Transcriptomic profiling of whole blood in 22q11.2 reciprocal copy number variants reveals that cell proportion highly impacts gene expression

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ABSTRACT

22q11.2 reciprocal copy number variants (CNVs) offer a powerful quasi-experimental "reverse-genetics" paradigm to elucidate how gene dosage (i.e., deletions and duplications) disrupts the transcriptome to cause further downstream effects. Clinical profiles of 22q11.2 CNV carriers indicate that disrupted gene expression causes alterations in neuroanatomy, cognitive function, and psychiatric disease risk. However, interpreting transcriptomic signal in bulk tissue requires careful consideration of potential changes in cell composition. We first characterized transcriptomic dysregulation in peripheral blood from reciprocal 22q11.2 CNV carriers using differential expression analysis and weighted gene co-expression network analysis (WGCNA) to identify modules of co-expressed genes. We also assessed for group differences in cell composition and re-characterized transcriptomic differences after accounting for cell type proportions and medication usage. Finally, to explore whether CNV-related transcriptomic changes relate to downstream phenotypes associated with 22q11.2 CNVs, we tested for associations of gene expression with neuroimaging measures and behavioral traits, including IQ and psychosis or ASD diagnosis. 22q11.2 deletion carriers (22qDel) showed widespread expression changes at the individual gene as well as module eigengene level compared to 22q11.2 duplication carriers (22qDup) and controls. 22qDup showed increased expression of 5 genes within the 22q11.2 locus, and CDH6 located outside of the locus. Downregulated modules in 22qDel implicated altered immune and inflammatory processes. Celltype deconvolution analyses revealed significant differences between CNV and control groups in T-cell, mast cell, and macrophage proportions; differential expression of individual genes between groups was substantially attenuated after adjusting for cell composition. Individual gene, module eigengene, and cell proportions were not significantly associated with psychiatric or neuroanatomic traits. Our findings suggest broad immune-related dysfunction in 22qDel and highlight the importance of understanding differences in cell composition when interpreting transcriptomic changes in clinical populations. Results also suggest novel directions for future investigation to test whether 22q11.2 CNV effects on macrophages have implications for brain-related microglial function that may contribute to psychiatric phenotypes in 22q11.2 CNV carriers.

1. Introduction

Deletions and duplications at the 22q11.2 locus occur in 1 in ~3000–4000 and 1 in ~1600 live births, respectively (Hoeffding et al., 2017; Olsen et al., 2018). They span a gene-rich region of chromosome 22 that includes multiple highly conserved, brain-expressed, protein-coding genes (Guna et al., 2015; Hiroi et al., 2013), and result in both convergent and divergent phenotypes (Hoeffding et al., 2017; Lin et al., 2020; Olsen et al., 2018). 22q11.2 deletions are associated with a multi-organ system phenotype that includes craniofacial and cardiac anomalies, immune dysfunction, and high rates of neuropsychiatric and neurodevelopmental disorders (McDonald-McGinn et al., 2015). Both copy number variant (CNV) types are associated with developmental delays and intellectual disability; however, cognitive deficits tend to be milder in 22q11.2 duplication (22qDup) carriers compared to 22q11.2 deletion (22qDel) carriers (Lin et al., 2020). Both 22qDel and 22qDup are associated with elevated rates of autism spectrum disorder (ASD) (Jacob A. S; Olsen et al., 2018. Vorstman et al., 2006; Wenger et al., 2016); however, only 22qDel confers elevated risk for psychosis (Li et al., 2016; Marshall et al., 2017; Monks et al., 2014; Niarchou et al., 2014; Rees et al., 2016; Schneider et al., 2014). Moreover, the only structural neuroimaging study thus far to analyze both 22q11.2 CNV groups showed that brain morphology differed meaningfully as a function of reciprocal genomic variation, with 22qDel carriers being more impacted (Lin et al., 2017). While understanding the biological mechanisms that lead to partially overlapping versus distinct phenotypes associated with reciprocal 22q11.2 CNVs remains a challenge, the use of high throughput transcriptomics offers the potential to gain new mechanistic insights.

Intermediate to genetic variation and downstream traits (i.e. measures of neuroanatomy, cognition and behavior), gene expression represents the transcriptional activity that underlies biological mechanisms (Coppola, 2011). Characterizing gene expression changes in 22qDel versus 22qDup carriers may enhance our understanding of how biological systems are disrupted by these major genetic perturbations to yield partially overlapping versus distinct phenotypes. Whole-transcriptome profiling allows for the unbiased interrogation of all genes in parallel, avoiding the limitations of targeted gene approaches (Zhang and Horvath, 2005). Moreover, as cellular processes often involve many genes acting in concert, it is also crucial to analyze genomic information at the level of coexpression (Allen et al., 2012; Kadarmideen and Watson-Haigh, 2012). Finally, integrating orthogonal behavioral phenotype measures can aid in functional interpretation of transcriptomic changes (Jalbrzikowski et al., 2015). This includes the incorporation of both quantitative and categorical measures of neurodevelopmentally relevant traits, such as those from brain imaging, cognitive assays, and psychiatric diagnoses.

Interpreting transcriptomic signal from heterogeneous bulk tissue like peripheral blood (Shen-Orr and Gaujoux, 2013) can be challenging because the gene expression levels detected by microarray or RNA sequencing can be greatly influenced by variation in cell type composition (Farahbod and Pavlidis, 2020). In studies of psychiatric populations, most transcriptomic studies to date examined differential gene expression in heterogeneous bulk tissue, either from brain or blood, and few accounted for cell type composition. However, without accounting for heterogeneity in the proportion of cell types comprising a given tissue, it is difficult to know whether disease-associated transcriptomic changes represent differences in the number of cells expressing certain genes, alterations in transcript levels within the cells themselves, or some combination of both. This may be particularly important in blood, where cell type variation is especially pronounced, with over a dozen distinct cell types for which abundance can vary up to 10–20-fold, even in healthy individuals (Adalsteinsson et al., 2012; Chikina et al., 2015; Farahbod and Pavlidis, 2020; Shen-Orr et al., 2010). To address this issue, computational methods have been developed to estimate cell type-specific proportions based on expression patterns of known marker genes (Chikina et al., 2015; Newman et al., 2015; Xu et al., 2013). It is not yet routine practice to account for cell type heterogeneity in studies of CNS disorders. However, when researchers accounted for cell composition differences in bulk tissue samples in idiopathic forms of ASD and schizophrenia (Toker et al., 2018; Wang et al., 2020), they gained additional insights into disease pathology.

