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Molecular biomarkers to track clinical improvement following an integrative treatment model in autistic toddlers

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Short Communication

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Abstract

Objectives: Identifying an objective, laboratory-based diagnostic tool (e.g. changes in gene expression), when used in conjunction with disease-specific clinical assessment, could increase the accuracy of the effectiveness of a therapeutic intervention. *Methods:* We assessed the association between treatment outcome and blood RNA expression before the therapeutic intervention to post-treatment (after 1 year) of five autism spectrum disorder (ASD) toddlers who underwent an intensive cognitive-behavioural intervention integrated with psychomotor and speech therapy. *Results:* We found 113 significant differentially expressed genes enriched for the nervous system, immune system, and transcription and translation-related pathways. Some of these genes, as *MALAT-1*, *TSPO*, and *CFL1*, appear to be promising candidates. *Conclusions:* Our findings show that changes in peripheral gene expression could be used in conjunction with clinical scales to monitor a rehabilitation intervention's effectiveness in toddlers affected by ASD. These results need to be validated in a larger cohort.

Significant outcomes

- This preliminary study shows promising candidates predicting therapy outcome in ASD.

Limitations

- The results need to be confirmed in a larger cohort due to the limited sample size.

Introduction

Autism spectrum disorder (ASD) is a genetically heterogeneous neurodevelopmental condition characterised by repetitive and stereotyped behaviours, restricted interests, and social and communication deficits (American Psychiatric Association, 2013). At present, there are no psychopharmacological therapies to treat all ASD core symptoms effectively. However, it is worldwide recognised that early detection and targeted intervention can significantly modify the ASD evolutionary trajectory by improving learning, communication, social skills, and underlying brain development. Indeed, during the first years of life, brain plasticity is exceptionally high (Kilinc, 2018) and can be modulated by external factors as *in primis* rehabilitation (Han *et al.*, 2018).

ASD guidelines point towards an early intensive treatment based on combined cognitive and behavioural therapies as the most effective pre-school children intervention. When deemed appropriate, cognitive-behavioural therapy is integrated with speech, psychomotor therapy, and specific parent and teacher training. Treatment duration varies from 20 to 40 h a week across to 1 to 4 years of the child's life (Reichow *et al.*, 2012). In ASD, the effectiveness of rehabilitation is routinely measured by administering clinical scales at various time points post-treatment. An emerging alternative and integrative approach is the use of biomarkers. Unlike clinical scales, biomarkers have the advantage of providing an objective measure as they reflect a given patient's current biological condition. Changes in gene expression appear to be promising biomarkers for monitoring and quantifying a psycho-behavioural therapy

intervention's success, as there is evidence that neural pathways may be modulated by external stimuli (Ishii *et al.*, 2014). No studies have investigated the association between gene expression changes and the response to the rehabilitation therapy in ASD to date.

Study aim

Here we assess genome-wide RNA expression changes following an intensive integrative treatment model in a small ASD toddler group. We aim at identifying reliable biomarkers that could be related to the therapy response.

Materials and methods

Detailed methods are reported in the Supplementary appendix.

Subjects

Fifteen ASD patients were recruited at the Centro Ricerca Cure (CRC) Balbuzie in Rome (Italy). Inclusion criteria for enrolment were a) patients who did not receive either cognitive and behavioural or medication treatment, b) age range 30–60 months, and c) availability of the patient's history from birth until the time of the diagnosis. The authors assert that all procedures contributing to this work comply with the relevant national and institutional committees' ethical standards on human experimentation and the Helsinki Declaration of 1975, as revised in 2008. A total of 10 patients were excluded from the study due to: refusal to participate ($n = 4$) and drop out for non-compliant treatment ($n = 6$). The final sample consisted of five subjects (one female, four males; mean age = 39.4 months; standard deviation = 2.7; range 32–45 months). ASD diagnosis was made using the Autism Diagnostic Observation Schedule (ADOS-2), the Vineland Adaptive Behavior Scales Second Edition (VABS-II), the Psychoeducational profile – Third Edition (PEP-3), and the Griffiths Mental Developmental Scales-Extended Revised (GMDS-ER). Psychodiagnostics tests were administered before the therapeutic intervention (time 0; T0) and 12 months of treatment (time 1; T1). Raters were blinded to child treatment status (pre-/post-intervention).

