target genes, it could be that this signal is

369 primarily driven by non-clozapine genes. To examine this, we used drug target gene lists from

370 drug-gene interaction databases as previously reported(Roth et al., 2000; Wagner et al., 2016).

371 We were able to extract 53 drug target gene-sets belonging to the N05A class of drugs with

372 antipsychotic actions, including clozapine. Across drug target gene-sets, we mapped all targets

373 to 104 unique genes of which 41 were detectable in our gene expression data but none

374 overlapped with differentially expressed genes identified. We found no evidence for SCZ risk to

375 be enriched in antipsychotic drug target genes overall (n = 96 genes, p = 0.96) nor with clozapine

376 targets excluded (n = 52, genes, p = 0.60). We did observe a strong concordance between the p-

377 values of individual drug gene-sets tested in our analysis and the p-values reported by the

378 previous study(Gaspar and Breen, 2017) (n = 50 drug gene sets, rho = 0.85, p = $1.49^{*}10^{-15}$),

379 indicating our analysis framework was able to reproduce previous findings at the level of individual

380 drug target gene-sets. Our analysis however does not observe an association between

381 schizophrenia genetic risk and clozapine-associated genes nor antipsychotic drug target genes.

16

382   *Genes upregulated after clozapine exposure show an association with GWAS signal of total*

383   *cholesterol and low-density lipoprotein.*

384   Next, we explored whether differentially expressed genes were enriched for genetic signal of

385   cholesterol- and cardiovascular disease-related traits. Using summary statistics of large GWASs

386   for each trait, we observe significant SNP-h^2 and a rich correlation structure between these traits

387   (Figure 4A and 4B). We then integrated the observed SNP-h2 with our detected clozapine

388   transcriptomic signatures. While no association remained significant after correction of multiple

389   testing (72 tests, p < 6.9^*10^{-4}), we did observe an increasing association between total cholesterol

390   and LDL heritability and clozapine gene-sets across more stringent thresholds of differentially

391   expressed genes (i.e. <FDR 5%, <FDR 1%, and Bonferroni correction (Figure 4C and Table S9).

392   Such trends were not observed for HDL or any of the other traits tested, including SCZ.
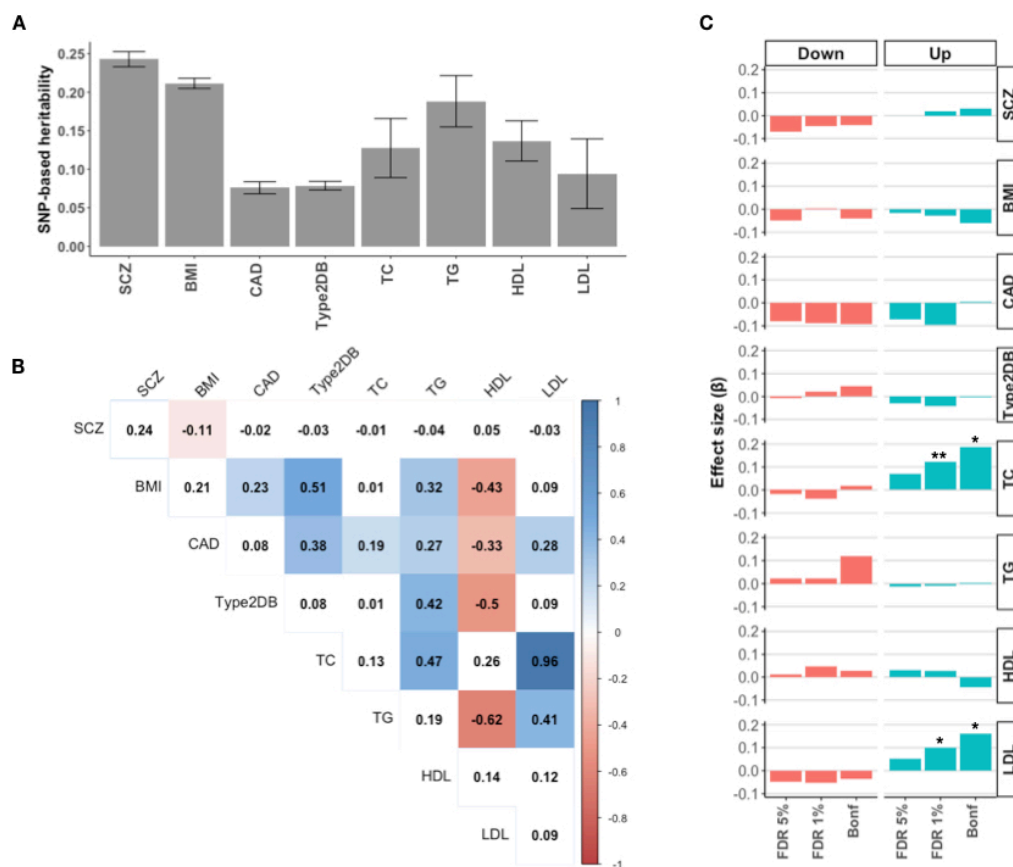
Figure 4. Clozapine gene-set analysis across cardiovascular traits. (A) SNP-based heritability estimates on the observed scale. (B) Genetic correlations between traits used in the analysis. The magnitude of the genetic correlation is only color-coded for estimates with p-value < 0.01. (C) MAGMA effect sizes (β) of GWAS trait associations with clozapine target genes are shown for down- and unregulated genes identified to be differentially expressed at FDR 5%, FDR 1%, and Bonferroni correction thresholds. Asterisks denote nominal significance of *P<0.05, **P<0.01. SCZ = schizophrenia, BMI = body mass index, CAD = coronary artery disease, Type2DB = type 2 diabetes, TC = total cholesterol, TG = triglycerides, HDL = high-density-lipoprotein, LDL = low-density-lipoprotein.

