August 2014

Translating Knowledge of Autism Spectrum Disorders to Action Through Tool Development and Exploration

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TRANSLATING KNOWLEDGE OF AUTISM SPECTRUM DISORDERS TO ACTION THROUGH DISCOVERY AND EXPLORATION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Healthcare Genetics

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by
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August 2014

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ABSTRACT

Translational processes are needed to move research development, methods, and techniques into clinical application. The knowledge to action framework organizes this bench to bedside process through three phases including: research, translation, and institutionalization without being specific to one disease or condition. The overall goal of this research is to bridge gaps in the translational process from assay development to disease detection through a mixed methods approach. A literature review identifies gaps associated with intestinal permeability and autism spectrum disorders. Mining social media related to autism and GI symptoms captures self-reported or observed data, identifies patterns and themes within the data, and works to translate that knowledge into healthcare applications. Development of novel tests can then examine relationships between zonulin levels, haptoglobin genotype, and autism spectrum disorders, and propose a paradigm shift in the use of proteomics and genomic diagnostic testing from clinical diagnosis to pre-symptomatic testing. Although results from this study do not find statistically significant relationships between zonulin and autism spectrum disorders, they do suggest clinical significance and the need to conduct larger studies. The discovery presents a novel approach for measuring intestinal permeability. Qualitative and quantitative methods collaboratively point toward implementation of molecular and data mining techniques in the development and evaluation of early diagnostic tests and interventions. Equally, the two methods working together drive the field forward in design and development to strengthen the outcomes.

Keywords: autism, zonulin, Radian6, haptoglobin, social media

ABBREVIATIONS

ASD	Autism Spectrum Disorder
ALI	Acute lung injury
ALT	Alanine aminotransferase
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
BBB	Blood brain barrier
CARS	Childhood Autism Rating Scale
CDC	Centers for Disease Control and Prevention
CF	Complement fixation
c-kit	V-KIT Hardy-Zuckerman 4 feline sarcoma viral oncogene
ctx	Cholera toxin
CXCR3	Chemokine (C-X-C motif) receptor 3
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorders
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assays
ER	Endoplasmic reticulum
GAPS	Gut and psychology syndrome
GARS	Gilliam Autism Rating Scale
gDNA	Genomic deoxyribonucleic acid
GI	Gastrointestinal
GLP-2	Glucagon-like peptide 2
GPBAR1	bile acid receptor
HP	Haptoglobin
HPR	Haptoglobin-related
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-6	Interleukin 6
IQR	Interquartile range
IRB	Internal Review Board
K2A	Knowledge to Action Framework
LM	Lactose mannitol
M	Mean
mRNA	Messenger ribonucleic acid
mi-RNA	micro-ribonucleic acid
MRC	British Medical Research Council
NCCDPHP	National Center for Chronic Disease Prevention and Health Promotion

Abbreviations (continued)

NP	Nurse practitioner
NS1	Non-structural protein 1 antibody
PAR2	Protease activated receptor 2
PCR	Polymerase chain reaction
PPI-3	Inositol 1,4,5-tris-phosphate
Pre-HP2	Pre-haptoglobin 2 or zonulin
PRNT	Plaque reduction and neutralization tests
RBMECs	Rat brain microvascular endothelial cells
RNA	Ribonucleic acid
SD	Standard Deviation
SPSS	Statistical package for the social sciences
STAT3	Signal transducer and activator of transcription 3
TJ	Tight junctions
US	United States
WHO	World Health Organization
ZOT	Zonulin occludins toxin

ACKNOWLEDGMENTS

First and foremost I want to thank the Lord Jesus Christ for granting me the knowledge and wisdom to make it through all the challenges up to this point. Without Him as a higher power in my life I would not have made it this far in my education and career. Secondly, I want to thank my husband for pushing me forward, dreaming with me, and reminding me to "take it easy" when times got rough. I also want to thank Michael for his patience, when mommy was stuck at her desk and not able to play. I love all of my family and I am very grateful to have each one in my life to play their own special role in this journey.

I would like to thank Dr. Julie Eggert, my advisor, for her belief in me and hours of wonderful conversation to make this dissertation a success. Additionally, I am very thankful for the members of my committee, Drs. Ann Wetsel, Brent Satterfield, Alex Feltus, and Matthew Boyer for their constructive criticism and advice.

I am also grateful for the support and advice from Dr. Veronica Parker, Dr. Jason Thrasher, Dr. Patricia Tate, Dr. Elisabeth Chismark, the Faculty and Staff from the Greenwood Genetic Center, and my colleagues in the Healthcare Genetic Doctoral Program.

I would like to acknowledge the financial contributions from the Center for Research on Health Disparities and Creative Inquiry.

DEDICATION

This dissertation is dedicated to Marlon and Michael for their patience, love, support, and faith in me.

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CHAPTER I

INTRODUCTION

Early diagnostics make up the engine core that thrusts public and private research through the translational process after discovery and development into clinical application. Qualitative and quantitative methods are equally important in driving research design toward conclusive experiences and evidence of disease states to strengthen research outcomes.

Exposure to bacteria can occur at any time from conception to adulthood. Even though childhood exposures may not be pathogenic, they may contribute to activation of disease pathways. For example, zonulin is a protein that regulates the epithelial permeability allowing macromolecules to pass into the blood stream (Tripathi et al., 2009; Wang, Uzzau, Goldblum, & Fasano, 2000). Normally this process assists in epithelial barrier protection from invading pathogens; however, the constitutive action of zonulin increasing epithelial permeability can also result in molecules passing through the barrier to initiate immune response and disease development (Drago et al., 2006; Duerksen, Wilhelm-Boyles, Veitch, Kryszak, & Parry, 2010). Studies have identified zonulin's specific roles in intestinal and blood brain barrier permeability (Cipriani et al., 2011; Diaz-Coranguez et al., 2013; Duerksen et al., 2010; Fasano et al., 2000; Lu et al., 2000; Wang et al., 2000). The type of permeability suggests zonulin may play a role in the pathological development of autism spectrum disorder (ASD) and related symptoms. ASD affects 1 in 68 children in the United States (U.S.) with no known cause or diagnostic test (Centers for Disease Control and Prevention [CDC], 2014). Interventions

for autism typically involve multiple levels of trial and error using medication, supplements, and an array of therapies with an annual expected lifetime cost of care at $2.4 million per individual (Buescher, Cidav, Knapp, & Mandell, 2014).

Using the Center for Disease Control and Prevention's (CDC) National Center for Chronic Disease Prevention and Health Promotion (NCCDPHP) 'Knowledge to Action' framework, this dissertation participates in discovery through practice (Wilson et al., 2011). The overall goal of this dissertation is to determine if it is possible to bridge gaps in the translational process from assay development to disease detection through a mixed methods approach.

Knowledge to Action Framework

The Knowledge to Action (K2A) framework used for this research project was created by the CDC to organize the processes from bench to bedside through three phases including research, translation, and institutionalization without being specific to one disease or condition (Wilson et al., 2011). See Fig 1.1.

Figure 1.1

NCCDPHP Knowledge to Action Framework for Public Health

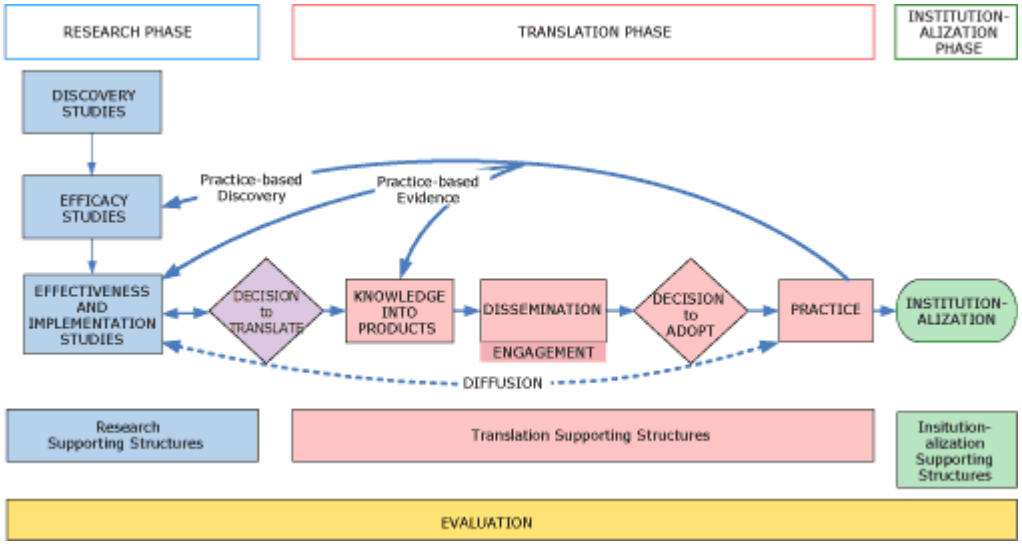

Figure 1.1 NCCDPHP Knowledge to Action Framework for Public Health. The figure illustrates the three phases of the NCCDPHP K2A framework used with permission from Wilson et al., 2011.

In this framework, each phase is made up of multiple components. For the purpose of this dissertation, the definitions used by the CDC's NCCDPHP K2A framework are embraced. The research phases reflected in the subsequent chapters are the focus of this dissertation project (Wilson et al., 2011).

The term 'research' refers to the first phase within the K2A framework and includes discovery, efficacy, effectiveness, and research supporting structures (Wilson et al., 2011). These guide the process of this dissertation research. Discovery encompasses epidemiological, behavioral, and biomedical studies (Wilson & Fridinger, 2008). Discovery is also comprised of initial ideas and thoughts that lead from synthesis of

literature to development of a research plan. Discovery entails investigations of literature, data, observations, and experiences. The discovery phase explores the literature to establish a background and path moving forward. Discovery studies provide proof-of-concept in order to acquire a larger research support network of organizations, scientists, sample sizes, and funding. Efficacy and effectiveness intertwines with discovery to explore where present interventions and testing fails or excels (Wilson & Fridinger, 2008). Efficacy refers to formative research, where attitudes, beliefs, and experiences can be explored and incorporated into development of diagnostic tests and interventions (Wilson & Fridinger, 2008). Efficacy allows the voice of the stakeholders to be incorporated into the research model (Wilson & Fridinger, 2008). Results from these initial experiments provide evidence to gather support from multiple disciplines and investors to launch a full investigation that will either lead to the decision to translate or continue with more discovery studies.

The K2A framework recognizes the need to incorporate all disciplines in the translational process in order to put knowledge into action (Wilson et al., 2011). Part of this translation includes measuring the gap between what is found in guidelines, the literature, and to practice (Kitson & Straus, 2010). Chapter II provides a review of the literature surrounding epithelial permeability, zonulin, and ASD. The review identifies a gap in the literature in studies associating intestinal permeability with ASD and serum zonulin levels in ASD individuals (D'Eufemia et al., 1996; De Magistris et al., 2010). When gaps provide useful and relevant knowledge, exploring these gaps through templates and tools can identify needs, gather resources, evaluate knowledge, discern

good from bad, and make information available to others (Turnbull et al., 2010). Social media data mining provides this capability when focused on a specific disease or list of symptoms. Chapter III is a discovery and efficacy study that looks for patterns among people with ASD and gastrointestinal (GI) symptoms in social media data. The social media research is part of discovery through collection of observations and experiences and it is an efficacy study through its compilation of attitudes and beliefs of the participants. Chapter IV follows with a discovery and efficacy study that measures zonulin levels and haptoglobin genotype of ASD individuals, newborns, and healthy controls to determine if zonulin can effectively detect the presence of ASD as an early diagnostic test. Effectiveness research expands the optimal discovery and efficacy studies to the translational phase through clinical trials and application (Wilson & Fridinger, 2008).

The clinical trials process follows a set of guidelines to ensure that a drug, medical device, or therapy works efficiently and effectively without harming patients. Each country has its own regulations that must be met before a product can pass this stage and be sold to consumers. Clinical trials focus on reproducibility of efficacy and effectiveness studies, and add double blind studies to eliminate possible researcher bias to the outcome (Hróbjartsson et al., 2013). Repetition is necessary because the response in humans may be different from those recorded from *in vitro* studies. Communication between stakeholders is pertinent at this point to identify the needs and risks of implementing scientific research into clinical practice (Droms, Ferguson, & Giuliano,

2014). Stakeholders might include parents, caregivers, individuals with ASD, insurance companies, clinicians, organizations, government agencies, or education sources.

Once the first phase of research is ended, the second phase of translation begins. The K2A framework delineates translation as: decisions to translate, knowledge into products, dissemination, engagement, decisions to adopt, practice, and translation supporting structures (Wilson & Fridinger, 2008; Wilson et al., 2011). Translation is the process by which an application is moved from the development phase into use in a clinical setting. The process may include extensive validation procedures that confirm the specificity and sensitivity of the test, as well as correlation studies with samples that have been previously tested by a validated testing method. In many cases there is movement between phases based on need to revisit the experimental processes related to the need to refine and improve methods as a result of unexpected consequences of implementation, advances in technology, environmental changes, or knowledge of genetic factors.

Research Phase of Zonulin Testing

The most common type of research in healthcare is quantitative research; however, qualitative research is also important in understanding the patient experience, assessment, diagnosis, and treatment. The early physicians, such as Hippocrates (460 B.C. -377B.C.), used observations and taste to determine disease states with a record of their findings in journals (Medvei, 1993). Visual observations continued to be the primary source of health assessment for diabetes until the first quantitative test was developed in 1841 by Carl Trommer; followed by the more well-known urine sugar test

developed by Hermann Fehling in 1848 (Medvei, 1993). As technology advanced and more tests were developed, quantitative research became embedded in healthcare.

Today, researchers question whether one type of research is enough to provide quality of care. Stuart and Wiles' (1997) comparisons of questionnaires and interviews suggest that use of only one type of research does not assess the complete understanding of information, and it provides misleading information when used alone. The lack of reproducibility between questionnaire and interview responses was significant ($p<0.008$), indicating a lack of information about patient needs, beliefs, and experiences necessary to facilitate self-management (Stuart & Wiles, 1997). Zhang and Creswell (2013) identify outcomes of mixed methods in convergence, complementarity, development, initiation, and expansion; whereas, the use of only quantitative methods fail to capture perspectives from broad, complex groups. Both quantitative and qualitative research is needed to develop and implement successful interventions and treatments. This dissertation uses both qualitative and quantitative research to provide a more complete view of the relationship between GI symptoms and ASD.

Quantitative and qualitative research looks at people and disease to identify a problem and develop a research plan to test a hypothesis. In the field of healthcare, stakeholders represent individuals and families affected by the same disease, those experiencing similar symptoms, those going through similar therapies, healthcare professionals, drugs, diseases, new technologies, or equipment. The stakeholders involved in this dissertation research include individuals with ASD, caregivers or family members, healthcare professionals, and private and public organizations. Each of these

stakeholders may be affected or represented in the data collected from social media outlets. The stakeholders are not always involved in the research groups, but they may be indirectly affected by the research outcomes. Differences can be seen in the methodology, data collection, and analysis of these groups. The term 'quality' has been recently defined in healthcare as "the degree to which policies, programs, services, and research for the population increase desired health outcomes and conditions in which the population can be healthy" (Honore et al., 2012, p.739). Table 1.1 provides a list of common and healthcare definitions for quality and quantity

Table 1.1

Quality verses Quantity Definitions

Quality	
Common Definitions	Medical Definitions
character	Accessibility and familiarity (Lee et al., 2013)
capacity	Test results (Lee et al., 2013)
vividness of hue	Evidence- based practice (Lee et al., 2013)
degree of excellence	cost effectiveness and service delivery (Lee et al., 2013)
Superiority	A management process, set of tools, and techniques used to meet healthcare needs (Riley et al., 2010)
acquired skill	how a group or person accepts rules, codes, laws, or conventions (Berger, 2013)
a distinguishing attribute	provides details of behavior, routines, needs, and personality characteristics (Madrigal & McClain, 2012)
social status	examines patterns and relationships in the data to develop and test hypotheses to generate theory or develop theory to explain data (Morse & Field, 1995)
role	Effectiveness, access and timeliness, capacity, safety, patient centeredness, and equity measurements (Committee on Quality of Health Care in America, Institute of Medicine, 2001)
feature	reaching a desired health outcome (Brilli et al., 2014)
Quantity	
Amount, estimate,	theory based, structured, systematic, planned, objective, logical, and

or number	dependent on scientific thought (Berger, 2013)

Table 1.1 Quality and Quantity Definitions. The definitions for both terms above are taken from Berger, 2013; Brilli et al., 2014; Committee on Quality of Health Care in America, Institute of Medicine, 2001; Lee et al., 2013; Madrigal & McClain, 2012; Morse & Field, 1995; Riley et al., 2010.

The combination of qualitative and quantitative measures increases the strength of the findings and moves science forward through the K2A framework to the translation phase. To begin the testing process, only quantitative research requires the formation of a research plan through the identification of a problem and development of a testable hypothesis. The strength of qualitative research can be found in description of the problem and understanding how the intervention or research plan will work. Models and diagrams can assist in conceptualization of the problem. These can be generated from qualitative or quantitative software programs. In qualitative research, the hypothesis may not be formed until data has been examined, at such point the hypothesis or research questions may be edited. Patterns and relationships in qualitative data can be used to develop and test hypotheses and to develop theories (Heyvaert, Maes, & Onghena, 2013; Morse & Field, 1995).

Qualitative research is designed around a set of open ended research questions, so the researcher can capture the viewpoint and context of the data being collected. Context is important to qualitative research because it holds the meaning of the data. Questions may differ depending on the design type, but they focus on understanding a single concept, discussing the results, and incorporating new-understandings and ethical, appropriate use of tests (Creswell, Hanson, Clark Plano, & Morales, 2007; Fetters, Curry, & Creswell, 2013). Qualitative research has multiple design types such as narrative

research, case study, grounded theory, phenomenology, and action research (Glesne, 2010). More than one design type can be used in an experiment or it can be mixed with quantitative methods (Fetters et al., 2013; Palinkas et al., 2013).

Limitations and questions exist when exploring the use of mixed methods research (Palinkas et al., 2013). The complexity of the data makes it difficult to analyze. One example is that text and behaviors may have multiple meanings within the same population; even more intensified across cultures and countries. Many times the question arises "is the research beneficial; is it a worthwhile cause?" Bendassolli (2013) discusses problems of induction of qualitative research explaining that in many circumstances adequate information does not exist to formulate a theory and therefore nothing can be tested. In addition, excessive amounts of data can overwhelm the analytical process; therefore, researchers need to know how to manage and analyze large data sets.

Automation has sought to resolve analysis problems of large data sets in multiple forms of research. Reliance on automation in qualitative data could lead to loss of context or meaning of the data; therefore, automation should be viewed as a tool to help the analyst interpret the data (Angus, Rintel, & Wiles, 2013). Another limitation shared between qualitative and quantitative research is researcher bias. Berger (2013) explains that when a person believes something should be done, he/she becomes selective and neglects fully informative data. Berger (2013) associates the term quality with evaluation, judgment, and taste. In qualitative research, this is also seen in data collected due to a person's motivation or personal interests in the research outcome.

Motivation and involvement by researchers and participants can also be beneficial in qualitative research (Barakat et al., 2013). Since qualitative research attempts to capture the context of the data, it can be very powerful and useful in describing the beliefs, desires, struggles, motivations, and responses to intervention of some groups (Nicolaidis et al., 2011). For example, individuals who are diagnosed with ASD often do not have a voice in research methodologies and designs. The autistic community has been compared to voices of lesbian, gay, bisexual, transgendered, deaf individuals as they represent individuals with a unique phenotype that is not shared by the rest of their families (Nicolaidis et al., 2011). Qualitative research can capture the voices of these minority groups without losing their passion and motivation to identification as a number. By listening to the voices of those with ASD and their families, researchers can incorporate their concerns and ideas into interventions and technologies (Nicolaidis et al., 2011; Rowe & Nevin, 2013).

Ethically speaking the inclusion of all stakeholder points-of-view is vital in developing technologies and/or resources that are both utilitarian and non-maleficent. Although quantitative research is assuming more non-invasive methods of collection, it does not maintain individual autonomy. In order to protect the anonymity of human subjects, all forms of research removes the identity of a person. The representation of autonomy and preservation of context in qualitative research may ultimately be its demise in protecting individuals or organizations. For example, a simple search or publication of reported data or phrases may inadvertently identify an individual, group, or organization.

Much detail in protection of human subjects and de-identification of data must take place in the development of both research methods.

The role of qualitative research in healthcare intervention is important and as a result guidelines and checklists have been created to provide a framework for development. In 2000, the British Medical Research Council (MRC) recognized that qualitative research could provide beneficial information for complex medical interventions (Medical Research Council, 2000). The MRC published guidelines in 2000 that proposed using qualitative research to identify mechanisms and determine effectiveness of interventions. These guidelines were updated in 2008 to focus on qualitative properties that maintain context of information (Medical Research Council, 2008). These guidelines help in development of a framework to approach qualitative research. Using these guidelines, this research incorporates them into the K2A framework.

Exploring Social Media

The research phase of this project is divided into two parts based on utilization of qualitative and quantitative methods to reach the *overall goal* of developing early diagnostic tests for ASD based on gastrointestinal symptoms. The qualitative portion of this research compares relationships between known physiological effects of zonulin and symptoms of ASD recorded through social media and phenotypic assessment. This study utilizes three months of public information to identify symptoms of ASD described through social media. Radian6 software designed to assist with queries of social media was used to collect the data. The ASD social media data was analyzed to identify

symptoms unique to autism implicating epithelial permeability physiological pathways related to increased risk of developing ASD. The qualitative portion of this research project focused on the following aims:

AIM 1: To determine themes and patterns of ASD from social media

AIM 2: To understand the impact of social media on self-management of ASD with co-morbid intestinal manifestations.

AIM 3: To identify common symptoms and/or thematic elements used through social media

The research seeks to answer the following research questions:

1. What symptoms are expressed in recorded social media data related to ASDs?
2. What are the themes related to ASD from social media?
3. What are the facilitators and barriers of social media use in relation to ASD?

Zonulin Pathway to Autism Spectrum Disorders

Although the pathology between ASD and GI symptoms has not been clearly defined, the Autism Research Institute (2013) reported that up to 70% of ASD individuals suffer with GI issues. The most commonly reported symptoms of ASD are social impairment, communication difficulties, and repetitive and stereotyped behaviors. Additionally, symptoms of stomach pain, diarrhea, constipation, acid reflux, vomiting, bloating, and food allergies have been reported (Chaidez, Hansen, & Hertz-Picciotto, 2014; Chandler et al., 2013; Mazefsky, Schreiber, Olino, & Minshew, 2013; National

Institute of Mental Health, 2011; Peters et al., 2014). Currently no diagnostic test is available to detect the presence or severity of ASD.

Zonulin, pre-haptoglobin (HP) 2, is a complex protein structure that regulates epithelial tight junctions, allowing macromolecules to pass across cell membranes into the blood stream causing altered immune responses (Wang et al., 2000; Tripathi et al., 2009). Although the relationship between ASD and the immune system is unclear, studies have identified associations between ASD and intestinal permeability (D'Eufemia et al., 1996; De Magistris et al., 2010), and between ASD males and HP genotypes (Aposhian, Zakharyan, Chowdhury, & Avram, 2006). However, no studies reported in the literature have directly measured zonulin levels in ASD populations. Therefore, there is a need to determine the role of zonulin in ASD.

The *long term goal* for this research is to provide a diagnostic assay that correlates the ASD diagnostic scale with different grades of GI severity. Based on pathophysiology of rising zonulin levels, the *overall objective* is to determine if there is a relationship between increasing or decreasing zonulin levels and ASD. The *central hypothesis* is that increases in zonulin concentrations in plasma are directly associated with ASD severity and that HP2-2 genotypes place an individual at a higher risk for developing ASD. The basis for this hypothesis is there is a correlation between intestinal permeability in individuals diagnosed with ASD, severity of ASD and symptoms of severe GI diseases (Adams, Johansen, Powell, Quig, & Rubin, 2011). Zonulin levels and haptoglobin genotypes will be determined from banked plasma and genomic deoxyribonucleic acid

(DNA) samples retrieved from the Greenwood Genetic Center. In order to test this hypothesis and accomplish the objectives, the following *specific aims* are proposed:

Aim 4: Determine if zonulin levels in plasma obtained from individuals diagnosed with an ASD (Group 1) or newborns (Group 2) are different from zonulin levels in controls of healthy individuals matched by age and/ or gender (Group 3). Zonulin levels will be quantified using an enzyme-linked immunosorbent assay. The *working hypothesis* is that increased zonulin levels in plasma are directly associated with the presence and severity of ASD.

Aim 5: Examine relationships between HP2 genotypes and increased zonulin levels in individuals with ASD (Group 1) or newborns (Group 2) compared to healthy controls matched by age and/or gender (Group 3). HP genotype will be determined using real time PCR. It is *postulated* that HP2-2 genotypes and zonulin will be increased in individuals with ASD.

Aim 6: Determine if zonulin levels are good predictors between ASD and healthy control groups. It is *postulated* that increased zonulin levels will predict the presence of ASDs.

The proposed research focused on identifying the role of zonulin or HP genotype in determining risk and severity of ASD. The results from this study will provide information for the development of a novel, quantifiable, diagnostic test to determine risk and/or severity of ASD. Additionally, the results will provide new mechanisms of interest for future preventive or therapeutic interventions.