The therapeutic intervention according to the treatment model used was based on: (1) 10 weekly hours of cognitive-behavioural therapy (naturalistic developmental behavioural intervention approach, five sessions of 2 h) integrated with 10 weekly hours of speech (2 sessions of 2 h) and psychomotor (3 sessions of 2 h) therapies; (2) parent support following diagnosis communication and parent training to implement specific programmes in the familiar context; and (3) psychoeducational intervention and meetings with teachers to facilitate child integration in the school context. The total number of hours per week was 20. Every child received treatment by the same operators during the entire study. Educational efforts focusing on autistic symptoms and their management were discussed in encouraging adherence to the treatment model. All parents were adherent to prescribed therapies and provided informed consent for their children, and the Ethical Committee approved the consent form.

RNA sequencing and data analysis

Whole blood was collected before and after 12 months of therapeutic intervention. RNA sequencing was conducted in one batch at NovoGene Corporation INC (https://en.novogene.com/; Sacramento, CA) using Globin-Zero Gold rRNA Removal Kit & NEB directional library. Reads were aligned to the human genome (GRCh37) using *STAR* v2.5 and summarised at the gene level using *FeatureCounts* 1.4.4. Gene expression differential analysis between post- and pre-treatment was conducted using *DESeq2* v1.14.1 with a paired model including RIN as a covariate and adjusting *p*-values using the false discovery rate (FDR). We sequenced a total of 487 Million (M) of reads (median: 47.3 M, range: 42.6–58.1 M), with an 89.9% mapping rate. Principal component analysis did not show any outlier (Fig. S1). The low responder patient was not included in the differential expression analysis.

We adjusted for RIN since we noted larger post-treatment group values, although not significant ($p < 0.500$). Additionally, we correlated the variation of ADOS-2 measurements with gene expression changes, computing a Pearson's correlation using the expression values adjusted for RIN, sex, and age.

Validation dataset

We hypothesised that associated genes with the treatment should be significantly different in our dataset (post- vs. pre-treatment) but have an opposite direction when comparing patients versus controls. We considered the RNA profiling meta-analysis conducted by Tylee *et al.* (2017) using whole blood data, considering "validated" genes if: (1) were significant at the unadjusted $p < 0.05$ in one of the two models (non-*sva* and *sva*), (2) with discordant log2 Fold Change (FC) direction with our results. We analysed an additional dataset (GSE18123) to investigate whether the differentially expressed genes (DEGs) detected in our study were related to age changes. Furthermore, we compared our results with an RNA-sequencing study from the dorsolateral prefrontal cortex (DLPFC) (Wright *et al.*, 2017) (GSE102741). Statistical enrichment between gene lists was conducted using Fisher's test and gene set enrichment analysis (GSEA).

Enrichment analysis

Genes identified as DEGs (adj $p < 0.05$) were further analysed by pathway analysis, and GSEA was conducted using the complete list of genes ranked by log2 FC. We referenced to the REACTOME database adjusting the *p*-values for multiple testing using the FDR method. Finally, we conducted a functional network analysis blood-specific using the *HumanBase* web tool (https://hb.flatironinstitute.org/gene).

Results

Clinical scales detected a global improvement in ASD symptoms following the therapeutic intervention. Compared to the baseline ADOS-2 scores (module 1), we showed a significant decrease in the Total Score ($p = 0.028$) and a nearly significant Repetitive Restricted Behaviors domain score ($p = 0.052$); a significant improvement of the GMDS-ER "Language" ($p = 0.039$), "Locomotor" ($p = 0.021$), "Eye and Hand Coordination" ($p = 0.024$), and "Performance" ($p = 0.008$) subscales scores and PEP-3 "Communication" score ($p = 0.039$). (Table S1). One patient (Fig. S1 and S2) displays an overall lower clinical response than the others. As expected, subjects with ASD showed adaptive behaviour impairment across all VABS-II domains. An improvement of adaptive behavioural functioning at follow-up was detected in all patients except for the low responder (improvement T1 score < 20% at the PEP-3 clinical scale).

We did not detect outliers (Fig. S3), and the differential expression analysis yielded a total of 113 DEGs (adj $p < 0.05$) (Table S2). Most of the genes were upregulated, indicating an increase in their

