Gastrointestinal Dysfunction, the MET receptor tyrosine kinase and Autism

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Phenotypic heterogeneity is a fundamental problem faced by efforts to understand the etiology of Autism Spectrum Disorder (ASD), and likely reflects underlying heterogeneity of genetic and non-genetic susceptibility and causative elements. In addition to the common triad of core impairments, subgroups of individuals with ASD also experience epilepsy, immune irregularities, or gastrointestinal dysfunction (GID). The MET receptor tyrosine kinase has been associated with ASD and is implicated in GI development and repair processes. We hypothesize that pleiotropy of the MET signaling system underlies the co-occurrence of ASD and GID. We seek to leverage the phenotypic heterogeneity of ASD by subsetting populations to enrich underlying genetic signals of risk and improve understanding of ASD etiology. Through this larger goal, we also aspire to develop novel diagnostic tools, interventions and treatments for patients with ASD and GID.

Humans are fundamentally social beings, each embedded in a dense network of social connections. A child with autism, however, never reaches out and remains as a singular node, leaving the surrounding web confused and hurt: we don't know how to interact with a solitary unit, and our natural efforts to connect are doomed to fail. Within the triad of core symptoms of autism (in this document the term autism refers to all autism spectrum disorders)—which include restricted interests and/or repetitive stereotyped behaviors, deficits in language development and communication, and abnormalities of reciprocal social interactions—it is the latter, the social phenotype, that is so difficult for us to comprehend and bear.

Individuals with autism have a number of comorbidities beyond the core triad of symptoms. Epilepsy is seen in one-third of individuals with autism, compared with 2% of a non-autistic population[1]; similarly, autism is often found with mild–to-severe mental retardation. Perhaps most intriguingly, it is anecdotally reported by parents and clinicians who interact regularly with children with autism that there is a high prevalence of gastrointestinal (GI) dysfunction in these children, ranging from chronic constipation and diarrhea to esophageal reflux. Scattered reports in the literature, discussed below, support these claims. Parents of children with autism, who must deal with chronic GI dysfunction in their children in addition to the emotional difficulty of raising a developmentally disabled child, are testaments to the resiliency of the human spirit. This review consolidates the relevant background material for a research project that seeks to understand the nature of these GI comorbidities. With this knowledge, there is the possibility of offering parents and caregivers a new and weighty intervention with two-fold significance: alleviating the GI symptoms can directly influence mood and behavior yielding an altered state that indirectly can increase the potential impact other interventions (behavioral, pharmacological, educational or otherwise) can have on the neurodevelopmental course of the disease.

The link between autism and GI dysfunction has a troubled history. In an original study ten years ago, authored by Wakefield and colleagues[2], twelve children were clinically examined for GI complaints and developmental regression that included the loss of language. Nine of the twelve were said to have autism and all twelve had intestinal abnormalities, including non-specific colitis and ileal-lymphoid-nodular hyperplasia. The authors concluded that these findings were "generally associated in time with possible environmental triggers"—namely, the MMR vaccine in eight of the twelve children. To explain the relationship between these three disparate themes—vaccines, GI dysfunction, and autism—the authors invoked "increased intestinal permeability" (also known as the "leaky gut hypothesis") and the "opioid excess" theory of autism to connect the distant dots. Their reasoning was that the measles component of the MMR vaccine had caused local inflammation in the gut, which altered intestinal permeability, allowing incompletely broken-down peptides to be readily absorbed, which then travel to the CNS where they "may exert central-opioid effects…leading to disruption of normal neuroregulation and brain

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development" and thereby cause autism.

Without ambiguity, the balance of the scientific enterprise has not supported Wakefield's interpretations. In a systematic review of 12 epidemiological studies that have investigated the possible role of the MMR vaccine in the etiology of autism, the overwhelming majority of conclusions do not support a causal link[3]. Although ten of the thirteen original authors have retracted their original interpretation[4], society has already experienced non-trivial consequences. Vaccine rates significantly decreased in the UK[5], and a measles outbreak infected thirty-four people in the US in 2005, primarily among children who were not vaccinated because of parental concerns for adverse events related to the vaccine, such as autism6. Prior to this outbreak, the last outbreak in the US was in 1996, and the disease was declared eliminated in the US in 2000. With this legacy and stigma, any future studies of GI dysfunction in autism must first address Wakefield's story, and then move on.

Despite the Wakefield morass, our group is interested in GI dysfunction in autism. We think that the gut of a child with autism contains important information about the brain of a child with autism. Our reasoning is summarized here and detailed below. We have shown a genetic association between autism and a functional variant in the promoter of *MET*, a receptor tyrosine kinase. Developmental and adult expression patterns of *MET* and related proteins show strong signals in the brain and gut, suggesting pleiotropy. Studies report, with limitations, an increased prevalence of GI dysfunction in children with autism. Finally, we have recently shown a genetic association between co-occurring GI dysfunction in autism and a functional variant of *MET*.