Summary

This chapter introduces the K2A framework and illustrates how each subsequent chapter brings this research from discovery to the translational phase. Chapter II synthesizes the literature in discovery of what is known and the gaps surrounding epithelial permeability related to zonulin and ASD. The information gained from the literature search identified a lack of zonulin testing among ASD individuals even though there were numerous publications which associated intestinal permeability and gastrointestinal symptoms with ASD. In order to fill this gap in literature, discovery and efficacy studies were planned and tested. Chapter III explores social media used to capture experiences, attitudes, and beliefs of individuals affected directly or indirectly by ASD and GI symptoms. The patterns and themes collected from mining social media supported future exploration. Chapter IV continued the discovery and efficacy studies by measure zonulin levels and haptoglobin genotype in individuals with ASD, newborns, and healthy controls.

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CHAPTER II

ZONULIN PATHWAY TO AUTISM SPECTRUM DISORDERS

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Abstract

Tight junctions are protein complexes regulated by zonulin. The modulation of the tight junctions allows macromolecules to pass, causing a disruption in homeostatic mechanisms that play important roles in the development of immunity and disease. This paper will explain the physiological mechanisms and molecular pathways through which zonulin is or could be associated with symptoms or disease especially Autism Spectrum Disorders. The hypothesis is increased serum zonulin levels and comparisons of the physiological effects of zonulin and phenotypic assessment data can be used to implicate severity of disease states. Furthermore, a model of the zonulin pathway to disease is presented.

Keywords: tight junctions, zonulin, prehaptoglobin2, autism, Autism Spectrum Disorder

Zonulin Pathway to Autism Spectrum Disorders

Tight junctions (TJ), or *zonulae occludentes,* are continuous intercellular spaces between epithelial cells that are composed of more than 40 proteins (Anderson & Van Itallie, 2009; Fasano, 2001). Presently these proteins consist of the occludins, claudins, junctional adhesion molecules, and cytoplasmic plaque proteins necessary for structural functions (Nusrat, Turner, & Madara, 2000). Specifically TJs have two main functions: they serve as a barrier to protect against microorganisms by allowing only molecules with radii less than 15Å or 3.5kDa to pass into the cell, and they transport fluids, macromolecules, and leukocytes in and out of body compartments (Fasano, 2008; Fasano, A., 2011; Tripathi et al, 2009). Zonulin has been identified as a 47-kDa protein that regulates the opening and closing of TJs (Wang, Uzzau, Goldblum, & Fasano, 2000; Tripathi et al., 2009). The modulation of TJs allows macromolecules to pass into the bloodstream, thus playing an important role in the development of immunity and disease. The purpose of this paper is to explain the physiological mechanisms and molecular pathways through which zonulin regulates tight junctions. Furthermore, a model of the zonulin pathway to disease will be presented.

The Zonulin Pathway

Fasano et al. (1991) identified zonulin occludens toxin (ZOT), an enterotoxin produced by *Vibrio cholerae*, as a regulator of TJ. Diarrhea in patients testing negative for cholera toxin raised suspicion that another toxin produced by *Vibrio cholerae* may be present (Fasano et al., 1991). The results from the study by Fasano et al. (1991) indicated that ZOT increased intestinal permeability by altering the structure of intercellular TJ,

which allowed water and electrolytes to leak into the lumen of the small intestine resulting in diarrhea. A follow-up study by Baudry, Fasano, Ketley, & Kaper (1992) identified three ZOT genes *CVD101*, *CVD108,* and *395-N1*. This study further defined ZOT as a 44.8kDa protein responsible for abdominal cramps, malaise, vomiting, headaches, and diarrhea. The *zot* gene was identified upstream of cholera toxin (*ctx)* genes with its terminal codon overlapping the *ctx* promoter (Baudry et al., 1992). ZOT binding was detected in jejunum and distal ileum, not the colon (Fasano, 2011; Fasano, 2008; Di Pierro et al, 2001). Affinity purified anti-ZOT antibodies tested on mammalian tissue in ussing chambers identified zonulin as a eukaryotic homolog to ZOT (Wang et al., 2000). In the same study, a comparison of adult and fetal intestinal tissue by micro-sequencing revealed that the N-terminal sequence of fetal zonulin (MLQKAESGGVLVQPG) is 60% identical to adult zonulin (EVQLVESGGXL) (Wang et al., 2000). The differences in the N-terminal sequences were believed to be due to fetal development, which also explained why the adult form regulated paracellular permeability while fetal forms regulated molecules between body compartments during embryogenesis (Wang et al., 2000). Since its first discovery, zonulin has been associated with several autoimmune diseases (Fasano, 2012).

Fasano (2011) connected zonulin and its associated diseases to chromosome 16 through two haptoglobin genes mapped to chromosome 16. A recent search in the University of California, Santa Cruz genome browser (http://genome.ucsc.edu/) using the human February 2009 (GRCh37/hg19) assembly identified the two genes as Haptoglobin (*HP*) mapped to chr16:72,088,508-72,094,955 and Haptoglobin-related protein (*HPR*)

mapped to chr16:72,097,125-72,111,145. Zonulin shows structural similarities to chymotrypsin and serine proteases which provide functions of reversibility, secretability, and presence in digestion, blood clotting, and complement (Fasano, 2011). The list of diseases linked to chromosome 16 included celiac disease, type 1 diabetes, asthma, multiple sclerosis, glioma, inflammation, and autism (Fasano, 2011; Swanwick, Larsen, Banerjee-Basu, & Banerjee-Basu, 2011).

A review of the related literature as of July 2014 indicates a list of diseases that are suggested to show association with between intestinal permeability and the zonulin pathway. A search in Academic Search Complete using the terms "intestinal permeability AND zonulin" yielded 68 articles. A brief review of the titles and abstracts excluded articles discussing intestinal permeability without the mention of the zonulin or disease association with genotypes of haptoglobin. Nineteen articles discussed a probable association between disease and intestinal permeability via the zonulin pathway.

Table 2.1

Diseases Associated with Zonulin

Physiological Category	**Disease**	**References**
Gastrointestinal System	Celiac	Duerksen, Wilhelm-Boyles, Veitch, Kryszak, & Parry, 2010; Fasano et al., 2000; Tripathi et al., 2009; Wang et al., 2000
	Colitis	Duerksen et al., 2010
	Crohn's Disease	Fasano , 2008
Respiratory System	Acute Lung Injury	Matthay et al., 2003
	Acute Respiratory Distress Syndrome	Matthay et al., 2003
	Asthma	Fasano, 2011
Nephrological System	Chronic Kidney Disease	Kelly et al., 2009

Neurological System	Schizophrenia	Paterson et al., 2007
	Alzheimers	Liu, Wang, Zhang, Wei, & Li, 2012
	Multiple Sclerosis	Fasano, 2011
Endocrine System	Obesity	Moreno-Navarrete et al., 2012; Zak-Gołąb et al., 2013
	Diabetes Mellitus Type 2	Jayashree et al., 2014; Moreno-Navarrete et al., 2012
	Type 1 Diabetes	Sapone et al., 2006; Watts et al., 2005
	Systemic Lupus Erythematosus	Pavon et al., 2006
Infectious Diseases	HIV	Liu et al., 2012
	HCV	Fasano, 2011
	Sepsis	Klaus et al., 2013
Cancers	Breast Cancer	Russo et al., 2013
	Glioblastoma	Skardelly et al., 2009
	Lung squamous carcinoma	Fasano, 2011
	Pancreatic carcinoma	Fasano, 2011

Table 2.1 Diseases Associated with Zonulin.

Zonulin exists in two forms: an *un-cleaved* form known as pre-haptoglobin 2 (pre-HP2) that binds to proteinase activated receptor 2 (PAR2) and epidermal growth factor receptor (EGFR), and a form *cleaved* by trypsin at Arg^{161} that seeks to bind hemoglobin (Tripathi et al., 2009; Fasano, 2012). The initial studies indicated that the two different forms of zonulin yield two different functions due to the folding of the protein, with only the un-cleaved form affecting epithelial permeability (Fasano, 2012; Tripathi et al., 2009). Current research suggests that both forms of zonulin affect permeability (Rittirsch et al., 2013). Additional research is needed to determine if permeability resulting from increased zonulin levels is due to tissue or model specific responses. Zonulin is heavily concentrated in the endoplasmic reticulum (ER) and golgi (Drago et al., 2006). The un-cleaved form of zonulin can be detected in human serum at a range of 80-208µg/mL (Tripathi et al., 2009).

Recent studies of micro-ribonucleic acid (miRNA) have shed light on transcription of haptoglobin. The discovery of an acute-phase response regulatory pathway shows that micro-RNA-18a can enhance interleukin 6 (IL-6) release from monocytes and immune cells causing phosphorylation and dimerization of s*ignal transducer and activator of transcription 3* (*STAT3*) in the liver (Brock, Trenkmann, Gay, Gay, Speich, & Huber, 2011; Gauldie, Richards, Harnish, Lansdorp, & Baumann, 1987; Wegenka, Buschmann, Lutticken, Heinrich, & Horn, 1993). STAT3 homodimers then move to the nucleus and bind to promoter regions of deoxyribonucleic acid (DNA) to stimulate transcription of IL-6 target genes haptoglobin and fibrinogen (Brock et al., 2011). Intracellular feedback loops maintain the expression of *STAT3* for transcription (Brock et al., 2011). The involvement of *STAT3* in transcription of haptoglobin suggests that mutations in *STAT3* may lead to interruptions in the zonulin pathway and maybe associated with other diseases and conditions. Research is needed to understand specific factors related to translation of RNA to zonulin protein.

To better understand and develop associations with disease it is important to begin with examining what triggers activation of the zonulin pathway. Activation of zonulin occurs through exposure to gliadin, bacteria, and viruses (Drago et al., 2006; Fasano, 2012, Guttman & Finlay, 2009). Gliadin is one of two protein products of gluten. Gliadin binds to chemokine (C-X-C motif) receptor 3 (*CXCR3*) which recruits an adapter protein MyD88 increasing intestinal permeability and activating the release of zonulin (Lammers et al., 2008). Zonulin interacts with different surface receptors to activate phospholipase C, hydrolyzing phosphatidyl inositol into inositol 1,4,5-tris-phosphate (PPI-3) and

diacylglycerol (DAG) (Fasano, 2001; Mowat, 2003; Brandtzaeg et al., 1989). DAG and PPI-3 mediate protein kinase C through different mechanisms. DAG can directly bind to protein kinase C, whereas PPI-3 causes the release of intracellular calcium leading to protein kinase C mediation (Drago et al., 2006; Visser, Rozing, Sapone, Lammers, & Fasano, 2009). In both cases, protein C mediation leads to polymerization of intracellular actin filaments leading to the reorganization and restructure of the cytoskeleton and epithelial barriers (Drago et al., 2006; Visser et al., 2009). Actin filaments function mainly in cell growth and shape. When actin filaments reorganize they can form fiber-like cables to provide shape and purpose to nerve cells or they can form track-like structures to transport molecules or microbial materials (Ivanov et al., 2010; Singh et al., 2010).

The reorganization of actin filaments can be visualized using human Caco2 cells and rat IEC6 cells. Caco2 cells from human colorectal adenocarcinoma in monolayers provide a model for small intestine enterocytes and tight junctions. One study exposed these two cell groups to gliadin to view the redistribution of proteins that make up the TJ complex (Drago et al., 2006). It is the N-terminal sequence of pre-HP2 that shares a common motif (GGVLVQPG) with the active ZOT fragment, binding to specific surface receptors that activate signaling pathways (Fasano, 2008).

Another study found the N-terminal sequences of Zot/zonulin binding proteins showed dose dependent binding in human brain, intestine, and heart tissues suggesting disease association with increased zonulin levels in these systems (Lu, Wang, Uzzau, Vigorito, Zielke, & Fasano, 2000). The serine protease characteristics and growth

hormone-like structures of pre-HP2 induce protease-activated receptor 2 (PAR2) dependent transactivation of epidermal growth factor receptor (EGFR) (Lammers et al., 2008; Fasano, 2012). Six spatially conserved cysteine residues in the ß-chain of pre-HP2 form three intramolecular disulfide bonds which are involved in epidermal growth factor activity. Concentrations of pre-HP2 ≥15 μg/mL are enough to activate *EGFR* (Tripathi et al., 2009). Activation and mutations in *EGFR* have been associated with lung adenocarcinoma, non-small cell lung carcinoma, breast cancers, colon cancers, and response to radiation and drug therapies (Ellina et al., 2014; McCarty, 2014; Nechushtan et al., 2014; Pan et al., 2014; Suda et al., 2014). Therefore, the association between zonulin and EGFR suggests involvement with cancers and drug therapies (McCarty, 2014; Suda et al., 2014).

The other triggers of zonulin activation occur by the unique TJ modifications caused by bacteria and viral pathogens (Guttman & Finlay, 2009). Figure 2.1 is a representation published by Guttman and Finlay (2009) demonstrating how different bacteria and viruses alter the TJ.

Figure 2.1

Bacteria and Virus Alteration of Tight Junctions

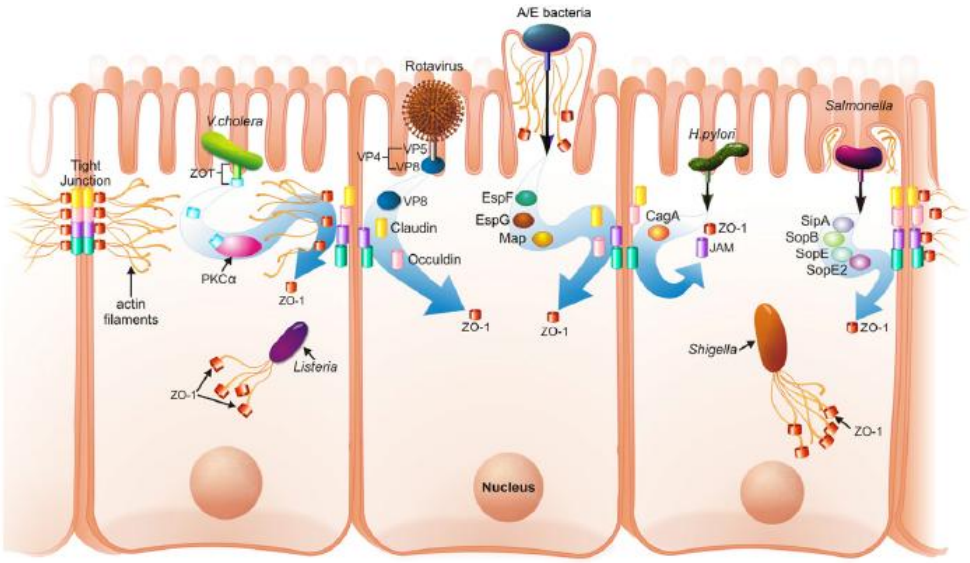

Figure 2.1 Bacteria and Virus Alteration of Tight Junctions. The figure shows how bacteria and viral pathogens alter the tight junctions (Guttman & Finlay, 2009).

The success of a bacteria or virus to penetrate the epithelial barrier or disassemble the TJ results in infection and inflammatory response (Guttman & Finlay, 2009). The attack on the epithelial barrier is two-sided. The lumen side is penetrated and altered through exposure to pathogenic organisms and toxins while the mucosal side is exposed to immune cell secretions of cytokines, proteases, and reactive oxygen species (Ivanov et al., 2010). Enteric microorganisms induce luminal secretion of zonulin in the small intestine and open the TJ (Fasano, 2008). Hydrostatic pressure gradients form, increasing water secretions into the lumen which flush bacteria from the intestine (Fasano, 2001; Fasano, 2008). These two mechanisms of zonulin activation provide a foundation to explore development of symptoms and diagnosis of disease.

Physiological System Involvement

Cell models, animal models, and human studies have identified physiologic mechanisms of zonulin and their association with disease. A review of the literature identifies four physiological systems and disease models that are disrupted due to increased epithelial permeability: Gastrointestinal (GI), Neurological, Oncology, Endocrine, and Respiratory Systems. Diseases associated with increased levels of zonulin have been suggested to be auto-immune; but, there is reason to consider that symptoms related to increased zonulin levels may be seen in other diseases not yet classified as having an autoimmune component (Fasano, 2011; Fasano, 2012). Further research is needed to elucidate these other diseases. However, symptoms and mechanisms related to increased zonulin have been described in GI, neurological, endocrine, and respiratory systems.

Gastrointestinal System

Zonulin was first generally identified as a regulator of intestinal permeability and has since been implicated in the pathogenesis of celiac disease and colitis (Wang et al., 2000; Fasano et al., 2000; Duerksen, Wilhelm-Boyles, Veitch, Kryszak, & Parry, 2010; Cipriani et al., 2011). In cases of Celiac disease, increased permeability in the small intestine allows gliadin to cross the lamina propria, activate an intramucosal immune cascade, and result in villous destruction and/or crypt heterotrophy (Duerksen et al., 2010). The intramucosal immune cascade is a first-line series of immune interactions for stimulation of immunoglobulin A (IgA) in response to foreign particles. Studies using real-time polymerase chain reaction (PCR) to quantitate zonulin expression levels found

increases with celiac disease activity (Tripathi et al., 2009). A study comparing permeability, antibody testing, and diet from zonulin levels in tissue found that zonulin is constitutively up-regulated in celiac disease patients, remaining elevated but not correlating well with histology (Duerksen et al., 2010). Comparisons between lab tests showed 100% sensitivity and specificity in antibody levels predicting abnormal of small intestine tissue compared to only 83% for permeability testing using lactose mannitol (LM) tests (Duerksen et al., 2010).

To date, all of the tests associating intestinal permeability with ASD have been measured using LM tests (De Magistris et al., 2010; D'Eufemia et al., 1996). The LM test involves oral administration of a solution of mannitol and lactulose with subsequent assessment of small intestine absorption measured by a six hour urine collection (Cooper, 1984). For children, LM testing has been used to determine allergies and intestinal permeability, but the methods of oral administration and collection are very difficult to obtain due to the use of diapers and inability of children to anticipate the need for urination and bowel movements. Enzyme linked immunosorbent assay (ELISA) testing of zonulin may provide novel methods for measuring intestinal permeability (Flanagan et al., 2014).

Comparison of tests showed significant relationships between permeability and serum zonulin levels in patients with destructive intestinal histology, but elevated levels of zonulin with normal intestinal histology limited the use of zonulin for clinical monitoring (Duerksen et al., 2010). Tissue specific differences in the ubiquitous expression levels of zonulin, long term exposure rates to increased serum zonulin, and

gene activation patterns associated with zonulin may explain why zonulin has not been useful in clinical monitoring. A study of zonulin levels in patients with gastroesphogeal reflux disease and normal healthy controls found ubiquitous expression of zonulin at 2.2-3.6ng/µg in the stomach and esophagus (Wex et al., 2009). The ubiquitous expression of zonulin is thought to be a result of its role in epithelial barrier function from pathogens. Further research is needed to identify gene interactions with serum zonulin and to determine if zonulin levels can be used for clinical monitoring.

Several studies have examined gliadin activation of zonulin and how serum zonulin levels can monitor celiac disease. A study by Fasano et al. (2000) showed that IgA anti-zonulin levels are increased in acute phase celiac disease at 21% ($p<0.0001$) but no increase is seen in IgG levels (Fasano et al., 2000). Baseline levels of zonulin remain increased for celiac patients compared to those in remission and healthy controls; dietary intervention always resulted in a return to baseline zonulin levels after 3-6 months of implementation (Fasano et al., 2000). The return to baseline levels indicate that increased permeability is reversible and dietary intervention may be a possible intervention upon presentation of disease.

Zonulin associated with colitis has also been studied in mice models. Bile acid receptor (GPBAR-1) knockout mice demonstrated increased expression of zonulin-1 mRNA, decreased expression of occludin messenger ribonucleic acid (mRNA) (p <0.05), and altered sub-cellular distribution along the apical border of colonocytes and vascular endothelial cells (Cipriani et al., 2011). Additionally, there was an increase in CD8+cells

and severity of colitis in the GP-BAR1 knockout mice associated with diarrhea and loss of mucous cells (Cipriani et al., 2011).

Neurological System

The blood brain barrier (BBB) is a complex system of membrane proteins that form TJ which facilitate control of the flow of molecules in and out of the central nervous system (Liu, Wang, Zhang, Wei, & Li, 2012). Physiological conditions associated with changes in the BBB include Alzheimer's disease, chronic inflammation, diabetes mellitus, *human immunodeficiency virus*, and abuse of drugs such as methamphetamine, cocaine, and morphine (Liu et al., 2012). Affinity column purification identified that zonulin binds to human brain tissue (Lu et al., 2000). This implies that zonulin may regulate the BBB allowing substances interrupt barrier function and alter the integrity of the actin filaments. In an *in vitro* BBB model using rat brain microvascular endothelial cells (RBMECs), zonulin increased the transmigration of human neural progenitor cell lines across the RBMECs and increased epidermal growth factor function (Diaz-Coranguez et al., 2013). This study was the first to show that pre-HP2 binds to a surface receptor that opens the BBB and activates a signal pathway that activates phospholipase C and protein kinase C, polymerizes actin, and contracts the perijunctional action-myosin ring allowing zonulin to alter the shape and function of nerve cells (Diaz-Coranguez et al., 2013; Ivanov et al., 2010). The role of zonulin in the BBB targets it in processes that direct migration of neural stem cells to sites of injury, which indicate its importance in medicinal interventions (Diaz-Coranguez et al., 2013).

Endocrine System

The endocrine system synthesizes and secretes chemicals into the body to regulate growth, metabolism, and sexual development (Coelho, Oliveira, & Fernandes, 2013). Within this system, increased zonulin levels have been associated with obesity and type 2 diabetes mellitus (Fasano et al., 2000; Jayashree et al., 2014; Moreno-Navarrete, Sabater, Ortega, Ricart, & Fernandez-Real, 2012; Zak-Gołąb et al., 2013). Obese individuals have a significantly higher concentration of zonulin than non-obese individuals (12.5±4.6 to 9.3±5.1, p=0.007) (Moreno-Navarrete et al., 2012). Associations between increased levels of zonulin and obesity were specifically related with increases in body mass index, waist to hip ratio, fasting insulin, fasting triglycerides, uric acid, IL-6, low levels of h*igh-density lipoprotein*-cholesterol, and decreased insulin sensitivity (Moreno-Navarrete et al., 2012). Another study by Zak-Gołąb et al. (2013) evaluated 50 obese individuals compared to 30 normal weight individuals. These researchers found that obese individuals had a mean zonulin level of 8.2ng/mL ($p < 0.001$) compared to a mean zonulin level of 5.4ng/mL in normal weight controls (Zak-Gołąb et al., 2013). The study also found positive correlations between plasma zonulin levels and age ($r = 0.43$, $p < 0.001$), body mass ($r = 0.30$, $p < 0.01$), body mass index ($r = 0.33$, $p < 0.01$), fat mass ($r = 0.31$, $p < 0.01$) and fat percentage ($r = 0.23$, $p < 0.05$) (Zak-Gołąb et al., 2013).

A study by Jayashree et al. (2014) confirmed elevated circulating serum zonulin levels (796 ± 55pg/mL, $p < 0.001$) in individuals with type 2 diabetes mellitus compared to healthy controls (3.96 ± 0.16pg/mL). These studies suggest that zonulin may play a role in metabolic disturbances. Further research is needed to determine if it is due to

increased intestinal permeability, loss of intestinal barrier function, or disruption in metabolic pathways.

Relationships between zonulin levels and diabetes have been explored using both animal and human studies. Animal models using rats determined relationships between zonulin, intestinal permeability, and glucose levels. Watts et al. (2005) found serum zonulin levels were correlated with intra-luminal zonulin, increasing prior to increases in glucose and development of type 1 diabetes. In the rat model studies, type 1 diabetes developed 25 days after zonulin increased intestinal permeability (Watts et al., 2005). In patients with type 1 diabetes, increased zonulin levels (0.83±0.05ng/mg) were higher than their "normal" relatives (0.62±0.07ng/mg) and healthy controls (0.21±0.02ng/mg) (Sapone et al., 2006). No correlations were found between serum zonulin levels and age, sex, diagnosis of type 1 diabetes, duration of type 1 diabetes, daily insulin dose, hemoglobin A_{1c}, or serum glucose levels (Sapone et al., 2006). This study provided evidence that increased intestinal permeability of type 1 diabetes was correlated to increased serum zonulin levels (Sapone et al., 2006).

Respiratory System

The development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) result from pneumonia, aspiration, trauma, sepsis, and associated disorders such as pancreatitis and drug overdose (Matthay et al., 2003). Lung permeability is typically measured by albumin leak to indicate severity as failure of treatment can lead to alveolar edema or hemorrhage (Rittirsch et al., 2013). A recent study exploring the unidentified molecular mechanisms of ALI found that administration

of zonulin antagonist AT-1001 reduced albumin leak by 67%; reduced the number of leukocytes (mostly neutrophils) by 25%; and reduced proinflammatory cytokines IL-6 and tumor necrosis factor α (TNF-α) (Rittirsch et al., 2013). Exposure to both forms of zonulin showed increased lung permeability in mouse models. Rittirsch et al. (2013) found that 20μg of pre-HP2 indicated a 3.2-fold increase in lung permeability and HP2 showed a 2.6-fold increase in lung permeability. The addition of zonulin to human serum activated complement demonstrating its role in barrier function (Rittirsch et al., 2013).

