

1 Subtypes of functional connectivity 2 associate robustly with ASD 3 diagnosis

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16
17 **Abstract** Our understanding of the changes in functional brain organization in autism is
18 hampered by the extensive heterogeneity that characterizes this neurodevelopmental disorder.
19 Data driven clustering offers a straightforward way to decompose this heterogeneity into
20 subtypes of distinguishable connectivity types and promises an unbiased framework to
21 investigate behavioural symptoms and causative genetic factors. Yet the robustness and
22 generalizability of these imaging subtypes is unknown. Here, we show that unsupervised
23 functional connectivity subtypes are moderately associated with the clinical diagnosis of autism,
24 and that these associations generalize to independent replication data. We found that subtypes
25 identified robust patterns of functional connectivity, but that a discrete assignment of individuals
26 to these subtypes was not supported by the data. Our results support the use of data driven
27 subtyping as a data dimensionality reduction technique, rather than to establish clinical
28 categories.

29 **Introduction**

30 Autism spectrum disorder (ASD) is a prevalent neurodevelopmental condition of impaired social
31 communication and restrictive behaviour, diagnosed in about 1% of children (*Lai et al., 2014; Baio*
32 *et al., 2018*), that is associated with extensive heterogeneity of behavioural symptoms and neuro-
33 biological endophenotypes (*Jacob et al., 2019; Lombardo et al., 2019*). Functional magnetic reso-
34 nance imaging (fMRI) has emerged as a promising technology to identify potential biomarkers of
35 functional connectivity (FC) in ASD and other psychiatric disorders (*Castellanos et al., 2013*). How-
36 ever, efforts to characterize the functional brain organization in ASD have so far largely focused
37 on case-control comparisons, thus ignoring the presumed heterogeneity of FC alterations (*Nunes*
38 *et al., 2019; Hahamy et al., 2015*).

39 Data driven cluster analysis has long been proposed as a solution to decompose the hetero-
40

41 genicity of behavioural symptoms in ASD into distinct subtypes (*Eaves et al., 1994; Beglinger and*
42 *Smith, 2001*), but these subtypes have proven difficult to distinguish in clinical practice (*Lord et al.,*
43 *2012*) and were recently abandoned in favour of the broader concept of an autism spectrum (*Amer-*
44 *ican Psychiatric Association. and DSM-5 Task Force., 2013*). The lack of progress towards repro-
45 ducible, brain based biomarkers of ASD (*Lombardo et al., 2019*) has renewed interest in clustering
46 methods to decompose the heterogeneity of brain alterations into distinct subtypes that are hy-
47 pothesized to underlie the multitude of behavioural symptoms.

48 To date, only a small number of studies have applied brain based subtyping to characterize the
49 neurobiological heterogeneity in ASD and relate it to behavioural symptoms (*Hong et al., 2019*).
50 Early work on subcortical volume alterations in ASD distinguished four subtypes, but did not find
51 significant differences of behavioural symptoms between them (*Hrdlicka et al., 2005*). A more re-
52 cent multi-modal analysis distinguished three subtypes of structural brain alterations in ASD and
53 found that core ASD symptoms could be much better predicted from the structural MRI data when
54 separate prediction models were trained on each subtype compared to the on the full, unstratified
55 dataset (*Hong et al., 2017*). Work on the heterogeneity of FC in individuals with ASD, attention deficit
56 hyperactivity disorder (ADHD), and NTC distinguished three FC subtypes among regions in the de-
57 fault mode network (DMN) and found that each subtype was associated with all three diagnostic
58 groups, indicating that these FC subtypes may be shared across diagnostic boundaries (*Kernbach*
59 *et al., 2018*). An analysis of whole-brain FC in ASD and neurotypical control (NTC) individuals distin-
60 guished two subtypes of diverging within- and between-network connectivity, but similarly showed
61 that the assignment of individuals to these subtypes was not associated to their clinical diagnosis
62 (*Easson et al., 2019*).

63 These initial findings of subtypes in ASD leave several important questions open. Firstly, stud-
64 ies have so far interpreted subtypes both as categories that individuals are discretely assigned to
65 (*Hrdlicka et al., 2005; Hong et al., 2017*) and as dimensions that each individual can have a contin-
66 uous measure of similarity with (*Kernbach et al., 2018; Easson et al., 2019*). However, the stability
67 of either of these two methods of assigning individuals to subtypes has not been systematically
68 established. Secondly, several previous studies have limited their investigation of subtypes to indi-
69 viduals who were already diagnosed with ASD (*Hrdlicka et al., 2005; Hong et al., 2017; Tang et al.,*
70 *2019a*). Whether subtypes associated with ASD symptoms are specifically found among these diag-
71 nosed individuals, or are also prevalent in the general population has not been clearly established.
72 Behavioural symptoms in ASD overlap with those of other neurodevelopmental disorders and also
73 extend into the general population (*Constantino and Todd, 2003; Grzadzinski et al., 2011*). Similarly,
74 neurobiological endophenotypes associated with ASD have been shown to exist among individu-
75 als with other neuropsychiatric disorders (*Park et al., 2018; Di Martino et al., 2013*). It is therefore
76 important to investigate whether subtypes identified in mixed samples of both ASD and NTC indi-
77 viduals show an association with ASD diagnosis and symptoms. Thirdly, none of the brain based
78 ASD subtypes reported in the literature have been replicated to date. The recent failure to replicate
79 promising reports of clinically meaningful neuroimaging subtypes in depression (*Drysdale et al.,*
80 *2017; Dinga et al., 2019*) has highlighted the importance of this limitation for the autism literature.

81 In this work, we aim to address these three gaps by applying a straightforward, unsupervised
82 subtyping approach to subdivide a heterogeneous sample of both ASD and NTC individuals by
83 their network based FC patterns. Firstly, we systematically evaluate the robustness of the subtype
84 maps, and the discrete and continuous assignment of individuals to them. Secondly, we determine
85 whether diagnosis naive subtypes of FC show an association with clinical ASD diagnosis at the
86 network level. And thirdly, we determine the generalizability of our findings by replicating them on
87 an independent dataset.

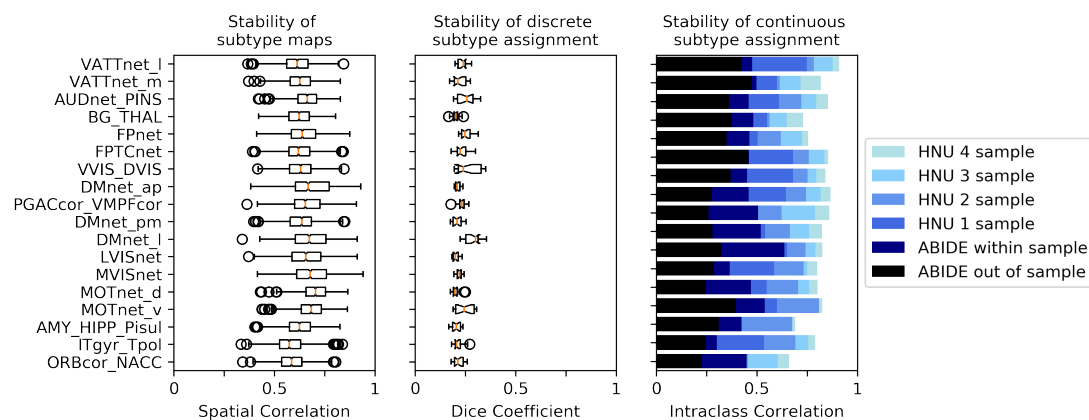


Figure 1. Robustness of subtyping outcomes across brain networks. Left: Stability of the FC subtype maps. Boxplots represent the range of the average similarity between FC subtype maps of the same brain network that were extracted from separate subsamples of the discovery dataset. Middle: Stability of discrete assignments of individuals to a FC subtype cluster. Boxplots represent the average overlap between the clusters an individual was assigned to in two different random subsamples. Right: Stability of continuous assignments of individuals to a FC subtype across repeated imaging sessions. Bar plots represent the average Intra-class Correlation between continuous subtype assignments computed on separate longitudinal imaging sessions. Different bar hues represent the stability of continuous subtype assignments extracted from out-of-sample subtypes (black), from within-sample subtypes (dark blue), within-sample subtypes in a general population data set where multiple scan sessions were combined to compute continuous subtype assignments (lighter shades of blue reflect more combined sessions).