Here, we characterized transcriptomic dysregulation in peripheral blood from individuals with reciprocal 22q11.2 CNVs while carefully considering the effect of cell type proportions and potential confounders such as batch and medication usage. While two prior studies profiled the blood transcriptome in 22qDel carriers with smaller sample sizes (Jalbrzikowski et al., 2015; van Beveren et al., 2012), neither study included 22qDup carriers nor adjusted for cell type composition or medication usage. Therefore, we characterized CNV-associated differential expression of individual genes and co-expression of genes (i.e., module eigengenes), before and after accounting for cell composition and medication. To explore whether transcriptomic changes in the context of 22q11.2 deletions and duplications relate to phenotypes associated with the CNVs, we also tested for associations between gene and eigengene expression with neuroimaging measures and behavioral traits (i.e., IQ and diagnosis of psychosis or ASD). This comprehensive approach aims to bridge the gap between transcriptomics, brain structure, and behavioral outcomes to generate testable hypotheses about the molecular effects of 22q11.2 CNVs.

2. Materials and methods

2.1. Participants

The sample consisted of 167 age and sex-matched individuals: 79 22qDel carriers, 20 22qDup carriers, and 68 demographically-matched controls (for demographics, see Table 1). CNV carriers were included if they had deletions or duplications that included the "critical" A-B low copy repeat (LCR) region of the 22q11.2 locus at minimum (Motahari et al., 2019). CNV status and breakpoints were determined via multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002); using the SALSA MLPA Probemix P250–B2 DiGeorge kit from MRC-Holland (Vorstman et al., 2006), a polymerase chain reaction (PCR)-based assay that is a gold standard method for determining copy number changes in humans. Patients were ascertained from a variety of sources, including the University of California at Los Angeles (UCLA) or Children's Hospital, Los Angeles Pediatric Genetics, Allergy/Immunology and Craniofacial Clinics, as well as local support groups and websites. Demographically comparable, typically developing comparison subjects were recruited from the same communities as patients via web-based

Table 1
Participant demographics.

	22q11.2 Deletion Carriers	Typically-developing Controls	22q11.2 Duplication Carriers
Sample Size	79	68	20
Age (SD)	16.9 (8.5)	18.5 (12.6)	17.4 (9.77)
Age Range	6 to 49	6 to 65	8 to 42
N, females (%)	39 (49.4%)	34 (50%)	7 (35%)
RIN (SD)[a,b]	8.52 (0.67)	8.86 (0.55)	8.40 (0.60)
Full-scale IQ (SD)[a,b,c]	76.9 (11.6)	110.0 (20.4)	96.6 (19.8)
N, Autism Spectrum Disorder (%)	43 (54.4%)	0	9 (45%)
N, Psychotic Disorder (%)	11 (13.9%)	0	0
N, Antidepressants	22	4	6
N, Antiepileptics	8	1	3
N, Benzodiazepines	8	0	0
N, Antipsychotics	10	1	2
N, Stimulants	9	2	6

[a] 22q-del ≠CTL (p < .05).
[b] 22q-dup ≠ CTL (p < .05).
[c] 22q-del ≠ 22q-dup (p < .05).

advertisements, flyers and brochures at local schools, pediatric clinics, and other community sites. Approximately 38% of the deletion carriers and 34% of the controls were included in a prior publication (Jalbrzikowski et al., 2015). The current study includes a substantially larger sample of both 22qDel and controls, as well as a novel cohort of 22qDup carriers.

Exclusion criteria for all study participants included significant neurological or medical conditions (unrelated to 22q11.2 CNVs) that might affect brain structure, history of head injury with loss of consciousness, insufficient fluency in English, and/or substance or alcohol abuse or dependence within the past 6 months. As we aimed to include a representative cohort of CNV carriers, patients with cardiac-related issues were not excluded, as this is a hallmark of 22q11.2 Deletion Syndrome. Healthy controls were free from significant intellectual disability and did not meet criteria for any psychiatric disorder, with the exception of attention deficit-hyperactivity disorder or a past episode of depression, due to their prevalence in childhood and adolescence (Ghandour et al., 2019; Sayal et al., 2018; Thapar et al., 2012). All participants underwent a verbal and written informed consent process. Participants under the age of 18 years provided written assent, while their parent or guardian completed written consent. The University of California at Los Angeles Institutional Review Board approved all study procedures and informed consent documents.

2.2. Peripheral blood sample preparation

RNA was extracted from whole blood using the PAXgene extraction kit (Qiagen) and stored at −80C for subsequent analysis. RNA quantity was assessed using Nanodrop (Nanodrop Technologies) and RNA quality was determined using the Agilent Bioanalyzer (Agilent Technologies) to quantify RNA fragmentation in each sample, creating an RNA integrity number (RIN); (Schroeder et al., 2006). Total RNA (200 ng) was amplified, biotinylated, and hybridized on Illumina HT12 v3 (48,803 total probes) or v4 (47,168 total probes) microarrays as per manufacturer protocol at the UCLA Neuroscience Genomics Core. Only probes shared between the 2 platforms were used in analyses (39,368 shared, annotated probes). Slides were scanned using an Illumina BeadStation and signal was extracted using the Illumina BeadStudio software (Illumina, San Diego, CA).

2.3. Structural neuroimaging

High-resolution structural magnetic resonance imaging (MRI) scans were acquired concurrently with blood measures for 49 22qDel carriers, 43 controls, and 21 22qDup carriers. Scanning was conducted on a 3T Siemens (Erlangen, Germany) TimTrio MRI scanner with a 12-channel head coil at the UCLA Brain Mapping Center or an identical 3T scanner, using identical acquisition parameters, at the UCLA Center for Cognitive Neuroscience. Details of scanning parameters have been described in prior publications (Lin et al., 2017); (see Supplementary Methods for details). Quality assessment procedures were applied by 2 raters blind to group status. We extracted cortical measures based on the Desikan-Killiany FreeSurfer atlas (Desikan et al., 2006). Segmented regions were visually inspected and statistically evaluated for outliers following standardized ENIGMA protocols (http://enigma.ini.usc.edu/protocols/imaging-protocols).

2.4. Clinical and neurocognitive assessments

IQ estimates were obtained using the Vocabulary and Matrix Reasoning subtests in the Wechsler Abbreviated Scale of Intelligence (Wechsler, 2012) or Wechsler Adult Intelligence Scale, Ed 4 (Wechsler, 2008) for 44 22qDel carriers, 42 controls, and 17 22qDup carriers. Supervised clinical psychology doctoral students administered psychodiagnostic evaluations to all study participants to assess for DSM psychiatric diagnoses (Structured Clinical Interview for DSM [SCID];

(First and Gibbon, 2004; Shaffer et al., 2000), and/or Computerized Diagnostic Interview Schedule for Children [C-DISC]; (First and Gibbon, 2004; Shaffer et al., 2000). Subjects were included as psychotic spectrum if they met SCID diagnostic criteria for a psychotic disorder including schizophrenia ($n = 4$), unspecified psychotic disorder ($n = 5$), schizoaffective disorder, depressed type ($n = 2$). To assess for ASD, the Autism Diagnostic Observation Schedule (ADOS) was administered to CNV carriers and the Autism Diagnostic Interview-Revised (ADI-R) was administered to their parent/primary caretaker (Lord et al., 2000). Participants were classified as having ASD, based on the ADI-R, if scores were above threshold for the Reciprocal Social Interaction domain, as well as either Communication Impairment or Repetitive Behaviors and Stereotyped Patterns (see Antshel et al., 2007; Antshel et al., 2006; Kaufmann et al., 2004). Scores from the ADOS and ADI-R were used to determine a consensus diagnosis of ASD. (PMID: 22962003).