Links Between Zonulin and ASD

Zonulin associations with disease are actively being explored to better understand the molecular pathways and mechanisms of proteins. Hallmarks of cancer and pathopysiological changes that are only characteristic of cancer show involvement of zonulin in different cancers. This work has evolved from effecting the onset of diarrhea during chemotherapy to zonulin involvement in therapy and radiation treatments (Russo et al., 2013). A study of breast cancer patients showed that GI symptoms (29% with diarrhea) increased on day 14 of chemotherapy compared to no GI symptoms at baseline (29.86±1.27 and 24.7±0.94, p=0.02) (Russo et al., 2013). The GI symptoms were accompanied by a day 14 increase in intestinal permeability (0.067%, p=0.001) (Russo et al., 2013). Over the 21 day chemotherapy treatment, total serum zonulin levels did not change; however, circulating levels of glucagon-like peptide 2 (GLP-2) and EGF showed significant decreases (p=0.0044 and p=0.004 respectively) until reaching a plateau at which point no further decrease was observed (Russo et al., 2013). The stability of

zonulin levels during treatment indicates they are independent of chemotherapy (Russo et al., 2013).

The study of zonulin levels in glioblastomas showed that zonulin expression controls intercellular communication in two ways: increased v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*c-kit*) expression and activation responsible for tumor aggression and effectiveness of tyrosine kinase inhibitor therapies (Skardelly, Armbruster, Meixensberger, & Hilbig, 2009). Results from human glioma studies provided evidence that zonulin expression correlated with tumor aggression more specifically than expression of *c-kit*, suggesting that zonulin levels may indicate the degree of disruption in the blood brain barrier and blood vessel walls (Skardelly et al., 2009). Further research is needed to determine associations between zonulin and genomic mutations that lead to cancer and suggestive therapy options.

A particular area of interest is the role of zonulin in Autism Spectrum Disorders. Autism Spectrum Disorder (ASD) describes an array of developmental brain disorders that are associated with multiple levels of symptoms, cognition, language, and behavior presentations. The Centers for Disease Control and Prevention (2014) indicates that 1 in 68 children in the United States have ASD. Symptoms of ASD include social impairment, communication difficulties, and repetitive and stereotyped behaviors (American Psychiatric Association, 2013). However, additional symptoms have been reported of food allergies, acid reflux, vomiting, bloating, stomach pain, diarrhea, and constipation (U.S. Department of Health and Human Services, National Institutes of Health, 2011). No studies in the literature report serum zonulin measurements of

individuals diagnosed with ASD, but many connections can be made based on overlapping mechanisms.

The GI and neurological systems demonstrate two examples that point toward the involvement of the zonulin pathway. Autism spectrum disorders have long been associated with GI issues. According to the Autism Research Institute (2013), 70 % of children with ASD have GI symptoms; however, these are sometimes difficult to recognize due to communication difficulties between the child, parents and healthcare providers. The spectrum of phenotypic expression and gene interactions related to ASD and GI issues make GI or ASD disorders challenging to diagnose. In a large registry-based study of 589 individuals, Wang, Tancredi, & Thomas (2011) reported 42% of children with ASD had GI problems including constipation and diarrhea compared with their unaffected siblings (12%). Interestingly, ASD and GI disorders, such as celiac disease, have increased at approximately the same rates over the past 10 years to a prevalence of 1 in 88 children (Centers for Disease Control and Prevention, 2012; Rubio-Tapia, Ludvigsson, Brantner, Murray, & Everhart, 2012). Table 2.2 identifies symptoms related to GI disorders and diagnostic evaluations in patients with ASD (Buie et al., 2010).

Table 2.2

Diagnostic Evaluation of GI Symptoms and Disorders in Individuals with ASDs

Symptoms	**Associated Gastrointestinal Disorder**
Sleep disturbance	GERD
Self-injurious behavior, tantrums, aggression, oppositional behavior	Constipation, GERD, gastritis, intestinal inflammation
Chronic diarrhea	Malabsorption, maldigestion
Straining to pass stool, hard or infrequent stool	Constipation

Abdominal discomfort	Constipation, GERD, intestinal inflammation, malabsorption, maldigestion
Flatulence or bloating	Constipation, food allergies, enteric infection
Any or all of the above	*Familial adenomatous polyposis, irritable bowel syndrome*

Table 2.2 Diagnostic Evaluation of GI Symptoms and Disorders in Individuals with ASDs adapted from Buie et al., 2010.

One of the symptoms, the amount of undigested food, raises concern for passage through the TJ and the physiological effects it may have on the body. Undigested food in the form of large immunogenic peptides or intact proteins may reach the lumen of the small intestine due to evasion of gastric fluid and proteolytic enzyme hydrolysis (Menard, Cerf-Bensussan, & Heyman, 2010). Undigested food particles may pass through the TJ stimulating an immune response. Similarly, drug molecules may pass through the tight junctions of the intestine leading to immune reactions, disease, or toxicity (Ju & Uetrecht, 2002). Drugs are classified based on solubility and permeability according to the Biopharmaceutics Classification System; permeability determination is made from pharmacokinetic studies, *in vivo* intestinal perfusion, *in vitro* permeability using tissue biopsies, and Caco2 monolayer studies (Chavda, Patel, & Anand, 2010).

The basis for determining zonulin expression levels in individuals with ASD is found by precedent of intestinal permeability in individuals diagnosed with ASD and correlations between severity of autism spectrum disorders and severe gastrointestinal diseases. D'Eufemia et al. (1996) identified that 43% of autistic patients displayed altered intestinal permeability using lactulose/mannitol (LM) tests. D'Eufemia et al. (1996) speculated that intestinal permeability increased the passage of food peptides through the gut leading to subsequent behavioral abnormalities.

De Magistris et al. (2010) performed tests on 90 children with Autism Spectrum Disorder and 146 of their first-degree relatives. Results revealed 36.7% of ASD patients and 21.2% of their first-degree relatives have abnormal intestinal permeability (De Magistris et al., 2010). Once again, intestinal permeability was determined by LM tests. For ASD patients and their first-degree relatives there was a 2-to 3-fold increase in lactulose compared with mannitol recovery (De Magistris et al., 2010). Interestingly, most of the ASD patients and relatives were negative for antibodies or antigens to food allergies (De Magistris et al., 2010). The toxic reactions without the presence of antibodies or antigens may indicate that zonulin association with gastrointestinal disease is a result of constitutive action of the toxic effects of the protein at increased levels.

Furthermore, the correlation between zonulin levels and severity of autism is supported by the results from Adams, Johansen, Powell, Quig, & Rubin (2011). These researchers found that children with more severe forms of autism are likely to have more severe gastrointestinal symptoms. Gastrointestinal symptoms were measured based on a GI severity index using a combined score of constipation, diarrhea, stool consistency, stool smell, flatulence, and abdominal pain. Children with higher Autism Treatment Evaluation Checklist scores had gastrointestinal severity index scores greater than 3 ($p=0.00002$) (Adams et al., 2011).

Neurological mechanisms are of major importance in linking ASD and zonulin, and supporting correlation between increased zonulin levels and severity of neurological symptoms. The study by Skardelly et al. (2009) implicated zonulin expression levels in disruption in the BBB and blood vessel walls suggested that zonulin may be correlated

with neurological levels of severity in individuals with ASD. Studies of zonulin may help determine if disruption of the BBB leads to disruption of brain development and signaling occurs *in utero* from maternal immune activation or during infancy. A connection can already be made through *signal transducer and activator of transcription 3* (*STAT3*) activation by IL-6. Using animal models, Parker-Athill et al. (2009) found that IL-6 mRNA levels remain elevated up to 24 weeks after birth suggesting *in utero* inflammatory pathogenic effects. Such a connection could lead to development of diagnostic testing for ASD and early diagnosis.

Conclusion

Identification of zonulin as a modulator of intestinal permeability has expanded the understanding of tight junctions. Research has implicated increases in zonulin to physiological effects in the Gastrointestinal, Neurological, Respiratory, Endocrine systems, and oncological diseases. The ability of zonulin to allow macromolecules and antigens to pass through the TJs in the intestine and the BBB challenge classical theories of inflammatory pathogenesis based solely on genetics and environmental triggers (Fasano, 2011). The regulation and reversal of intestinal permeability by zonulin proposes a new theory that pathogenesis, in general, is not self-perpetuating but can be interrupted (Fasano, 2011). Based a review of the literature published thus far, a model is proposed that uses increased serum zonulin levels and comparisons of the physiological effects of zonulin and phenotypic assessment data to implicate severity of disease states. Figure 2.2 shows the proposed zonulin pathway to disease.

Figure 2.2

New Zonulin Model of Pathogenesis

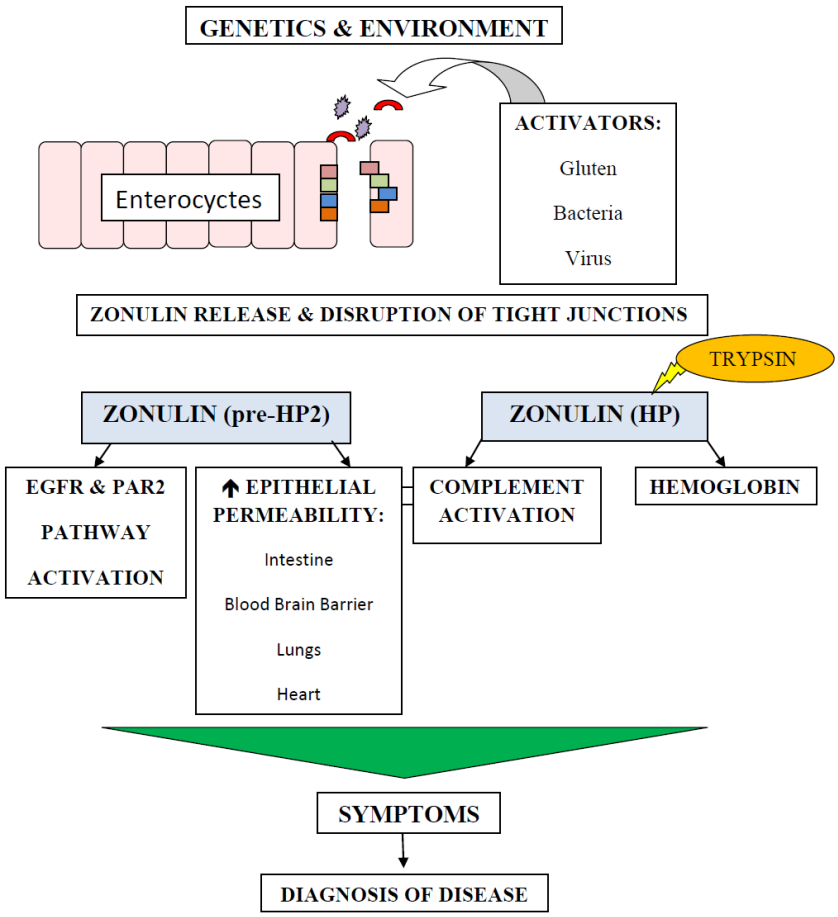

Figure 2.2 illustrates the zonulin pathway that produces symptoms and diagnosis of disease.

Additional research is needed to understand the zonulin pathway and its associated diseases. It will be important to identify ranges of zonulin, highlight specific symptoms or a phenotypic threshold for disease, identify gene interactions and environmental triggers, and indicate zonulin regulation of drug metabolism. The need for understanding zonulin's role in protein molecular pathways and genomic interactions is important.

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CHAPTER III

EXPLORING AUTISM AND GI SYMPTOMS USING RADIAN6

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Abstract

Autism spectrum disorder (ASD) is a developmental disorder that is accompanied by an array of symptoms affecting behavior, communication, cognition, and multiple organ systems. Early diagnosis of ASD is being sought to reduce the severity of symptoms, increase daily function, and maintain quality of life. Social media such as Twitter, Facebook, LinkedIn, chat rooms, and blogs present resources to the public that allow them to capture experiences and symptoms of autism or other diseases in real time. This study explores and captures self-reported material posted in social media using Radian6 to identify patterns and themes related to autism and GI symptoms for the purpose of translating that knowledge into healthcare applications at the individual and corporate levels.

Keywords: social media, ASD, autism, autism spectrum disorders, Radian6

Exploring Autism and GI Symptoms using Radian6

Autism spectrum disorder (ASD) is a developmental disorder that is accompanied by a myriad of symptoms affecting behavior, communication, cognition, and multiple organ systems (American Psychiatric Association, 2013). Many genes have been implicated with risk associated with disorder development, but the etiology of autism remains unknown (Gilling et al., 2013; Handrigan et al., 2013; Liu et al., 2013; Sarachana & Hu, 2013). Focus has recently moved toward early detection of ASD, which is pertinent to minimize symptoms and maintain quality of life. With no diagnostic test available, early detection relies autism rating scales and evaluation checklists that rely on behaviors and observations made by parents, caregivers, or healthcare professionals (Geier, Kern, & Geier, 2013; Nah, Young, & Brewer, 2014).

The development of social media provides an open platform to witness a variety of self-reported experiences related to autism. A recent study by Mazurek (2013) identifies 79.6% of 108 ASD individuals used social media sites to connect with others. In another study, Mazurek and Wenstrup (2013) found children with ASD spent little time using social media and more time playing video games than their siblings who followed typical growth and development guidelines. Additionally, a qualitative study was performed using chat logs of twelve young people diagnosed with attention-deficit/hyperactivity disorder and ASD (Ahlstrom & Wentz, 2014). The chat logs identified two themes of ‘fighting against an everyday life lived in vulnerability” and “struggling to find a life of one’s own” (Ahlstrom & Wentz, 2014). These studies using internet and social media suggest that these devices or applications may provide methods

for diagnosing, monitoring, or treating ASD (Ahlstrom and Wentz, 2014; Mazurek, 2013; Mazurek & Wenstrup, 2013). Topics focusing on communication, management, and health related concerns dominate social media and findings indicate that after initial participation, engagement of social media decreases over time with autistic individuals (Hong, Yarosh, Kim, Abowd, & Arriaga, 2013).

Studies surrounding autism and gastrointestinal (GI) symptoms have focused primarily on self-report from questionnaires and surveys (Kang, Wagner, & Ming, 2014). Internet based approaches to ASD intervention consistently indicate increases in quality of life (Garcia-Villamisar and Dattilo, 2010; Wentz et al., 2012). Evidence suggests that facilitating interaction with media, exercise, games, crafts, and events decreases stress levels and increases quality of life for individuals with ASD (Garcia-Villamisar and Dattilo, 2010; Wentz et al., 2012). A review of the related literature was unable to identify publications directly mention using social media as a translational tool to study ASD for the purpose of targeting pathways of disease mechanisms or for developing new interventions and approaches. The purpose of this study was to explore and capture self-reported material posted in social media in order to identify patterns and themes related to autism and GI symptoms for the purpose of knowledge translation to healthcare application.

Methodology

A non-experimental exploratory study was conducted using Radian6 to mine social media data. Radian6 software allows for data mining across multiple social media outlets such as Facebook, Twitter, blogs, and Media. Data was captured at the end of

each month from September 1, 2013 to November 30, 2013. Parameters in Radian6 were set to collect only data in the English language in the United States (U.S.). All media types were included. The study data was present in the public domain, free of subscription or login requirements. Following the rules of repeated entry elimination by elastic matching identification, data was excluded if it was a repeated entry having ≥90% identity with a previous post (Zhao et al., 2013). In addition, information was excluded if: (1) the source required membership dues, site registration, or login; (2) the hit was an advertisement, sub-headline only, or random text with no contextual information; or (3) the entry contained ≥ 90% sexually related or derogatory comments or content.

Filtering Criteria

Search terms entered in Radian6 were based on a literature review of gastrointestinal symptoms commonly seen in ASD individuals. Search terms of GI symptoms were combined with "AND CONTATINS AUTISM" and were separated with "OR." For example, the search terms read ("food allergies" AND CONTAINS "autism") OR ("nausea" AND CONTAINS "autism"). The search strand continued until all GI symptoms had been added. See Table 3.1 for a list of GI symptoms seen in patients with autism, which were used as keywords.

Table 3.1

GI Symptom Keywords for Radian6 Search Criteria

1. Food Allergies
2. Nausea
3. Reflux
4. Digestion
5. Infection (Bacteria, parasite, OR yeast)
6. Bloating OR Flatulence

7. Abdominal pain
8. Bowel obstruction
9. Constipation
10. Diarrhea
11. Stool
12. Cancer

The keywords were based on common symptoms related to the GI system and ASD. It was difficult to define a boundary of vocabulary used by the public for autism or GI problems due to the spectrum of symptoms. The keywords provide a boundary within social media to reduce the data collection from over a million hits using only autism as a search term, to a collection of 6878 data entries.

Data Analysis

Using the specified filtering criteria, Radian6 yielded 6878 entries. The first stage of data analysis indicated that the majority of social media information was being dispersed through Facebook (49%) and Blogs (21%).

Figure 3.1

Dispersion of Information in Social Media

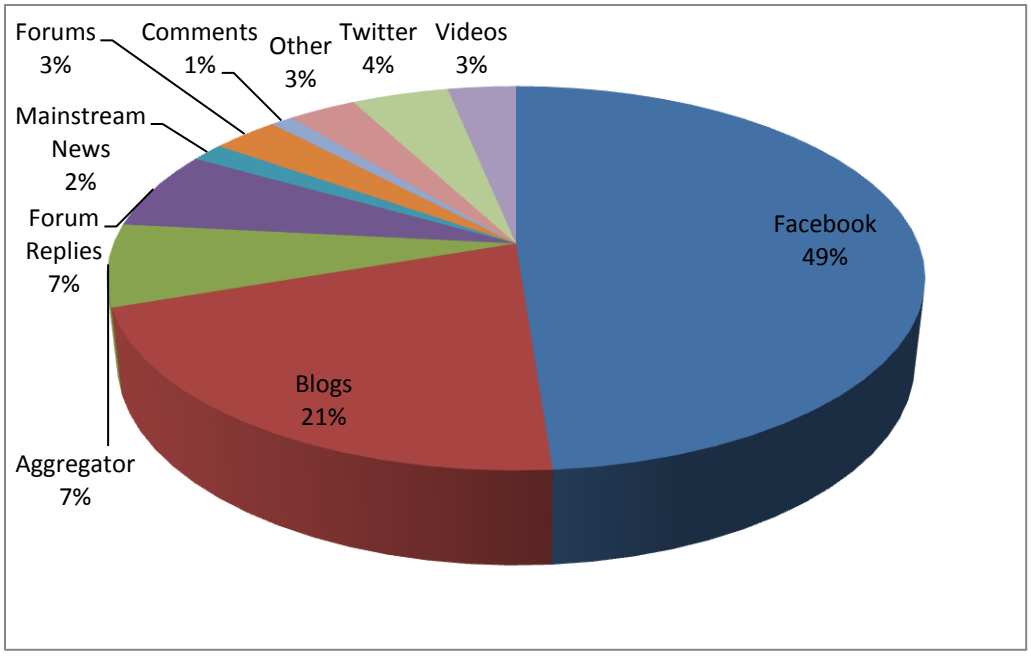

Figure 3.1 Dispersion of Information in Social Media. [The pie graph above shows the percentage of hits per media outlet across a total of 6878 entries from Radian6 for months September, October, and November. The coded data was not used for the dispersion diagram in order to represent which type of social media would most likely be seen by the public when searching for information. The information shows that Facebook tends to be the most common form of social media discussing autism and GI symptoms. Radian6 also produced comment counts for each month of data collection. Across all three months of data, interventions were most frequently commented on, with topic of gluten being in the top 2 for all three months. Baby development was also included within the top 5 topics of comment across all three months.]

In order to prepare the data for analysis, it was exported from Radian6 into Excel in the form of a .csv file at which point each entry underwent manual examination. Each entry was accessed in full via its corresponding web address. A research team member read the entry and coded it based on contents. Entries were tagged for deletion if they did not meet the inclusion criteria as discussed above. The "FIND" function in Excel assisted

in the identification of repeated entries, which could be identified by web address, contents, or author. Entries failing to meet the criteria were discarded before upload into QSR International's NVivo 10 software for further analysis. Of 6878 entries identified in social media, only 1930 hits contained contextual information related to autism and GI symptoms. The remaining 1930 social media hits after initial cleaning in Excel were dispersed among several media providers. The top four media providers included Facebook (49.9%), wordpress (13.7%), generic blogs (11.2%), and forums (10.5%). Twitter posts contained the most repeated content which was tagged for removal from the data set.

Manual examination of the 1930 hits in Excel yielded 20 coded categories. These categories included links to "autism", "pathology", "no links to autism", "symptoms", "interventions", "consequences", "feelings", "siblings", "age at diagnosis", "age at onset", "coping mechanisms", "testing", "intestinal permeability", "co-morbid diseases", "retraction of autism diagnosis", "lacks deficits needs", "questioning", "family history", "ASD improvements", and "indications of pharmacogenomic testing". Each entry was coded for one or more category based on the contextual information. The "no links to autism" category was included to capture all stake holder points of view, and to address the final quantity of these hits was enough to statistically support the null hypothesis. After coding three months of information, it was determined that some categories could be collapsed to better represent the data. The "co-morbid diseases" category was combined with the "symptoms" category, the "retraction of autism diagnosis" category was combined with "ASD improvements," and cases within the "indications of

pharmacogenetic testing" category were re-coded to either interventions or symptoms. The final category of symptoms was intended to look at all symptoms associated with ASD and to answer the research question, "what symptoms are expressed in recorded social media data related to ASDs?" Additionally, the categories were used to identify themes related to ASD from social media. Categories of "lack deficit needs", "ASD improvements", and "interventions" were intentionally directed at answering the research question, "what are the facilitators and barriers of social media use in relation to ASD?" The re-categorized data was uploaded into QSR NVivo for further content analysis.

In NVivo, tree nodes were created to catalogue categories into hierarchical structures, and other categories were collapsed into more generalized categories. In total, 18 categories emerged from the 1930 social media hits. The 18 categories had a combined 24,127 coded references. The term "coded references" refers to words or phrases from each hit that are coded to a particular node or multiple nodes. The term "node" is used synonymously with category. The final categories included "siblings", "feelings", "proposed links", "family history", "pathology", "coping mechanisms", "consequences", "media provider", "no link to autism", "lack deficit needs", "age at onset", "intervention", "questions", "improvements", "symptoms", "age at diagnosis", "abilities", and "quotes". The following includes the major findings for each category.

Results

Siblings

The category of "siblings" contained 64 out of 1930 hits across the three months with 1943 coded references. Siblings were defined as a brother or sister relationship,

adopted or blood relative related to an individual with autism. The references coded into the "sibling" category referred to a number of children in the family identifying affected or not, gender, or symptoms. The most frequently mentioned terms were words that indicated autism (autism, ASD, or affected) was present among children (children, kids, sibling, or siblings) for weighted percentages of 15.86% (55 counts) and 10.37% (36 counts) respectively. Most families had one or two children with both terms represented at 3.17% (11 counts) each and of these, boys were most commonly represented (2.59% or 9 counts). These findings lend support to the prevalence that boys are five times more likely to be affected by autism than girls (Centers for Disease Control and Prevention, 2014). The word cloud generated by QSR NVivo for "siblings", as shown in Figure 3.2, illustrated words most commonly coded under the sibling node.

The size of each word in the word cloud is proportional to the number of times that word appears in the node. The word count also increases the weighted percentage a word has within a node. The word cloud provides a visual interpretation of the numerical values without having to insert lengthy tables, and for the purposes of qualitative research the word cloud offers a more contextual interpretation of the data.

Figure 3.2

Siblings Word Cloud

Figure 3.2 Siblings Word Cloud. [The word cloud illustrates autism, affected, children, siblings, two, one, and kids were the most frequently counted words.]

Feelings

Feelings represented the emotions, thoughts, and physical response related to autism reported through social media hits. This category contained 178 out of 1930 hits with 1936 coded references. The majority of hits was self- reports from caregivers or parents of individuals with autism; indicated by word counts that included child

(weighted percentage of 1.28% or 40 counts) or children (weighted percentage of 0.45% or 14 counts). Figure 3.3 is a word cloud produced from all the hits within the "feelings" category.

Figure 3.3

Feelings Word Cloud

Figure 3.3 Feelings Word Cloud. [The word cloud shows the words autism, child, know, like, people, parents, and time are most frequently counted.]

Many of the caregivers or parents expressed concern, frustrations, or reactions to the diagnosis and management of autism. One of the most important terms in the "feelings" category was to know which terms took on multiple meanings. The term "know" was mentioned 28 times for a weighted percentage of 0.90%, but it yielded many different meanings as shown in the paraphrased excerpts below:

Caregivers or parents know what is best for his/her child

Tell us [caregivers and parents] something we do not know already

> I [individual with autism] can choose the best for myself because I want to be well.
>
> Call me an autistic person, but do not define me by my disease as a person with autism. I have a name so introduce me by my name so that people can know who I am.
>
> Nothing could be worse than watching my child in pain. My child bites, pinches, and hits his/her body to the point of bleeding, bruising, and swelling.

The term "know" took on many different forms. First, the act of "knowing" from the perspective of a parent or caregiver, described understanding their child's response to treatments or interventions. In many cases, the parent or caregiver identified the symptoms of autism first and then sought out an explanation. This suggests a need for an instrument to record home experiences and log response to interventions. Some parents make daily logs that record their child's response to foods, medications, and therapies. These become valuable to help the clinicians manage the treatment of autism. This resource also points toward the importance of a strong relationship and communication between the caregiver, parent and the clinicians. In almost all cases the parent or caregiver fulfills the role of patient advocate.