88 Results

89 Subtype maps are stable

90 We first aimed at evaluating the robustness of subtype maps. Subtype maps are the spatial FC
 91 profiles corresponding to each identified subtype in the brain. For this purpose, we repeated the
 92 subtype analysis on random subsamples of 50% of the discovery dataset. We then matched the
 93 subtype maps of each seed network across subsamples, based on the highest similarity between
 94 pairs of maps. The average spatial Pearson correlation between matched subtype maps was $\bar{r} =$
 95 $0.65 (0.034SD)$ across all seed networks and subsamples. We observed small variations across seed
 96 networks: from $r = 0.58 (0.081SD)$ for the inferior temporal gyrus seed network up to $r = 0.7$
 97 $(0.069SD)$ for the dorsal motor network (see **Figure 1**). We thus showed that the subtype maps of
 98 the identified subtypes were robust to random perturbations in the dataset.

99 Discrete individual subtype assignments are not stable

100 To evaluate the robustness of discrete assignments of individuals to a subtype, we compared the
 101 overlap of the subtypes that an individual was part of between pairs of random subsamples. Be-
 102 cause each subsample contained a random selection of individuals, we constrained our analysis
 103 to individuals that were included in both subsamples. The overlap was measured by the Dice co-
 104 efficient (*Dice, 1945*). The average overlap of discrete subtype assignments was low at $Dice = 0.22$
 105 $(0.025SD)$. That is, 22% of the subtype neighbours of an individual in one subsample would on
 106 average also be subtype neighbours of this individual in another subsample. The range of over-
 107 lap between subtypes was $Dice = 0.2 (0.018SD)$ for the auditory network to $Dice = 0.28 (0.046SD)$
 108 for the medial visual network (see **Figure 1**). We thus showed that the discrete assignment of an
 109 individual to a subtype was not robust to random permutations of the data in our simulation.

110 Continuous individual subtype assignments are stable

111 We evaluated the replicability of continuous, individual subtype assignments for each seed net-
 112 work. To do so, we computed the intraclass correlation coefficient (ICC, *Shrout and Fleiss, 1979*)

113 of the continuous assignment across repeated scan sessions. The observed ICC coefficients were
114 interpreted (*Cicchetti, 1994*) as

115 **poor** if less than 0.4
116 **fair** up to 0.59
117 **good** up to 0.74
118 **excellent** if larger than 0.75

119 When subtypes and the corresponding individual assignments to these subtypes were computed
120 on data from the same individuals (within-sample replicability), the average ICC across seed net-
121 works was fair at $ICC = 0.46$ ($0.073SD$). The range of the stability of continuous subtype as-
122 signments across networks was $ICC = 0.3$ for the amygdala-hippocampal complex network to
123 $ICC = 0.63$ for the lateral default mode network.

124 To evaluate the replicability of continuous subtype assignments, we repeated this analysis in a
125 separate, general population dataset, wherein 10 scan sessions were available for each individual.
126 In this data set, the average ICC of continuous subtype assignments was fair at $ICC = 0.57$ (0.094)
127 when each assignment was computed on a single session. When estimating continuous assign-
128 ments on the average of multiple sessions, the ICC increased markedly: good ($ICC = 0.68$, $SD =$
129 0.09) for 2 sessions, good ($ICC = 0.75$, $SD = 0.071$) for 3 sessions, and excellent ($ICC = 0.80$, $SD =$
130 0.067) for 4 sessions.

131 Finally, we evaluated the replicability of continuous subtype assignments for subtypes that were
132 computed on independent data (out of sample replicability). To this end, we computed subtypes
133 on the discovery sample and estimated continuous subtype assignments for individuals in the lon-
134 gitudinal mixed patient-control sample. Here the average ICC was poor at $ICC = 0.33$ ($SD 0.072$)
135 with a range of $ICC = 0.23$ in the inferior temporal gyrus to $ICC = 0.48$ in the medial ventral at-
136 tention network. We thus showed that the replicability of continuous subtype assignments ranges
137 from poor to excellent as a function of the amount of available data per individual and whether
138 subtypes and continuous subtype assignments are computed on the same data.

139 **Subtypes are robust to nuisance covariates and parameter changes**

140 We then conducted the FC subtype analysis in the discovery dataset for each seed network. FC
141 subtypes were identified according to two criteria: an average spatial dissimilarity below 1, and a
142 minimum number of 20 individuals within each subtype. Across all seed networks, we identified 87
143 FC subtypes, with an average of 5 per network. These FC subtypes captured on average 97% of in-
144 dividuals in the sample (see also *Appendix 1*). We tested whether continuous subtype assignments
145 were driven by head motion, age, or recording site and found no significant linear associations with
146 these covariates (see also *Appendix 1*). Lastly, we evaluated whether our results were influenced
147 by the choice of the dissimilarity threshold by repeating the subtyping and subsequent analysis
148 steps for different levels of dissimilarity thresholds. We found that our results were robust across
149 dissimilarity thresholds but that higher thresholds led to the inclusion of smaller proportions of
150 the sample (see Fig 1 in *Appendix 3*).

151 **Subtypes show association with ASD diagnosis**

152 We next investigated whether any of the identified FC subtypes naturally captured interindividual
153 variance of clinical ASD symptoms. To test this question, we computed the continuous assignment
154 of all individuals in the discovery dataset to the identified subtypes. We then tested for a linear
155 relationship between continuous subtype assignments and ASD diagnosis (i.e. ASD or NTC) and
156 measures of ASD symptom severity (i.e. calibrated ADOS severity scores).

157 We identified 11 FC subtypes for which the continuous assignment of individuals were signifi-
158 cantly associated with the clinical diagnosis of ASD, after correction for multiple comparisons (p_{adj}
159 reflects the false discovery rate adjusted p-values, see Methods for details). That is, ASD and
160 NTC individuals differed significantly in their continuous assignments with these subtypes. NTC

161 individuals showed significantly stronger assignments than ASD individuals with 5 of the 11 sub-
162 types. These protective subtypes originated from seed networks in the ventral motor network
163 ($T = 3.79, p_{adj} = 0.0037, d = -0.42$), the auditory network ($T = 4.25, p_{adj} = 0.0018, d = -0.52$), the
164 dorsal motor network ($T = 4.15, p_{adj} = 0.0018, d = -0.49$), the medial ventral attention network ($T =$
165 $3.49, p_{adj} = 0.0091, d = -0.39$), and the downstream visual network ($T = 3.23, p_{adj} = 0.0196, d = -0.38$).

166 ASD individuals showed significantly stronger continuous assignments than NTC individuals
167 with 6 of the 11 subtypes. These risk subtypes originated from seed networks in the ventral motor
168 network ($T = 2.91, p_{adj} = 0.0330, d = 0.32$), the dorsal motor network ($T = 3.8, p_{adj} = 0.0369, d =$
169 0.39), the downstream visual network ($T = 2.94, p_{adj} = 0.0330, d = 0.28$), the amygdala-hippocampal
170 complex ($T = 2.75, p_{adj} = 0.0488, d = 0.27$), the fronto-parietal control network ($T = 2.92, p_{adj} =$
171 $0.0330, d = 0.29$), and the lateral default mode network ($T = 3.17, p_{adj} = 0.0204, d = 0.30$). We thus
172 showed that a subset of the identified FC subtypes naturally captured some variance of the clinical
173 ASD diagnosis. We did not find an association between continuous subtype assignments and ASD
174 symptom severity beyond the effect of the clinical diagnosis (see **Appendix 2**).

175 **Subtype associations with ASD diagnosis replicate moderately**

176 We next investigated how reproducible the discovered association between FC subtypes and ASD
177 diagnosis was in an independent dataset. For each of the subtypes that showed a significant associ-
178 ation with ASD diagnosis in the discovery dataset, we computed the continuous assignment for the
179 individuals in the independent replication dataset. In this way, we tested the out of sample repro-
180 ducibility of the observed association effect. We tested different degrees of replication: whether
181 the observed effect in the replication sample was significant after correction for multiple compar-
182 isons, significant at an uncorrected $p < 0.05$, whether the estimated magnitude of the effect fell
183 within the 90% confidence interval of the effect size estimate in the discovery sample, and whether
184 the effect in the replication sample had the same direction as the one estimated in the discovery
185 sample. We found that all effects of association with diagnosis in the replication sample had the
186 same direction as in the discovery sample. The effect size estimates in the discovery sample were
187 correlated at $r = 0.91$ with the effect size estimates in the replication sample. The magnitude of the
188 estimated effects in the replication sample was on average 63% of those estimated in the discovery
189 sample, and nine out of eleven effect size estimates fell within the 90% confidence intervals of the
190 effect size estimates in the discovery sample. Five of those effects were significant at $p < 0.05$, and
191 two of those were significant at $p_{adj} < 0.05$ (**Figure 2 b**). We thus showed that the association be-
192 tween subtypes and ASD diagnosis observed on the discovery dataset was moderately replicable
193 in the independent replication dataset.