The etiology of autism has a large genetic component. Classic studies of twins showed 92% concordance for autism in monozygotic twins, compared with 10% in dizygotic twins[7,8]. With a population prevalence of one in 150 and a male to female ratio of approximately 5:1[9], the recurrence of autism in siblings (2-8%) is noteworthy[10]. Some of the first genome-wide linkage studies identified chromosomal region 7q, among others, as a site of likely autism vulnerability genes[11,12]—a region which includes the *MET* gene.

Prior to investigating *MET* as a susceptibility gene in autism, our lab had an interest in the role played by MET and its only known ligand, Hepatocyte Growth Factor (HGF), in the development of the cerebral cortex[13,14]. In these studies, we showed that altered MET/HGF signaling in the cortex led to decreased counts of interneurons due to abnormal migration of these cells from the ganglionic eminence during development. Because *MET* is located at 7q31, and because the cerebral cortex shows abnormalities in autism[15], our lab investigated the possibility of MET as an autism vulnerability gene.

In an initial screen using temperature gradient capillary electrophoresis, several SNPs were identified in the coding and non-coding regions of the *MET* gene, which has 21 exons over 125-kb[16]. We investigated the transmission of each SNP using the family-based association test (FBAT), which compares the expected versus observed transmission from parent to affected offspring. Compared to the G allele, the C allele of common variant rs1858830, which is located 20-bp upstream of the *MET* transcriptional start site, was significantly over-transmitted to autism-affected offspring in a combined sample of 743 families ($p < 0.001$). Comparing genotypic frequencies in cases to unrelated controls, the rs1858830 C/C genotype had a relative risk for autism diagnosis of 2.27 (95% CI 1.41 to 3.65), compared to the G/G genotype. It was hypothesized that because of the variant's location in the promoter, that it was functionally important for gene transcription. In transcription assays with a luciferase reporter construct driven by the *MET* human promoter, constructs with the C allele were only half as effective as the constructs with the G allele in driving transcription. Using bioinformatics, it was predicted that the G and C alleles would have different transcription factor binding profiles. Electrophoretic mobility shift assays and subsequent supershift assays comparing the two alleles demonstrated different binding of the transcription factors SP1 and PC4.

In a subsequent study of postmortem brain tissue of individuals with autism compared to matched controls, MET protein levels were found to be decreased two-fold in affected individuals[17]. Moreover, when mRNA levels for other genes involved in the MET signaling pathway were examined, components that activate MET signaling were significantly increased in cases compared to controls. It was proposed that long-term compensatory changes are responsible for this upregulation of mRNAs, suggesting alterations of the entire MET signaling system in autism, rather than only within MET alone. These data combined with the initial genetic findings to provide substantial support for altered MET signaling in autism susceptibility.

Based on the findings of altered mRNAs of MET signaling pathway components described above, a subsequent study investigated genetic association between these components and autism[18]. In this study, the *MET* rs1858830 C allele association found previously was replicated in a new sample of 101 affected families. Additionally, the rs344781 variant T allele in the promoter of *PLAUR* was shown to be

significantly associated with autism in two ways: through over-transmission tested by FBAT ($p < 0.01$), and in frequency tested by case-control analysis ($p < 0.01$). This variant gave a relative risk of 1.93 (95% CI 1.12 to 3.31) for genotype T/T and 2.42 (95% CI 1.38 to 4.25) for genotype C/T compared to genotype C/C. *PLAUR* encodes the receptor for the urokinase plasminogen activator (uPA), which is responsible for cleaving the inactive precursor of HGF into its active form. Using a similar luciferase reporter assay as described above, the T allele induced transcription to a greater degree than the C allele, suggesting functional significance of this variant. Finally, variant rs13238709 C allele in *SERPINE1* was shown to be significantly associated with autism by FBAT transmission studies ($p < 0.05$). Unfortunately, case-control analysis did not support this association. *SERPINE1* encodes the plasminogen activator inhibitor-1, which can suppress cleavage of the inactive precursor of HGF by uPA. This study demonstrates that multiple elements of the MET signaling pathway can confer risk for autism.