Secondly, the term "know" referred to understanding or wanting to know more about autism as a disease. Even though there is a plethora of information available on the internet regarding autism, parents or caregivers often question the reliability of the information. As pointed out in the "lack deficit needs" category, information from the internet can be contradictory in nature and not always scientifically based. It may be difficult for parents or caregivers to: (1) locate reliable and scientifically based information; (2) understand ASD because of the extensive use of scientific or clinical

jargon; and (3) comprehend the full meaning of the information due to the emotional and physical stress they are experiencing. Additionally, social media suggests that most parents want to know and understand their child(ren); which can be difficult due to problems with communication and isolation. Parents want to know what their child likes and does not like, and they want to know and understand their pain. From the data entries, several examples of communication devices or methods emerged as helpful tools in relaying messages between the individual with autism and other individuals. For example, sign language or picture boards were found to be useful in stimulating communication at home.

Another perspective mentioned in social media was that of the affected individual. The desire of the affected individuals to feel normal was present in social media entries. On a personal level, individuals with autism want to be treated equally with non-affected individuals on a personal level. They appreciated others treating them as normal not as different and including them in daily activities with other children their age or introducing them to new people without identifying their disease. This reinforces the stigma of having a mental disorder (Link & Phelan, 2013; Pescosolido, Medina, Martin, & Long, 2013; Szeto, Luong, & Dobson, 2013; Thoits, 2013). Individuals with autism desire respect as an individual and they want to be included in a discussion about the future interventions. According to this data, individuals with ASD want and should have a voice in the outcome of their future.

People with autism see the same world in a different way

Some of the strongest feelings represented in the contextual data are displayed in table 3.2.

Table 3.2

Types of Feelings and Weighted Percentages

Type of Feeling	Weighted Percentage (%)
need or needs	0.44
fear	0.42
believe	0.29
hard	0.29
want	0.29
bad	0.26
denial	0.26
good	0.26
guilt	0.26
pain	0.26
think	0.26
try	0.26
care	0.22
cause	0.22
help	0.22
love	0.22
talk	0.22
change	0.19
difficult	0.19
find	0.19
give	0.19
health	0.19
joy	0.19
stress	0.19
wrong	0.19

Table 3.2 Types of Feelings and Weighted Percentages.

Many of the feelings either surround the initial reaction of diagnosis or the feeling is a reaction to demands and comments of society. The following is an example of a combined reaction that clearly expresses the feelings of the parent.

> The doctor gave the diagnosis and I [the caregiver] became overwhelmed with fear, guilt, and extreme sadness. I was nauseated from the news… feeling like I had let my child down. I felt like it was fault and that my child's future would be hindered by this diagnosis. It was the worst feeling in the world. Comforting and reassuring words were not accepted. My gut feelings told me to seek a second opinion and reject this diagnosis. I refused to accept that autism is a permanent disorder and that nothing can be done. I tried to think of ways to keep my child from getting sick, ways to improve the current state, and ways to help others who are in the same situation. I immediately began to research autism. I read everything I could find related to healing Autism.
>
> My spouse was working multiple jobs to provide for our family. I stayed home with my children and worked from home. One of the hardest, darkest times in my life was learning of our child's diagnosis of autism. No one seemed to understand… We heard all kinds of comments from people. Most of the things we heard were negative about our parenting or about our children. The comments made it seem like we had done something wrong- that this was our fault. I searched in desperation for an answer. We mourned the ideas and aspirations that our child would never achieve.

Proposed Links

Proposed links was defined as elements suspected to have a relationship with autism, but remain under scientific investigation. The items listed under this category produced much debate among respondents. Proposed links contained 1344 out of 1930 hits with 1934 coded references. Below is the word cloud produced for the "proposed links" category.

Figure 3.4

Proposed Links Word Cloud

Figure 3.4 Proposed Links Word Cloud. [The word cloud shows that symptoms, food, vaccines, gut, brain, and children were the most frequently counted words associated with proposed links with autism.]

Interestingly, the raw data counts listed autism (536 counts or 7.79%), gut (82 counts or 1.19%), vaccines (77 counts or 1.12%), and children (72 counts or 1.05%) as the top four words counted under the category of proposed links. Further evaluation of the word counts, revealed that 174 of 1344 counts contained terms synonymous with gastrointestinal function or location followed by terms for vaccines (143 counts) and children (98 counts). The coded hits suggest there is a relationship between the GI system and ASD. In this study, the majority of coded entries under gastrointestinal terms refer to symptoms related to autism or the GI system present during early years before diagnosis of autism. It is unclear from the data whether autism or GI symptoms occur first, or if a causal relationship exists.

Unlike the references coded for gastrointestinal, the majority of hits under vaccines stemmed from debate surrounding the well known and re-tracted article published in the Lancet by Wakefield *et al* (1998) and warning labels produced by vaccine manufacturers. For the purpose of this study, vaccines are acknowledged as a potential mechanism by which an individual can be exposed to bacteria or viruses which trigger a cascade of events that lead to diverse symptoms. However, these pathways require more scientific research to implicate them with the onset of autism (Tomljenovic, Blaylock, & Shaw, 2014).

Family History of Individuals with ASD

Family history accounted for 62 of 1930 hits with 1932 coded references. Family history was defined as self described symptoms, exposures, diseases, or events that occurred in the life of a person who indicates a relationship with an autistic proband. Mothers were the most frequently reported (18 counts or 1.82%) in relationship with the proband and among mothers pregnancy (15 counts or 1.51%) was most frequently discussed as having a possible connection to the affected individual. Autoimmune disease (7 counts or 0.71%, diabetes 3 counts or 0.30%), depression (6 counts or 0.61%), pain (6 counts or 0.61%), gastrointestinal (bowel 5 counts or 0.50%, digestive 3 counts or 0.30%, celiac 3 counts or 0.30%, constipation 3 counts or 0.30%), allergies (4 counts or 0.40%), arthritis (4 counts or 0.40%), fibromyalgia (4 counts or 0.40%), and cancer (4 counts or 0.40%) were most frequently reported diseases and symptoms reported in family history. Most of the discussion on family history was by families attempting to find an explanation as to why their child developed ASD.

> Our family does not fit any genetic models of inheritance, since no one on either side of our families is autistic.

The use of pitocin (6 counts or 0.61%) was the most frequently discussed medication listed in family history believed to result in a child with autism. One of the particular problems related to the use of pitocin was the cited "unregulated cap on the highest level of pitocin allowed." A recent study by Gregory *et al* (2013) has found that the risk of having a child with autism increased with induction and augmented childbirth. Therefore, a complete pregnancy and birth history are critical.

Figure 3.5

Family History Word Cloud

Figure 3.5 Family History Word Cloud. [Mother and family have the highest word counts associated with autism and family history.]

Pathology

Pathology identified 43 of 1930 hits (1931 coded references) with the most common pathology of autism being related to the gut (gut 12 counts or 1.78%, intestinal 5 counts or 0.74%, digestion 4 counts or 0.59%, gastrointestinal 2 counts or 0.30%) or bacteria (8 counts or 1.19%) compared to genetic inheritance (DNA 4 counts or 0.59%, gene 2 counts or 0.30%, genes 3 counts or 0.45%, genetic 3 counts or 0.45%). The results suggest that social media participants believe genetics play a large role in the pathology of autism. The relationship between gastrointestinal genes and their relationship to autism appears to be strong based on pathology key words. Some examples of GI pathways mentioned in social media include the following:

> Failure of digestion that initiates a cascade leading to cognitive decline
>
> Mucosal surface damage of the gastrointestinal tract leading to immune suppression
>
> Genetic predispositions and environmental exposures leading to unhealthy gut flora, inflammation, and leaky gut

Figure 3.6

Pathology Word Cloud

Figure 3.6 Pathology Word Cloud. [Bacteria and gut are the most counted words associated with autism pathology.]

Coping Mechanisms

Coping mechanisms include 53 of 1930 hits with 1931 coded references. Coping mechanisms are defined as means by which families or individuals deal with problems and situations related to autism. The main way families and individuals cope with autism is through support systems. People (7 counts or 1.01%), kids or children (9 counts or 1.30%), and parents (5 counts or 0.72%) are the top three terms representing support systems. A support system provides sharing (4 counts or 0.58%) and time (4 counts or 0.58%) to help the families and individuals deal with their stresses related to autism. People also find resolve in things (5 counts or 0.72%), which most frequently includes some form of writing activity (writing 3 counts or 0.43%, Facebook 3 counts or 0.43%, blogging 2 counts or 0.29%). Paraphrased notes from social media hits indicate the

writing activity allows the people to share their lives, dispel myths about autism, and share what they have learned.

Figure 3.7

Coping Mechanisms Word Cloud

Figure 3.7 Coping Mechanisms Word Cloud. [The highest ward counts for coping mechanisms are people, parents, kids, and things.]

Consequences

Consequences were defined as the result of or an effect of any action related to autism. There were 74 consequences out of 1930 hits with 1931 coded references. Most consequences effected children (children 9 counts or 1.27%, child 8 counts or 1.13%) or families as a whole (8 counts or 1.13%). Consequences resulted from the topic of vaccinations (13 counts or 1.82%), which was two sided. Those who received vaccinations most frequently reported side effects which lead to controversial belief that vaccines lead to autism. Secondly, the lack of vaccinations (4 counts or 0.56%) has

resulted in increased exposure, presence of diseases that could be prevented (e.g. measles 0.84% or 6 counts) and life-threatening dangerous complications.

Figure 3.8

Consequences Word Cloud

Figure 3.8 Consequences Word Cloud. [The highest word counts for consequences were children, family, child, people, measles, and vaccines.]

No Link to Autism

There were 55 of 1930 hits that stated that there were no causative links with autism. Topping this list was vaccines (35 counts or 7.69%) which were clearly indicative of the retraction and lack of scientific evidence that vaccines cause autism. Celiac (8 counts or 1.76%) was also included in this list due to recent publication that found no evidence of celiac in autism subjects.

Figure 3.9

No Link to Autism Word Cloud

Figure 3.9 No Link to Autism Word Cloud. [Vaccines had the highest word count from the no link to autism category.]

Lack Deficit Needs

The category of "lack deficit needs" is defined as resources related to autism that are absent, scarce, or insufficient to meet the needs. This category was made up of 188 of 1930 hits with 1329 coded references. The category was further broken down into the following subcategories.

Table 3.3

Lack Deficit Needs Sub-Category References

Sub-Category	**Number of Coded References**
Problems with medical interpretation	63
Need for testing or treatment	43
Cost	36
Problems diagnosing	29
Treatment failure	25
Misconceptions	20

Access to healthcare	18
Parental interference	14
Need for information	14
Legal representation	11
Access to specialized education	11
Employment	5
Abuse	4
Lack of support	4
Failure of prescription compliance	3
Need for proper labeling	2
Unapproved uses of treatment	2
Failure of assistance program	1
Missing person	1

Table 3.3 Lack Deficit Needs Sub-Category References. [The numbers represent the coded references within the node.]

The majority of complaints about gaps were focused on meeting the needs of children with autism, which included deficiencies in doctors (35 counts or 1.16%) and medical care (16 counts or 0.53%). Problems with pediatricians were noted specifically in the word count eight times. Of the deficiencies, misdiagnoses and costs or money topped the list, 0.23% (7 counts) and 0.23% (7 counts) respectively. Examples related to healthcare include problems with medical interpretation (coded 63 times), lack of testing or treatment (coded 43 times) and excessive cost of care (coded 36 times). The following are paraphrased entries from social media that capture these complaints:

> The medical approach to medicating individuals with autism relies on trial and error due to the lack of pharmacogenetic testing. Dosages are adjusted differently for each person, and one medication may be ineffective or have negative effects while others are helpful.
>
> Medical expenditures for individuals with ASD are very costly, reaching an average five times greater than those without ASD.
>
> Pediatricians are reluctant to diagnose ASD early.
>
> Physicians are not trained to find and treat the underlying causes of ASD.

Health professionals lack training to deal with ASD.

Figure 3.10

Lack Deficit Needs Word Cloud

Figure 3.10 Lack Deficit Needs Word Cloud. [The largest word counts for lack deficits and needs were children, child, symptoms, doctors, and medical.]

Many complaints mentioned the use of a trial and error approach to treatment, and some hits even expressed a need for better education and training for healthcare professionals. Items in the "problems diagnosing" sub-category referred to the inability to distinguish autism symptoms from other diseases like attention deficit disorder, thyroid disorders, Lyme disease, Rett syndrome, and 22q13.3 deletion syndrome. Treatment failure focused on side effects, resistance, or tolerance of drugs.

Examining the sub-category of "employment" indicated ineffective interventions for autism made it difficult for these individuals to obtain and keep employment. The sub-category of "employment" showed 100% negative outcomes for employment of autistic individuals so it was moved from abilities to the lack deficit need category. All of

the coded references expressed problems in holding a job citing "numerous terminations" from "being too sensitive to having difficulty concentrating."

Sub-categories of abuse, lack of support, parental interference, and failure of compliance or from assistance programs help explain from where problems emerge. Failure of compliance refers to care givers, parents, or autistic individual's adherence to prescribed treatment programs either with medication, diet, or therapies. Parents express difficulty in forcing children with food aversions or sensitivities to take medicines or maintain diets. Several ways were offered throughout the data to resolve this problem: mixing medicine in foods, making the medicines available in different forms (i.e. tablets, liquids, sub lingual forms, or by injections), or pharmacogenetic testing to prevent trial and error.

Parental interference occurred when parents or caregivers did not see response in their child or they saw adverse events in response to intervention methods. In some cases the caretaker failed to see the symptoms or regression, which led to delayed exposure or changes in intervention. In other instances, the caretaker failed to see an improvement and would seek out new interventions or add supplemental or unapproved interventions to care for their child. Feelings of denial and desire to give the child the best can fuel parental actions and create counter effects from what is intended. The following are summarized excerpts that describe the extent of parents over reacting and interfering in their child's treatment.

> The doctor blames my child's symptoms on autism without ruling out other causes.

The parent or caregiver refuses to take his/her child back to the pediatrician because of a statement made stereotyping parents of ASD children wanting a diagnosis for financial assistance.

Some parents try to please their child with ASD so much that the child can get away with anything. In extreme cases the child completely rules the house with no parental control.

The parents refused to have their child evaluated for ASD at a young age because they felt the child's symptoms were minimal.

An individual with ASD wants to feel independent and gain respect from his/her parents.

Age at Onset

The age of onset was defined as the self-reported age at which the parent or caregiver noticed symptoms that were non-typical leading up to a diagnosis of autism. The age of onset category was made up of 61 out of 1930 hits with 1283 references. The category was subcategorized to capture the age of onset at birth (18%), 12 months or less (39.3%), 24 months or less (31.1%), and 36 months or less (6.6%).

Age at Diagnosis

The age of diagnosis was self-reported in 44 of 1930 hits with 540 coded references. The age of diagnosis was sub-categorized into age in years at diagnosis.

Table 3.4

Age at Diagnosis Sub-categorization into Years

Age in Years	**Number of References Coded for Age**
1 year or less	10
2 years	12
3 years	11
4 years	5
6 years	1
7 years	2

8 years	0
9 years	1
10+ years	3

Table 3.4 Age at Diagnosis Sub-categorization into Years.

The youngest individual diagnosed with autism was five months of age and the oldest was 17 years of age. Most frequently children were diagnosed around one year of age. Figures 3.11 and 3.12 illustrate age of onset and age of diagnosis. A gap is visible between age of onset around 12 months and age of diagnosis at two years.

Figure 3.11

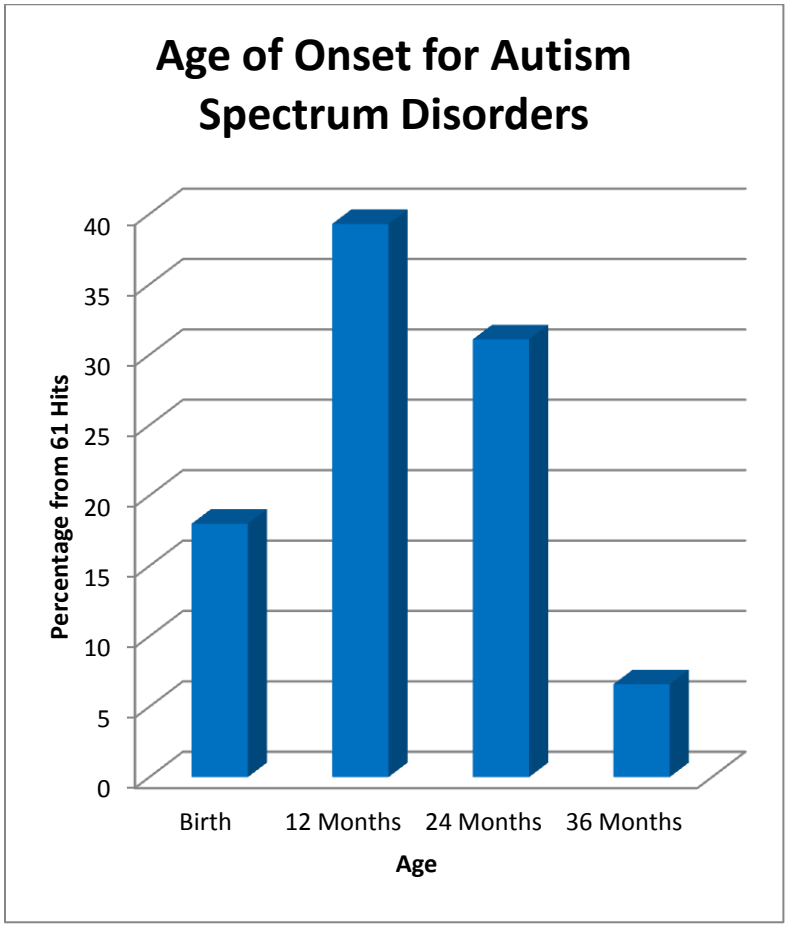

Figure 3.11 Age of Onset for Autism Spectrum Disorders.

Figure 3.12

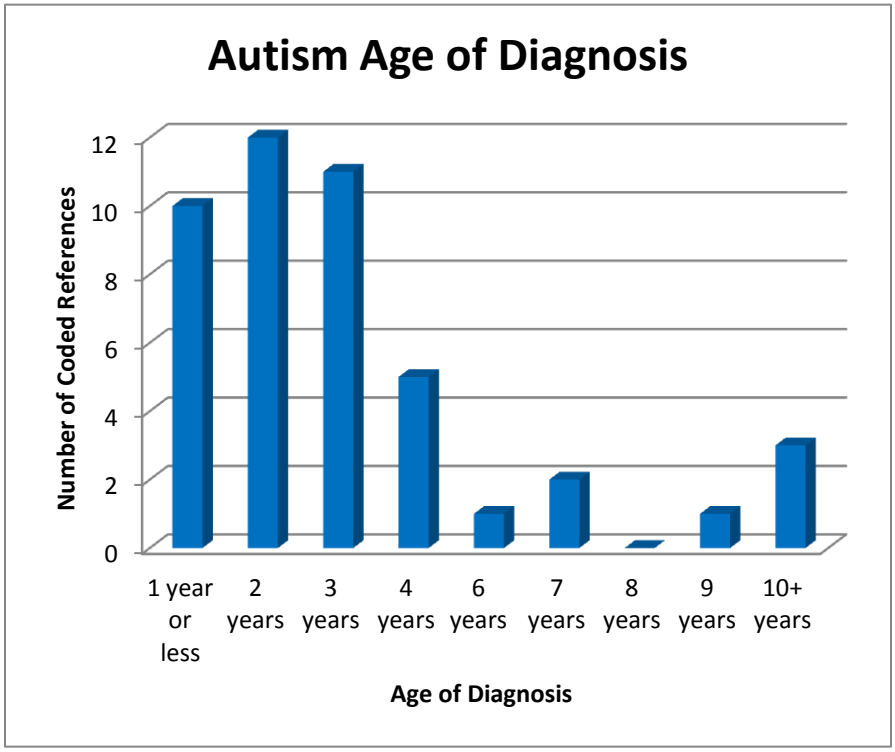

Figure 3.12 Autism Age of Diagnosis

Interventions

The category labeled interventions contained 878 of 1930 hits with 1260 references. Interventions were defined as something that occurs between two points in time as related to autism. The word count summary identifies diet (164 counts or 1.65%), therapy (123 counts or 1.23%), and gluten (59 counts or 0.59%) to be the top three ways to intervene once autism is suspected in a child. The word cloud is shown below.

Figure 3.13

Interventions Word Cloud

Figure 3.13 Interventions Word Cloud. [The words with the highest count included diet, therapy, child, children, gluten, food, and supplements.]

Interventions were sub-categorized as “actions”, “supplements”, “diet”, “therapies”, “medications”, “support”, “specialists”, “medical testing”, “education”, “tools”, “devices”, “warnings”, or “treatment resistance”. The most common “action” was early intervention, which seemed to lead to better outcomes through the disease process. The following are some paraphrased examples from the social media hits:

> Early detection of ASD can protect caregivers, parents, and affected individuals from severe consequences

> Young children respond faster to interventions than older children, which improve their quality of life long term.

Of course, early intervention could not be achieved without helps from other resources or

combined actions.

From the sub-category of actions, “helps” were counted 24 times (0.60% weighted percentage) as the most important term used to define action. Examples of help include:

helping an individual with ASD with reading and language

helping an individual with ASD re-learn important cues

helping the public understand autism

helping promote studies on gene-environment interaction

Other terms synonymous with help were also indicated as actions of importance such as advocate or give. Much of the action listed in the entries was two-folded focusing on learning, gaining knowledge, or relaying positive messages either for the caregiver, family, or affected individual. In either situation the one acting to provide an intervention would also gain from his/her actions through personal growth.

For the individual with autism, one of the themes seen under this category referred to learning or obtainment of skills. Life skills are important to the person in overcoming developmental delays, obtaining employment, learning, and being able to function with everyday tasks. One post in social media discussed the impact of life skills on the individual sharing that experience and coping skills empower students to solve problems and recognize stress levels. As with any form of intervention, support is needed to obtain and maintain the resources to make a difference in the disease processes.

Support is another sub-category of interventions containing 119 coded references. Support is dependent on the actions of many individuals. The word count shows that

support is influenced most by support groups (3.12%), family (1.40%), books (1.25%), parents (2.18%), community (0.78%), Facebook (0.47%), and friends (0.47%). Although specialists were sub-coded separately, they need to be included to provide medical support and guidance toward treatment options. The most sought after types of specialists were: chiropractors (3.72%), developmental pediatricians (3.10%), and neurologists (1.86%). This number was heavily influenced by the increased number of social media entries by chiropractors versus other medical specialists.

It was apparent, within social media, that parents and caregivers have sought out unconventional treatments for their children due to deficits in healthcare and failure of treatments. Many of these unconventional treatments utilize complementary or alternative treatments (Akins et al., 2014). Supplements (288 references) or diets (281 references). Oil (3.0%), magnesium (1.64%), vitamins (4.09%), enzymes (1.45%), glutathione (1.45%), and amino acids (1.09%) were the most frequently mentioned forms of supplements. Several dietary changes were reported as effective in reducing symptoms related to autism. These diets included elimination of gluten, casein, dairy, and/or sugar. Elimination of these four items either individually or in some combination made up 125 hits. Many people had self-reported success with the Gut and Psychology Syndrome (GAPS) nutritional program (2.52%), commonly referred to as the GAPS diet, which is a combination of diet, supplementation, and detoxification. Other diets of mention were organic (1.03%), fermented foods (0.84%), and ketogenic diets (0.84%). Usually, the incorporation of diet and therapy was seen as beneficial in treating symptoms of autism.

Therapeutic measures and medications differed based on the severity and

symptoms manifested in the individual. Speech (3.93%), behavioral (2.62%), occupational (2.62%), chelation (1.31%), sensory (1.16%), biomedical (1.02%), music (1.02%), physical (1.02%), play (1.02%), and stem cell therapies (1.02%) were just a few of the therapies mentioned in social media. Much emphasis was placed on the success of applied behavioral analysis (2.62%), which is a comprehensive form of therapy that focuses on modifying behaviors. Although therapy alone can positively impact behavioral patterns, sometimes the presence of co-morbid diseases or severity of autism symptoms requires the use of medications as coded in 135 references. Medications most frequently used by autistic individuals include antibiotics (4.15%), marijuana (3.9%), Miralax (1.95%), Diflucan (1.22%), Risperdal (1.22%), Ritalin (1.22%), Nystatin (0.98%), and antidepressants (0.98%). Of course medications used are linked with symptoms.

While devices made up only 30 of the 1260 references for interventions, posts demonstrated a need for more integration based on the positive outcomes that have resulted from their use. Communication (6.14%) was the number one use for devices, such as iPads (7.89%), computers (2.63%), and special lenses (2.63%). Software applications were discussed due to their personalization to the user's needs. Integrated devices in healthcare were of benefit to the clinicians as well. One example from social media described how computer automation helps pediatricians focus on the individual and the personalized needs of each patient.

Devices were also supportive in emergency situations. One example of this described in social media is the development of a communication book designed for first

responders that is valuable when a person cannot verbalize how they feel. It is evident from the posts on social media that devices help improve symptoms and provide important ways to communicate in general and in emergency situations. The use of such devices support a child's education, medical care, and monitoring of patient's needs related to personalized medicine. Devices bridge the gap between treatment and symptoms by making the symptoms more apparent through day to day records and allowing treatment responses to be monitored more easily.