194 **Subtypes with similar risk for ASD show similar spatial patterns of FC alterations**

195 We noticed that the spatial pattern of protective subtype maps appeared similar, despite repre-
196 senting connectivity profiles from different seed networks (**Figure 3 a, b**). Similarly, the subtype
197 maps of risk subtypes all appeared to show below average connectivity. We therefore investigated
198 whether subtypes with the same direction of association with ASD diagnosis (i.e. protective and risk
199 subtypes) shared similar FC profiles and whether this also extended to the continuous assignments
200 of individuals to these subtypes. We found that protective subtypes exhibited a highly convergent
201 pattern of FC alterations ($\tilde{r}_{spatial} = 0.81$, where \tilde{r} reflects the median spatial correlation across sub-
202 type pairs) that was distinct from those of risk subtypes ($\tilde{r}_{spatial} = -0.3$). The spatial similarity among
203 risk subtypes was less pronounced ($\tilde{r}_{spatial} = 0.3$) than that of protective subtypes. This finding ex-
204 tended to continuous subtype assignments that were more strongly correlated among protective
205 subtypes ($\tilde{r} = 0.62$) than among risk subtypes ($\tilde{r} = 0.21$), and anti-correlated between protective and
206 risk subtypes ($\tilde{r} = -0.25$). By dividing all subtype maps into the 18 seed networks, we observed that
207 the shared spatial pattern of protective subtypes was characterized by overconnectivity with uni-
208 modal sensory brain networks, and underconnectivity with the basal ganglia and fronto-parietal
209 network (green hues, **Figure 3 c**). By contrast, the shared spatial pattern of risk subtypes was char-

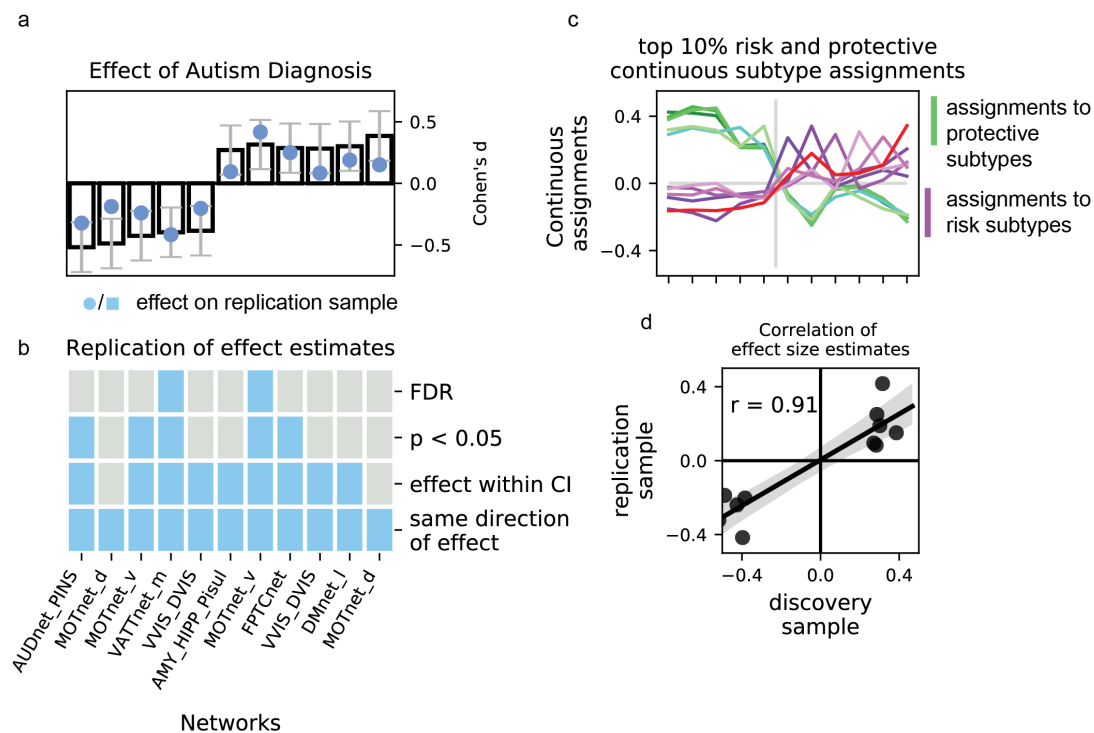


Figure 2. Association of continuous subtype assignments and diagnosis. a) Bar plots represent the standardized group difference (Cohen's d) of continuous subtype assignments between NTC individuals and ASD patients. Negative value reflect greater similarity of neurotypical control subjects with the subtype, positive values reflect greater similarity of ASD patients with the subtype. Error bars reflect the 95% confidence interval of the effect size estimates. The effect size observed in the independent replication data set is shown as a blue dot. b) Matrix showing the degree of replication in the independent replication dataset of the observed association with diagnosis for each of the 11 protective and risk subtypes. Each row corresponds to a bar-plot in a). From top to bottom, the degrees of replication are: FDR: full replication of the effect after FDR correction, $p < 0.05$: replication of the effect for uncorrected statistics, effect within CI: observed effect size in the replication sample falls within the 95% confidence interval of the observed effect in the discovery sample, direction: observed effects in the discovery and independent replication sample go in the same direction. c) Graph illustrating the similarity of continuous subtype assignments across risk and protective subtypes. The average continuous subtype assignments of the top 10% of individuals with the highest similarity with a protective (green shades) or risk (red shades) subtype are displayed across all identified protective (left side) and risk (right side) subtypes. An individual may belong to the top 10% in more than one subtype. d) Correlation plot of the observed effect sizes in the discovery and independent replication datasets. The black line represents the correlation of effect sizes, the grey shaded area reflects the estimated 95% CI of the linear fit.

Figure 2-source data 1. Table of the unthresholded association T-test between continuous subtype assignments and ASD diagnosis on the discovery dataset.

Figure 2-source data 2. Table of the unthresholded association T-test between continuous subtype assignments and ASD diagnosis on the validation dataset.

acterized by pervasive underconnectivity (red hues, *Figure 3 c*). We thus showed that subtypes associated with a similar risk of ASD diagnosis exhibited similarities of FC alteration and continuous assignments, and that these similarities were more pronounced for protective subtypes than risk subtypes.

Discussion

ASD is characterized by a heterogeneity of symptoms and neurobiological endophenotypes (*Nunes et al., 2019; Dickie et al., 2018; Jacob et al., 2019*) among the affected individuals. Data driven, unsupervised subtyping appears as a natural approach to decompose the heterogeneity in ASD and to identify subtypes of functional brain connectivity. Here, we first sought to evaluate how stable and reproducible subtypes of FC are when derived from a heterogeneous sample of both neurotypical and autistic individuals. We then investigate whether fully data driven subtypes are associated with a clinical diagnosis of ASD. Our results suggest that data driven imaging subtypes are moderately reliable on currently available datasets and show a weak to moderate association with the clinical diagnosis of ASD, that generalizes to independent replication data.

Functional connectivity subtypes are stable

Our systematic evaluation of the robustness of subtype maps, and the discrete or continuous assignments of individuals to them, establishes a foundation on which to understand previous incidental findings on the robustness (*Easson et al., 2019*) or non-reproducibility (*Dinga et al., 2019*) of subtype analyses. The FC patterns of the subtypes identified in our analysis were found to be robust to perturbations of the discovery data set. This observation fits with previous studies that reported stable imaging subtypes in ASD (*Hong et al., 2017; Easson et al., 2019; Tang et al., 2019b*). By contrast, we found that making a discrete assignment of individuals to the identified subtypes was not robust to perturbations. Few papers have investigated the robustness of discrete subtype assignments explicitly. One recent study attempted to replicate a high profile report of clinically predictive FC subtypes among depressed patients (*Drysdale et al., 2017*) but found that the asserted discrete subtypes were not sufficiently supported by an independent dataset (*Dinga et al., 2019*). The authors concluded that the data instead supported a more parsimonious model of continuous neurobiological axes.

In the wider ASD literature, the robustness of discrete subtype assignments has been more comprehensively investigated for symptom based subtypes. Several symptom based subtypes of autism have been proposed in attempts to provide more homogeneous diagnostic criteria. However, the distinction between these subtypes was also not found to be well supported by replication attempts which has led the field to merge sub-diagnoses of autism under the label of autism spectrum disorder (*Lord et al., 2012; Volkmar and McPartland, 2014*).