The expression patterns of MET and HGF in development and adulthood suggest that MET signaling is pleiotropic—it is not only important in brain development and function, but also in the GI system. An early study found robust expression of both MET and HGF throughout mid and late embryonic development in the mouse[19]. From E10 to E18, both transcripts are present in developing kidney, intestine, lung, liver, pancreas, stomach and muscle. In most cases, MET is found in epithelial tissues and HGF in mesenchymal tissues. Another study looked earlier in development, from E6.5 to E10 in the mouse, and found *MET* and *HGF* expression in the intermediate primitive streak, notochord, and importantly, later neural crest cells[20]. Replicating previous findings, they also showed that later at E13, *MET* and *HGF* are expressed in the developing lungs, liver, and gut. Importantly, similar expression was seen of both *MET* and *HGF* transcript and protein in human fetal tissue, aged 7-24 weeks gestational age, in the developing GI system[21]. Dynamic expression patterns were seen in different tissues—including the esophagus, stomach, small and large intestine, liver, and pancreas—throughout development. Looking in adulthood, another study found similar expression of HGF in human and rat tissues that included the digestive, renal, and reproductive systems[22]. Taken together, these studies demonstrate that MET signaling is also important outside of the brain, in both development and adulthood.

There are scattered reports in the literature that examine the prevalence of GI dysfunction in autistic populations, but many studies are limited in sample size, have inadequate control groups, or lack independent replication. A critical review[23] of these reports concludes that, after failing to find any replicative and rigorous studies, they "found no evidence on which to base a confident statement whether GI symptoms are more common in children with than without autism." Among the five studies they found worth reviewing, prevalence of GI symptoms among individuals with autism ranged from 9% to 84%. It should be noted that, since Wakefield's original study, most studies investigating the intersection of GI dysfunction and autism have intended to address the issue of measles virus causing autism, rather than the significance of GI dysfunction alone. Since that review, one well-executed, prospective study has shown a significantly increased prevalence of GI symptoms in individuals with autism when compared to two matched control groups[24]. The autism group had a prevalence of 70%, compared to a typically developing group with 28% and another group with other developmental delays at 42%.

Another recent study, although with limitations, has interesting findings for the issue of GI dysfunction in autism[25]. The authors report a GI dysfunction prevalence of 23% in a convenience sample of 172 children with autism enrolled in a pharmacology study. The limitations of the study are many: there are no matched controls, there is no indication that this sample is generalizable to the population of individuals with autism at-large, the clinical expertise of a gastroenterologist was not consulted, and their method of assaying GI dysfunction was through either a retrospective medical history review or a short structured questionnaire designed to monitor drug side effects. However, even with those limitations, this is a fascinating paper for what it suggests in the preliminary data it provides. In addition to a medical history, study participants were also asked to complete assessments to characterize their social and cognitive development, as well as levels of anxiety and social withdrawal. Because this GI study was in the context of a larger pharmacology study, responsiveness to treatment was also monitored. The preliminary data in this paper shows that children with autism and GI problems, compared to those without GI problems, have greater levels of anxiety and social withdrawal. Additionally, for children in the risperidone arm of the study, those without GI dysfunction were two times more likely to respond to drug treatment, compared to those with GI problems. These data suggest that GI comorbidities in autism can have important effects on treatment response, and overall disposition, which could ultimately impact the success of other interventions for children with autism.

With these data taken together, our lab tested the reasonable hypothesis that the autism-associated *MET*

promoter variant has an increased association in individuals with co-occurring autism and GI dysfunction[26]. To test this hypothesis we gathered data from an existing research database and gene bank, which yielded 992 individuals in 214 families with a complete medical history and GI condition report. In this sample, 41% of individuals with autism had GI conditions, significantly more than in parents (24%) and unaffected siblings (9%). Although for this analysis the presence of GI condition was scored as a binary outcome (present or not), in this sample the majority of GI conditions in individuals with autism is distributed amongst diarrhea (28%), constipation (33%), and gastroesophageal reflux (5%). When the functional variant in the promoter of *MET* was examined in a subset of 62 families not included in the original study16, the significant association of the C allele with autism was replicated. Additionally, in the 214 family samples, the C allele was significantly associated with the presence of GI conditions. When the 214 family samples was stratified into families with at least one affected child with co-occurring autism and a GI condition, a subsample of 118 families was identified. In these 118 families, the *MET* C allele was significantly associated with co-occurring autism and GI conditions. A potential weakness of this study was that it relied on retrospective, parent-reported GI symptoms. To address this, an additional sample stratification was performed: a subset of 64 families were identified in which at least one child with autism and co-occurring GI symptoms were present, as well as at least one sibling affected with autism but not GI symptoms. The *MET* C allele was significantly over-transmitted to offspring with co-occurring GI symptoms. Because parents were unaware of their offspring's allelic status at rs1858830, this suggests that the association of the *MET* C allele with co-occurring GI conditions and autism is not due to parental reporting bias. This study brings together several themes discussed above to demonstrate that MET signaling is important in a subset of individuals with co-occurring autism and GI conditions, and might reflect a common underlying genetic vulnerability for both central and peripheral pathologies.