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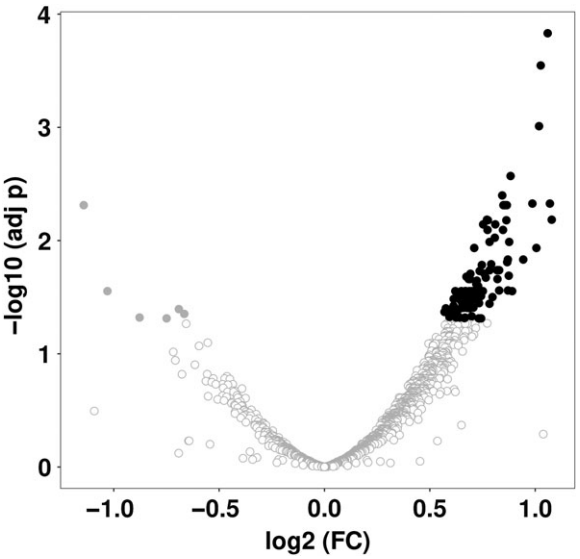

Fig. 1. Volcano plot representing the results of the differential expression analysis (post- vs. pre-treatment). Differentially expressed genes (adj-$p < 0.05$) are reported in black (upregulated) and grey (downregulated).

expression associated with the treatment (Fig. 1). The enrichment of our 113 DEGs in the list from Tylee et al. (2017) ($q < 0.05$ in the *sva* or *non-sva* model: $n = 2{,}175$) was statistically significant (Fisher's exact test: $p = 0.0017$). A total of 22 genes were significantly differentially expressed in the meta-analysis by Tylee et al. (2017), 11 of them significant at the genome-wide level (Table S2). We considered these genes as top candidates because (1) they are significantly associated with the therapy and because (2) they display the same trend in controls versus ASD in the meta-analysis by Tylee et al. (2017). The patients' treatment effect should drive expression changes more like non-affected than affected individuals. Finally, none of these 22 genes were significantly associated with age in the ASD patients from dataset GSE18123 (Table S2), demonstrating our results are not correlated with the age variation between pre-treatment and post-treatment. After re-analysis of the DLPFC brain dataset (Wright *et al.*, 2017), we did not find DEGs between ASD and controls. A total of 104 genes overlapped with our DEGs list, with most of them discordant for log2 FC (65.4%), confirming the trend observed in the whole blood dataset. Only 12 genes were significant before correction, all with log2 FC in the opposite direction than our results (Table S2). Finally, we observed a non-significant enrichment of our 113 DEGs (mostly upregulated) across genes downregulated in ASD in the DLPFC dataset (Fig. S4) ($p = 0.343$; normalised enrichment score = −1.022), confirming the findings in the whole blood dataset with the fold change direction.

The correlation between the changes post- versus pre-treatment in expression profiling and the ADOS-2 variation for all the five patients showed the extent of moderate/strong correlation coefficients (Pearson's r) for the DEGs in comparison to the non-DEGs (Fisher's exact test: $p < 1.0\text{E-}6$). No genes showed a statistically significant correlation. Pearson's r values and log2 FC showed a significant correlation between each other ($R = 0.718$; $p < 2.2\text{E-}16$) (Fig. S5).

We conducted pathway analysis using the 113 DEGs detecting 51 significant pathways (Table S3). GSEA yielded 47 significant pathways (Fig. 2; Table S4). Finally, we conducted a functional network analysis detecting 5 modules enriched for 13 Gene Ontology processes (Fig. S6; Table S5). All these analyses converged upon three main biological processes, including (1) nervous system, (2) immune system, and (3) gene transcription and translation. Nervous system-related pathways included Eph–ephrin signalling, signalling by ROBO receptors, EPHB-mediated forward signalling, semaphorin interactions, RHO GTPases activate WASPs and WAVEs, and axon guidance (Tables S3 and S4). The 22 genes associated with therapy and showed the same trend in the controls of the meta-analysis by Tylee *et al.* (2017) (Table S2) are included in these pathways. Indeed, the *CFL1* gene, the *ACTB* gene, and the *RPL28* gene are present in many nervous system-related pathways where they mediate axon guidance and migration during neurodevelopment and synapsis and dendritic spine remodelling postnatally. Genes such as *ACTB* and *CFL1* and the other significant FCGR3A gene are also found within phagocytosis-related immunological pathways as regulation of actin dynamics for phagocytic cup formation Fc gamma receptor-dependent phagocytosis. These genes display an increase in their expression post-therapy and are usually downregulated in ASD versus controls. Finally, we checked the expression of these candidate genes in blood and brain using GTEx data, observing that most of the genes have a relevant expression also in the brain (Table S6; Fig. S7 and S8).

Discussion

To our knowledge, this is the first study that attempts to correlate gene expression changes with the outcome of an integrative treatment model in ASD toddlers. The identification of genetic predictors of prognosis following cognitive-behavioural therapy could have important clinical implications. Firstly, "genetic therapy biomarkers" could be used to track clinical improvements in ASD-affected children in conjunction with clinical scales by increasing the accuracy of a therapeutic intervention's effectiveness. Furthermore, unlike clinical scales, gene expression changes are patient-specific, and thus they could provide an insight into the complex ASD physiopathology of that particular subject. This information could, in turn, be used to design personalised therapies.