Symptoms

In this study, "symptoms" included a wide range of physical or mental appearance, reaction, and diagnosed diseases or disorders that are apparent in individuals who also have a self-reported diagnosis of autism. The symptoms in the data were defined in both lay and medical terms and with or without supportive laboratory results, demographic or family history information. From social media, 692 of 1930 hits were coded under "symptoms", which were further divided into sub-categories.

Table 3.5

Sub-Categories of Symptoms

Sub-Categories	**Number of References Coded**
Behaviors	534
Gastrointestinal	482
Communication	329
Emotion	179
Allergies	168
Senses	142
Infection	132
Mental processes	130
Feeding Problems	105
Pain	97
Nervous System	97

Sleep	86
Laboratory Levels	80
Immune System	71
Skin	62
Respiratory	59
Delayed Development	48
Genetics	45
Nutritional Deficiency	37
Vision	30
Ear Nose Head	29
Inflammation	29
Ambulation	29
Adverse Reactions	25
Height and Weight	24
Muscles	23
Relationships	20
Potty Training	19
Fatigue	18
Regression	17
Detoxification	15
Circulation	15
Personality	13
Methylation	13
Limbs and Joints	12
Renal Function	12
Birth	11
Cardiac	10
Dental	8
Progression with Age	7
Jaundice	6
Failure to Thrive	6
Bone	5
Level of Care	4
Discipline	4
CFS/Fibromyalgia	3
Cancer	3
Reproductive Organs	3
Death	3
Frequency of Illness	2
Liver	1

Table 3.5 Sub-Categories of Symptoms.

Behaviors, gastrointestinal, and communication problems covered 71.1, 69.6, and 47.5% respectively, of the symptoms identified in social media. The word cloud for symptoms specified the problems associated with autism even further.

Figure 3.14

Symptoms Word Cloud

Figure 3.14 Symptoms Word Cloud showing increased word counts of constipation, food, diarrhea, allergies, and general problems with autism.

Food allergies (138 counts or a weighted percentage of 1.19%), constipation (122 counts or 1.05%), diarrhea (97 counts or 0.84%), and pain (79 counts or 0.68%) were the most frequently used words to describe symptoms. For gastrointestinal problems alone constipation, diarrhea, and reflux were the main symptoms seen in autism patients.

Questions

The questions category included 122 of 1930 hits with 820 coded references. This category was sub-categorized into questioning causes and links, clinicians, ethics,

information, insurance, and interventions. Many of the questions focused on legitimacy of treatments, safety, and sought to find information from others who have experienced similar situations. Questions asking for definitions of autism were left unanswered by the respondents.

Other emphasis was placed on the role that parents and caregivers should take by questioning diagnoses and suggested intervention methods, leaving a heavy weight of skepticism among the autistic community. It is evident from the posts that people are seeking answers, and expectations are high for legal representatives, organizations, and government agencies to respond. The following are paraphrased excerpts from social media hits that express the questions being raised:

> Why do we see increases in cancer, autism, and allergies but human DNA sequences have not changed at all?
>
> How does a parent know if their child with ASD has leaky gut syndrome?
>
> Why, doctor, is this happening to my child?
>
> Which risk outweighs the other: the chance that a child will be autistic from vaccination, or the chance that a child will die from a disease that could be prevented?
>
> What should society do if the prevalence of ASD is not enough to get government to fund research or programs for those with autism?
>
> How do you deal with an autism diagnosis?

Figure 3.15

Questions Word Cloud

Figure 3.15 Questions Word Cloud showing increased word counts of questioning especially among children as related to autism.

Improvements

Improvements were defined as reductions in symptoms as a result of intervention. The self-report from social media resulted in 75 of 1930 hits with 787 coded references. Improvements were sub-categorized into allergy reduction, behavior improvements, communication improvements, dermatological improvements, endocrine improvements, gastrointestinal improvements, growth improvements, hearing, infections, insurance coverage, medication, neurological, nutrition, parenting, personal care, reduced adverse events, reduced inflammation, renal improvements, sleep patterns, toilet training. The overall word count for improvements indicates that there are less symptoms (8 counts or 1.08%) which include behavior (7 counts or 0.95%), language or speech (14 counts or 1.9%), cognition (5 counts or 0.68%), contact (5 counts or 0.68%), eye (5 counts or

0.68%), focus (5 counts or 0.68%), school (5 counts or 0.68%), and skills (5 counts or 0.68%).

Figure 3.16

Improvements Word Cloud

Figure 3.16 Improvements Word Cloud illustrates increased word counts of improvements in symptoms, behavior, language, speech, and diet.

Out of the word lists of improvements, diet is counted 7 times with a weighted percentage of 0.95%, suggesting that it has a strong relationship with improved outcomes. From the sub-coding, neurological improvements had 41 coded references, communication 38 coded references, behavior 27 coded references, and gastrointestinal 10 coded references of specific examples of improvements from interventions. Out of all the improvements, no one intervention had the same effect among the individuals with autism. Positive response to treatment was best achieved when issued on a personal level. Unfortunately, in all cases the improvements were not seen until after much trial and error. Without

going into symptoms and interventions which have been discussed earlier, the following are some paraphrased examples of improvement that was commonly seen throughout social media.

> Our child is doing great and is more engaged. He/she is talking and socializing more.
>
> Our child has a better skin color, weighs more, is taller, and has a healthy appetite. The ear infections and chronic diarrhea are gone, and the thyroid has normalized. We see much improvement in his/her behavior and school work.
>
> We have noted improvements in learning, socializing, focusing, eating habits, productivity, awareness, speech, attention, counting, labeling, following directions, eye contact, and understanding non-verbal communication. Additionally, we have seen a developing interest in others, better adaptation with change, and having a natural curiosity about the world.
>
> We placed our child with ASD on biomedical therapy. After several months of therapy, she/he was evaluated by a group of independent professionals who had never met him/her before. The speech and occupational therapists could not detect ASD in our child. When the laboratory reports came back normal the biomedical therapist said we no longer needed their services.

When implementing a diet, one rather unique method for families to offer treatment and show support was for the entire family to follow the nutritional plan. In some instances, siblings also showed improvement in acting out, learning, and reduction in their own symptoms related to a disease other than autism, such as attention deficit disorder or allergies.

Abilities

Abilities are defined in this study as the possession of skills or talents that the individual with autism displays. Out of 1930 hits only 18 defined specific abilities. The most common passions focused on music (7 counts or 9.23%), math (3 counts or 3.95%), and art (2 counts or 2.63%). Other areas of interest included sewing, dance, computers,

science, and horses. One theme defined through the posts was developing and utilizing an individual's special abilities to make them self sustainable. For example, organizations exist that assist individuals in obtaining skills, improving their lifestyle, and providing them with future employment by taking their interests such as art to create practical commodities.

Figure 3.17

Abilities Word Cloud

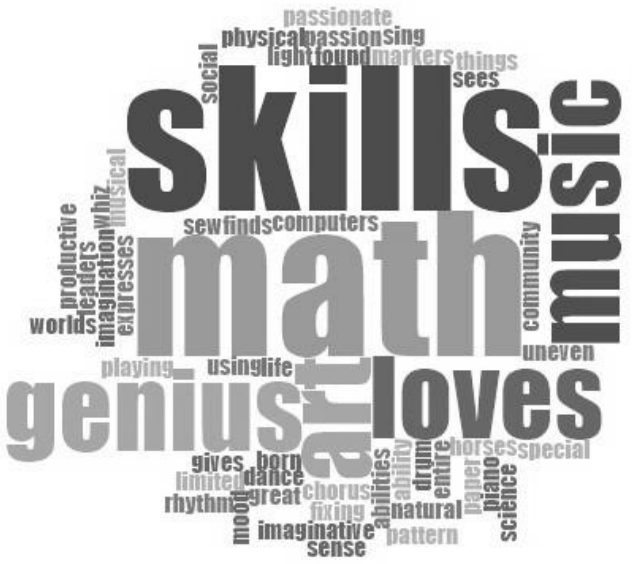

Figure 3.17 Abilities Word Cloud shows the largest word counts were skills, math, music, art, genius, and love.

Statistical Analysis

Cluster analysis was performed in NVivo to group together nodes based on words in common. All nodes were selected and clustered by word similarity using a Pearson correlation coefficient similarity metric. A Pearson correlation close to -1 indicates the

items are not similar, and a Pearson correlation close to +1 shows the items are more similar to each other. The following is a diagram of nodes clustered by word similarity:

Figure 3.18

Nodes Clustered by Word Similarity

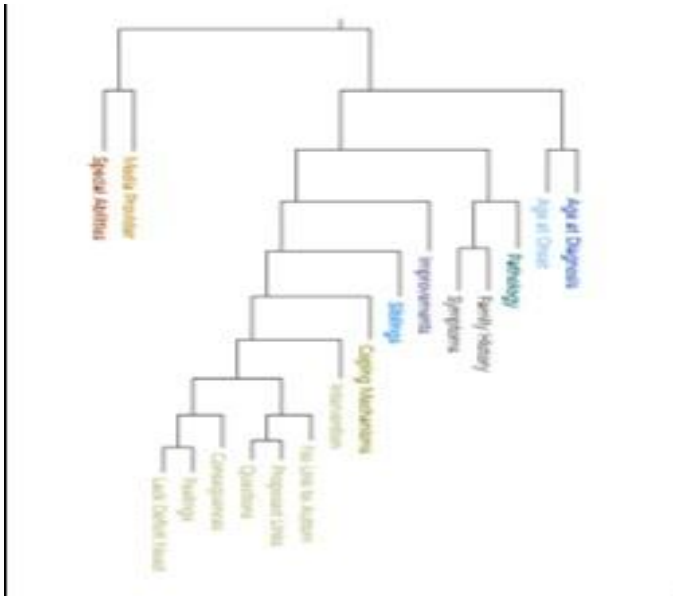

Figure 3.18 Nodes Clustered by Word Similarity produced by QSR NVivo 10.

Table 3.6

Pearson Correlation Between Nodes

Node A	Node B	Pearson correlation coefficient
Questions	Proposed Links	0.8185
Proposed Links	No Link to Autism	0.8120
Questions	Lack Deficit Need	0.7532
Lack Deficit Need	Feelings	0.7121
Proposed Links	Lack Deficit Need	0.7001
Questions	No Link to Autism	0.6958
Questions	Feelings	0.6635
Lack Deficit Need	Intervention	0.6238
Questions	Intervention	0.6057
No Link to Autism	Lack Deficit Need	0.5991
Feelings	Coping	0.5931

	Mechanisms	
Lack Deficit Need	Consequences	0.5693
Proposed Links	Feelings	0.5630
Questions	Consequences	0.5581
Feelings	Consequences	0.5556
Proposed Links	Consequences	0.5523
Proposed Links	Intervention	0.5402
Lack Deficit Need	Coping Mechanisms	0.5357
Proposed Links	Pathology	0.5169
Intervention	Feelings	0.5154
No Link to Autism	Consequences	0.4890
Questions	Family History	0.4825
No Link to Autism	Feelings	0.4825
Questions	Coping Mechanisms	0.4768
Siblings	Lack Deficit Need	0.4764
Siblings	Questions	0.4754
Feelings	Family History	0.4731
Siblings	Proposed Links	0.4645
Intervention	Consequences	0.4631
Proposed Links	Family History	0.4596
Lack Deficit Need	Family History	0.4489
Coping Mechanisms	Consequences	0.4375
No Link to Autism	Intervention	0.4343
Family History	Consequences	0.4213
Intervention	Coping Mechanisms	0.4195
Symptoms	Questions	0.4152
Symptoms	Proposed Links	0.4135
Siblings	Feelings	0.4123
Symptoms	Lack Deficit Need	0.3997
Intervention	Improvements	0.3978
Siblings	No Link to Autism	0.3952
Proposed Links	Coping Mechanisms	0.3952
Questions	Pathology	0.3918
Symptoms	Intervention	0.3912
Lack Deficit Need	Improvements	0.3793
No Link to Autism	Family History	0.3728
Symptoms	Feelings	0.3706
Symptoms	Family History	0.3694
Intervention	Family History	0.3636
Symptoms	Improvements	0.3567
No Link to Autism	Coping	0.3500

	Mechanisms	
Siblings	Consequences	0.3490
Age at Onset	Age at Diagnosis	0.3473
Siblings	Intervention	0.3446
Questions	Improvements	0.3374
Pathology	No Link to Autism	0.3355
Siblings	Family History	0.3340
Family History	Coping Mechanisms	0.3300
Proposed Links	Improvements	0.3279
Pathology	Lack Deficit Need	0.3261
Symptoms	Consequences	0.3201
Symptoms	Pathology	0.3159
Improvements	Feelings	0.3136
Siblings	Age at Onset	0.3129
Pathology	Consequences	0.3050
Siblings	Coping Mechanisms	0.3045
Symptoms	Age at Onset	0.2945
Pathology	Feelings	0.2906
Pathology	Intervention	0.2893
Symptoms	Coping Mechanisms	0.2879
Improvements	Consequences	0.2803
Lack Deficit Need	Age at Onset	0.2753
Pathology	Family History	0.2723
Siblings	Age at Diagnosis	0.2685
No Link to Autism	Improvements	0.2571
Improvements	Family History	0.2495
Proposed Links	Age at Onset	0.2445
Symptoms	No Link to Autism	0.2444
Family History	Age at Onset	0.2378
Consequences	Age at Onset	0.2359
Siblings	Pathology	0.2298
Siblings	Improvements	0.2284
Questions	Age at Onset	0.2227
Improvements	Coping Mechanisms	0.2173
Improvements	Age at Onset	0.2168
Symptoms	Siblings	0.2156
Feelings	Age at Onset	0.2086
Pathology	Coping Mechanisms	0.2028
Intervention	Age at Onset	0.1985
Pathology	Improvements	0.1781
No Link to Autism	Age at Onset	0.1713

Coping Mechanisms	Age at Onset	0.1512
Pathology	Age at Onset	0.1503
Lack Deficit Need	Age at Diagnosis	0.1393
Questions	Age at Diagnosis	0.1151
Family History	Age at Diagnosis	0.1117
Proposed Links	Age at Diagnosis	0.0978
Consequences	Age at Diagnosis	0.0937
Feelings	Age at Diagnosis	0.0902
Symptoms	Age at Diagnosis	0.0873
Coping Mechanisms	Age at Diagnosis	0.0858
Intervention	Age at Diagnosis	0.0835
No Link to Autism	Age at Diagnosis	0.0767
Improvements	Age at Diagnosis	0.0732
Symptoms	Special Abilities	0.0669
Media Provider	Coping Mechanisms	0.0577
Special Abilities	Improvements	0.0554
Special Abilities	Intervention	0.0488
Special Abilities	Coping Mechanisms	0.0416
Pathology	Age at Diagnosis	0.0345
Special Abilities	Feelings	0.0272
Special Abilities	Age at Onset	0.0268
Special Abilities	Consequences	0.0262
Media Provider	Feelings	0.0196
Special Abilities	Family History	0.0090
Special Abilities	Pathology	0.0079
Special Abilities	Lack Deficit Need	0.0070
Special Abilities	No Link to Autism	0.0059
Special Abilities	Questions	0.0011
Special Abilities	Proposed Links	0.0007
Media Provider	Intervention	-0.0010
Media Provider	Age at Diagnosis	-0.0017
Siblings	Media Provider	-0.0020
Media Provider	Lack Deficit Need	-0.0021
No Link to Autism	Media Provider	-0.0021
Special Abilities	Media Provider	-0.0021
Media Provider	Age at Onset	-0.0031
Proposed Links	Media Provider	-0.0032
Pathology	Media Provider	-0.0039
Media Provider	Improvements	-0.0039
Questions	Media Provider	-0.0045
Special Abilities	Age at Diagnosis	-0.0047
Media Provider	Consequences	-0.0053

Media Provider	Family History	-0.0055
Special Abilities	Siblings	-0.0056
Symptoms	Media Provider	-0.0065

Table 3.6 Pearson Correlation between Nodes depicted as decreasing Pearson Coefficient values.

Cluster analysis between sub-categories of questions and proposed links indicates a high positive correlation between questioning causes and links and proposed links (r=0.8572), proposed links and no link to autism (r= 0.811967), and questions and lack deficit need (r= 0.7532).

Discussion

Analysis of social media entries for autism identified patterns and themes related to GI symptoms. A pattern of symptoms leading to a diagnosis of autism included infection at a young age, appearance of altered bowel function (e.g. constipation, diarrhea), increased behavioral problems, and diagnosis of ASD at least a year or two after onset. From the data, onset appears after birth and before 12 months of age. However, autism is often not diagnosed until two or three years of age which leads to frustration and skepticism for the current healthcare system. A need for early diagnostic testing is seen as desirable by parents, caretakers, and clinicians as long as it is effective and is accompanied by financial assistance. Types of financial assistance were identified in the data set as insurance, reimbursements, or tax deduction. Early diagnostics of particular interest, from the data, included tests that do not directly diagnose autism as a disease, such as pharmacogenetic testing and allergy testing. These tests provide direction in intervention application and monitoring. Patterns within the data also indicate

pathways of interest that would lead to development of such tests and earlier diagnosis of autism.

Based on word counts and reoccurring self-reports of symptoms, the pathway identified in the data starts with an infection before twelve months of age. Infection is followed by or accompanied with bowel abnormalities which over time result in behavioral changes and increased co-morbid symptoms. The diagnosis of autism follows this progression years later.

Three major themes emerge from the data: (1) proposed links to autism; (2) symptoms related to food allergies and GI issues; and (3) interventions. Proposed links to autism make up the largest theme found in social media. The links offer a glimpse into what are believed to be causative suggestions for ASD. The majority of participants in social media search for causes and answers related to ASD. In many cases one participant will share symptoms of ASD to either communicate with others experiencing the same symptoms or to seek advice for successful interventions. With no specific medication to treat ASD, parents and clinicians typically try different approaches to relieve symptoms.

In the categories of proposed links, family history, pathology, and symptoms the gut or gastrointestinal tract is one of the most frequently used words; indicating a relationship between the gut and autism. Dietary measures and therapy (e.g. physical, occupational, behavioral, etc.) are the most commonly used interventions mentioned in social media. The data suggests that with a comprehensive family history, log of symptoms and reactions, and genetic information, a personalized treatment plan may be applicable to ASD. It also suggests a need to study multivariate patterns of inheritance as

related to GI disorders, autoimmune diseases, and allergies. Epigenetic and pharmacogenetic testing is also identified beneficial in the treatment and understanding of ASD. The extensive amount of trial and error that occurs during the treatment process suggest that implementation of pharmacogenetic testing may improve quality of life and lead to more direct positive outcomes.

While social media offers many perspectives on autism which may be beneficial, it must be recognized that the software used to mine and analyze the data and the research team are limited in their abilities. Radian6 collected a wealth of data using the parameters identified, but it was limited in its ability to identify repeated entries or false leads in the data series. This resulted in the research team manually coding each entry and eliminating repeated posts and entries not related to the search criteria. Due to the size of the dataset and extensive time required to manually code the information, inexperienced research team members were not able to tolerate the research load along with their academic obligations. Therefore, the analysis from only one coder may present bias in the results and lend itself to lack of inter and intra reproducibility of the study. To reduce the effect of researcher bias, all the coded entries were uploaded into NVivo and an auto-code was performed based on the selected nodes. The entries were then sub-categorized and re-coded if needed in NVivo. For future studies, it would be beneficial to develop a tool compatible with Radian6 and NVivo that would tag repeated entries and perform a secondary scan of the data for search criteria requirements. It would also be recommended to have at least three people to code the data to ensure interrater reliability or agreement among the results without bias.

Several factors presented as limitations to this study which could be improved in future research designs. First, the study lacks inter rater reproducibility due to the issues with coder retention. Future studies should seek funding to offer incentives and hire experienced qualitative research coders in order to prevent coders from dropping out of the research project. Second, the search terms entered into Radian6 produced an extensive amount of false leads which had to be removed manually. The results from Radian6 in addition to the use of manual coding increased risks of error. The development of syntax to identify repeated entries would reduce the number of false leads. Also, identification of specific search terms and the way in which they are entered into Radian6 may reduce the need for manual coding. One suggestion would be to test combinations of search terms and entry methods over a few days to determine the best fit model for a larger study. The data set could also be statistically analyzed using pathway modeling to determine if certain symptoms or categories could be used to model ASD using social media. Future studies could then focus on using social media to model other forms of disease and interventions.

Several advantages exist for using social media data posted in the public domain. The first advantage is that it does not remove the person who providing the information from their typical environment. Social media preserves the data in its original context. Posts in the public domain reduce harm due to confidentiality breaches. It is assumed that when a person posts information in the public domain it is intended for the public to view and interpret on an individual basis. Information posted in the public domain allows equal access to anyone who wishes to analyze it, and it gives the research subject the

opportunity to regulate which information they wish to share and what they do not want to share.

Conclusions

Mining social media data provides a tool for translating experiences, symptoms, and reactions of disease into applicable categories that may be further studied as new pathways, interventions, or approaches to the disease. The purpose of this study was to explore social media in order to identify patterns and themes related to autism and GI symptoms. Eighteen categories emerged from the data set; the top three categories yielding themes of proposed links to autism, symptoms related to food allergies and GI issues, and interventions. The self-reported symptoms and pathology in social media indicate a pattern of infection at a young age that leads to GI symptoms and continues to progress, leading to layers of symptoms and eventually an autism diagnosis.

Previous studies have identified associations between constipation, behavioral problems and ASD (Ibrahim et al., 2009; Kral et al., 2013). This study supports the previous associations through increased word counts for GI symptoms, behavior problems, and autism. Major complaints of GI symptoms included changes in bowel behavior and constipation after an infection at a young age. The pathology represented by the data of a pathogen interrupting the epithelial barrier and creating a cascade of symptoms is suggestive of the proposed gut to brain pathway. The data shows that layers of symptoms accumulate as well as adverse reactions to treatment leading to delayed diagnosis.

Social media data gives individuals with autism a voice and it provides direction for future research and intervention. This research shows that social media data can identify suspected pathways of disease through identification of commonly reported symptoms, onset, and progression over time. Social media provides an excellent opportunity for participants to log experiences of disease over time and record treatment responses. Examples shared through personal experiences can benefit families to recognize symptoms and successful treatment options. Social media gives individuals and families with autism a means of support and mechanism to share their experiences.

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CHAPTER IV

ZONULIN LEVELS IN NEWBORNS AND CHILDREN WITH AUTISM SPECTRUM DISORDERS

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Autism Insights

This manuscript highlights basic scientific research to explore the etiology and development of early diagnostic testing for autism spectrum disorders.

Abstract

Zonulin is a precursor for the protein haptoglobin 2 and functions to regulate epithelial tight junctions. Modulation of the tight junctions allows macromolecules to pass into the blood stream, causing a disruption in homeostatic mechanisms that play important roles in the development of immunity and disease. Symptoms related to epithelial barrier permeation have been correlated with autoimmune diseases and cancer, but there is reason to believe that similar gastrointestinal and neurological symptoms may be seen in persons with autism spectrum disorder (ASD). This study aims to determine if increased zonulin levels or haptoglobin genotypes are directly associated with ASDs among three groups: individuals diagnosed with ASD and gastrointestinal symptoms, newborns, and healthy controls. Results indicate a low negative correlation exists between zonulin concentrations and the three groups in addition to the haptoglobin genotypes and the groups. More studies are recommended to explain the presence of zonulin in 10% of ASD individuals and 20% of newborns compared to the lack of zonulin in the control group.

Keywords: autism, zonulin, intestinal permeability, haptoglobin, newborns

Zonulin, pre- haptogolobin 2 (pre-HP2), is a complex protein structure that regulates epithelial tight junctions, allowing macromolecules to pass into the blood stream causing altered immune responses (Tripathi et al., 2009; Wang et al., 2000). Enterocytes of the small intestine release zonulin into the lumen when triggered by bacteria, parasites, or gliadin (Fasano, 2012). Zonulin exists in two forms: an un-cleaved form known as pre-HP2 that binds to protease activated receptor 2 (PAR2) and epithelial growth factor receptor (EGFR). The second form is cleaved by trypsin at Arg^{161} to form either an α-2 or ß subunit of haptoglobin (*HP*) that seeks to bind hemoglobin but is unable to bind to EGFR (Fasano, 2012; Tripathi et al., 2009). Original studies indicate that the two different forms of zonulin yield two different functions due to the folding of the protein, with only the un-cleaved form affecting intestinal epithelial permeability (Fasano, 2012; Tripathi et al., 2009). Current research suggests that both forms of zonulin affect permeability, but the association between zonulin and autism spectrum disorder (ASD) is not known (Rittirsch et al., 2013).

A literature search was conducted in PubMed using the search terms "zonulin," "intestinal permeability," "pre-haptoglobin 2," and "epithelial permeability" from 2000-2014. The results yielded 11060 publications. The search criteria was then refined to include autism using the search terms "intestinal permeability AND autism," "zonulin AND autism," and "pre-haptoglobin 2." The results found 96 articles from 2000-2014. Article titles and abstracts were screened for inclusion of individuals with ASD, eliminating articles focused on animal studies, drug development, and emphasis on other developmental diseases.