We may reconcile the seemingly conflicting findings of robust subtypes on the one hand and non-reproducible discrete subtype assignments on the other, when we consider that the similarity of an individual with each subtype is a continuous measure. For individuals who are equally similar to two different subtypes, a small change of the connectivity profile of either of the subtypes may be enough for them to be assigned to the other subtype if a discrete choice is forced. By contrast, the continuous similarity measure would not change drastically. An emerging body of literature therefore conceptualizes subtypes as latent dimensions that can be expressed to varying degrees in each individual (*Kernbach et al., 2018; Tang et al., 2019b; Easson et al., 2019*). Our own results support this view: we find that unlike discrete subtype assignments, continuous measures of an individuals' similarity with each subtype are moderately robust and can be very robust when more data is available per individual to compute the continuous assignment. The ICC of continuous subtypes assignments computed on separate data was low but consistent with previous reports of the robustness of single session seed based FC measures (*Shehzad et al., 2009*). When the continuous subtype assignments were computed based on the average FC of multiple scan sessions per

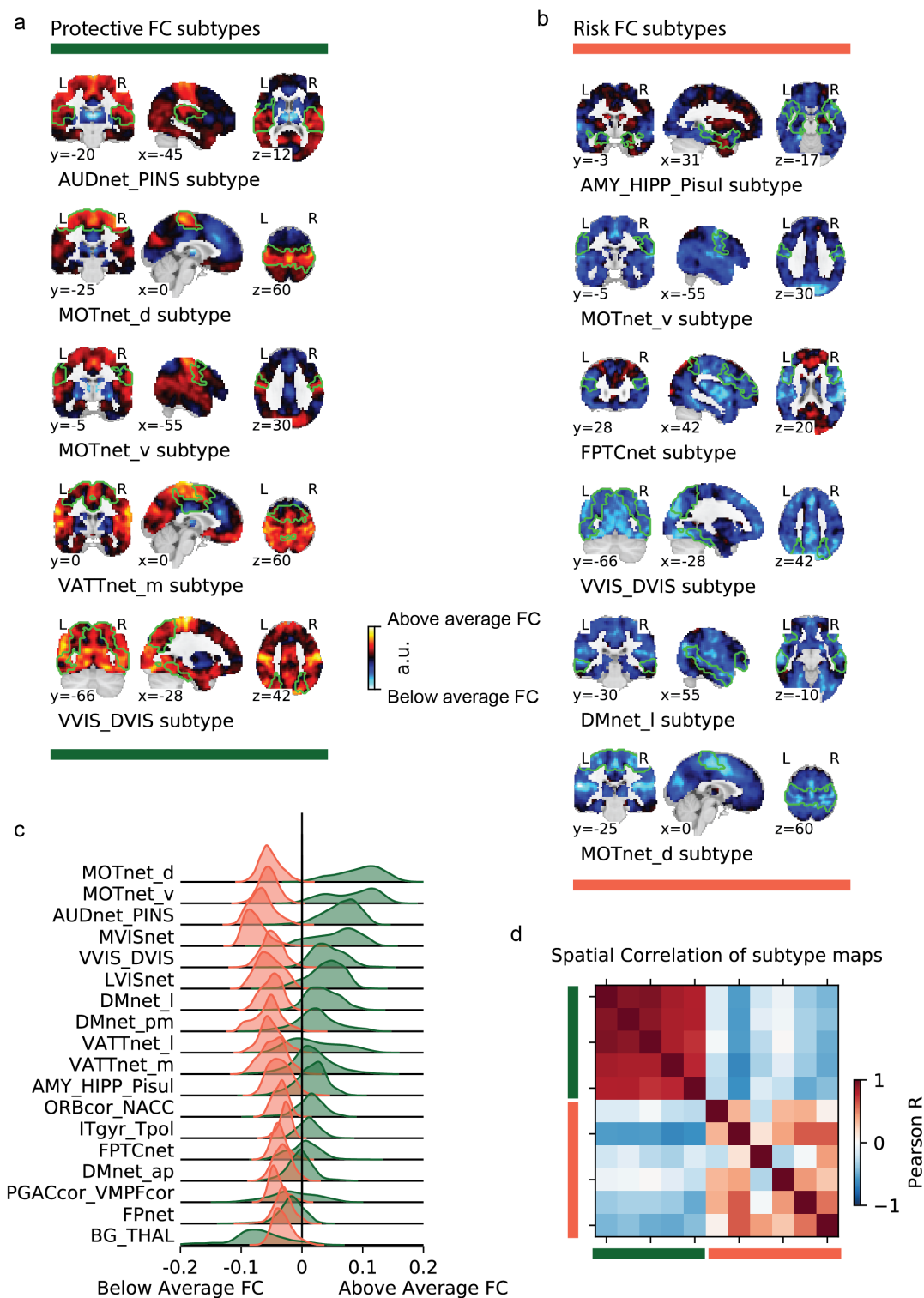


Figure 3. Overview of risk and protective subtype maps. Maps of protective (a) and risk (b) subtypes (corresponding seed networks are outlined with a thin green boundary on the map). c) Decomposition of the average protective (green) and risk (red) subtype map into 18 brain networks. d) Spatial correlation between subtypes. Protective (green) and risk (red) subtypes are denoted by colored bars along the correlation matrix.

258 individual, we found high to very high robustness measures that were in line with the well estab-
259 lished link between scan length and FC reliability (*Gordon et al., 2017*). It is reasonable to assume
260 that the generalizability of the associations between continuous subtype assignments and clinical
261 ASD diagnosis we have reported here could be increased if longer or repeated scan sessions were
262 available for the replication sample. Based on our findings we may thus conclude that continuous
263 measures of individual subtype assignment that reflect the similarity of an individual with several
264 subtypes provide a better representation of the data.

265 **Subtypes moderately, but reproducibly, associate with ASD diagnosis**

266 The majority of previous subtyping analyses in ASD have been constrained to patients that were
267 already diagnosed with ASD (*Hrdlicka et al., 2005; Hong et al., 2017; Tang et al., 2019b*). We have in-
268 stead used an unsupervised clustering approach to identify diagnosis-naive subtypes of FC across
269 autistic and neurotypical individuals in order to determine whether they would associate with ASD
270 diagnosis. Our results showed that these subtypes were significantly associated with a clinical diag-
271 nosis of ASD, and that the observed effects were small to moderate, ranging between $d = 0.3$ and
272 $d = 0.5$ (or from $r = 0.15$ to $r = 0.24$ when expressed as a correlation coefficient) on the discovery
273 sample, with reduced effect sizes identified in an independent replication sample.

274 Our effect sizes are comparable to those reported by other imaging based subtypes in ASD,
275 which have all estimated association with diagnosis in their discovery sample (i.e. have not been
276 replicated on independent data). A recent study (*Kernbach et al., 2018*) investigated the hetero-
277 geneity of FC in mixed data of ASD, NTC, and attention deficit hyperactivity disorder (ADHD), a
278 common comorbidity of ASD individuals (*Rommelse et al., 2010*). The authors identified one FC
279 endophenotype that was weakly associated with ASD ($r = 0.15$) but extended both to ADHD and
280 NTC individuals. Another study on structural imaging subtypes among ASD (*Hong et al., 2017*) pa-
281 tients found that ADOS severity scores could be better predicted from structural cortical alterations
282 when individuals were first divided into three subtypes ($r = 0.47$ compared to $r = -0.12$ when the
283 association was computed without regard for subtypes), although the prediction performed worse
284 for calibrated ADOS severity ($r = 0.25$). The magnitude of the association between data driven sub-
285 types and clinical diagnosis in our analyses is therefore comparable to what has been previously
286 reported by other imaging based subtypes analyses of ASD. Weak-to-moderate associations be-
287 tween data driven subtypes and clinical diagnosis thus seem robust to the employed subtyping
288 method, at least in the currently limited number of published studies.