Though we are aware of the small sample size of the present study, we detected a total of 113 DEGs, and they were significantly enriched in the meta-analysis by Tylee et al. (2017). We selected 22 candidates who displayed the same trend in the same study's controls (Tylee et al., 2017). The same trend was observed in post-mortem brain RNA expression, although the enrichment across downregulated genes was not statistically significant. Nearly all these genes (20 out 22) were upregulated post-treatment. *CFL1*, *RPL28,* and *ACTB* genes appear to be good candidates to track clinical improvement. These genes are also expressed in the brain at a considerable level and play a role in post-synaptic development contributing directly to dendritic spine development and morphogenesis (Rust *et al.*, 2010; Pontrello *et al.*, 2012). Indeed, many genes are expressed both in brain and in peripheral blood mononuclear cells, because they have pleiotropic effects and are involved in complex brain–immune interactions (Afridi *et al.*, 2021).

The long non-coding *MALAT-1*, which was found significantly downregulated after treatment, is another attractive biomarker. MALAT-1 is also highly expressed in neurons where it regulates synaptogenesis-related gene expression (Quan *et al.*, 2017). The loss of MALAT-1 leads to decreased synaptic density, whereas its overexpression increases synaptic density (Bernard *et al.*, 2010).

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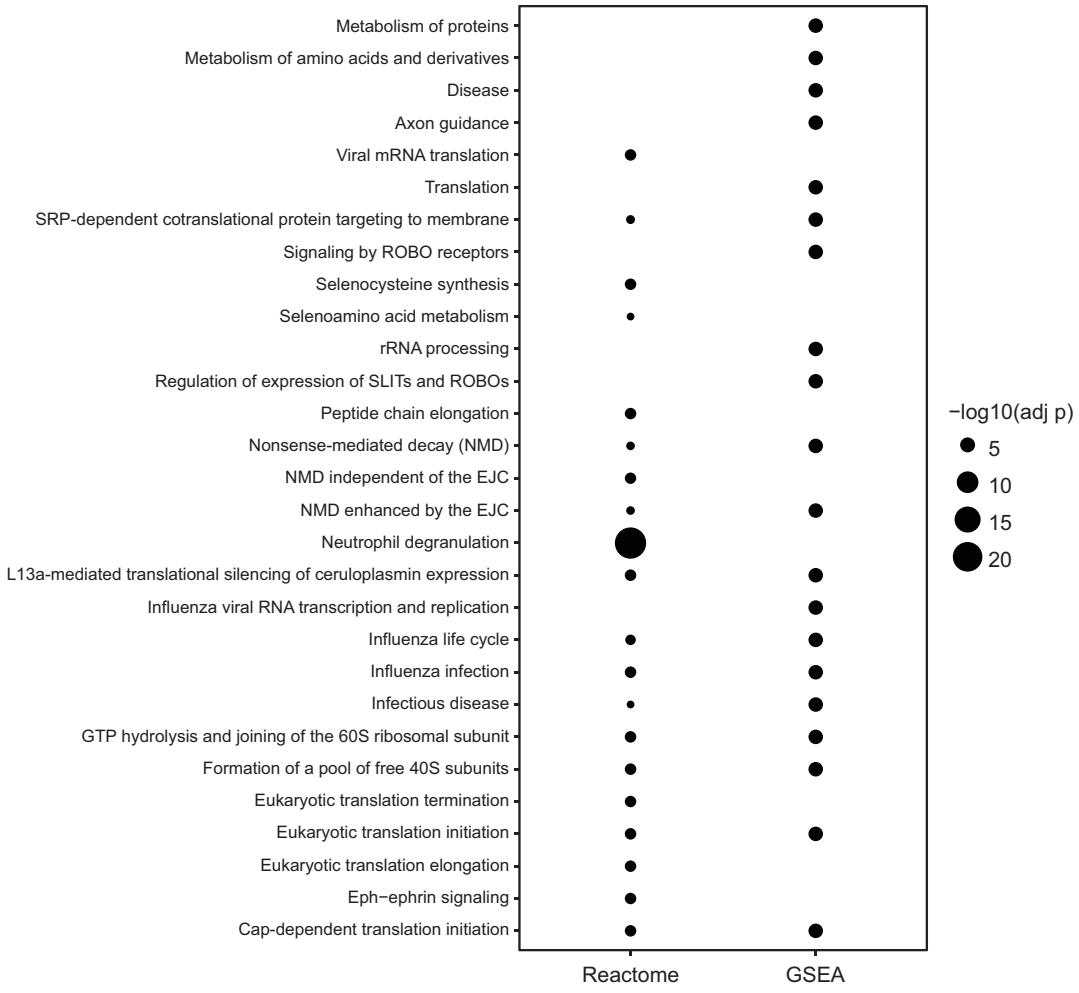