393 **Discussion**

394 We used an *in vitro* cell system of LCLs to study molecular effects of clozapine exposure to better

395 understand the mechanisms involved in adverse effects of psychotropic drug use and make

396 several important observations. First, genome-wide gene expression profiling of cells exposed to

397 clozapine showed strong activation of cholesterol metabolism and deactivation of genes involved

398 in cell cycle processes. These findings align with our observation of changed levels of DNAm

399 upstream of the LDL-R and CCNF genes after clozapine exposure. Second, DNAm analyses

400 suggest that effects of clozapine are time-dependent indicating that time of drug exposure is an

401 important experimental variable to take into account when studying clozapine. Third, integration

402 of *in vitro* transcriptomic signatures with human tissues in GTEx highlight liver tissue and several

403 immune and endocrine tissues as possible downstream effectors of clozapine exposure. Finally,

404 genetic analysis of the results suggests that clozapine-response in LCLs is independent from

405 schizophrenia disease risk and depleted from known drug targets of antipsychotic drugs, while it

406 is likely linked to the genetic architecture involved in cholesterol and low-density lipoprotein levels.

407

408 *In vitro* gene expression changes after exposure to clozapine have been reported before for

409 various cell lines. Here, we for the first time applied a genome-wide analysis of gene expression

410 in lymphoblastoid cell lines in response to clozapine exposure. The central role of cholesterol

411 metabolism in the response to clozapine is concordant with previously reported studies performed

412 in other cell types and for different antipsychotic drugs(Ferno et al., 2011; Foley and Mackinnon,

413 2014). Even though most of these were based on candidate genes rather than by genome-wide

414 analyses, the results consistently implicate cholesterol metabolism in response to antipsychotic

415 drugs (Choi et al., 2009; Fernø et al., 2009, 2005; Hu et al., 2010; Lauressergues et al., 2012,

416 2011, 2010; Liu et al., 2009; Raeder et al., 2006; Vik-Mo et al., 2009; Yang et al., 2007, 2009). At

417 the gene network level, clozapine-associated genes group to clear gene clusters. In addition to

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418   cholesterol metabolism, we observed mitochondrial and nucleosome pathways to be upregulated

419   after clozapine exposure, while cell cycle processes were downregulated, for example.

420

421   A small number of studies have identified a link between genetic markers in genes involved in

422   cholesterol metabolism and adverse effects of antipsychotic treatment, helping to further

423   substantiate these findings (Chowdhury et al., 2011; Yang et al., 2016, 2015). Additional studies

424   suggest that alterations in cholesterol metabolism by antipsychotic drugs may contribute to the

425   beneficial effects in treating psychosis. Cholesterol is extremely important in brain development

426   and in sustaining neuronal connections and myelination(Dietschy, 2009). Furthermore, Le Hellard

427   et al. described an association between genes important in cholesterol metabolism (*SREBP1* and

428   *SREBP2)* and schizophrenia(Dietschy, 2009; Le Hellard et al., 2010), which was confirmed in a

429   schizophrenia GWAS(Schizophrenia Working Group of the Psychiatric Genomics Consortium,

430   2014).

431

432   In addition to significantly upregulated genes, we also identified 284 genes that are downregulated

433   in the presence of clozapine, with significant enrichment for genes involved in cell cycle pathways.

434   A small number of studies have shown differing gene expression levels in cell cycle genes in

435   patients with schizophrenia, compared to healthy controls (Gassó et al., 2017; Lin et al., 2016;

436   Okazaki et al., 2016; Wang et al., 2010; Yang et al., 2017). A similar connection with antipsychotic

437   drug effects has not been reported before. It is possible that genes involved in cell cycle play a

438   role in the etiology of schizophrenia, making it a possible target of antipsychotic drugs as

439   clozapine. Conversely, the described effect may be a direct consequence of clozapine toxicity.