It is known that intestinal permeability is present in individuals with ASD. Lactose mannitol testing by D'Eufemia et al (1996) and De Magistris et al. (2010) have

identified intestinal permeability in ASD individuals at 43% and 36.7% respectively. Furthermore, the correlation between zonulin levels and severity of autism is supported by the results from Adams et al. (2011) that show children with more severe forms of ASD are likely to have more severe gastrointestinal (GI) symptoms. Children with higher Autism Treatment Evaluation Checklist scores had GI severity index scores greater than 3 ($p=0.00002$) and lower levels of total short chain fatty acids (Adams et al., 2011). A large registry-based study of 589 individuals by Wang, Tancredi, and Thomas (2011) reported 42% of children with ASD had GI problems including constipation and diarrhea compared with their unaffected siblings (12%). Additionally, a more recent meta-analysis from research studies in peer-reviewed journals shows that GI symptoms of diarrhea, constipation, and abdominal pain are greater in children with ASD compared to children in control groups (McElhanon, McCracken, Karpen, & Sharp, 2014).

Without measuring zonulin specifically, protein marker and genotyping exploration in individuals with ASD has indicated significant concentration differences in proteins related to zonulin. Of greatest interest are studies surrounding HP, a form of zonulin cleaved by trypsin and mapped to chromosome 16 (Fasano, 2011; Fasano, 2012; Swanwick et al., 2011; Tripathi et al., 2009). Aposhian et al., (2006) showed that HP plasma concentration was decreased in autistic male children (29.5±7.0 SE mg/dL) in comparison to healthy controls (91.8±14.8SE mg/dL, $p=0.001$). Additionally, Aposhian et al. (2006) identified an increase in HP2-2 genotypes in males with ASD (75%) compared to normal controls (40%). These results indicate that further testing is needed to determine relationships between HP and zonulin concentrations in serum, as well as relationships between protein concentrations and genotype. It may be possible that serum concentrations of HP are decreased due to decreased cleavage of zonulin, which

would indicate an increase in zonulin in the pre-HP2 form. Therefore, the *central hypothesis* of this research project is that increases in zonulin concentrations of serum are directly associated with ASD severity and that *HP2-2* genotypes place an individual at a higher risk for developing ASD.

Symptoms reported in ASD individuals implicate physiological mechanisms of zonulin in the onset of the disease. Symptoms of ASD include social impairment, communication difficulties, and repetitive and stereotyped behaviors; however, additional symptoms have been reported of stomach pain, diarrhea, constipation, acid reflux, vomiting, bloating, and food allergies (U.S. Department of Health and Human Services, National Institutes of Health, 2011). A review of the literature provides a list of diseases that show association between intestinal permeability and the zonulin pathway. Table 4.1 summarizes a list of these physiological pathways associated with zonulin and known disease states.

[Insert Table 4.1]

The literature identifies five physiological systems that are disrupted due to increased epithelial permeability: GI, Neurological, Oncology, Endocrine, and Respiratory Systems. Diseases associated with increased levels of zonulin have been suggested to be auto-immune, but there is reason to believe that symptoms related to increased zonulin levels may be seen in other diseases not yet classified as such. This dissertation research broadens the investigation to look at relationships between ASD, *HP* genotypes, and quantitative zonulin levels through the following aims:

1. Determine if zonulin levels in plasma obtained from individuals diagnosed with an ASD (Group 1) or newborns (Group 2) are different from zonulin levels in controls of healthy individuals matched by age and/ or gender (Group 3).

2. Examine relationships between HP2 genotypes and increased zonulin levels in individuals with ASD (Group 1) or newborns (Group 2) compared to healthy controls matched by age and/or gender (Group 3).
3. Determine if zonulin levels are good predictors between ASD and healthy control groups.

Methodology

A non-experimental cohort comparison design of banked, convenience plasma sample and matching genomic deoxyribonucleic acid (gDNA) sample from the Greenwood Genetic Center was used in this dissertation study. Enrollment consisted of three groups: Group 1- individuals ages 6-15 years with ASD (n=10), Group 2- newborns (n=10), and Group 3- healthy gender and age matched controls to the autism group (n=10). The number of samples collected for the study was based on the Clinical Laboratory Improvement Amendments 88' standards for clinical validation studies and the volume of samples obtained by the Greenwood Genetic Center on an annual basis. A minimum of 200uL of banked plasma and matching 20uL of gDNA was available for each group. Each sample was previously characterized through medical history and genetic testing to confirm the diagnosis of ASD. The healthy controls were also obtained from the Greenwood Genetic Center collection. These healthy controls were previously established as negative for ASD through medical history and genetic testing. All samples and data were identified only by laboratory accession numbers.

Inclusion criterion for diagnosed ASD individuals included a confirmed diagnosis of ASD as defined by the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) criteria (American Psychiatric Association, 2013). Although the DSM has been updated to version V, the banked samples were collected under the DSM-IV criteria and

were measured by Autism Rating Scales developed under the same DSM criteria. Autism Rating Scales were not specified due to the lack of standardization across the field. The autism rating scales could include any one of twelve scales available, in order to measure mental health symptoms, measure progress of interventions, and establish methods of communication (Massachusetts General Hospital, 2010). Exclusion criterion for groups 1 and 2 included type 1 diabetes, multiple sclerosis, celiac disease, and rheumatoid arthritis. This project was granted approval by the Clemson University Institutional Review Board under exempt status.

All samples were received at the Clemson University Healthcare Genetics laboratory. Upon receipt, the tubes were logged into a sample accession database. Plasma and gDNA received did not have any subject identifiers and were only labeled with a laboratory accession number, date of receipt, and laboratory technician initials. Information accompanying each sample was labeled with only laboratory accession numbers which link it to the sample received with no subject identifiers. The accompanying information included ethnicity, gender, and presence/absence of autism spectrum disorder from medical records. The samples were analyzed upon receipt.

Design for Zonulin Measurements

Research shows that intestinal permeability is present in ASD and that it is linked with severity of ASD (Adams et al., 2011). Intestinal permeability is defined as the capacity by which substances can penetrate the epithelial surfaces of the intestine (Montalto et al., 1997). Two theories have been developed to explain the permeation routes: transcellular permeation through small pores and paracellular permeation through channels. This dissertation research focuses on the paracellular route by which substances travel through the tight junctions. In order to objectively measure intestinal

permeability, the first aim of this project targets the product of the mechanism that regulates intestinal permeability by quantifying zonulin in the plasma. Plasma zonulin levels in individuals with ASD have not been documented in the literature. The first aim was developed to answer the following questions: 1) what are the levels of plasma zonulin levels among the ASD population in comparison to normal healthy controls? 2) Is there a difference in plasma zonulin levels between ASD individuals with GI symptoms and without GI symptoms?

The *working hypothesis* is that increased zonulin levels in plasma are directly and significantly associated with the presence of ASD, and higher levels of plasma zonulin are specifically related to GI symptoms. In order to test this hypothesis, zonulin levels were quantified using an enzyme-linked immunosorbent assay (ELISA) from MyBioSource. This assay determined zonulin levels in plasma by measuring color changes that result from a series of steps.

First, a reaction plate was coated with polyclonal anti-zonulin antibodies and samples were added that were mixed with biotinylated zonulin tracer. Each sample was run in duplicate. Free target antigens in the samples competed with the biotinylated zonulin to bind to antibodies on the plate. Then streptavidin-labeled peroxidase antibody bound to the zonulin tracer, tetramethylbenzidine was added and the enzyme activity was stopped, yielding a color change. Absorbance readings were analyzed at 450nm and 540nm using a Tecan Infinite M200 absorbance plate reader.

Design for Haptoglobin Genotyping

One study has determined *HP*2-2 phenotypes are associated with ASD in males (Aposhian et al., 2006). *HP* is a protein coding gene involved in hemoglobin binding and catalytic activity. Genotyping can be used to determine the type of inherited alleles which

are then suggested to predict phenotype. *HP* genotyping- although used to predict increased risk of type 2 diabetes, sickle cell disease, vascular diseases, and cancers- is not commonly tested for in individuals with ASD (Cahill et al., 2013; Haas et al., 2011; Mandato et al., 2012; Purushothaman et al., 2012; Santos et al., 2011; Shi et al., 2012; Speeckaert et al., 2011; Speeckaert et al., 2012). DNA testing was not done because no studies have indicated a clinically relevant use for *HP* genotyping in relation to ASD. Since zonulin is pre-haptoglobin2 and it occurs in two forms, it seems genetic testing would be beneficial to determine the statistical relationship of each form with ASD.

The objective of specific aim two targets how genetics place an individual at greater risk to acquire ASD. Specific aim two was developed to answer the following questions: 1) Do genotypes of *HP* significantly differ between the ASD population in comparison to normal healthy controls? 2) Does a specific HP genotype place one at a higher risk of acquiring ASD? 3) How do plasma zonulin levels and *HP* genotype uniquely contribute to ASD?

It was *postulated* that *HP2-2* genotypes would be significantly more abundant in individuals with ASD compared to healthy controls, and would place individuals with a *HP2-2* genotype at a higher risk of acquiring ASD. This hypothesis was tested by genotyping *HP* from gDNA using real-time polymerase chain reaction (PCR). Preliminary studies were conducted to develop and validate a *HP* assay to target three common genotypes of *HP*: *HP1-1, HP1-2*, and *HP2-2*. Primers and probes were designed using *HP* sequences *AC004682* and *M69197* obtained from the National Center for Biotechnology Information nucleotide database. The probe designs target *HP1* and *HP2* using proprietary rapid probe technology courtesy of Co-Diagnostics for research purposes. Probes and primers were synthesized by Integrated DNA Technologies.

[Insert Table 4.2]

The preliminary validation indicates that the HP assay is able to detect presence or absence of HP1, HP2, and HP1/2 genotypes with no cross reactivity using a minimum input of 1ng gDNA. Detection limit analysis and reproducibility was determined from dilutions at 200fM, 20fM, 2fM, 0.2fM, and 0.02fM of each HP synthetic control in duplicate reactions. The estimated reproducibility between runs for HP1 targets is 0.855, with a 95% confidence interval (0.474, 0.982) and for HP2 targets is .990, with a 95% confidence interval (0.953, 0.999). Estimating reproducibility within a run, for HP1 the ICC = 0.984, with a 95% confidence interval (0.889, 0.998) and for HP2 the ICC = 0 .990, with a 95% confidence interval (0.929, 0.999). T-tests were used to analyze the cycle threshold values between single plex and multiplex assay using a significance value of $p < .05$. The t-tests identified no significant differences in cycle thresholds between single plex and multiplex assays, indicating that the plexing of primers and probes together does not significantly affect the cycle threshold detection.

[Insert Figure 4.1 and Figure 4.2]

The multiplexed HP genotyping assay was performed on a CFX96 Real-time System using reagents acquired from Bio-Rad Laboratories (Hercules, California). Each gDNA sample was run in duplicate in a 20-uL reaction containing 10uL Sso Advanced Universal Probes Supermix (Bio-Rad) and 1-100ng of DNA. The optimal thermal cycling conditions included an initial denaturation step at 95˚C for 2 minutes followed by 40 cycles of denaturation at 95˚C for 30 seconds and annealing and extension at 59˚C for 30 seconds. Given that the positive controls indicate a growth curve before cycle 40 and the negative controls indicate no growth curve, samples indicating a growth curve between cycles 10 and 40 will be positive for the respective haptoglobin genotype. A

growth curve in the FAM channel indicates *HP1* allele, a growth curve only in the TET channel only indicates *HP2* allele, and those showing growth curves for both FAM and TET are heterozygous for *HP1*/2 alleles. If the positive control shows no growth curve or the negative control shows a growth curve, the results are invalid and the test should be repeated using new controls.

Results

Several forms of statistical analysis were used to interpret the data. ELISA curve fit was performed using CurveExpert Professional version 2.0. All other analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 21.0 (IBM Corporation, Released 2012). For each analysis, the categorical variables were dummy coded and then all variables were examined for outliers, missing values, normal distribution, and homogeneity. For specific aim one, a correlation analysis was performed between zonulin and the three groups. The results indicate a non-significant low positive correlation exists between plasma zonulin concentrations and the three groups (r= 0.206, p= 0.275), and a non-significant low negative correlation between haptoglobin genotypes and the three groups (r= -0.164, p= 0.387). The same type of analysis was used for specific aim two to determine the relationship between zonulin levels and haptoglobin genotypes. Very little to no correlation was found between zonulin and haptoglobin genotypes (r= 0.046, p= 0.808). In this case, the null hypothesis is accepted that there is no relationship between zonulin and sample groups, there is no relationship between haptoglobin genotype and sample groups, and there is no relationship between zonulin levels and HP genotypes.

A one-way ANOVA was used to test for differences in zonulin levels between the groups. The significance level of p< .05 was used for the ANOVA. The results show no

significant differences in the means between the three groups, $F(2, 27) = 0.883$, $p = 0.425$. Table 4.3 shows the mean zonulin levels among groups of children with ASD, newborns, and healthy controls.

[Insert Table 4.3]

Once again the null hypothesis is accepted that there are no differences in zonulin levels among the three groups.

A binary logistic regression with a significance level of $p = 0.05$ was run to determine specific aim three. A test of the model was not statistically significant from the constant, indicating that zonulin is not a reliable predictor between groups with autism and healthy controls (chi square = 1.176, $p = 0.278$ with df = 1).

Finally, Pearson's chi square analysis was performed to determine the relationships between the categorical data. Chi square tests were run between groups and each of the variables including HP genotype and zonulin level (dichotomously coded high, medium, low, very low, or zero). Statistical significance of < .05 was used for each chi square test. The number of subjects within each group as a function of zonulin level is shown in figure 5.2. Zonulin levels were identifiable in 20% of autism individuals (at low levels of 2.5450 and4.9280 ng/mL), 20% of newborns (at high levels of 75.2014 and very low levels of 0.2710ng/mL), and 50.0% of normal controls (at very low levels between 0.0060-0.9981 ng/mL. The assumptions for Pearson Chi-Square were violated with 75% having an expected count less than 5 so the likelihood ratio values were used. The association between zonulin levels and the three groups were significant, X^2 Likelihood Ratio (6, N=30) = 15.277, p = 0.018. Newborns were more likely to have high levels of zonulin than the other groups, autism groups were more likely to have low levels of

zonulin than other groups, and controls were more likely to have lower levels of zonulin than autism or newborn groups.

[Insert Figure 4.3]

The number of subjects within each group as a function of haptoglobin genotype is shown in figure 4.4. Heterozygous alleles of haptoglobin were present in 100% of individuals with autism, 90% of newborns, and 90% of healthy controls. The other 10% of newborns and 10% of controls were homozygous for haptoglobin 1 alleles. The assumptions for Pearson Chi-Square were violated with 50% having an expected count less than 5 so the likelihood ratio values were used. The association between haptoglobin genotype and the three groups were not significant, X^2 Likelihood Ratio (2, N=30) = 1.692, p = 0.429.

[Insert Figure 4.4]

Discussion & Conclusion

Even though no statistically significant levels were reached from this study, the absence and low levels of zonulin in healthy controls and medium to high levels of zonulin in individuals with ASD and newborns suggest the possibility of clinical significance. The zonulin levels that were present within each sample group raise suspicion that clinical differences in zonulin levels exist when excess zonulin does not clear the system shortly after birth. The high levels of zonulin in newborns compared to the low levels in controls suggest that zonulin decreases over time after birth except in the presence of disease. It is unclear whether increased levels of zonulin at young ages cause disease. These suspicions could only be objectively tested with a larger number of samples for each group. More research is recommended to investigate the role of intestinal permeability and ASD. It is evident from previous research that GI symptoms

are more common among individuals with ASD than normal controls (Adams et al., 2011; Buie et al., 2010). One of the major factors influencing the outcome of this dissertation research project was the small sample size and limited funds. With more funding, a larger sample size could be obtained lending itself to a better statistical outcome. Other potential limiting factors include reversal mechanisms of the gut which function to create a barrier to potential pathogens, tissue specificity of zonulin, or unknown variables that are not accounted for in this study (Fasano, 2011). Reversal of zonulin in gut function has been documented in cases of celiac disease (Drago et al., 2006). Studies suggest that intestinal permeability increases and then returns to baseline over time, but the baseline levels between disease – ASD included- and normal controls remain significantly different (Drago et al., 2006; Tripathi et al., 2009). Since ASD studies documenting dietary interventions of diet indicate a plateau effect, this reversal mechanism has a potential to interfere with direct linear relationships between zonulin and ASD (Whiteley et al., 2010).

A study by Aposhian et al. (2006) indicates a connection between *HP2-2* genotypes and ASD, and preliminary data from social media suggests relationships exist between GI symptoms and ASD. However, in this study there was no significant differentiation in HP genotype among the three groups. Differences related to ethnicity were unachievable due to the availability of samples. The results from this study contrasted greatly from previous findings by other researchers, in that a majority of samples from this study are heterozygous for HP. The differences in HP genotype related to ASD may be the result of gender or the presence of gastrointestinal symptoms. The results related to ethnicity from this study are inconclusive due to small numbers of ethnic groups. A larger study would need to be repeated to provide better statistical

evidence of using HP genotype to predict risk of ASD and to define the role that ethnicity plays in HP genotyping and disease. For example, future studies might include more than just *HP* genotype but other genotypes associated with ASD and/or zonulin.

Finally, the unknown always presents a possible problem in research but it can only be dealt with upon discovery. In this case, zonulin has not been found to be a useful predictor of ASD, but the results are of significant interest in directing future research. Other mechanisms in the zonulin pathway may also be of interest using genome wide association or methylation studies among ASD. The ground work has been established that intestinal permeability is present in ASD and that GI symptoms are linked to severity, but there may be unknown factors that account for these relationships that are not currently known. For this reason, it is suggested that the study be replicated with increased sample numbers to detect zonulin levels within subgroups of ASD specific only to GI disorders or other potential mechanisms that are shared between intestinal permeability and ASD.

Acknowledgements

Samples were provided in kind by the Greenwood Genetic Center, Greenwood, SC.

Funding sources

Support for this project was provided from funding from the Center for Research on Health Disparities at Clemson University.

Author Contributions

Idea conception and experimental design: RG. Analyzed the data: RG. Wrote the first draft of the manuscript: RG. Made critical revisions and approved final version: RG. The author has reviewed and approved the final manuscript.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

Tables

Table 4.1

Diseases Associated with Zonulin		
Physiological Category	**Disease**	**References**
Gastrointestinal System	Celiac	Duerksen et al., 2010; Fasano et al., 2000; Tripathi et al., 2009; Wang et al., 2000
	Colitis	Duerksen et al., 2010
	Crohn's Disease	Fasano , 2008
Respiratory System	Acute Lung Injury	Matthay et al., 2003
	Acute Respiratory Distress Syndrome	Matthay et al., 2003
	Asthma	Fasano, 2011
Nephrological System	Chronic Kidney Disease	Kelly et al., 2009
Neurological System	Schizophrenia	Paterson et al., 2007
	Alzheimers	Liu et al., 2012
	Multiple Sclerosis	Fasano, 2011
Endocrine System	Obesity	Moreno-Navarrete et al., 2012; Zak-Gołąb et al., 2013
	Diabetes Mellitus Type 2	Jayashree et al., 2014; Moreno-Navarrete et al., 2012
	Type 1 Diabetes	Sapone et al., 2006; Watts et al., 2005
	Systemic Lupus Erythematosus	Pavon et al., 2006
Infectious Diseases	HIV	Liu et al., 2012
	HCV	Fasano, 2011
	Sepsis	Klaus et al., 2013
Cancers	Breast Cancer	Russo et al., 2013
	Glioblastoma	Skardelly et al., 2009
	Lung squamous carcinoma	Fasano, 2011
	Pancreatic carcinoma	Fasano, 2011

Tables (continued)

Table 4.2

Haptoglobin Primer & Probe Design		
ID	**Tm (°C)**	**Sequences 5'-3'**
HP1 Forward Primer	59.3	gcaagctcccctcatttct
HP1 Reverse Primer	59.7	actcaggcaatgatgtcacg
HP2 Forward Primer	60.5	aatctgagctccagccagtg
HP2 Reverse Primer	59.1	agctgctctgcacatcaatc
*HP*1 Probe	63.3	[FAM] atggtcctgaaagcccagaggtcgaaggacca [DABCYL]
*HP*2 Probe	64.7	[TET] tagcccctagcccttttcaatgggct [DABCYL]

Table 4.3

Descriptive Statistics

Dependent Variable: zonulin

Group ID	Mean	Std. Deviation	N
Autism	0.7523	1.6786	10
Controls	0.2122	0.3274	10
Newborn	7.5472	23.7714	10
Total	2.8372	13.7041	30

Table legends

Table 4.1 Diseases Associated with Zonulin. A literature review search in PubMed acquired in August, 2013 identified the systems and diseases associated with zonulin.

Table 4.2 Haptoglobin primer and probe design. The design illustrates the proprietary hybridization probe design based on Co-Diagnostic's rapid probe technology. The fluorophores were selected to use in a complimentary fashion for multiplexing capability.

Table 4.3 Descriptive Statistics. This chart shows the mean zonulin levels among the groups of individuals with ASD, newborns, and healthy controls.

Figures

Figure 4.1

Real-time PCR of HP1 Dilution Series

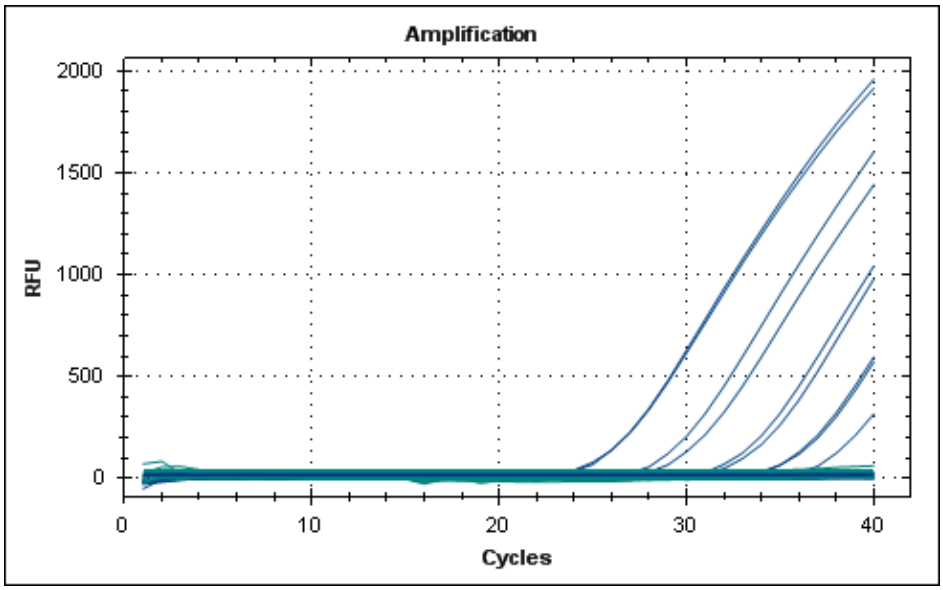
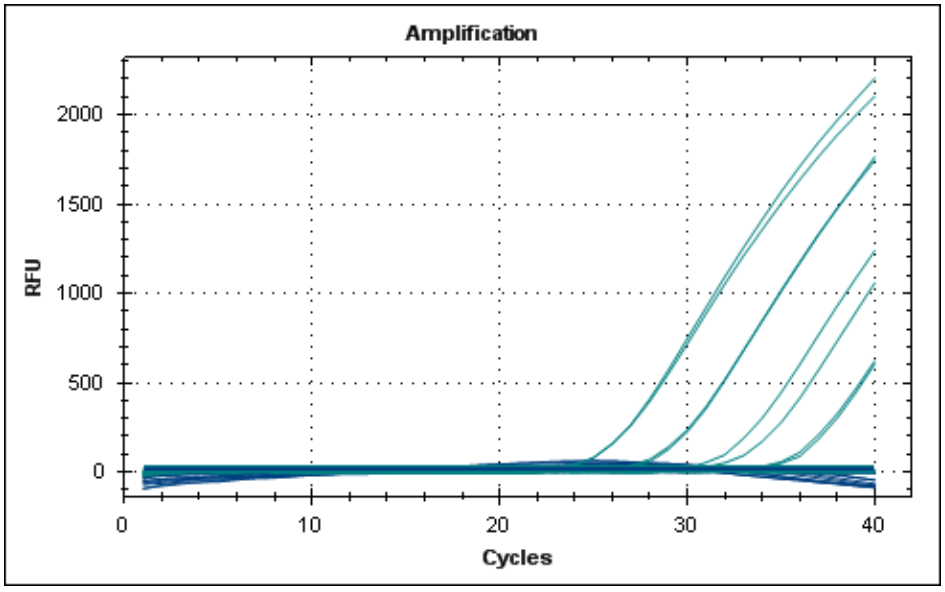


Figure 4.2 Real-time PCR of HP2 Dilution Series



Figures (continued)

Figure 4.3

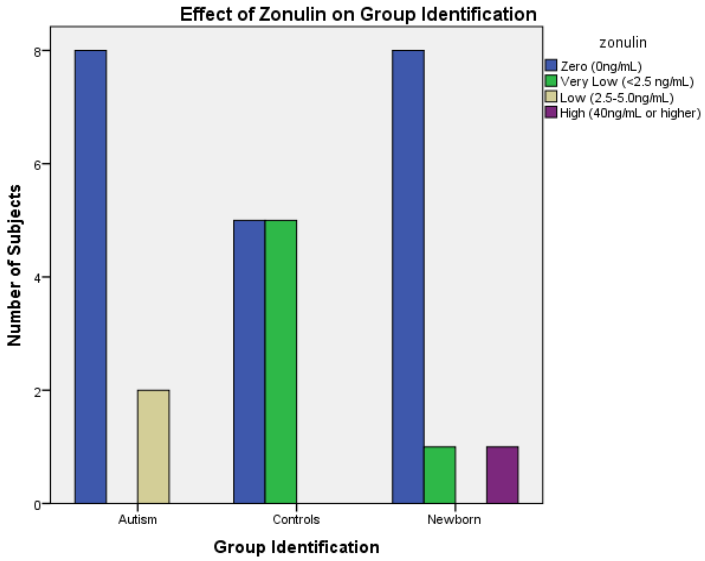


Figure 4.4

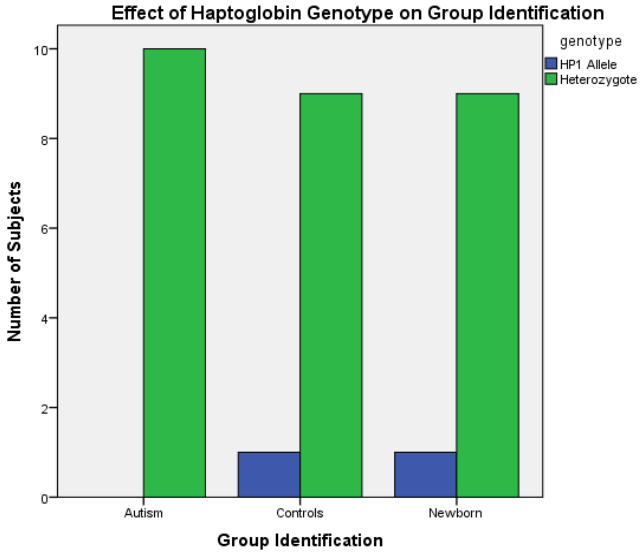


Figure Legends

Figure 4.1 Real-time PCR of HP1 Dilution Series. The plot shows the cycle thresholds and growth curves for a HP1 control serial dilution at 200fM, 20fM, 2fM, 0.2fM, and .02fM concentrations.