289 Very few imaging based subtype analyses have been replicated on independent data, and to
290 our knowledge none have so far been replicated successfully (*Dinga et al., 2019*). The replication of
291 our results on independent data therefore establishes a novel benchmark of reliability for imaging
292 based subtype analyses in ASD. We found that the observed effect sizes of the association between
293 FC subtypes and clinical ASD diagnosis strongly correlated between the discovery dataset and the
294 independent replication dataset ($r = 0.91$), however effect sizes in the replication data were on
295 average only 2/3rds the the magnitude of those in the discovery data. This reduction of effect
296 sizes on the replication data is expected as it reflects the inherent bias of significance testing to
297 select larger effects and further underlines the importance of reporting original findings together
298 with independent replications for an unbiased estimate (*Vul and Pashler, 2012*). Because no other
299 imaging subtype analysis in ASD has been independently replicated to date, our results have to be
300 interpreted in the context of replication attempts in the ASD case-control literature. The largest
301 case-control analysis of FC alterations to date (*Holiga et al., 2019*) reported FC group differences
302 between ASD and NTC individuals with effect sizes between $d = 0.46$ and $d = 0.6$, similar in size to
303 our own results of FC subtype associations with clinical ASD diagnosis. Using several large replica-
304 tion samples, the authors then showed that these results were reproducible in independent data,
305 however with similarly depressed effect sizes (i.e. $d \approx 0.2$).

306 The comparison with the large case-control study by Holiga and colleagues may also serve to
307 illustrate the conceptual advantage of a subtyping approach over the traditional case-control de-

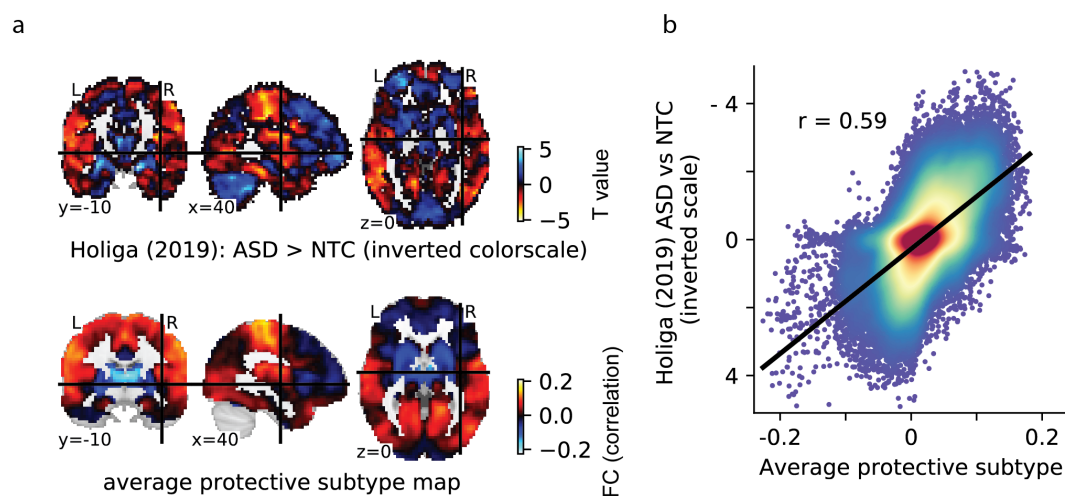


Figure 4. Comparison of the average protective subtype map to a case-control signature. a) The spatial map of a large sample size case-control contrast between ASD and NTC individuals (top row), compared to the average spatial map of the protective subtypes identified on our data (bottom row). Note that because of the opposite nature of the two contrasts (i.e. ASD > NTC for the case-control contrast and NTC > ASD for the protective subtype map), the color scale for the case-control map has been inverted for better comparability. b) Plot of the voxel-wise spatial correlation between the (inverted) case-control contrast map and the average protective subtype map. The blue to red color gradient reflects the density of voxels represented in each area of the graph.

308 sign for the investigation of ASD related alterations of FC. An incidental finding of our analysis was
309 that protective subtypes converged onto a network of mutually overconnected and predominantly
310 unimodal brain networks (see *Figure 3*). The spatial pattern of this overconnectivity profile is visu-
311 ally very similar to the case-control pattern of differences FC ASD and NTC individuals reported
312 in the study of Holiga et al (see *Figure 4*). We computed the spatial correlation between the two
313 patterns at $r = -0.6$, as high or higher than the reported replicability of the case-control pattern
314 itself (between $r = 0.3$ and $r = 0.6$, depending on the replication sample). Because the case-control
315 results of Holiga are at least in part based on the same data that were used in our study, the strik-
316 ingly high spatial correlation and the similarity of effect sizes suggests that case-control studies
317 may not capture ASD specific FC alteration patterns but are rather driven by the most prevalent
318 FC subtypes in the data, that are themselves not strongly linked to ASD. The fact that our analysis
319 identified several distinct and non-overlapping FC subtypes that were reproducibly associated with
320 moderately increased risk of ASD further illustrates the conceptual limitation of computing group
321 averages across data known to be highly heterogeneous (*Ecker and Murphy, 2014; Lombardo et al.,*
322 *2019*).

323 Taken together, our results suggest that unsupervised FC subtypes associate with clinical ASD
324 diagnoses at a level similar to case-control studies and have comparable reproducibility. They also
325 likely provide a more comprehensive and informative representation of the underlying neurobio-
326 logical heterogeneity.

327 Limitations

328 Our findings are limited by the amount of data available per individual. We found that continuous
329 assignments of individuals to subtypes became more stable, the more data was available per indi-
330 vidual. One reason for this observation could be that the FC pattern associated with an individual
331 is in fact not static across time but rather represents an average of a number of dynamic states
332 (*Allen et al., 2014*). How long an individual spends in each dynamic state can be a reproducible
333 trait (*Choe et al., 2017*) and may provide additional insight into the relationship of connectivity and

334 ASD symptoms (*Rashid et al., 2018*). It is therefore possible that by averaging longer time series
335 for each individual, we get a better approximation of that individual's preferred dynamic state. A
336 promising direction for future research will be the investigation of dynamic FC subtypes in ASD.
337 Datasets providing longer time series per individual will facilitate these inquiries.

338 Our results have focused only on individuals with ASD. Given the extensive evidence of overlap
339 of symptoms (*Grzadzinski et al., 2011*) and neurobiological phenotypes between ASD and other
340 neurodevelopmental disorders (*Sha et al., 2019*), a fruitful avenue for future research will be to
341 extend this approach to investigate cross-diagnostic subtypes of FC (*Elliott et al., 2018*).

342 **Conclusions**

343 Our findings suggest that unsupervised clustering of heterogeneous imaging data is well suited to
344 identify subtypes that are reproducibly associated with clinical symptoms. The low to moderate
345 effect size of the observed association makes it clear that subtypes of FC will not replace or provide
346 better clinical categories than the current diagnostic system. The instability of discrete assignments
347 of individuals to subtypes and the small to moderate effect sizes associated with these subtypes do
348 not lend themselves to make meaningful predictions about the clinical prognosis of an individual.
349 However, we find that the associations between subtypes and clinical diagnosis do generalize to
350 independent data and that the risk subtypes we identified in our dataset capture non-redundant
351 profiles of FC dysfunction. Both of these observations point towards a promising avenue for future
352 research: data driven subtyping appears well suited as an efficient method to summarize high
353 dimensional, heterogeneous data while retaining clinically meaningful variation. These properties
354 make FC subtypes good candidates as features for multivariate, supervised predictive learning
355 models to predict clinical diagnosis.

356 **Methods and Materials**

357 **Discovery sample**

358 The discovery sample consisted of imaging data from the ABIDE 1 dataset ($N = 388$, $N_{ASD} =$
359 194 , $Age = 17.04$, (7.08) , from 7 recording sites) and ASD individuals were matched with neurotyp-
360 ical controls on age ($Age_{ASD} = 17.0$, (7.28) ; $Age_{NTC} = 17.04$, (6.89)) and head motion ($FD_{ASD} =$
361 $0.17mm$, (0.048) ; $FD_{NTC} = 0.16mm$, (0.041)). The full ABIDE 1 dataset includes 1112 individuals from
362 20 imaging sites ($N_{ASD} = 539$, $age = 17.04$, (8.04)) of which 948 are male. Due to the strong sex
363 imbalance of the data, we limited our analysis to male individuals. After preprocessing of the imag-
364 ing data, 557 individuals ($272ASD$, $Age = 16.65$, (6.75)) from 13 imaging sites were found to pass
365 our quality control criteria. We then matched the NTC and ASD individuals at each site by age
366 and head motion through propensity score matching without replacement (*Rosenbaum and Ru-*
367 *bin, 1985*). The matched sample included 478 individuals ($239ASD$, $Age = 16.67$, (6.67)). We further
368 excluded the 5 imaging sites with fewer than 20 matched individuals, leaving 388 individuals for
369 the final discovery sample.