2.5. Microarray-based gene expression analysis data: pre-processing and statistical overview

Raw data were processed with the lumi package (Du et al., 2008) in the R statistical environment (version 3.5.2; R Foundation for Statistical Computing, Vienna, Austria). Only samples with a RNA integrity number (RIN) of 7 or greater were included (Gallego Romero et al., 2014) (refer to Table 1). Signal intensity was normalized with variance stabilizing transformation (Lin et al., 2008), and interarray normalization was done using robust spline regression normalization. Probes with a detection threshold of $p > .01$ or that were unannotated were dropped. Duplicated probes for the same transcript were also dropped using the collapseRows function (Miller et al., 2011) from the WGCNA package using the default maxMean approach, resulting in expression measurements for 14,013 unique genes. Finally, 6 participant outliers ($>|3|$ SD) were removed based on connectivity z-scores (Dong and Horvath, 2007).

As psychotropic medication usage is known to influence peripheral blood gene expression (Flanagan and Dunk, 2008; Stübner et al., 2004), we also included categories of psychotropic medications used by CNV carriers in a subset of our analyses. Categories included antidepressants, antiepileptics, antipsychotics, benzodiazepines and stimulants (Table 1). Batch effects were controlled for using the 'removeBatchEffect' from limma (Ritchie et al., 2015). For analyses additionally controlling for cell type proportions and/or medication effects, these variables were modeled simultaneously with batch using 'removeBatchEffect', to avoid introducing biases that can occur when sequentially removing confounded variables which are associated with our variable of interest and each other (i.e., CNV group status (Aschard et al., 2017)). Age, sex, and RIN were included as covariates for all statistical models involving gene expression data.

2.6. Cell type proportion estimation

Cell type estimation relies on the observation that variation in expression of cell type-specific marker genes is correlated with the abundance of the cell type in which they are expressed and has been validated in multiple studies (Kuhn et al., 2011; Mancarci et al., 2017; Newman et al., 2015; Patrick et al., 2020). Cell type proportions were estimated using the LM22 reference dataset and CIBERSORT (Kuhn et al., 2011; Mancarci et al., 2017; Newman et al., 2015; Patrick et al., 2020). The LM22 reference dataset consists of 547 genes that discriminate between 22 mature human hematopoietic populations that were isolated from peripheral blood or in vitro cultures. It includes 7 T-cell types, naïve and memory B-cells, plasma cells, natural killer (NK) cells, and myeloid subsets including monocytes, macrophages, dendritic cells, mast cells, and granulocytes. Proportions were logit-transformed after applying an adjustment for values of 0 (Smithson and Verkuilen, 2006). A linear model was used to test for associations between cell-type proportion and CNV status and brain and behavioral measures, with confounding covariates first removed using residualization.

2.7. Differential expression

Genes were assessed transcriptome-wide for differential expression using linear models in the limma package (Ritchie et al., 2015). Pairwise contrasts compared controls vs 22qDel, controls vs. 22qDup, and 22qDup vs 22qDel. Empirical Bayes-moderated p-values generated by limma were used to adjust for multiple testing across all genes; genes with an adjusted p-value < .05 (Benjamini-Hochberg FDR correction) were considered significantly differentially expressed (DE). Potential group differences in gene expression were assessed prior to, and after adjusting expression data for differences in cell-type proportions. This analysis was done both ways in order to provide comparability to prior studies in which cell-type proportions were generally not accounted for. Genes with significant DE were functionally annotated using gene ontology (GO) with g:Profiler (Raudvere et al., 2019), with moderate hierarchical filtering (i.e., best term per parent) and a minimum query/term overlap size of 5 genes. A custom background was set to all genes analyzed in the present study.

2.8. Weighted gene Co-expression network analyses (WGCNA) and enrichment of gene sets from WGCNA modules for cell-type specific expression

WGCNA was used to identify modules of co-expressed genes using standard parameters (Langfelder and Horvath, 2008). Briefly, an adjacency matrix was created by computing the correlation between the expression level of each gene with every other gene using signed biweight midcorrelations and applying a soft power of 12. The adjacency matrix was transformed into a topological overlap matrix (TOM), which sums the connection strength of each gene with every other gene and unsupervised clustering was implemented with the 'Dynamic Hybrid' tree-cutting method to identify modules of co-expressed genes (cut height = 0.995, deepSplit = 3, minimum module size = 30). Module eigengenes were computed from the first principal component of the expression values of the genes in each module, and correlated modules were merged using a dissimilarity threshold of 0.2. Modules were functionally annotated using GO with g:Profiler (Raudvere et al., 2019) as well as for cell-type specificity using the pSI package (Dougherty et al., 2010; Xu et al., 2013) and the cell-type markers from the LM22 reference dataset from CIBERSORT (see Supplementary Methods for details). The first principal component of each module (i.e., module eigengene) was used to summarize the expression of each module. Co-expression modules were generated and tested for potential differences in expression by CNV group prior to, and after adjusting for, differences in cell-type proportions and medication status.

2.9. Associations with neuroimaging, IQ, and psychiatric phenotypes

We assessed potential interaction effects between gene expression and CNV group after accounting for effects of batch, cell-type proportion, and medication, on trait measures of mean cortical thickness, total cortical surface area, and IQ using linear models. The linear model included: trait ~ age + sex + CNV group*gene expression.

To assess for significant main effects of gene expression on CNV group-residualized outcomes, traits were residualized for CNV group status because the 22q11.2 CNV affects both gene expression and the clinical traits (Lin et al., 2017, 2020) and could thus drive spurious associations (Aschard et al., 2017). We use a linear model with cell type and medication-adjusted expression measures, age, and sex as predictors and group-residualized neurobehavioral traits (i.e., cortical thickness, total cortical surface area, and IQ) as the outcome measures. To assess the association between cell type proportion and the same 3 outcome measures, we applied the same model using medication- and batch-adjusted cell type proportion as predictors to assess interaction effects with CNV group on unadjusted outcomes and for main effects on CNV group-residualized outcomes. The linear model included: group

residualized trait ~ age + sex + gene expression. The same linear models including the interaction effect or main effect were used to test for the association between clinical phenotypes and cell-type-adjusted WGCNA module expression or cell type proportions. Only genes and modules that showed significant differential expression between any two groups were included in these analyses in an attempt to characterize potential functional consequences of differentially-expressed genes or modules.