Figure 4.2 Real-time PCR of HP2 Dilution Series. The plot shows the cycle thresholds and growth curves for a HP2 control serial dilution at 200fM, 20fM, 2fM, 0.2fM, and .02fM concentrations.

Figure 4.3 Effect of Zonulin on Group Identification. The graph shows the zonulin levels among the number of subjects within each group.

Figure 4.4 Effect of Haptoglobin Genotype on Group Identification. The graph shows the haptoglobin genotype among the number of subjects within each group.

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CHAPTER V

CONCLUSION

Translational research brings knowledge from multiple disciplines together and sets that knowledge into action within clinical practice. Using the knowledge to action framework, this project has moved through multiple phases from translation to research. As this research clearly indicates, this translational process is littered with much trial and error moving continuously from the translation phase back to the research phase. It almost seems as if research and translation is a continuous cycle with only an occasional, random institutionalization output. None-the-less, without continuous research as demonstrated through the previous four chapters lives would not be saved from disease and healthcare would not progress. Chapter I summarized how the research within this project fits within the knowledge to action framework. The chapter provided conclusions from each experiment and future directions to push forward toward institutionalization.

In chapter I, the knowledge to action framework was introduced along with brief summaries of how each of the subsequent research chapters would fit within the framework. This chapter defined translation as the process by which an application is moved from the development phase into action within the clinical setting. It introduced Chapter II, an article that reviews the current status of zonulin associated intestinal permeability and ASD and indicates a gap in early diagnostic testing that can be developed for clinical use.

Chapter III explored social media for themes and patterns of autism and GI symptoms collected using Radian6. It was anticipated that the themes presented first hand by individuals with autism, care takers, and friends or family through social media can implicate relationships and themes of disease that stimulate the need for further research. Three major themes were identified: 1) proposed links to autism, 2) symptoms related to food allergies and GI

issues, and 3) interventions. The pathways of disease present through social may be further analyzed to create pathway models for ASD and other diseases. Additionally, the information obtained through social media data may be used to create novel interventions. In this case, it is projected that preliminary exploration of ASD symptoms reported in social media indicate large numbers of GI dysfunction, GI symptoms, early onset of bacterial infection and food allergies. The research extended this investigation to look at quantitative relationships between ASD, HP genotypes, and quantitative zonulin levels.

Although ELISA testing has been used as a "gold standard" method of testing for many diseases, this research suggested that molecular testing may prove to be a more sensitive, specific, and rapid way of testing for disease at an earlier stage. Real-time PCR testing can detect the presence of virus at early onset even before symptoms develop. The results from zonulin and haptoglobin testing for ASD showed no significant relationship between zonulin levels, haptoglobin genotype, and the presence of ASD. However, the presence of GI symptoms and the extremely high zonulin (>40ng/mL) in 10% of ASD and presence of zonulin at 2-5ng/mL in newborns compared to no zonulin detection in healthy controls suggest more research is needed. A larger sample population combined with more medical history and genome sequencing may provide answers needed to explain the mechanisms of zonulin and etiology of ASD. The qualitative social media data mining of ASD and GI symptoms using Radian6 supported the need for more genetic testing, specifically related to epigenetic and pharmacogenetic testing. The qualitative data indicated that genetic testing could lead toward more personalized treatment options whether or not it could be used for early diagnosis.

The need to reduce trial and error that occurs during the treatment process was a repeated theme throughout each chapter under no boundaries of disease type. In each case, genetic testing and a comprehensive patient history were the common factors with the ability to reduce trial and

error while maintaining quality of life. Of interest was the ability of qualitative data mining of social media to come to such conclusion regarding genetic testing and the ability to suggest a model of disease pathology that may be tested experimentally. A follow up study using the qualitative data would be to create a pathway model designed to use data collected from social media to model any disease pathway. All research suggests the need for a comprehensive family history and log of symptoms and reactions to obtain an early diagnosis. Like many diseases, ASD show patterns of early onset symptoms that may or may not be readily visible to the human eye. Therefore, effective early diagnostic testing is desirable to detect the presence of disease or specific symptoms with accompanying recommendations for treatment. Pharmacogenetic testing presents a viable option for diseases that do not currently have a confirmatory diagnostic test in that it can help determine treatment options based on the body's capacity to metabolize specific substances. Conclusively early diagnostic testing is desirable as long as the cost for such testing is minimal.

Testing for zonulin presents a new innovative method of detecting intestinal permeability. To date all of the tests associating intestinal permeability with ASD have been measured using LM tests (De Magistris et al., 2010; D'Eufemia et al., 1996). The LM test involves oral administration of a solution of mannitol and lactulose with subsequent assessment of small intestine absorption measured by a six hour urine collection (Cooper, 1984). For children, LM testing has been used to determine allergies and intestinal permeability, but the methods of oral administration and collection are very difficult to obtain due to the use of diapers and inability of children to anticipate the need for urination and bowel movements. Although this research detected no significant relationship between zonulin levels and ASD, it does not discount the use of zonulin ELISA tests as a replacement for LM testing. The research here presents the use of zonulin ELISA testing and HP genotyping as novel early diagnostic method for intestinal

permeability. The implementation of these methodologies represents a paradigm shift in the use of proteomic and genomic diagnostic testing from clinical diagnosis to pre-symptomatic testing. Early diagnostic testing may prevent or reduce altered developmental endpoints and may specifically define the severity of the related disease. Next steps in the development of early diagnostic testing would be to conduct experiments to compare the use of serum zonulin testing to LM testing to identify which is most effective and to understand their application on a global scale. It may also be of interest to incorporate this testing of diagnostic techniques with the current autism rating scales and treatment regimens to monitor adverse events. It is inevitable for testing methods to remain in a cycle of research and translation. Genetic recombination events and mutations do not stop with time they continue to produce new phenotypes; therefore, all testing standards, even "gold standards," need to be constantly re-evaluated to ensure they perform at optimal efficiencies. Finally, early diagnostic tests simply measured from blood collection could result in a rapid turn-around-time for reporting clinical results, future development of point-of-care assays, and provide direction and significance for continuing zonulin research.

An extension of zonulin research in relation to ASD is warranted by the differences in the zonulin concentrations between ASD individuals (M= 0.7523, SD = 1.6786), newborns (M= 7.5472, SD = 23.7714), and healthy controls (M= 0.2122, SD = 0.3274). With a larger samples size, it is possible that zonulin levels between groups will be significant. Future studies suggest repeating this exact experiment with at least 30 samples per group and extending the research to explore differences between ASD individuals with and without GI symptoms. Another aspect is to further explore zonulin levels from birth to adulthood to see how the levels may fluctuate related to changes in diet from nursing or formula to solid foods, and to study levels of zonulin from birth to adulthood in relation to disease development. Of additional interest would be

development of symptoms or disease over time in relation to the zonulin levels. Genome wide sequencing of all of these individuals may also help understand gene interactions related to intestinal permeability and patterns of inheritance. In addition to exploring zonulin levels, pharmacogenetics and epigenetics of ASD should be explored.

The array of symptoms and levels of severity related to ASD make it hard for a single intervention to work for all individuals. Pharmacogenetics and epigenetic studies of ASD provide opportunities to understand metabolism and methylation patterns. Based on results from social media, pharmacogenetic testing should focus on medications related to pain, neurological conditions, and gastrointestinal symptoms. The use of pharmacogenetic testing would make personalization of treatment an option to avoid adverse events and frustration experienced as a result of trial and error interventions.

In conclusion, future research will have translational importance in the development of novel early diagnostic testing for the prevention and aggressive treatment of disease. The development of diagnostic methodologies will lead to interventions that will improve quality of life for both the affected individuals and their care takers.

APPENDICES

APPENDIX A: GLOSSARY OF TERMS

TERM	DEFINITION	REFERENCE
Research Phase	The first phase within the knowledge to action framework that includes discovery, efficacy, effectiveness, and research supporting structures.	Wilson et al., 2011
Translation Phase	The second phase within the knowledge to action framework that includes translation, decisions to translate, knowledge into products, dissemination, engagement, decisions to adopt, practice, and translation supporting structures.	Wilson et al., 2011
Translation	The process of putting research into clinical practice, which is not dependent on a certain method of implementation or intervention.	Wilson et al., 2011
Early Diagnostics	A method that indicates presence/absence, risk, or severity of a disease or condition prior to the manifestation of symptoms or outside of the traditional guidelines for diagnosis.	

APPENDIX B: INSTITUTIONAL REVIEW BOARD APPLICATIONS AND ATTACHMENTS

Exempt Review Application
Clemson University (CU) Institutional Review Board (IRB) **(Version 9.7.2012)**
Clemson University IRB Website

Office use only ☐ Approved Exemption Category _____	Protocol Number:
_____ Signature of IRB Chair / Designee	_____ Date

1.	**Developmental Approval:** If you already have developmental approval for this research study (you should know if you do), please give the IRB protocol number assigned to the study. More information available here.

2.	**Research Title:**	Social Media: A functional tool for Autism Spectrum Disorders
	If different, title used on consent document(s)	
	If class project, include course number and title	NURS3980, Creative Inquiry

3.	**Principal Investigator (PI):** The PI must be a member of the Clemson faculty or staff. You cannot be the PI if this is your thesis or dissertation. The PI must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.	
	Name: Julie Eggert, PhD, RN, GNP, AOCN	☒ Faculty ☐ Staff
	Department: Nursing	E-mail: jaegger@clemson.edu
	Campus address: 528 Edwards Hall, Clemson, SC 29634-0743	Phone: 864-640-1869
		Fax:

4.	**Co-Investigator(s):** Co-Investigators must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.		
	Name: Rebecca A. Garcia		E-mail: ragarci@g.clemson.edu
	Department: Nursing		Phone: 864-421-3757
	☐ Faculty ☐ Staff	☒ Graduate student ☐ Undergraduate student	☐ Other. Please specify.
	Name: D. Matthew Boyer, PhD		E-mail: dmboyer@g.clemson.edu
	Department: Education		Phone: 864-656-0355
	☒ Faculty ☐ Staff	☐ Graduate student ☐ Undergraduate student	☐ Other. Please specify.

5.	**Additional Research Team Members:** All research team members must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.
	☒ List of additional research team members included. Form available here.

6. **Research Team Roles:** Describe the role of each member of the research team (everyone included in Items 3, 4 and 5), indicating which research activities will be carried out by each particular member. Team members may be grouped into categories.

   Description: Rebecca Garcia will design the project, collect data, and analyze the results. Erika McMillion will collect data and assist in analysis. Dr. Julie Eggert will oversee the project and provide advice or editing as needed. Dr. Boyer will provide guidance for data analysis and coding as needed.

7. **Email Communications:** If you would like one or two of your team members (in addition to the PI) to be copied on all email communications, please list these individuals in the box below.

Name: Rebecca Garcia	E-mail: ragarci@g.clemson.edu
Name:	E-mail:

8. **Study Purpose:** Provide a brief description of the purpose of the study. Use lay language and avoid technical terms. IRB members not familiar with the area of research must understand the nature of the research. Upon conclusion of the study, how will you share your results (e.g., academic publication, evaluation report to funder, conference presentation)?

   Description: This query of public information using Radian6 will explore the ways that social media is used to report physical symptoms of Autism Spectrum disorders. Specifically it is known that modulation of the intestinal tight junctions allows macromolecules to pass into the bloodstream causing gastrointestinal and neurological symptoms. So far the symptoms have been correlated to autoimmune diseases and cancer, but there is reason to believe that similar gastrointestinal and neurological symptoms may be seen in persons with autism. This study will use 3 months of public information to identify symptoms of autism described through social media. A software (Radian6) designed to assist with queries of social media will be used to assist with data collection. Radian6 data from social media will be analyzed to identify symptoms unique to autism implicating pathways to increased risk of this disorder. The aim of this study is to understand the impact of social media on self-management of ASD with co-morbid intestinal manifestations. Analysis of the data from the public domain will include identification of common symptoms and/or thematic elements used through social media for the self-management of ASD with gastrointestinal issues. The examination of relationships between concerns of ASD with co-morbid intestinal abnormalities and conversation threads will also be compared. All unique identifiers will be removed and quotes will be paraphrazed to protect companies and individuals. The results will be submitted for peer reviewed journal publication and conference presentation.

9. **Anticipated Dates of Research:**

   Anticipated start date (may not be prior to IRB approval; may be "upon IRB approval"): upon IRB approval

   Anticipated completion date (Please include time needed for analysis of individually identifiable data): 12 months after start date

10. Funding Source: Please check all that apply.

☐ Submitted for internal funding
☐ Internally funded
☐ Submitted for external funding
Funding source, if applicable (Do not use initials): _____
Proposal number (PPN) for the Office of Sponsored Programs: _____
Name of PI on Funding Proposal: _____
☐ Externally funded
Funding source, if applicable (Do not use initials): _____
Proposal number (PPN) for the Office of Sponsored Programs: _____
Name of PI on Funding Proposal: _____
☐ Intend to seek funding From whom? _____
☒ Not funded

11. Support provided by Creative Inquiry Initiative: ☒ Yes ☐ No

If yes, all Creative Inquiry students will be members of the research team, please see item # 5.

12. Other IRB Approvals:

Has this research study been presented to any other IRB? ☐ Yes ☒ No

Where? _____ When? _____

If yes, what was their decision? ☐ Approved ☐ Disapproved ☐ Pending

Please attach a copy of any submissions, approvals, or disapprovals from other IRBs.

13. Exempt Review Checklist: To determine whether this study meets the federal requirements for exemption [45 CFR 46.101], please complete the following checklist. This will indicate if your study can be exempted from IRB continuing review.

The Federal Code [45 CFR 46.101] permits research activities in the following six categories to be exempted. Please check the relevant exemption category / categories.
The Federal Office of Human Research Protections has made Decision Charts available here to help in determining whether a particular study falls within a particular Exemption Category.

	Categories of Research Activities Exempt from Continuing Review
☐	**B1**. Research conducted in established or commonly accepted educational settings, involving normal educational practices, such as: a. research on regular and special education instructional strategies, OR b. research on the effectiveness of or the comparison among instructional techniques, curricula, or classroom management methods. NOTE: Survey and interview procedures with minors are exemptible if the activities fall within this category.
☐	**B2**. Research involving the use of educational tests (cognitive, diagnostic, aptitude, achievement), survey procedures, interview procedures or observation of public behavior, UNLESS: a. the information obtained is recorded in such a manner that human participants can be identified, directly or through identifiers linked to the participants; AND

	b. any disclosure of the human participants' responses outside the research could reasonably place the participants at risk of criminal or civil liability or be damaging to the participants' financial standing, employability, or reputation. NOTE: Survey and interview techniques which include minors are not exempt. Observation of the public behavior of minors, if the researcher is not a participant, is exempt.
☐	**B3**. Research involving the use of educational tests (cognitive, diagnostic, aptitude, achievement), survey procedures, interview procedures, or observation of public behavior **that is not exempt under Category B2**, if: a. the human participants are elected or appointed public officials or candidates for public office, or b. federal statute(s) require(s) without exception that the confidentiality of the personally identifiable information will be maintained throughout the research and thereafter.
☒	**B4**. Research, involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that participants cannot be identified directly or through identifiers linked to the participants.
☐	**B5. NOTE: Please contact the IRB office before selecting this category since use of this exemption must be initiated by the agency head of the federal funder.** Research and demonstration projects which are conducted by or subject to the approval of appropriate Federal Department or Agency heads, and which are designed to study, evaluate, or otherwise examine: a. public benefit or service programs; or b. procedures for obtaining benefits or services under those programs; or c. possible changes in or alternatives to those programs or procedures; or d. possible changes in methods or levels of payment for benefits or services under those programs.
☐	**B6**. Taste and food quality evaluation and consumer acceptance studies, a. if wholesome foods without additives are consumed, OR b. if a food is consumed that contains a food ingredient at or below the level and for a use found to be safe, or agricultural chemical or environmental contaminant at or below the level found to be safe, by the Food and Drug Administration or approved by the Environmental Protection Agency or the Food Safety and Inspection Service of the U.S. Department of Agriculture.

14. If you selected Exemption Category B4, please complete questions a through g below:

a. Provide a detailed description of the data or specimens and what information will be used. Radian6 data from public domain searches will be collected as *.csv files, screen shots, and data collector written observations.

b. What is the source of the data or specimens? Radian6 information collected from the Internet in public domain.

c. Are the data or specimens publicly available without restriction or password? (That is, can the general public obtain the data or specimens? Data are not considered publicly available if access is limited to researchers.)
Yes ☒ No ☐
If yes, please contact the IRB staff for consultation. You may not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46.102).

d. If the data or specimens are not publicly available, how are you obtaining permission to access these or to

use them for research purposes? Data from Radian 6 is publicly available via internet search.
Please attach a copy of the correspondence or agreement granting you permission.

e. How will you receive the data or specimens (e.g., electronic file, access to hard copy records at record-holder's institution, test tube)? Electronic file transfer from Radian6 as *.csv files or screen shots and data collector observation from Radian6 data.

f. How are the data or specimens identified when they are made available to you?
1) ☐ Direct Identifier (e.g., subject name, address, social security number).
a) Will you record any direct identifiers that are available to you? Yes* ☐ No ☐
b) Will you have access to the data from home or office? Yes* ☐ No ☐

2) ☐ Indirect Identifier (e.g., an assigned code that could be used by the investigator or the source providing the data or specimens to identify a subject, such as a pathology tracking number or a tracking code used by the source).
a) Will you or a team member have access to the data set code key? Yes* ☐ No ☐
If you will receive data with indirect identifiers only, please contact the IRB staff for consultation. You may not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46.102).

3) ☒ No Identifier (i.e., neither the researcher nor the source providing the data or specimens can identify a subject based upon information provided with the data or specimens).
If it will be impossible for anyone to identify subjects based upon information provided with the data or specimens, you will not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46). Please contact the IRB staff for confirmation.

g. Will any data or specimens be collected from participants after the submission of this application? (Data or specimens are considered to "exist" if ALL the data or specimens to be used for the research have been collected prior to the submission of this application.)
Yes* ☐ No ☒

*Your research does not qualify for exemption from IRB review under Exemption Category B4.

PLEASE NOTE: If you are applying for exemption only under Exemption Category B4, please skip to question 22.

15. Study Sample: (Groups specifically targeted for study)

Describe the participants you plan to recruit and the criteria used in the selection process. Indicate if there are any special inclusion or exclusion criteria.

NOTE: If individuals who are incarcerated will be participants, your research is not exemptible. Please complete the Expedited / Full Review Application.

Description: This exploratory query of public data will use the Radian6 software located in the Social Media Learning Center (SMLC) at Clemson University to collect public data over a 3 month period. The software will search for "Autism Spectrum Disorder AND Gastrointestinal Symptoms" or any synonyms or shorthand terms for the search words. This data from Radian6 will further determine at least 2 representatives from each of the following entities: foundations, health institutions, traditional media, and persons directly/indirectly affected by ASD. At this point, the data collected will include screen shots of social media and internet pages from each organization that are tagged by the Radian6 software, export data as *.csv files from the Radian6 software, and data collector notes and interpretation recorded in a journal. The content will be uploaded into NVivo for descriptive analysis and coding. Unique identifiers for individuals and companies will be removed from all data collected from each organization (this may include removal of screen names, logos, or other personal information). All quotes will be paraphrased to protect the identity of human subjects and

organizations. The data collected from this project will not be distributed in public forum. The following inclusion and exclusion criteria must be met by each entity:
Inclusion Criteria:
1. The entity must have a primary focus or targeted project focused on ASD with mention of gastrointestinal issues.
2. The entity must have some form of social media: a link, group, blog page, ongoing conversation, or an inquiry system directly connected to their webpage.
3. Information from each entity must be in the public domain.

Exclusion Criteria:
1. Information will be excluded from sources that require membership dues, site registration, or websites that are created and supported for personal interests.
2. Information from organizations or institutions will be excluded if social media information appears to be left in a dormant state for a period of more than 3 months.
3. Information will be excluded for extensive derogatory comments or content.
4. Information will be excluded from items tagged in the search that provide false leads, such as advertisement or sub-headline on a page discussing other information.

Preference will be given to entities that focus on ASD with gastrointestinal issues. Research will be performed online as information is distributed in public domain.

Collected, coded, and analyzed data for this project will be stored in a password protected computer in a locked office. Due to the fact that the original data is public domain it may be possible to access it from any computer with Internet.

Age range of participants: Any Projected number of participants: 1,000,000

- ☒ Employees
- ☒ Students
- ☒ Minors (under 18) [1]
- ☒ Pregnant women [1]
- ☐ Fetuses / neonates [1]
- ☒ Educationally / economically disadvantaged [1]
- ☒ Minors who are wards of the state, or any other agency, institution, or entity [1]
- ☒ Individuals who are incarcerated [2]
- ☒ Persons incompetent to give valid consent [1]
- ☒ Other–specify: Elders or anyone with access to public information
- ☒ Military personnel

[1] State necessity for using this type of participant: it is a possibility because it is public information available through the Internet

[2] Please note that research involving prisoners (incarcerated individuals) requires full board review. Please submit an Expedited / Full Board Review Application and a Prisoner Research Addendum (available here).

16. Study Locations:

- ☒ Clemson University
- ☐ Other University / College _____
- ☐ School System / Individual Schools _____
- ☐ Other – specify _____

You may need to obtain permission if participants will be recruited or data will be obtained through schools, employers, or community organizations. Are you required to obtain permission to gain access to people or to access data that are not publicly available? If yes, provide a research site letter from a person authorized to give you access to the participants or to the data. Guidance regarding Research Site Letters is available here.

- ☒ Research Site Letter(s) not required.
- ☐ Research Site Letter(s) attached.
- ☐ Research Site Letter(s) pending and will be provided when obtained.

17. Recruitment Method:

Describe how research participants will be recruited in the study. How will you identify potential participants? How will you contact them? **Attach a copy of any material you will use to recruit participants (e.g., advertisements, flyers, telephone scripts, verbal recruitment, cover letters, or follow-up reminders).**

Description: No contact with participants will be required. All material for the study will be collected using Radian6 and the information will be that of companies, organizations, or individual persons placed in public domain.

18. Participant Incentives:

a. Will you pay participants? ☐ Yes ☒ No

Amount: $_____ When will money be paid?: _____

b. Will you give participants incentives / gifts / reimbursements? ☐ Yes ☒ No

Describe incentives / gifts / reimbursements: _____

Value of incentives / gifts / reimbursements: $_____

When will incentives / gifts / reimbursements be given?: _____

c. Will participants receive course credit? ☐ Yes ☒ No

d. Will participants receive extra credit? ☐ Yes ☒ No

If yes, an equivalent alternative to research participation must be provided and described in your informed consent document(s).

19. Informed Consent:

a. Attach a copy of the informational letter or consent script you plan to provide to your participants (and their parents or guardians, if applicable). Consent Document Templates

b. Will you use concealment (incomplete disclosure) or deception in this study? ☐ Yes ☒ No
If yes, please see guidance regarding Research Involving Deception or Concealment here, submit a copy of the Additional Pertinent Information / Permission for Use of Data Collected in a Research Study form you will use, and provide a justification in the following space for this use of concealment or deception. _____

20. Procedures:

a. What data will you collect? Data will include *csv files, screen shots, and internet pages tagged by the Radian6 software. It will also include data collector notes and interpretation recorded in a journal.

b. Please describe in detail the process each participant will experience and how you will obtain the data. I will obtain the data from searches using Radian6 software, which will cover a 3 month period. For example, the first collection will include the previous ~90 days.

c. How many participation sessions and how much time will be required for each participant, including follow up sessions? None

d. How will you collect data?