370 **Replication sample**

371 The replication sample consisted of imaging data from the ABIDE 2 dataset ($N = 300$, $N_{ASD} =$
372 150 , from 7 imaging sites) and ASD individuals were matched with neurotypical controls on age
373 ($Age_{ASD} = 12.0$, (4.05) ; $Age_{NTC} = 12.3$, (4.59)) and head motion ($FD_{ASD} = 0.17$, (0.053) ; $FD_{NTC} =$
374 0.16 , (0.048)). The full ABIDE 2 dataset includes 1114 individuals from 19 imaging sites ($N_{ASD} =$
375 521 , $Age = 14.86$, (9.16)) of which 856 are male. Analogous to the discovery sample we limited
376 our analysis to male subjects. After preprocessing of the imaging data, 587 individuals ($N_{ASD} =$
377 273 , $Age = 13.94$, (5.9)) from 16 imaging sites were found to pass our quality control criteria. These
378 individuals were then matched by age and head motion within each site through propensity score
379 matching without replacement. The matched sample included 424 individuals ($N_{ASD} = 212$, $Age =$
380 13.66 , (5.25)). We further excluded 9 imaging sites with fewer than 20 matched individuals, leaving
381 300 individuals for the final replication sample.

382 **Longitudinal sample 1**

383 The first longitudinal test sample was taken from a subset of individuals in ABIDE 2 for whom
384 multiple scan sessions were available. In ABIDE 2, longitudinal imaging data are available for 168
385 individuals from 4 imaging sites ($N_{ASD} = 88$, $Age = 21.24$, (15.45)) of which 154 are male. Analogous
386 to the discovery and replication sample, we limited our analysis to male individuals. After prepro-
387 cessing of the imaging data, 84 individuals ($N_{ASD} = 42$, $Age = 14.58$, (6.29)) from 3 imaging sites were
388 found to pass our quality control criteria. We selected the two imaging sites with the largest num-
389 ber of acceptable individuals (ABIDEII-OHSU_1 and ABIDEII-IP_1) and randomly selected individuals
390 at each site to enforce equal sized groups of NTC and ASD. Where more than 2 acceptable imaging
391 scans were available for an individual, the 2 scans with the lowest average head motion were se-
392 lected. The final longitudinal test sample consisted of 68 individuals ($N_{ASD} = 34$, $Age = 13.46$, (5.79))
393 from 2 imaging sites.

394 **Longitudinal sample 2**

395 The second longitudinal test sample consisted of individuals in the general population Hangzhou
396 Normal University dataset (http://dx.doi.org/10.15387/fcp_indi.corr.hnu1) released by the consor-
397 tium for reliability and reproducibility (Zuo *et al.*, 2014). The final sample included 26 individuals
398 ($N_{male} = 14$, $Age = 24.58$, (2.45)) that were each scanned 10 times at 3 day intervals over the course
399 of a month. We selected the 26 individuals (out of a total of 30 available individuals) for which all
400 resting state scans passed visual quality control.

401 **Clinical scores and symptom severity**

402 The individuals from the ABIDE 1 and ABIDE 2 samples included in this study were diagnosed with
403 ASD by expert clinicians based on either the Autism Diagnostic Observation Schedule (ADOS) (Lord
404 *et al.*, 2000; Gotham *et al.*, 2007) or the Autism Diagnostic Interview - Revised (Lord *et al.*, 1994). The
405 ADOS provides a total sum of ratings of observation items in the ADOS subdomains that reflects
406 the severity of observed symptoms but is primarily intended for diagnostic purposes. Calibrated
407 ADOS severity scores have been proposed as a standardized, research appropriate measure of
408 symptom severity that is comparable across ADOS modules and is less dependent on demographic
409 factors such as age (Gotham *et al.*, 2009). Few individuals in the discovery ($N=109$, 93 ASD) and
410 replication (88 ASD) samples had calibrated ADOS severity scores. We therefore also investigated
411 associations with the raw ADOS total scores that were available in larger numbers in the discovery
412 ($N = 213$, $N_{ASD} = 182$) and replication ($N = 157$, $N_{ASD} = 148$) sample.

413 **Imaging data preprocessing**

414 All imaging data were preprocessed with the NeuroImaging Analysis Kit (NIAK) version 1.13 (Bellec
415 *et al.*, 2011). The preprocessing pipeline was executed inside a Singularity (version 2.6.1) software
416 container (Kurtzer *et al.*, 2017) to facilitate the reproducibility of our findings. Preprocessing of
417 the functional imaging data consisted of the following steps: Head motion between frames was
418 corrected by affine realignment with a reference image (median image across frames). The mag-
419 nitude of framewise head displacement (FD) was estimated from the time course of the affine
420 realignment parameters (Power *et al.*, 2012). The reference image was then coregistered into the
421 MNI152 stereotaxic space (Evans *et al.*, 1994) through an initial affine alignment with the individ-
422 ual anatomical T1 image and a subsequent, non-linear coregistration of the T1 image with the MNI
423 template. A high-pass temporal filter (0.01 Hz) was fitted to the whole time series by discrete cosine
424 transform to remove slow time drifts. Time frames with excessive head motion ($FD > 0.4mm$) were
425 then censored by removing the affected frame, as well as the preceding and the two succeeding
426 frames from the time series (Power *et al.*, 2012). Nuisance covariates were then regressed from the
427 remaining time points: the previously estimated discrete cosine basis functions, the average signal
428 in conservative masks of the white matter and lateral ventricles, and the first principal components

429 (accounting for 95% of variance) of the six rigid-body motion parameters and their squares (*Lund*
430 *et al., 2006; Giove et al., 2009*).

431 **Quality control of imaging data**

432 We controlled the quality of preprocessed data manually and through quantitative cut off values.
433 Data were visually checked by a trained rater following a standardized QC protocol (*Benhajali et al.,*
434 *2020*) with a structured QC tool (*Urchs et al., 2018*). Individuals were excluded for coregistration
435 failure and for incomplete brain coverage of the field of view of the functional data. During the
436 visual QC we noticed that a large number of individuals in both the discovery and replication sam-
437 ple had incomplete field of view coverage of the cerebellum. We chose to remove all cerebellar
438 networks from our analyses in order to include these individuals. Individuals were also excluded
439 from the analysis if fewer than 50 time frames remained after motion censoring or if the average
440 framewise displacement exceeded 0.3 mm.

441 **Functional connectivity estimation**

442 We estimated the seed based FC maps of 18 non-cerebellar seed networks defined in the MIST_20
443 functional brain atlas (*Urchs et al., 2017*). The MIST_20 atlas represents large, spatially distributed
444 subcomponents of canonical FC networks. The seed to voxel FC maps were estimated as the Pear-
445 son correlation between the average time series signal of a seed network and the time series of
446 all gray matter voxels in the brain (excluding the cerebellum). Within each sample separately, the
447 individual seed FC maps were centered to the group mean and known sources of variance of non-
448 interest were regressed for each voxel at the group level: linear effects of age, head motion and
449 imaging site. As a consequence, the individual seed FC maps in each sample represented the resid-
450 ual variance around the group mean after accounting for these factors.

451 **Subtyping of functional connectivity**

452 To identify communities of individuals with similar seed FC patterns we computed the spatial cor-
453 relation of all pairs of subjects in the discovery sample, separately for each seed network. We
454 expressed the dissimilarity between pairs of individual seed FC maps as the absolute value of 1 -
455 their spatial correlation. The 18 subject by subject dissimilarity matrices (one per seed network)
456 thus contained values between 0 (no dissimilarity or a spatial correlation of 1) to 2 (perfect dissim-
457 ilarity or a spatial correlation of -1) with 1 denoting no spatial relationship (a spatial correlation of
458 0).

459 For each seed network separately, we characterized communities of individuals with similar
460 seed FC maps by hierarchical agglomerative clustering of the dissimilarity matrix for each seed
461 network using the unweighted average distance linkage criterion (*Müllner, 2011*). We applied two
462 criteria for the identification of seed FC communities: 1) the average dissimilarity between seed
463 FC maps in a community could not be greater than 1, and 2) the community had to have at least
464 20 members. This allowed for small subsets of individuals with distinct seed FC patterns to not be
465 assigned to any communities. Assigning individuals to subtypes in this way is a discrete process
466 and we therefore refer to these assignments as discrete subtype assignments.

467 Within each seed FC community, we estimated the average seed FC map across all community
468 members. This map reflected the subtype of seed FC shared by the community members and we
469 refer to these maps as the subtype map.

470 Finally, we computed the spatial similarity of each individual in the discovery sample with the
471 identified seed FC subtypes by spatial correlation of the individual seed FC map with the corre-
472 sponding seed FC subtype map. The estimated spatial correlation coefficient is a continuous mea-
473 sure of an individual's similarity with each of the subtypes and we therefore refer to it as a continu-
474 ous subtype assignment. Each individual had continuous subtype assignments for each identified
475 subtype, ranging from -1 (perfect anticorrelation of the individual and the subtype seed FC map)
476 to +1 (perfect correlation of the individual and subtype seed FC map).