Fig. 2. Dot plot showing the top 20 REACTOME pathways enriched (adj-$p < 0.05$) using a hypergeometric test and gene set enrichment analysis (GSEA).

Upregulation of MALAT-1 has been associated with many pathological conditions as neuropathic pain (Chen *et al.*, 2019), alcohol use disorder (Kryger *et al.*, 2012), as well as many different types of cancer (Cheng *et al.*, 2018). Interestingly, the meta-analysis results by Tylee *et al.* (2017) show upregulation of this lncRNA also in ASD compared to controls, whereas the opposite trend is seen in our study post-treatment. Finally, the translocator protein (*TSPO*) also appears as a potential peripheral marker as it is upregulated post-treatment, whereas it is generally downregulated in the blood of subjects with neuropsychiatric conditions (Barichello *et al.*, 2017), including ASD patients (Tylee *et al.*, 2017).

The action exerted by hormones released by the hypothalamus/hypophysis axis could partially explain the RNA changes we observed in the blood after treatment. Indeed, peripheral genome-wide expression in PMBCs is the results of not just one or a few hormones, rather of a complex array of many hormones, cytokines, metabolites of human and gut microbiote origin, and environmental substances reaching the blood stream through multiple entry paths into the organism and physical variables (body temperature, blood pressure, etc). Genome-wide transcriptomics, though complex in itself, is thus simpler to interpret, than the vast array of physiological and pathogenic modulators present at any given time in a living organism.

Many studies have documented plastic changes in the brain after psychotherapy in several neuropsychiatric conditions as major depressive disorder, obsessive-compulsive disorder, panic disorder, post-traumatic stress disorder, and borderline personality disorder. Kandel (1998) put forth the hypothesis that psychotherapy could lead to gene expression changes by modifying the strengths of synaptic connections and neuron morphology. Since then, several neuroimaging studies have attempted to measure the success of a psychotherapy intervention in major depressive disorder at a more molecular level by measuring serotonin and dopamine transporters' densities and their receptors in specific brain regions (Lehto *et al.*, 2008; Hirvonen *et al.*, 2011).

More recently, some groups have examined the relationship between genome-wide expression changes and psychological treatment. Coleman *and colleagues* (2017), in patients affected by anxiety disorder, found no significant change in gene expression following cognitive-behavioural therapy and at 6 months follow-up. Interestingly, one of the top genes that displayed a change in expression was the high-affinity IgE receptor *FCER1G* gene, which is known to be involved in allergic response and immunity. This gene was significantly upregulated in post-treatment in our study, whereas it is typically downregulated in ASD/control studies (Tylee *et al.*, 2017). Indeed, many studies have linked immune dysregulation to psychiatric conditions (Gibney & Drexhage, 2013; Gandal *et al.*, 2018), including ASD (Garbett *et al.*, 2008).

Different forms of post-transcriptional, translational, and post-translational control can profoundly affect function downstream

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from the point of observation used in this study. Despite this relative disadvantage compared to proteomics, transcriptomics nonetheless is rich of informative content. In fact, plenty of contributions in the current neuropsychiatric literature aim to find valuable molecular biomarkers including microRNA and lncRNA (long not coding RNA). A recent work (Sehovic *et al.*, 2020) conducted in salivary samples of ASD children and typically devoloping children identifies six differentially expressed microRNAs. Remarkable alterations of lncRNA expression have been reported in psychiatric conditions such as schizophrenia, autism, and depression (Rusconi *et al.*, 2020). Furthermore, changes in PMBC mRNA expression associated with psychotherapy have been identified in major depression (Kéri *et al.*, 2014) and post-traumatic stress disorder (Levy-Gigi *et al.*, 2013; Kéri *et al.*, 2014).

In conclusion, we reported the extent of potential blood RNA biomarkers of cognitive-behavioural therapy outcome in ASD. Despite the limited sample size, the results for 22 genes were validated in a meta-analysis including 626 ASD and 447 non-affected.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/neu.2021.12

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Conflict of interest. None.

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