To test for potential associations between diagnoses of psychosis or ASD and the expression of each gene or WGCNA-derived module eigengene, we fit linear models with the limma package using batch, cell-type proportion, and medication adjusted gene expression data and WGCNA modules. Potential group differences in medication- and batch-adjusted cell-type proportions were also assessed using linear models. Since psychosis is associated with 22qDel only (Li et al., 2016; Marshall et al., 2017; Rees et al., 2016), the linear models testing for group differences in subjects with (n = 11) or without psychosis (n = 68) were restricted to 22qDel subjects. To maximize power for this analysis we also assessed for cell composition differences and differential gene expression (using cell type and medication-adjusted gene expression data) after incorporating data for 22qDel patients from an additional site, Utrecht University Medical Center (additional 25 22qDel without psychosis and 6 22qDel with psychosis), processed using the identical pipeline described above and including an additional covariate for site (Fiksinski et al., 2017). Thus, this analysis included a total number of 110 22qDel subjects (17 with psychosis and 93 without a psychosis diagnosis). For ASD, which is associated both with 22qDel and 22qDup (Olsen et al., 2018; Wenger et al., 2016), the linear model included an interaction term between CNV status and ASD diagnosis, as well as the main effects of CNV status and ASD diagnosis.

False discovery rate correction was used to correct for multiple comparisons and performed per analysis (i.e., across genes, eigengenes, cell-types, and clinical phenotype measures of IQ, cortical thickness, surface area, or ASD/psychosis diagnosis). All results were considered significant at an FDR-corrected q < .05. Code for analyses is available on request.

3. Results

3.1. 22q11.2 CNVs significantly alter gene expression

For gene expression that was unadjusted for cell type proportion or medication status, 22qDel carriers showed 390 genes with significant DE relative to controls (251 downregulated, 139 upregulated Fig. 1A, Table 2, Data Table S1). This included 28 downregulated genes within the 22q11.2 locus, with the remaining DE genes outside the locus. GO analysis of DE genes implicated biological adhesion, as well as immune pathways including regulation of T cell activation and adaptive immune response. Relative to controls, six genes were significantly upregulated in 22qDup carriers, including 5 genes within the 22q11.2 locus and 1 gene outside the locus, CDH6 (Fig. 1C; Data Table S2), which encodes the cadherin-6 protein that is involved in cell adhesion and differentiation, including for platelet aggregation in blood and angiogenesis in developing brain (Dunne et al., 2012; Krishna and Redies, 2009). Between 22qDel and 22qDup carriers, there were 141 significantly DE genes, 117 downregulated and 24 upregulated, including 28 22q11.2 genes (Fig. 1B; Data Table S3). GO analysis of DE genes between 22qDel and 22qDup carriers implicated cell adhesion and response to wound healing.

3.2. 22q11.2 CNVs alter expression of co-expression modules

Without adjusting for cell type proportion, WGCNA identified 28 modules of co-expressed genes in the full dataset of 22qDel carriers, 22qDup carriers, and control subjects (see Supplementary Fig. S1 for full module dendrogram). Three out of 28 module eigengenes showed significant DE between groups (see Fig. 2). Specifically, for the dark red module, all three groups differed from one-another, with 22qDel carriers
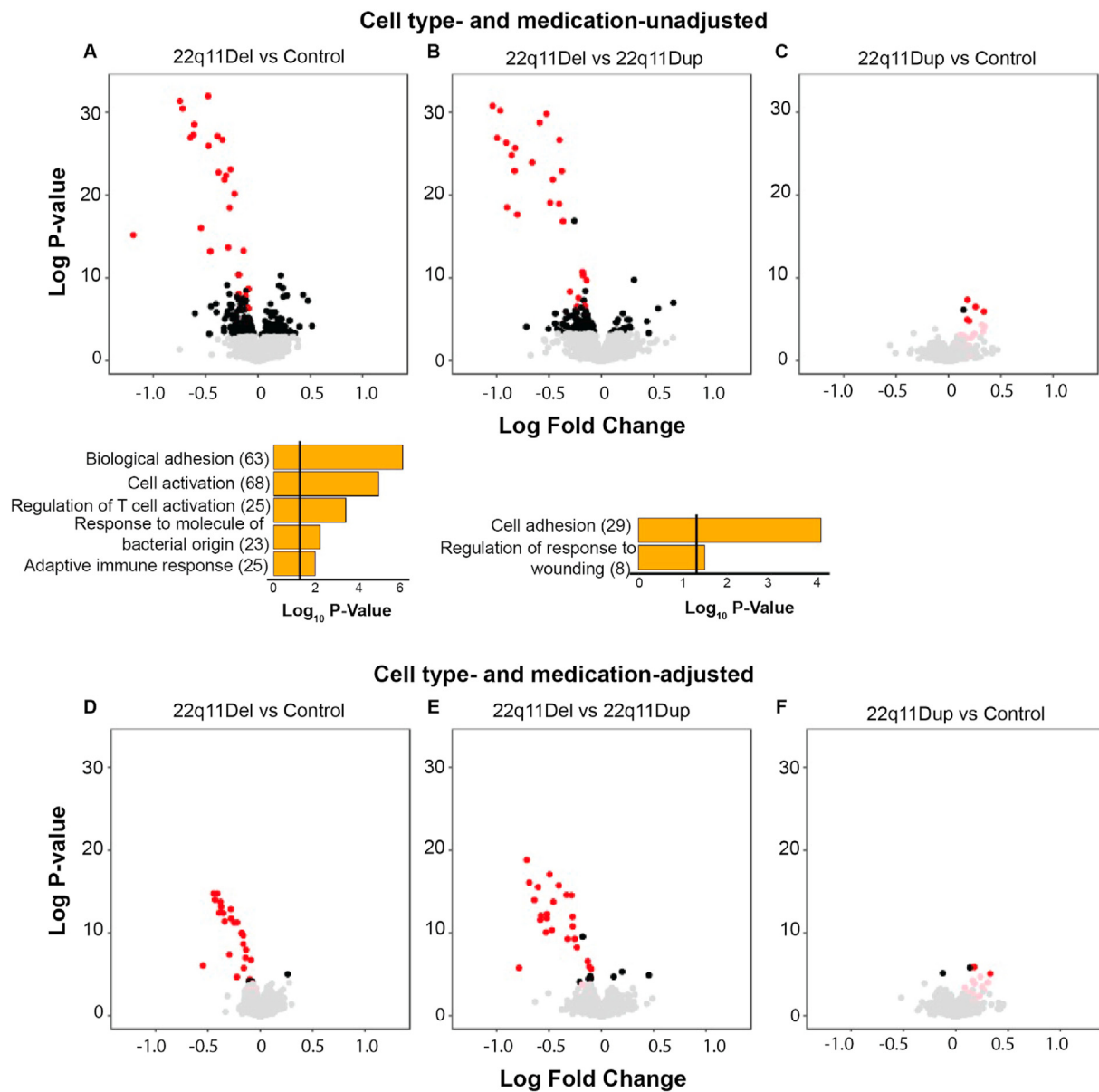
Fig. 1. Volcano plots of differential gene expression across the genome for each pairwise contrast. Panels A–C represent group differences in gene expression data that is unadjusted for cell type or medication, while panels D–F represent group differences in gene expression data that is adjusted for both cell type and medication. Panels A, D show 22qDel vs Control, panels B, E show 22qDel vs 22qDup, and panels C, F show 22qDup vs Control contrasts. Red dots represent genome-corrected, significant DE genes within the 22q11.2 locus. Black dots represent genome-corrected, significant DE genes outside the 22q11.2 locus. Light red dots represent nonsignificant 22q11.2 DE genes. Light black dots represent nonsignificant, non-22q11.2 DE genes. Significant DE genes were submitted to gene ontology (GO) enrichment analysis and the top 5 GO terms are displayed. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showing the lowest, 22qDup carriers showing the highest, and controls showing intermediate expression. GO analyses indicated that the dark red module was enriched for genes involved in platelet-associated pathways; the module was also enriched for mast cell, B cell, dendritic cell and M0 macrophage markers as well as neutrophils, plasma cells, and gamma delta T cell markers (Fig. 3). Platelet activation is increasingly recognized to initiate activation of both innate and adaptive immunity through interactions with and/or activation of multiple cell-types, including mast cells, dendritic cells, B cells, T-cells, and neutrophils (Ali et al., 2015; Cognasse et al., 2007; Karhausen et al., 2020; Koupenova et al., 2018) Thus, the dark red module appears to reflect immune-related sequelae. The medium purple3 module similarly showed differential expression between all three groups, with the 22qDel group showing the lowest, controls showing intermediate, and the 22qDup