477 **Stability analysis**

478 Before we investigated the three aspects of FC subtypes (subtype maps, and discrete and contin-
479 uous assignments) in detail, we wanted to determine the robustness of these metrics to perturba-
480 tions of the discovery data. We used two approaches: 1) to determine the robustness of discrete
481 subtype assignments and subtype maps, we conducted a stratified subsampling scheme on our dis-
482 covery sample, 2) to determine the robustness of continuous subtype assignments, we computed
483 the within subject stability of continuous subtype assignments across repeated scan sessions for
484 individuals in the longitudinal sample.

485 We randomly selected 1000 stratified subsamples of half of our discovery sample while preserv-
486 ing the equal ratio of ASD patients and NTC. Within each subsample, we repeated the full subtype
487 characterization procedure: group level regression of nuisance sources of variance, characteriza-
488 tion of communities of similar residual seed FC maps, estimation of seed FC subtype maps. The
489 number of unique pairs of subsamples was large (≈ 500.000) and there was considerable overlap
490 of individuals between subsamples. Therefore, we randomly selected 1000 unique pairs of sub-
491 samples to estimate the robustness of the subtype community membership and subtype maps to
492 perturbations in the data.

493 We determined the robustness of discrete subtype assignments by computing the similarity of
494 the communities an individual was assigned to within two subsamples using the Dice coefficient
495 (*Dice, 1945*). For each pair of subsamples A and B, we first identified the intersect of individuals
496 (i.e. those individuals that were present in both subsamples). For each individual we then com-
497 puted the Dice coefficient of the communities it was assigned to in sample A and sample B. The
498 Dice coefficient here computes the ratio of twice the number of individuals shared between both
499 communities over the total number of individuals in both communities. Thus, if all community
500 neighbours of an individual in sample A were also community neighbors of that individual in sam-
501 ple B, then the Dice coefficient will be 1. Conversely, if none of the community neighbours of an
502 individual in sample A were community members of that individual in sample B, then the Dice co-
503 efficient will be 0. We computed the average Dice coefficient across all individuals shared between
504 a pair of subsamples.

505 We determined the robustness of the subtype maps by examining the spatial correlation of
506 subtype maps extracted in each pair of subsamples. For each pair of subsamples A and B, we
507 computed the spatial correlation of all subtype maps in sample A with all subtype maps in sample
508 B. If subtype maps were robustly identified, then we would expect that for each subtype map in
509 sample A we can find at least one subtype map in sample B that is very similar. We therefore
510 searched (with replacement) for each subtype map in sample A the subtype map in sample B with
511 the highest spatial correlation. Since the number of subtypes extracted in each subsample was
512 determined by the data, we allowed for subtype maps in sample B to be a match for multiple
513 subtype maps in sample A. We then took the average of the maximal spatial similarity between
514 subtype maps of sample A and B as a measure of the robustness of the subtype maps.

515 We computed the robustness of the continuous subtype assignments as the intraclass corre-
516 lation coefficient between repeated scan sessions of the same individual. We first investigated
517 the robustness of assignments to subtypes that had been identified on data from a separate scan
518 session but of the same sample (within sample robustness). Using the longitudinal sample 1, we
519 identified FC subtypes for each network on scan session 1, and computed seed based FC maps
520 for all individuals on the remaining two scan sessions. Independently for each scanning session
521 we then centered the seed FC maps to the group mean and regressed covariates of non-interest
522 for each voxel. The residual seed FC maps were then used to compute the continuous subtype
523 assignments for the FC subtypes identified on scan session 1. The replicability of these contin-
524 uous subtype assignments across the two remaining scan sessions was then estimated with the
525 intraclass correlation coefficient.

526 Using the longitudinal sample 2, we tested whether continuous subtype assignments were

527 more robust if they were computed on larger amounts of data per individual. Again, we identi-
528 fied FC subtypes for each network on the first scan session and computed individual seed FC maps
529 on the remaining nine scan sessions. Within each scan session, the individual seed FC maps were
530 then centered to the group mean and nuisance covariates were regressed. Two average residual
531 seed FC maps per individual were then computed by averaging across sets of 2, 3, and 4 scan
532 sessions. We then computed the continuous subtype assignments with the FC subtypes and es-
533 timated their replicability for the different number of averaged scan sessions with the intraclass
534 correlation coefficient.

535 Finally, we computed the out of sample robustness of continuous subtype assignments based
536 on the repeated scan session in the longitudinal sample 1 with the FC subtypes identified on the
537 complete discovery sample. Again, the robustness was measured with the intraclass correlation
538 coefficient of continuous subtype assignments across scan sessions.

539 **Association with autism diagnosis**

540 We explored whether seed FC subtypes existed for which the presence of an autism diagnosis
541 explained a significant amount of variance of the continuous subtype assignments. We tested
542 this for each subtype by comparing the means of continuous subtype assignments between ASD
543 individuals and NTC with a general linear model with diagnosis as the explanatory factor. As we
544 had taken care to ensure equal sizes of individuals in both diagnostic categories, we did not use
545 a correction for unequal variances. The estimated p-values were corrected at a false discovery
546 rate (FDR) of 5% across all subtypes using the Benjamini and Hochberg method (*Benjamini and*
547 *Hochberg, 1995*). We report the standardized group difference (Cohen's d) between diagnosis and
548 continuous subtype assignments as a measure of the effect size of the association with the clinical
549 diagnosis. We continued investigating subtypes for which a significant difference of continuous
550 subtype assignments between ASD patients and NTC was found in the discovery sample.

551 Within the set of subtypes that showed a significant association with ASD diagnosis we investi-
552 gated whether spatial similarity with the subtype map explained additional variance of the sever-
553 ity in clinical symptoms. Because symptom severity and the clinical ASD diagnosis were highly
554 correlated, and because healthy individuals had compressed or missing scores for most severity
555 measures, we only tested this association in individuals with a diagnosis of ASD. We investigated
556 the linear relationship between continuous subtype assignments and severity estimates for the
557 calibrated ADOS severity scores (*Gotham et al., 2009*) and also for the raw ADOS total scores. We
558 reported the correlation between symptom scores and continuous subtype assignments as a mea-
559 sure of the effect size of the association with symptom severity after correction for multiple com-
560 parisons using FDR.

561 **Replicability**

562 We tested the replicability of the associations between seed FC subtypes and ASD diagnosis in an
563 independent replication sample. Within the replication sample we computed individual seed FC
564 maps for the 18 non-cerebellar MIST_20 seed networks, centered the seed FC maps to the replica-
565 tion sample group average and regressed variance of non-interest due to age, head motion and
566 imaging site for each voxel. For the residual seed FC maps, we computed the continuous subtype
567 assignment scores with the subtypes identified in the discovery sample. For those subtypes that
568 showed significant associations with ASD diagnosis in the discovery sample, we then investigated
569 the difference in continuous subtype assignment scores between ASD and NTC individuals in the
570 replication sample.

571 **Robustness of findings to changes in the subtyping pipeline**

572 Although we did not explicitly specify the number of subtypes to be identified for each seed net-
573 work, it was implicitly determined by the maximum dissimilarity parameter and the structure of
574 the subject by subject dissimilarity matrix. In order to understand how robust our findings were to

575 changes in this parameter, we repeated all analysis steps (i.e. the identification of subtypes, the test
576 for associations with ASD symptoms, and the generalization to the independent replication data)
577 for different values of the maximum dissimilarity parameter. To measure the spatial similarity of
578 subtype maps identified for different dissimilarity parameters we computed their pairwise spatial
579 correlation. We then compared the number of identified subtypes and the observed associations
580 with ASD symptoms and their generalization to independent data qualitatively.

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725 Appendix 1

726 **Subtypes capture majority of individuals in the dataset**

727 Subtypes for each seed network were identified according to two criteria: the average spa-
728 tial dissimilarity within a subtype was no larger than 1 and at least 20 individuals were part
729 of the subtype. Across all 18 seed networks, we identified 87 FC subtypes in the discovery
730 dataset. In each seed network we identified between 3 (medial visual network) and 6 sub-
731 types (lateral visual network) that satisfied these criteria (the median number of subtypes
732 was 5). On average across networks, 97% of the individuals in the discovery dataset were as-
733 signed to a subtype (see also Figure 5). The largest number of individuals not assigned to any
734 subtype was 19 in the inferior temporal gyrus network, and all individuals were assigned to
735 subtypes in the ventral somatomotor and perigenual anterior cingulate seed networks. The
736 average number of individuals in a subtype was $\bar{N} = 79.4, (13.2SD)$. We thus show that the
737 majority of individuals in the discovery dataset contributed to the identified 87 FC subtypes.