Taking all of these data into account, there are several possible biological mechanisms that could explain MET's involvement in GI dysfunction in autism. The development of the enteric nervous system (ENS) could be altered, leading to abnormal function in adulthood. The story of the RET receptor tyrosine kinase in Hirschsprung's disease (HSCR) could be an interesting analogy to MET. HSCR is a congenital disease caused by deficient ENS innervation of the terminal gut. One in 5000 children are affected by this disease and it is usually diagnosed within the first hours after birth, with physical findings of an inability to pass stool, distended abdomen, and vomiting which can lead to tonic contraction of the terminal gut, obstruction, and proximal distention if left untreated[27]. Human mutations in *RET* coding regions are associated with HSCR[28], which are now understood to account for 50% of familial and 15-35% of sporadic cases of the disease. In mice with mutated RET kinase activity, the two primary results are gut aganglionosis and renal agenesis[29]. Conspicuously, the kidney defects are not a common finding in HSCR, suggesting complete ablation of RET activity is an imperfect model of the disease. It has also been recently shown that non-coding mutations in a RET enhancer region are associated with HSCR, suggesting the importance of *RET* dosage in the etiology of the disease[30].

A recent study integrated these facts and demonstrated through a series of *RET* mutants that decreasing *RET* expression to one-third normal levels produces an accurate model of HSCR[31]. These mice lack renal defects, exhibit distal gut aganglionosis, and show incomplete penetrance and a male bias (which are both seen in HSCR[32]). In these mice, neural crest cell (NCC) precursors of ENS cells have altered migration and survival, causing aberrant innervation of the gut. These findings have important advances for the HSCR field: it demonstrates the importance of *RET* dosage and suggests a threshold that could be reached by many different ways; it is a better model of the human disease, addressing obvious inconsistencies in previous models; it demonstrates a combination of migration and survival are responsible for the observed aganglionosis; and finally it clarifies the role of a receptor tyrosine kinase in the development of the ENS.

There is evidence that MET is important in some aspects of NCC development as well. One study showed that transgenic mice which over-express HGF in a variety of tissues characteristically develop ectopic melanocytes[33]. These mice develop melanosis in various parts of the CNS, including the brain, meninges, and spinal cord, as well as hyper-pigmented skin. Interestingly, the authors comment that although abnormal melanocyte development was easily observed, other neural crest derivatives might also be affected in these mice. In passing, they note that intestinal obstruction is conspicuous in these mice and could be related to altered ENS development. Through the studies discussed here, there is a possibly interesting parallel for the story of MET and GI dysfunction. While it might not be the same underlying biology, the story of RET demonstrates how altered NCC and ENS development can impact GI function.

Altered MET signaling could also contribute to GI dysfunction by impacting normal epithelial repair processes in the gut. Recent studies have

demonstrated that HGF can promote epithelial repair in rodent models of GI disorders. One study used a well-characterized model of ulcerative colitis (UC) in rats and showed that exogenous administration of recombinant HGF significantly improved several measures of GI dysfunction[34]. The animals were fed dextran sulfate sodium (DSS) for several days, which is known to induce a phenotype similar to UC, and then continuously administered HGF intraperitoneally. Without HGF, there were decreases in body weight and colon length, and epithelial erosions present—all seen in human UC—associated with DSS administration. HGF administration, in contrast, prevented all of these measures of pathology, suggesting HGF/MET signaling plays an important role in GI epithelial repair in this rodent model of UC. Another study used a rodent model of inflammatory bowel disease with intravenous administration of HGF, and showed decreases in diarrhea and gut inflammation[35]. These studies demonstrate an important possibility for how altered MET signaling could be contributing to GI dysfunction in autism.

Altered ENS development and perturbed epithelial repair are only two of many different possible ways in which MET signaling could be contributing to GI dysfunction in autism. Children with autism can have sensory issues which could lead to behaviorally-mediated GI dysfunction. It is not difficult to imagine a child who has strong tactile aversions refusing to go to the bathroom, leading to chronic GI issues. Additionally, the core feature of restricted interests could drive some children to have poor nutrition, again leading to GI dysfunction. Many of the psychoactive drugs prescribed to children with autism have known GI side-effects[23] which could also contribute to GI dysfunction. For each child, it is possible that any combination of or interaction between these or other possible mechanisms could underlie their GI dysfunction.

We believe the autistic phenotype is deeply heterogeneous, and that by focusing on a clinically significant subgroup of affected individuals, we will boost our genetic signal by focusing on a common underlying biological mechanism. We do not see GI dysfunction as a separate clinical issue for individuals with autism. As investigators, we see it as an opportunity to increase our understanding of the etiology of autism: altered MET signaling, in our hypothesis, affects multiple systems in parallel through a common genetic etiology. As clinicians, we see GI dysfunction as another important element that affects some individuals, making each person unique. With increased understanding of GI dysfunction in autism, we will be able to offer better therapy and interventions to individuals with autism.

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FURTHER INFORMATION

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