group showing the highest expression. The medium purple3 module was not significantly enriched for any GO terms but showed weak enrichment for markers of three T-cell subtypes (e.g. CD8, CD4 memory resting, and CD4 naive; Fig. 3). Given that 25 of the 30 genes in the module are located within the 22q11.2 locus, this module largely reflects differential expression of 22q11.2 genes between CNV carriers and controls. Finally, 22qDel carriers also showed significantly reduced expression of the dark green module relative to controls and 22qDup carriers, who did not differ from one another. This module did not show significant GO enrichment; however, it was highly enriched for markers of multiple T-cell subtypes (Fig. 3). Significantly reduced expression of this module in 22qDel carriers is consistent with long-standing evidence of thymic dysplasia and resulting T-cell deficits in 22q11.2 deletion syndrome (Crowley et al., 2018).

Table 2

Significant differentially-expressed (DE) genes before and after cell type and medication adjustment. Percentages across each row indicate the percent of DE genes compared to the total DE gene number at the top of each column.

Cell type and medication-unadjusted	22qDel vs Control	22qDel vs 22qDup	22qDup vs Control
Total DE gene	390	141	6
Upregulated genes	139 (36%)	24 (53%)	6 (100%)
Downregulated genes	251 (64%)	117 (47%)	0
22q11.2 genes	28 (7%)	28 (20%)	5 (83%), all upregulated
Outside 22q region	362 (93%)	113 (80%)	1 (17%; CDH6)
Cell type and medication-adjusted	**22qDel vs Control**	**22qDel vs 22qDup**	**22qDup vs Control**
Total DE gene	27	32	4
Upregulated genes	1 (4%)	3 (9%)	3 (75%)
Downregulated genes	26 (96%)	29 (91%)	1 (25%)
22q11.2 genes	24 (89%), all downregulated	24 (75%), all downregulated	2 (50%; C22orf39, ZDHHC8) upregulated
Outside 22q region	3 (11%; HIST1H2BD, FUT7, EDAR)	8 (25%)	2 (50%; CDH6, SAPCD2)

### 3.3. Proportions of T cell, mast cell, and macrophage subtypes differed between CNV groups

After finding differential expression in genes and module eigengenes that were related to immune function and cell types, we sought to directly assess potential differences in cell type composition between groups. After residualizing cell proportions for medication usage, there were significant groupwise differences in 5 cell types at a corrected $q \leq$ 0.05: resting mast cells, activated mast cells, CD8 T cells, M0 macrophages, and M1 macrophages (Fig. 4). Specifically, 22qDel carriers had significantly decreased CD8 T cell proportions compared to controls, consistent with prior findings of T-cell deficits and thymic dysplasia in 22q11.2 Deletion Syndrome (Crowley et al., 2018; McLean-Tooke et al., 2008; Morsheimer et al., 2017), as well as marginally decreased proportions compared to 22q11Dup carriers. Interestingly, 22qDel carriers showed an increased proportion of activated mast cells and a corresponding decrease in resting mast cells relative to both controls and 22qDup carriers. Mast cells are selectively activated in response to multiple triggers to release mediators that regulate immune/pro-inflammatory cell-types including CD8 T cells and macrophages (Krystel-Whittemore et al., 2015). Finally, 22qDel carriers also showed significantly increased M1 macrophage proportions compared to controls and 22qDup carriers, while 22qDup carriers showed increased proportions of M0 macrophages relative to 22qDel carriers and controls. Together, this suggests broad dysregulation of multiple immune-related cell-types in 22qDel carriers and milder changes in cell composition in 22qDup carriers.

Because of these cell type proportion differences between groups, differential expression analysis and WGCNA were re-assessed following cell type adjustment in order to assess: i) the extent to which differences in cell type proportion accounts for the prior described differential gene and module eigengene expression findings; and ii) any potential pan-cellular effects of the CNVs on DE.