738 **Subtypes are not driven by nuisance covariance**

739 To ensure that subtypes were not driven by variation of non interest, we tested for linear
740 associations between the continuous assignment of individuals to each subtype, and head
741 motion and age by Pearson correlation. We also tested whether recording sites were over-
742 represented in subtypes above chance level with a chi-square test. We found no signifi-
743 cant linear relationship between continuous assignments of individuals to subtypes and
744 in-scanner head motion, and age for in any seed network. In addition, we found that the
745 distribution of imaging sites across subtypes did not differ significantly from chance. We
746 thus show that subtypes in the discovery dataset were not significantly driven by variance
747 sources of non-interest.

748 Appendix 2

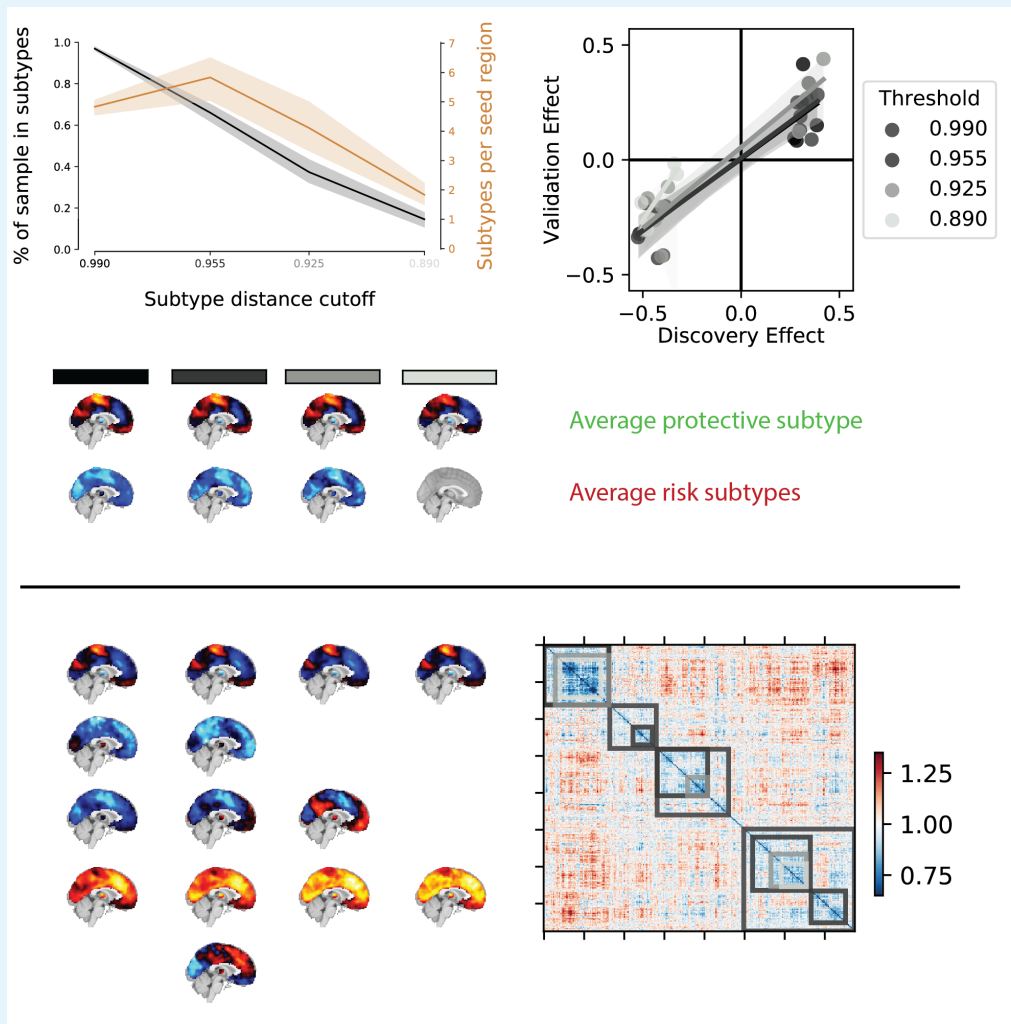
749 **No added effect of ASD symptom severity beyond diagnosis**

750 We also investigated whether any of the identified subtypes captured variation due to ASD
751 symptom severity beyond the observed effects of ASD diagnosis. The distinction between
752 the effects of ASD diagnosis and additional effects of ASD symptom severity is necessary
753 because ASD symptom severity measures are by definition strongly correlated with ASD di-
754 agnosis. Calibrated ADOS symptom severity scores were available for 109 individuals from
755 four recording sites in the discovery dataset (NYU, UCLA, KKI, USM). Of those, only 16 were
756 NTC. In the independent replication dataset, calibrated ADOS severity scores were available
757 for 88 individuals from five recording sites (NYU_1, OHSU_1, SDSU_1, KKI_1, GU_1). All of
758 the 88 individuals were ASD individuals. We therefore limited our analysis to ASD patients.
759 When controlling for the effect of clinical diagnosis in this way, we did not find significant ad-
760 ditional effects of symptom severity in any subtypes. Repeating this analysis for the ADOS
761 raw total scores (that were available for 182 and 148 ASD individuals in the discovery and
762 replication sample respectively) likewise resulted in no significant association with continu-
763 ous subtype assignments for the identified risk and protective subtypes. We thus showed
764 that beyond the effects of clinical ASD diagnosis, there was no significant added effect of
765 symptom severity captured by the FC subtypes.

766 Appendix 3

767 Effects are robust to changes in the subtyping method

768 We identified subtypes of FC that satisfied two criteria: a maximal average dissimilarity of
769 the connectivity patterns of individuals contributing to the subtype, and a minimal number
770 of individuals. Although this process did not explicitly specify the number of subtypes
771 to be identified, we sought to understand how robust our findings were to changes in the
772 subtyping criteria. We therefore repeated the complete subtype analysis (i.e. identification
773 of subtypes, association with ASD diagnosis, and generalization on independent data) for
774 different values of maximal within-subtype dissimilarity. This analysis revealed that sub-
775 type maps remained highly similar across different values of the dissimilarity criterion with
776 subtypes for the most part contracting and only rarely splitting into subcomponents (see
777 Figure 4). We found highly consistent spatial patterns of protective and risk subtypes and
778 effects of association with clinical ASD diagnosis did generalize to the independent replica-
779 tion dataset at equal rates. We thus conclude that our findings were robust to changes in
780 the parameters of the subtyping analysis.



783 **Appendix 3 Figure 1.** Overview of the robustness of subtype associations with ASD diagnosis to
784 changes in the distance cutoff parameter. a) Average percentage of sample assigned to any subtype
785 (black line) and average number of identified subtypes (orange line) for different levels of distance
786 cutoff parameters. Shaded areas show range of values across all seed networks. Note that both the
787 number of subtypes and the average percentage of the sample described by these subtypes sharply
788 drops off for increasingly stringent FC distance thresholds (black to light grey shaded values on the
789 horizontal axis). b) Correlation of the effect size of subtype association with ASD diagnosis in the
790 discovery and replication data for different levels of distance threshold parameters. Lighter colors
791 reflect more stringent distance cutoff thresholds. Note that the reproducibility of the observed effect
792 sizes remains largely unaffected for small changes of the distance threshold parameters. c) Average
793 subtype maps of protective (top row) and risk (bottom row) for different levels of cutoff parameters.
794 The shaded squares correspond to distance cutoff levels in a) and b). Note that the spatial pattern of
795 the average subtype maps are highly preserved across different thresholds. d) Breakdown of subtype
796 maps across different levels of thresholds illustrated by the example of the dorsal somato-motor
797 seed network. Rows correspond to subtypes and columns correspond to threshold levels. Note that
798 the spatial pattern of individual subtype maps are highly preserved across increasingly stringent
799 threshold levels and that subtypes are rarely split into subsets but rather the total number of
800 subtypes is reduced (x denotes subtypes removed by an increase in the distance threshold). e)
801 Breakdown of subtypes across threshold levels illustrated by the example of the subject by subject
802 dissimilarity matrix of the dorsal somato-motor seed network. Grey shaded overlays reflect the
803 subtype solutions at different dissimilarity thresholds.