440   We have shown before that clozapine, in high concentrations, has a direct effect on viability of

441   lymphoblastoid cell lines (de With et al., 2015). Downregulation of genes involved in cell cycle

442   processes could thus be a direct (toxic) effect of clozapine. The toxic effects of clozapine and its

443   metabolites have been associated with clozapine-induced agranulocytosis(de With et al., 2015;

19

444   Lahdelma et al., 2010; Pereira and Dean, 2006; Williams et al., 1997). We note that for this study,

445   we used LCLs, which are blood-derived cells, but from a different progenitor cell and with different

446   cellular functions than neutrophils. The question remains whether findings from *in vitro* LCL can

447   be directly extrapolated to *in vivo* effects of clozapine on neutrophils.

448

449   While we found strong effects of clozapine exposure at the gene expression level, we did not

450   observe similar global effects at the level of DNA methylation. We did find 3 differently methylated

451   CpGs located within genes implicated by our gene expression analysis, including the LDL

452   receptor gene. These effects, however, were not observed after 96h of clozapine exposure.

453   Additionally, we observed overall enrichment of regulatory and immune pathways in the top

454   associated DNAm probes. Although previous studies have indicated that antipsychotic drugs may

455   induce changes in DNA methylation (Burghardt et al., 2016; Houtepen et al., 2016; Kinoshita et

456   al., 2017; Rukova et al., 2014), we did not find evidence for immediate large effects on DNA

457   methylation based on CpG sites assayed in our experiments. Possibly, subtle changes in DNA

458   methylation patterns play a regulatory role in the observed gene expression changes but a larger

459   sample size is needed to decipher these changes(Jones, 2012). In addition, a wider range of

460   clozapine concentrations *in vitro* may provide finer experimental resolution to observe subtle

461   epigenetic changes and identify key regulatory drivers. DNA methylation is one type of epigenetic

462   regulation and assaying other regulatory mechanisms, such as histone modification or RNA

463   regulation, may provide further insights into the regulatory dynamics that drive widespread

464   changes in gene expression (Allis and Jenuwein, 2016). Lastly, we observed DNA methylation

465   changes to be time-dependent. While the clinical meaning of this remains speculative, our findings

466   do suggest that the existing literature should be evaluated in the context of the duration of drug

467   exposure. In addition, future studies could gain more insights into the function of clozapine when

468   modeling drug exposure time as a variable in their analyses.

469

470   To further explore our gene expression findings, we investigated their association with genetic

471   susceptibility of schizophrenia. We did not observe significant enrichment of schizophrenia

472   heritability across clozapine-associated genes. Two previous studies did report an association

473   between schizophrenia heritability and antipsychotic target genes(Gaspar and Breen, 2017;

474   Skene et al., 2018). Their approach, however, differed from ours. While they used lists of

475   antipsychotic target genes originating from pharmacological databases, we used a list of

476   experimentally derived gene sets after *in vitro* exposure to clozapine in LCLs. The genes we found

477   to be differentially expressed did not overlap with antipsychotic drug target genes used in these

478   two previous studies, which may explain the discrepancy in findings. If we assume that the

479   clozapine-induced gene expression differences are a proxy for adverse effects, the lack of

480   evidence of the enrichment analysis suggests that the genetic architecture of disease

481   susceptibility is likely independent from susceptibility to adverse effects. We explored this further

482   by examining the enrichment of genetic signal linked to cholesterol and cardiovascular disease-

483   related traits such as body mass index, coronary artery disease, type 2 diabetes, and triglyceride

484   levels. We observed a nominally significant increased enrichment for total cholesterol and LDL

485   genetic signal in upregulated genes in response to clozapine exposure. While these findings did

486   not survive multiple testing correction and should be interpreted with caution, the observed

487   cholesterol and LDL heritability enrichment increased as we narrowed down on the most strongly

488   associated clozapine genes suggesting that this association warrants further investigation.

489   Mapping heritability from large-scale population studies to *in vitro* experimental systems can then

490   serve as a powerful approach to study biological pathways through integration of polygenic

491   disease risk (Ori et al., 2019). Metabolic adverse effects are often observed in patients using

492   clozapine (and antipsychotics in general) and improving our understanding of the mechanisms

493   that underlie these adverse effects is an imperative area of research.