3.4. Cell type proportion strongly impacts differential expression at the single-gene level

After adjusting gene expression for cell type proportion, DE was less pronounced between groups in magnitude and number of genes. While
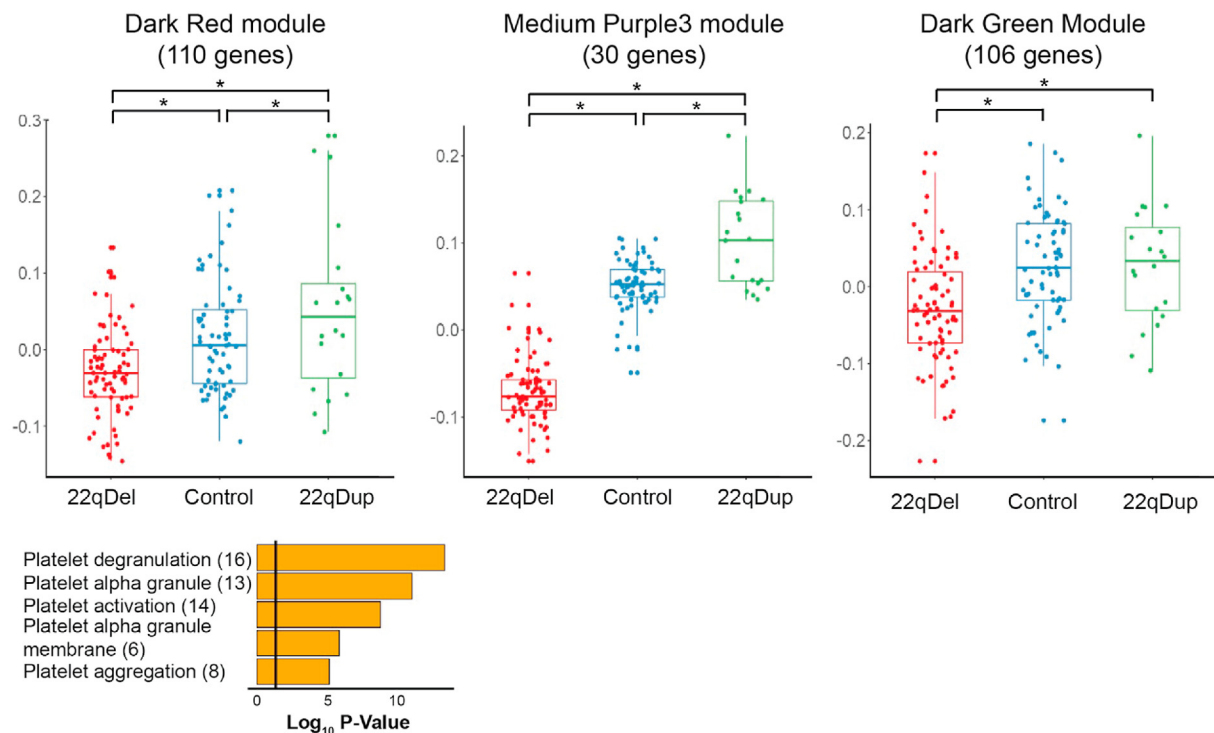
Fig. 2. Module eigengene expression for the 3 modules (of 28 modules) that showed corrected group differences. These modules were from gene expression that was not adjusted for cell type or medication status. Bars with an asterisk indicate significant pairwise group differences at FDR corrected q ≤ .05. Modules that were significantly different between groups were submitted to gene ontology (GO) enrichment analysis, and up to 5 associated GO terms are displayed. The medium purple module largely consisted of genes within the 22q11.2 locus. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
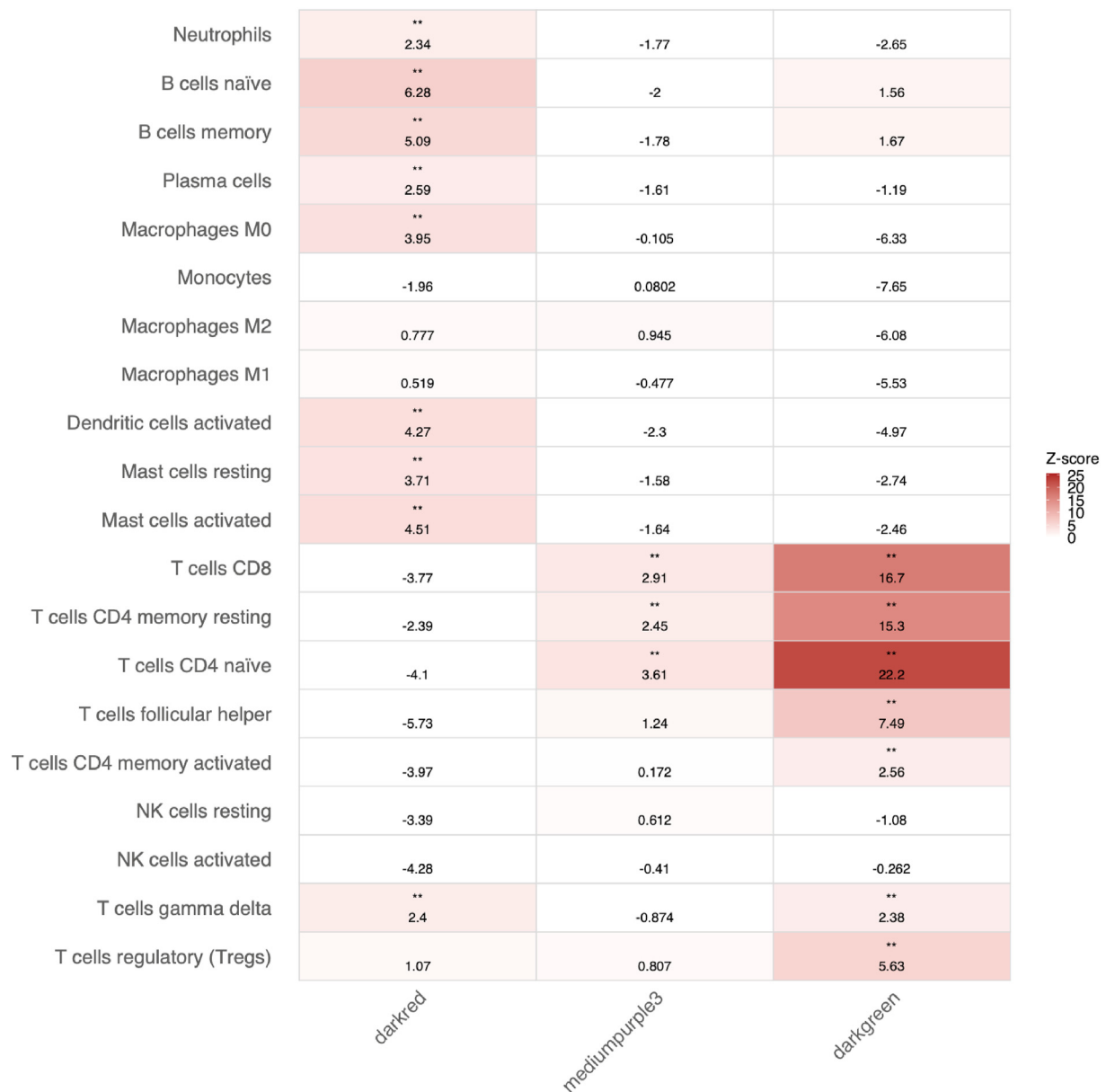
Fig. 3. Cell type enrichment of the unadjusted module eigengenes that significantly differed between CNV groups. This heatmap displays the enrichment of the gene set for each module for cell type specificity. Cell types are ordered by hierarchical clustering of average expression profiles. Modules are ordered by hierarchical clustering of eigengene values. The full enrichment results for all modules can be found in Supplementary Fig. S2. ** = significant at FDR-adjusted p < .05.

24 and 25 genes within the 22q11.2 locus remained significantly downregulated in 22qDel compared to controls and 22qDup, respectively, the number of significantly DE genes outside the 22q11.2 locus decreased from 362 to 3 for 22qDel versus controls (Data Table S4) and from 113 to 8 for 22qDel versus 22qDup (Data Table S5). For 22qDel vs. controls, this included downregulation of the non-22q11.2 genes, FUT7, which encodes an enzyme that allows white blood cells to accumulate at a site of inflammation (Zhang et al., 2018), and EDAR, which encodes a receptor that can activate NF-Kappa-B transcription factors which are involved in inflammatory and immune responses (Morlon et al., 2005), and significant upregulation of HIST1H2BD, which encodes a histone protein that is part of the H2B family (Kari et al., 2013). For the 22qDup versus control comparison, the number of significantly differentially expressed genes within the locus dropped from 5 to 2, while those outside the locus increased from 1 to 2. Specifically, the non-22q11.2 cadherin-6 encoding gene, CDH6 remained significantly upregulated in 22qDup after controlling for cell-type proportions, and SAPCD2, which is

involved in regulating cell division (Chiu et al., 2016), was downregulated in 22qDup compared to controls (Data Table S6). Thus, while some genes remained significantly differentially expressed between CNV groups and controls, the above-described differences in T cells, mast cells and macrophages appear to largely underlie the broader transcriptomic changes detected between groups.

3.5. No group differences in WGCNA module eigengene expression were observed after adjusting for cell-type proportion and medication usage

Applying WGCNA to cell-type proportion and medication-adjusted gene expression data identified 29 modules of co-expressed genes in the full dataset of 22qDel carriers, 22qDup carriers, and control subjects. Consistent with cell-types being a major driver of gene co-expression and with cell proportion changes being a major driver of transcriptomic changes in 22qDel carriers, none of the modules derived from cell-proportion adjusted expression data were significantly differentially

Fig. 4. Blood cell type proportions across groups. Box plots overlaid with scatterplots of cell-type proportion values that have been adjusted for medication usage for 20 cell types across each group. Bars with an asterisk indicate significant pairwise contrast at Benjamini-Hochberg FDR corrected q ≤ 0.05. Due to the low expression of some cell types, a linear model was applied to logit-transformed cell type percentages after residualizing for potential batch and medication confounds to handle non-normality of values and presence of zeroes.

expressed between groups.

3.6. Highly similar results in unrelated sample of 22qDel, 22qDup, and controls

A subset of 22qDel and control or 22qDup and control participants were related to one another. Restricting analyses to only unrelated individuals by randomly selecting one individual per family across groups (22qDel n = 78, Control n = 39, 22qDup n = 12) yielded a highly similar pattern of group differences for gene expression (Figure S3, A-C) and module eigengene expression (Fig. S4), as well as cell proportion (Fig. S5), including after adjusting gene expression data for estimated cell proportions and medication status (Fig. S3, D-F; Table S1). However, macrophages M1 no longer differed significantly between groups after controlling for multiple testing (Fig. S5).

3.7. No significant differences for DE, WGCNA, and cell type proportions detected in 22q11 CNV subjects with ASD or psychosis

No significant main effects or interactions were found between 22qDel and 22qDup carriers with and without ASD in cell type proportions (FDR adjusted *p-values* > .05), nor in cell type- and medication-adjusted WGCNA module eigengene expression or DE (Data Tables S7 and S8).

Similarly, no significant differences were found in cell type proportion, nor cell type- and medication-adjusted module eigengene expression or DE for 22qDel carriers with and without psychosis (Data Table S9). Incorporating cell type and medication-adjusted gene expression data for 22qDel patients from the Utrecht site (total 22qDel without psychosis n = 93, total 22qDel with psychosis n = 17) yielded similar results, with no significant group differences in cell type proportions or gene-expression (FDR adjusted *p-values* > .05).

3.8. No associations between IQ or structural MRI characteristics and adjusted expression of DE genes, module eigengenes, or cell type proportions

There were no significant interactions between cell type-adjusted gene expression and CNV group on IQ, mean cortical thickness, or total cortical surface area. There were also no significant associations between DE genes and group-residualized IQ, mean cortical thickness, or total cortical surface area.

There were no interaction effects of adjusted module expression with group on IQ, mean cortical thickness, or total cortical surface area, nor significant main effects of adjusted module expression on group-residualized IQ, mean cortical thickness, or total cortical surface area.

Finally, there were no significant interactions of cell type proportion with group on IQ, mean cortical thickness, or total cortical surface area, nor main effect associations of cell type proportion on group-residualized IQ, mean cortical thickness, or total cortical surface area.

4. Discussion

This study is the first, to our knowledge, to comprehensively characterize transcriptome-wide gene dosage effects of peripheral blood gene expression from reciprocal 22q11.2 CNV carriers. We observed robust DE in 22qDel carriers compared to controls, as previously reported (Jalbrzikowski et al., 2015; van Beveren et al., 2012), and showed for the first time significant DE between 22qDup carriers versus 22qDel carriers and controls. Notably, the proportion of estimated T-cells, mast cells, and activated macrophages also differed between 22qDel and the control and 22qDup groups, and 22qDup carriers additionally showed increased proportions of undifferentiated macrophages compared to 22qDel carriers and controls. Differences in gene expression between groups were substantially reduced after adjusting for cell composition differences, with the strongest remaining differences between 22q11.2 CNV carriers and controls being differential expression of genes within the 22q11.2

locus. For 22qDel carriers, this suggests that the widespread DE of genes outside the 22q11.2 locus largely reflects downstream changes in cell proportions rather than more specific changes in gene expression within cells or across cell types.

A small number of genes were significantly differentially expressed in 22qDup carriers compared to controls. This included the cadherin 6 (CDH6) gene, located on chromosome 5, which was differentially expressed both before and after controlling for differences in cell proportion. Given the role of CDH6 in cell adhesion and central nervous system morphogenesis (Paulson et al., 2014), its dysregulation, in addition to dysregulation of genes within the 22q11.2 locus, could contribute to broader phenotypes in 22qDup. This overall small number of DE genes partially reflects our power limitations for analyses comparing 22qDup carriers and controls relative to analyses comparing 22qDel carriers and controls; however, the log-fold change in gene expression for 22qDup carriers was also attenuated compared to that observed for 22qDel carriers. This is consistent with the milder phenotypes observed in 22qDup compared to 22qDel carriers, a pattern that has been observed for other reciprocal copy number variants (Douard et al., 2021; Girirajan et al., 2012; Rosenfeld et al., 2013), and with prior findings that the molecular consequences of gene duplications are more variable and context-dependent than gene deletions (Hurles et al., 2008).