494

495 An important advantage of *in vitro* experimental models is the controlled laboratory environment,

496 which not only decreases the signal-to-noise ratio in the collected data but also allows for precise

497 manipulation of the model in follow up work. While our current results are consistent with previous

498 findings in other cell types(Choi et al., 2009; Fernø et al., 2009, 2005; Hu et al., 2010;

499 Lauressergues et al., 2012, 2011, 2010; Liu et al., 2009; Raeder et al., 2006; Vik-Mo et al., 2009;

500 Yang et al., 2007, 2009), it remains unclear whether LCLs are an appropriate cell type to capture

501 the molecular changes that are most relevant for studying adverse effects of antipsychotics in

502 patients. To gain insight into the transferability of the *in vitro* clozapine-response in LCLs to

503 functions of human tissues, we investigated how clozapine-associated genes are expressed

504 across GTEx tissues. As gene expression patterns have been shown to have significant degree

505 of sharing across human tissues(Buil et al., n.d.; GTEx Consortium et al., 2017) effects discovered

506 in one tissue may thus be informative for other tissues. We observed that preferentially expressed

507 genes in GTEx-LCL tissue overlap the most with clozapine genes identified in LCLs *in vitro*, with

508 whole blood ranked as second highest tissue. Preferentially expressed genes however, represent

509 only a subset of the clozapine genes. Using all associated genes, we demonstrated that

510 upregulated clozapine genes have significantly different average expression in liver, muscle, lung,

511 and testis tissue. This suggests multi-tissue downstream effects through clozapine treatment,

512 which fits its clinical presentation of a diverse set of adverse effects that have been reported (Iqbal

513 et al., 2003). Our finding of higher expression in the liver is in line with the observed cholesterol

514 gene ontology signature of upregulated clozapine genes in our assay. The liver is a central player

515 in the function of cholesterol in the body and clozapine-related hepatotoxicity has furthermore

516 been reported in cases of treatment with the drug (Keane et al., 2009; Kellner et al., 1993). We

517 observe no differences for any of the GTEx brain tissues.

518

519 On top of *within tissue* effects, we also performed a more explorative analysis to examine

520 clozapine gene expression-based similarity *between tissues* and found specific tissue pairs that

22

521   were significantly different in tissue similarities. Here, we highlight some interesting observations.

522   We observe a decrease in similarity for tissues involved in immune (spleen), endocrine (testis,

523   ovary, adrenal and thyroid gland) and metabolic (adipose) functioning. The spleen and testis in

524   particular stand out with a significant change in dissimilarity with many tissues. The spleen is the

525   largest secondary lymphoid organ in the body and hosts a wide range of immunological functions,

526   including the storage of leukocytes (Lewis et al., 2019). While it remains speculative if splenic

527   dysfunction could for example lead to agranulocytosis, our findings do solicit for more research

528   on the mechanism between clozapine and splenic function. An intriguing, and perhaps also

529   surprising, finding is the implication of the testis among analysis of genes downregulated by

530   clozapine. The testis has two primary functions; to produce sperm and to produce hormones, in

531   particular testosterone, which is a sex steroid synthesized from cholesterol (Eacker et al., 2008).

532   Genes downregulated by clozapine are enriched for cell division processes and cell proliferation

533   is an important function of testicular cells (Sohni et al., 2019). While little is currently known about

534   how clozapine or other antipsychotics may affect testicular function in adult humans, our finding

535   does again point to cholesterol-related biology. In addition to the testis, we also observe deviation

536   in between-tissue similarity for several other endocrine tissues. As endocrine abnormalities are

537   known causes of human obesity, the identified transcriptomic profile of clozapine response

538   together with downstream tissue effects may point to new avenues to study clozapine-induced

539   weight gain (Baptista, 1999). Together, these findings suggest that clozapine disproportionately

540   affects the function of specific tissues. Our results furthermore demonstrate how experimental

541   molecular signatures can be integrated with external genomic datasets, such as the GTEx project,

542   to help translate in vitro findings to human biology.

543

544   We used LCLs as an *in vitro* model for studying molecular effects of clozapine exposure in an

545   effort to improve our understanding of antipsychotic-induced adverse effects. Genome-wide gene

546   expression profiling demonstrated a robust up-regulation of cholesterol metabolism and down-

23

547   regulation of cell cycle pathways, with only limited changes in DNA methylation profiles. We did

548   not find evidence that genes up- or down-regulated during clozapine exposure were enriched for

549   genetic variation associated with schizophrenia. On the other hand, the observed enrichment

550   signal with the genetic basis of total cholesterol and LDL levels and multi-tissue involved across

551   immune, endocrine, and metabolic functions may provide important leads linked to antipsychotic

552   drug induced metabolic adverse effects. The necessary challenge of large-scale, systematic

553   prospective patient cohort studies of adverse effects of clozapine and other AP remains, while *in*

554   *vitro* studies such as ours provide only glimpses of what may be relevant.

Declarations

Competing interest

The authors declare no conflict of interest.

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