Genes within the 22q11.2 locus which still showed significantly decreased expression in 22qDel carriers compared to controls after adjusting for cell type proportion may reflect pan-cellular effects of the deletion that could contribute to the broad systemic pathology of 22q11.2 deletion syndrome. These findings may reflect genuine non-cell type-specific effects, which could reflect gene regulatory effects of some genes within the locus, such as DGCR8, which has been hypothesized to play an important role in the 22q11.2 Deletion Syndrome phenotype (Forsyth et al., 2020; Merico et al., 2014; Stark et al., 2008). However, we cannot definitively rule out the possibility that they represent residual effects of cell type composition, batch, or other unaccounted-for confounding variables. To better understand cell-type specific effects of 22q11.2 CNVs, future studies could incorporate single-cell RNA sequencing on purified cell populations obtained through sorting approaches such as fluorescence activated cell sorting (FACS) or magnetic activated cell sorting (MACS (Sutermaster and Darling, 2019)). Future development of "digital sorting" methods for gene expression may also allow us to impute cell-type specific expression from bulk tissue. Such methods are well developed for methylation (Rahmani et al., 2019) but remain challenging to apply to gene expression data.

We observed several differences in cell type composition that are supported by a rich literature showing mild-to-moderate T cell deficits in 22qDel carriers (Crowley et al., 2018; Gennery, 2012), resulting in a spectrum of immune dysfunction, including infection and autoimmunity issues (Derfalvi et al., 2016; Jawad et al., 2001). To our knowledge, differences in activated macrophage and mast cell abundance between 22q11.2 CNV carriers have not been previously reported; however, interactions between T-cells, mast cells, and macrophages are well-documented as part of a coordinated immune response (Krystel-Whittemore et al., 2015). In addition, low abundance cell populations such as mast cells and macrophages must be purified from whole blood using flow cytometry, making them more challenging to study than more abundant cell populations.

Notably, macrophages in peripheral blood share many properties with microglia, the resident macrophages of the brain, which suggests that the 22q11.2 deletion could also affect microglia function. Microglia interact with virtually all CNS components, are critical for brain development, tissue integrity, and neuronal activity, and also refine cortical circuits by regulating synaptic pruning (Li and Barres, 2018; Paolicelli et al., 2011). Mounting evidence implicates microglial dysfunction leading to chronic neuroinflammation in the pathogenesis of schizophrenia as well as ASD (De Picker et al., 2017; Koyama and Ikegaya, 2015; Laskaris et al., 2016; Petrelli et al., 2016; Takano, 2015), although no differences in cell proportion were observed between CNV carriers

with vs. without ASD or psychosis in the present study that survived correction for multiple testing. Nevertheless, our identification of several potentially novel cell type composition differences warrants future investigation. Recent work has established in vitro organoid models of 22q11.2 deletion and found evidence of neuronal defects (Khan et al., 2020). This experimental context would provide an ideal environment to test whether and to what extent microglial dysfunction, particularly in synaptic pruning, is observed in the 22q11.2 deletion syndrome.

We did not find any significant associations between cell type proportion, cell type-adjusted gene expression, or cell type-adjusted module expression with psychiatric phenotypes, nor with IQ or major neuroanatomic characteristics. It is possible that previous reports of clinical associations with module expression reflected differences in cell type composition (Jalbrzikowski et al., 2015; van Beveren et al., 2012), which were not assessed, or the more liberal significance threshold used in prior studies compared to the current study. Larger samples of 22q11.2 CNV carriers with psychosis and ASD diagnoses are needed in order to identify reliable transcriptomic changes associated with these phenotypes.

Our study has some parallels to expression quantitative trait loci (eQTL) studies, which inform one of our most significant limitations in connecting CNV-associated gene expression changes in blood to potential effects in the brain, especially regarding DE of genes outside the 22q11.2 locus. Like analyses of the transcriptomic effects of CNVs, eQTL studies seek to identify the effects of genetic variation on gene expression. While our focus is on rare pathogenic genetic variants, eQTL studies examine the effects of many common variants. The significant differences that we observe in expression of genes within the 22q11.2 locus are comparable to *cis*-eQTLs, which identify common single nucleotide and structural variants that affect expression of proximal genes. Conversely, the significant but sparse differences that we observe in expression of genes outside the 22q11.2 locus are analogous to *trans*-eQTLs, which identify variants that affect distal gene expression. eQTL studies have shown that while *cis*-eQTLs are often conserved across many cell types and tissues, *trans*-eQTLs are more tissue- and cell-type-specific (GTEx Consortium, 2020). This suggests that the molecular effects of 22q11.2 deletions on the genes in the locus are also conserved across many cell types and tissues, which is consistent with the broad, multi-systemic clinical phenotype associated with 22q11.2 Deletion Syndrome. Given these insights from eQTL studies, that a subset of 22q11.2 genes are known to be highly-conserved and broadly-expressed (Guna et al., 2015), and that 22q11.2 deletion confers a multisystemic phenotype (McDonald-McGinn et al., 2015), this may limit our ability to extrapolate tissue and cell type specific *trans*-effects of the 22q11.2 CNVs. This reinforces the importance of in vitro and in vivo modeling and post-mortem sample collection to complement this work and gain a more complete understanding of the broad effects of 22q11.2 CNVs across many tissues and cell types.

5. Conclusions

In sum, while we found that the 22q11.2 deletion significantly altered gene expression across the genome, these differences were substantially attenuated after adjustment for cell type heterogeneity. We also extended findings regarding cell type differences between 22q11.2 deletion carriers and controls beyond known T cell differences to include mast cell and macrophage subtypes. Notably, no study has yet characterized the 22q11.2 duplication in terms of transcriptomic dysregulation. We identified milder changes in gene expression in 22qDup carriers compared to controls.

While there are challenges inherent in using peripheral blood to study brain-related diseases, given that broadly-expressed genes are an important class of genes for neurodevelopmental disorders like intellectual disabilities or ASD (Courchesne et al., 2020; Kasherman et al., 2020), and that there is correspondence between brain and blood tissues (Qi et al., 2018; Sullivan et al., 2006; Tylee et al., 2013), investigating blood expression patterns can still be valuable. Indeed, immune dysfunction is also becoming increasingly studied in the pathology of psychiatric

disorders (Capuron and Miller, 2011; Irwin and Miller, 2007; Jones and Thomsen, 2013; Misiak et al., 2019), and peripheral blood is a readily accessible tissue for studying immune effects of the 22q11.2 deletion and other rare genetic variants implicated in psychiatric disease. The methodology established here demonstrates how information can be extracted from existing blood "omics" datasets to generate testable hypotheses with the ultimate goal of advancing our understanding of the pathophysiology of psychiatric and neurodevelopmental disorders.

Declaration of competing interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2021.100386.

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