☐ in-person contact	☐ telephone
☐ snail mail	☐ email
☒ website	☒ other, describe Radian6

Please include copies of surveys, interview questions, data collection tools and debriefing statements. If survey or interview questions have not been fully developed, provide information on the types of questions to be asked, or a description of the parameters of the survey / interview. Please note: finalized survey or interview instruments will need to be reviewed and approved by amendment, before implementation.

e. Will you audio record participants? ☐ Yes ☒ No
f. Will you video record participants? ☐ Yes ☒ No
g. Will you photograph participants? ☐ Yes ☒ No

If you will audio or video record or take identifiable photographs of participants, please consult the IRB's Guidance on the Use of Audio / Video Recording and Photography here. Please include all the information addressed by this guidance document in the application and, where appropriate, in the consent document(s).

21. Protection of Confidentiality: Describe the security measures you will take to protect the confidentiality of the information obtained. Will participants be identifiable either by name or through demographic data? If yes, how will you protect the identity of the participants and their responses? Where will the data be stored and how will it be secured? Who will have access to the data? How will identifiers be maintained or destroyed after the study is completed?

Description: Unique identifiers for individuals and companies will be removed from all data collected from each organization (this may include removal of screen names, logos, or other personal information). All quotes will be paraphrased to protect the identity of human subjects and organizations. The data collected from this project will not be distributed in public forum. Data collected will be stored in NVivo for analysis, *.csv files on a drive for conversion to analysis computers (suitable for file sizes), and written data collector notes in a jounal that will be transcribed to NVivo and computer drive. Only the research team and PI will have access to the collected data and analysis. Once all unique identifiers have been removed the new data file will be stored in a separate file. All data will be collected and maintained on a password protected computer in a locked office. After completion of the study all data may be deleted from all hard drives and analysis devices.

22. PI Signature:

I have reviewed this research protocol and the informed consent document(s), if applicable. I request approval of this research study by the IRB of Clemson University.

Conflict of Interest Statement:

Could the results of the study provide an actual or potential financial gain to you, a member of your family, or any of the co-investigators, or give the appearance of a potential conflict of interest?

☒ No.

☐ Yes. I agree to disclose any actual or potential conflict of interest prior to IRB action on this study.
Financial Conflict of Interest Policy for PHS / NIH Supported Research
Financial Disclosure Policy for All Other Sponsored Programs
Disclosure Statement for All Other Sponsored Programs

Signature of Principal Investigator _____ Date _____

(hard-copy signature only needed if application will not be submitted via PI's email account)

Submission Instructions: Exempt applications are processed as received. There is no deadline for submitting exempt applications for review. Please allow seven to ten business days for processing.

Please submit this application and all associated documents from the Principal Investigator's (PI's) email address to the IRB staff. Receipt of the application electronically from the PI will qualify the application as a signed electronic submission. Alternatively, the signed, hard-copy application may be mailed or delivered to the Office of Research Compliance, 223 Brackett Hall, Clemson, SC 29634-5704.

Request for Amendment
Clemson University Institutional Review Board (IRB) **(Version 10.28.2011)**
Clemson University IRB Website

Office use only

For Expedited and Full Protocols

☐ Approved ☐ Disapproved

For Exempt Protocols

☐ Validated as continuing to meet the criteria for Exempt status
☐ Not validated as continuing to meet the criteria for Exempt status

Signature of IRB Chair / Designee _____ Date _____

Protocol Number:	IRB2013-340
Research Title:	Social Media: A Functional Tool for Autism Spectrum Disorders"
Principal Investigator:	Julie Eggert, PhD, RN, GNP, AOCN

1. **Type of Amendment Request:** Check all that apply. Be sure to attach any new or revised documents, with changes highlighted or electronically shaded.

☐ Protocol Change or Amendment
☐ Change to Subject Selection Criteria
☐ Subject Recruitment Methods
☐ Other (please specify): _____
☐ Change to Data Collection Tools or Procedure
☐ Consent Form Changes
☒ Editorial/Administrative/Personnel Changes

2. **Summary:** Provide a brief description of changes and rationale.

Description: The only change is to add an additional research team member, Bohua Wu, to help code the extensive amount of data collected. By adding this team member, we will be able to maintain our deadlines.

☐ I am the principal investigator. I am submitting this form electronically and this submission constitutes my signature.

Principal investigator signature: _____ Date: _____

Expedited / Full Board Review Application for Data Set Analysis
Clemson University (CU) Institutional Review Board (IRB) **(Version 9.7.2012)**
Clemson University IRB Website

Office use only	Protocol Number:
Approved ☐ Expedited ☐ Full Board	Expiration date: _____
_____ Signature of IRB Chair / Designee	_____ Date

Level of Review (Questions 13 & 14 determine if the protocol can be expedited): ☐ Expedited ☐ Full Board

1.	**Developmental Approval:** If you already have developmental approval for this research study (you should know if you do), please give the IRB protocol number assigned to the study. More information available here.

2.	**Research Title:**	Zonulin Pathway to Autism Spectrum Disorders
	If different, title used on consent document(s)	
	If class project, include course number and title	

3.	**Principal Investigator (PI):** The PI must be a member of the Clemson faculty or staff. You cannot be the PI if this is your thesis or dissertation. The PI must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.	
	Name: Julie Eggert, PhD, RN, GNP, AOCN	☒ Faculty ☐ Staff
	Department: Nursing	E-mail: jaegger@clemson.edu
	Campus address: 528 Edwards Hall, Clemson, SC 29634-0743	Phone: 864-640-1869
		Fax:

4.	**Co-Investigator(s):** Co-Investigators must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.		
	Name: Rebecca A. Garcia		E-mail: ragarci@clemson.edu
	Department: Nursing		Phone: 864-421-3757
	☐ Faculty ☐ Staff	☒ Graduate student ☐ Undergraduate student	☐ Other. Please specify.
	Name:		E-mail:
	Department:		Phone:
	☐ Faculty ☐ Staff	☐ Graduate student ☐ Undergraduate student	☐ Other. Please specify.

5.	**Additional Research Team Members:** All research team members must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.
	☐ List of additional research team members included. Form available here.

6. **Research Team Roles:** Describe the role of each member of the research team (everyone included in Items 3, 4 and 5), indicating which research activities will be carried out by each particular member. Team members may be grouped into categories.

   Description: Rebecca Garcia will design the project, collect data, and analyze the results. Dr. Julie Eggert will oversee the project and provide advice or editing as needed.

7. **Email Communications:** If you would like one or two of your team members (in addition to the PI) to be copied on all email communications, please list these individuals in the box below.

Name: Rebecca Garcia	E-mail: ragarci@clemson.edu
Name:	E-mail:

8. **Study Purpose:** Provide a brief description of the purpose of the study. Use lay language and avoid technical terms. IRB members not familiar with the area of research must understand the nature of the research. Upon conclusion of the study, how will you share your results (e.g., academic publication, evaluation report to funder, conference presentation)?

   Description: Zonulin is a complex protein structure that regulates epithelial tight junctions in the intestine (Tripathi et al., 2009). Increased zonulin levels in human sera allow macromolecules to pass through tight junctions causing altered immune responses leading to a variety of auto-immune diseases. De Magistris et al. (2010) discovered that abnormal intestinal permeability is present with autism spectrum disorder (ASD). This study will determine if increased levels of zonulin are directly associated with an increased risk of autism spectrum disorders, it will identify correlations among zonulin levels and the available observational and quantitative scores used to diagnose the level of autism as suitable for the age range, and it will look at variations among race in phenotypic assessment data and zonulin levels. The National Database for Autism Research (NDAR) will be accessed to compare phsyiological symptoms of autism with those associated with zonulin. The NDAR is a collection of previously collected data including demographic and genomic data from families and affected individuals with autism spectrum disorder. Once obtaining Clemson IRB approval, we will need to submit application for access to the NDAR database. By looking for data such as gender, ethnicity, weight, height, BMI, hip to waist ratios, genomic/proteomic profiles, autism rating scales, symptoms, and comorbid disease, we will be able to identify common traits between autism and zonulin. The results are expected to determine if zonulin testing could be used to identify a population of autism with GI disorders. The results will be published and submitted for conference presentation.

9. **Anticipated Dates of Research:**

   Anticipated start date (may not be prior to IRB approval; may be "upon IRB approval"): upon IRB approval

   Anticipated completion date (Please include time needed for analysis of individually identifiable data): one year from approval date

10. **Funding Source:** Please check all that apply.

☐ Submitted for internal funding
☒ Internally funded
☐ Submitted for external funding
Funding source, if applicable (Do not use initials): _____
Proposal number (PPN) for the Office of Sponsored Programs: _____
Name of PI on Funding Proposal: _____
☐ Externally funded
Funding source, if applicable (Do not use initials): _____
Proposal number (PPN) for the Office of Sponsored Programs: _____
Name of PI on Funding Proposal: _____
☐ Intend to seek funding From whom? _____
☐ Not funded

11. Support provided by Creative Inquiry Initiative: ☐ Yes ☒ No

If yes, all Creative Inquiry students will be members of the research team, please see item # 5.

12. Other IRB Approvals:

Has this research study been presented to any other IRB? ☐ Yes ☒ No

Where? _____ When? _____

If yes, what was their decision? ☐ Approved ☐ Disapproved ☐ Pending

Please attach a copy of any submissions, approvals, or disapprovals from other IRBs.

13. Level of Risk: Does this project include any procedures that present more than minimal risk to the participants? (A project is considered to present minimal risk if the probability and magnitude of harm or discomfort anticipated in the research are not greater than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations.)
☐ Yes ☒ No

If your study presents no more than minimal risk to participants, your study may be eligible for expedited review.

14. Expedited Review Categories: The Code of Federal Regulations [45 CFR 46.110] permits research activities to undergo expedited review if they fall within the following category.
The Federal Office of Human Research Protections has made Decision Charts available here to help in determining whether a particular study may be reviewed using Expedited Review Procedures.

Research involving materials (data, documents, records, or specimens) that have been collected or will be collected solely for non-research purposes (such as medical treatment or diagnoses).

Does your study fall within this category? Yes ☒ No ☐

15. Data Set(s):

a. What are the types of data? Data, documents, records that have been previously collect and submitted to NDAR

b. What is the source of the data? NDAR

Note: If your study involves prisoners, please complete and attach the Prisoner Research Addendum.

c. Are the data publicly available? (That is, can the general public obtain the data? Data are not considered publicly available if access is limited to researchers.)
Yes ☐ No ☒
If yes, please contact the IRB staff for consultation. You may not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46.102).

d. If the data or specimens are not publicly available, how are you obtaining permission to access these or to use them for research purposes? Must have CLemson IRB approval to apply for NDAR access
Please attach a copy of the correspondence or agreement granting you permission.

e. Does the data holder require IRB review of your study in order to release the data to you?
Yes ☒ No ☐

f. How will the data be made available to you (e.g., electronic file, access to hard copy records at record-holder's institution)? electronic files through password protected database

g. How are the data or specimens identified when they are made available to you?
1) ☐ Direct Identifier (e.g., subject name, address, or social security number).
2) ☒ Indirect Identifier (e.g., an assigned code that could be used by the investigator or the source providing the data or specimens to identify a subject, such as a pathology tracking number or a tracking code used by the source).
If you will receive data with indirect identifiers only, please contact the IRB staff for consultation. You may not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46.102).
3) ☐ No Identifier (i.e., neither the researcher nor the source providing the data or specimens can identify a subject based upon information provided with the data).
If it will be impossible for anyone to identify subjects based upon information provided with the data or specimens, you will not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46). Please contact the IRB staff for confirmation.

16. Protection of Confidentiality: Describe the security measures you will take to protect the confidentiality of the information obtained. Will participants be identifiable either by name or through demographic data? If yes, how will you protect the identity of the participants and their responses? Where will the data be stored and how will it be secured? Who will have access to the data? How will identifiers be maintained or destroyed after the study is completed?

Description: All subjects will have an assigned identifier that has been assigned by the NDAR curators and not identifiable to our researchers. The data collected will be stored on a password protected computer that is in a locked and secure office. Only the PI and the research team member will have access to the data for the duration of the study. Upon completion of the study all files will be deleted.

17. Risk / Benefit Analysis:

a. Describe all potential risks (before protective measures are put into place) and benefits for this study. Risks can include physical, psychological, social, legal or other risks connected with the proposed procedures. Benefits can include benefits to the participant or to society in general.

Description: Potential risk includes public exposure of medical record data. The benefits include a better understanding of zonulin and autism spectrum disorders, possibly leading to use of zonulin testing as a diagnostic test for autism.

b. Describe the procedures to be used to protect against or minimize potential risks. Assess the likely effectiveness of these procedures.

Description: All subjects will have a unique identifier that is not traceable by the research team. All data will be stored on a password protected and secured computer.

18. Agreement, Statement of Assurance, and Conflict of Interest Statement by the PI:

I have reviewed this research protocol and the consent form, if applicable. I have also evaluated the scientific merit and potential value of the proposed research study, as well as the plan for protecting human participants. I have read the Terms of Assurance held by Clemson University and commit to abiding by the provisions of the Assurance and the determinations of the IRB. I request approval of this research study by the IRB of Clemson University.

I understand that failure to adhere to any of these guidelines may result in immediate termination of the research. I also understand that approval of this research study is contingent upon my agreement to:

1. Report to the IRB any adverse events, research-related injuries or unexpected problems affecting the rights or safety of research participants (All such occurrences must be reported to the IRB within three (3) working days.);
2. Submit in writing for IRB approval any proposed revisions or amendments to this research study;
3. Submit timely continuing review reports of this research as requested by the IRB; and
4. Notify the IRB upon completion of this research study.

Conflict of Interest Statement:

Could the results of the study provide an actual or potential financial gain to you, a member of your family, or any of the co-investigators, or give the appearance of a potential conflict of interest?

☒ No.

☐ Yes. I agree to disclose any actual or potential conflict of interest prior to IRB action on this study.
Financial Conflict of Interest Policy for PHS / NIH Supported Research
Financial Disclosure Policy for All Other Sponsored Programs
Disclosure Statement for All Other Sponsored Programs

_____ _____

Signature of Principal Investigator Date

19. Statement of Assurance by Department Chair (or supervisor if PI is Department Chair):

I have reviewed this research protocol and the consent form, if applicable. I verify this proposed research study has received approval in accordance with department procedures. I have evaluated the plan for protecting human participants. I have read the Terms of Assurance held by Clemson University and commit to abiding by the provisions of the Assurance and the determinations of the IRB. I request approval of this research study by the IRB of Clemson University.

_____

Department Chair or supervisor if PI is Department Chair (Printed Name)

_____ _____

Signature of Department Chair Date

Exempt Review Application
Clemson University (CU) Institutional Review Board (IRB) **(Version 6.1.2013)**
Clemson University IRB Website

Office use only	Protocol Number:
☐ Approved Exemption Category _____	Expiration date: _____
_____ Signature of IRB Chair / Designee	_____ Date

1.	**Developmental Approval:** If you already have developmental approval for this research study (you should know if you do), please give the IRB protocol number assigned to the study. More information available here.

2.	**Research Title:**	Prehaptoglobin 2 ELISA and Haptoglobin Genotyping for Autism Spectrum Disorder
	If different, title used on consent document(s)	
	If class project, include course number and title	

3.	**Principal Investigator (PI):** The PI must be a member of the Clemson faculty or staff. You cannot be the PI if this is your thesis or dissertation. The PI must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.	
	Name: Julie Eggert, PhD, RN, GNP, AOCN	☒ Faculty ☐ Staff
	Department: Nursing	E-mail: jaegger@clemson.edu
	Campus address: 528 Edwards Hall, Clemson, SC 29634-0743	Phone: 864-640-1869
		Fax:

4.	**Co-Investigator(s):** Co-Investigators must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.		
	Name: Rebecca Garcia		E-mail: ragarci@clemson.edu
	Department: Nursing		Phone: 864-421-3757
	☐ Faculty ☐ Staff	☒ Graduate student ☐ Undergraduate student	☐ Other. Please specify.
	Name: Patricia Tate, PhD		E-mail: ptate@clemson.edu
	Department: Nursing		Phone: 864-656-3666
	☐ Faculty ☒ Staff	☐ Graduate student ☐ Undergraduate student	☐ Other. Please specify.

5.	**Additional Research Team Members:** All research team members must have completed IRB-approved human research protections training. Training will be verified by IRB staff before approval is granted. Training instructions available here. CITI training site available here.
	☐ List of additional research team members included. Form available here.

6. **Research Team Roles:** Describe the role of each member of the research team (everyone included in Items 3, 4 and 5), indicating which research activities will be carried out by each particular member. Team members may be grouped into categories.

   Description: Rebecca Garcia has designed the primers and porbes for the genotyping experiment. She will perform all laboratory testing and analyze the results. Dr. Julie Eggert will oversee the project and provide advice or editing as needed. Dr. Patricia Tate will oversee laboratory compliance, use of equipment, ordering supplies, and receiving supplies or samples.

7. **Email Communications:** If you would like one or two of your team members (in addition to the PI) to be copied on all email communications, please list these individuals in the box below.

Name: Rebecca Garcia	E-mail: ragarci@clemson.edu
Name: Patricia Tate	E-mail: ptate@clemson.edu

8. **Study Purpose:** Provide a brief description of the purpose of the study. Use lay language and avoid technical terms. IRB members not familiar with the area of research must understand the nature of the research. Upon conclusion of the study, how will you share your results (e.g., academic publication, evaluation report to funder, conference presentation)?

   Description: The study will determine prehaptoglobin 2 levels in serum from banked samples, and it will determine haptoglobin genotype of banked genomic DNA samples. The results from each test will be statistically analyzed between banked samples positive for autism spectrum disorders and normal healthy controls. The samples will be obtained from the Greenwood Genetic Center as banked serum and genomic DNA that has been striped of all identifiers. The samples will be received with untrackable sequential numbers and indication of control or ASD status, ethnicity, age, gender and GI symptoms (if available). The results will be published in a peer reviewed journal and submitted for poster presentation.

9. **Anticipated Dates of Research:**

   Anticipated start date (may not be prior to IRB approval; may be "upon IRB approval"): upon IRB approval

   Anticipated completion date (Expiration date will be determined by the date entered, maximum three years for initial approval with optional extensions. Please include time needed for analysis of individually identifiable data.): 5/01/2015

10. **Funding Source:** Please check all that apply.

    ☐ Submitted for internal funding
    ☒ Internally funded
    ☐ Submitted for external funding
    Funding source, if applicable (Do not use initials): _____
    Proposal number (PPN) for the Office of Sponsored Programs: _____
    Name of PI on Funding Proposal: _____

☐ Externally funded
Funding source, if applicable (Do not use initials): _____
Proposal number (PPN) for the Office of Sponsored Programs: _____
Name of PI on Funding Proposal: _____
☐ Intend to seek funding From whom? _____
☐ Not funded

11. Support provided by Creative Inquiry Initiative: ☐ Yes ☒ No

If yes, all Creative Inquiry students will be members of the research team, please see item # 5.

12. Other IRB Approvals:

Has this research study been presented to any other IRB? ☐ Yes ☒ No

Where? _____ When? _____

If yes, what was their decision? ☐ Approved ☐ Disapproved ☐ Pending

Please attach a copy of any submissions, approvals, or disapprovals from other IRBs.

13. Exempt Review Checklist: To determine whether this study meets the federal requirements for exemption [45 CFR 46.101], please complete the following checklist. This will indicate if your study can be exempted from IRB continuing review.

The Federal Code [45 CFR 46.101] permits research activities in the following six categories to be exempted. Please check the relevant exemption category / categories.
The Federal Office of Human Research Protections has made Decision Charts available here to help in determining whether a particular study falls within a particular Exemption Category.

	Categories of Research Activities Exempt from Continuing Review
☐	**B1**. Research conducted in established or commonly accepted educational settings, involving normal educational practices, such as: a. research on regular and special education instructional strategies, OR b. research on the effectiveness of or the comparison among instructional techniques, curricula, or classroom management methods. NOTE: Survey and interview procedures with minors are exemptible if the activities fall within this category.
☐	**B2**. Research involving the use of educational tests (cognitive, diagnostic, aptitude, achievement), survey procedures, interview procedures or observation of public behavior, UNLESS: a. the information obtained is recorded in such a manner that human participants can be identified, directly or through identifiers linked to the participants; AND b. any disclosure of the human participants' responses outside the research could reasonably place the participants at risk of criminal or civil liability or be damaging to the participants' financial standing, employability, or reputation. NOTE: Survey and interview techniques which include minors are not exempt. Observation of the public behavior of minors, if the researcher is not a participant, is exempt.

☐	**B3**. Research involving the use of educational tests (cognitive, diagnostic, aptitude, achievement), survey procedures, interview procedures, or observation of public behavior **that is not exempt under Category B2**, if: a. the human participants are elected or appointed public officials or candidates for public office, or b. federal statute(s) require(s) without exception that the confidentiality of the personally identifiable information will be maintained throughout the research and thereafter.
☒	**B4**. Research, involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that participants cannot be identified directly or through identifiers linked to the participants.
☐	**B5. NOTE: Please contact the IRB office before selecting this category since use of this exemption must be initiated by the agency head of the federal funder.** Research and demonstration projects which are conducted by or subject to the approval of appropriate Federal Department or Agency heads, and which are designed to study, evaluate, or otherwise examine: a. public benefit or service programs; or b. procedures for obtaining benefits or services under those programs; or c. possible changes in or alternatives to those programs or procedures; or d. possible changes in methods or levels of payment for benefits or services under those programs.
☐	**B6**. Taste and food quality evaluation and consumer acceptance studies, a. if wholesome foods without additives are consumed, OR b. if a food is consumed that contains a food ingredient at or below the level and for a use found to be safe, or agricultural chemical or environmental contaminant at or below the level found to be safe, by the Food and Drug Administration or approved by the Environmental Protection Agency or the Food Safety and Inspection Service of the U.S. Department of Agriculture.

14. If you selected Exemption Category B4, please complete questions a through g below:

a. Provide a detailed description of the data or specimens and what information will be used. banked serum and genomic DNA with no unique identifiers along with indication of control or ASD status, ethnicity, age, gender and GI symptoms (if available)

b. What is the source of the data or specimens? Greenwood Genetic Center banked samples

c. Are the data or specimens publicly available without restriction or password? (That is, can the general public obtain the data or specimens? Data are not considered publicly available if access is limited to researchers.)
Yes ☐ No ☒
If yes, please contact the IRB staff for consultation. You may not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46.102).

d. If the data or specimens are not publicly available, how are you obtaining permission to access these or to use them for research purposes? Permission obtained through correspondence with the Greenwood Genetic Center
Please attach a copy of the correspondence or agreement granting you permission.

e. How will you receive the data or specimens (e.g., electronic file, access to hard copy records at record-holder's institution, test tube)? Aliquot tubes with no identifiers that are labeled with random numbers and indication of control or ASD status, ethnicity, age, gender and GI symptoms (if available)

f. How are the data or specimens identified when they are made available to you?

1) ☐ Direct Identifier (e.g., subject name, address, social security number).
   a) Will you record any direct identifiers that are available to you? Yes* ☐ No ☐
   b) Will you have access to the data from home or office? Yes* ☐ No ☐

2) ☒ Indirect Identifier (e.g., an assigned code that could be used by the investigator or the source providing the data or specimens to identify a subject, such as a pathology tracking number or a tracking code used by the source).
   a) Will you or a team member have access to the data set code key? Yes* ☐ No ☒
   If you will receive data with indirect identifiers only, please contact the IRB staff for consultation. You may not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46.102).

3) ☐ No Identifier (i.e., neither the researcher nor the source providing the data or specimens can identify a subject based upon information provided with the data or specimens).
   If it will be impossible for anyone to identify subjects based upon information provided with the data or specimens, you will not be conducting research involving human subjects as defined in the federal regulations governing research involving human subjects (45 CFR 46). Please contact the IRB staff for confirmation.

g. Will any data or specimens be collected from participants after the submission of this application? (Data or specimens are considered to "exist" if ALL the data or specimens to be used for the research have been collected prior to the submission of this application.)
   Yes* ☐ No ☒

*Your research does not qualify for exemption from IRB review under Exemption Category B4.

PLEASE NOTE: If you are applying for exemption only under Exemption Category B4, please skip to question 22.

15. Study Sample: (Groups specifically targeted for study)

Describe the participants you plan to recruit and the criteria used in the selection process. Indicate if there are any special inclusion or exclusion criteria.

NOTE: If individuals who are incarcerated will be participants, your research is not exemptible. Please complete the Expedited / Full Review Application.

Description: Participants are individuals with diagnosis of autism spectrum disorder and healthy controls including newborns and children.

Age range of participants: newborn-65yrs Projected number of participants: 30

☒ Employees	☒ Students	☒ Minors (under 18) [1]
☐ Pregnant women [1]	☒ Fetuses / neonates [1]	☒ Educationally / economically disadvantaged [1]
☒ Minors who are wards of the state, or any other agency, institution, or entity [1]		☐ Individuals who are incarcerated [2]
		☒ Persons incompetent to give valid consent [1]
☐ Other–specify: _____		☐ Military personnel

[1] State necessity for using this type of participant: Minors, newborns, disadvantaged, incompentent persons were chosen for this study because autism is typically diagnosed between 18-24 months. All ages were included due to the undetermined age of onset of autism spectrum disorders and the importance of ellucidating the use of the tests for diagnostic purposes. I have checked disadvantaged and incompetent persons due to the inclusion of these with the severity of autism which may not be identifiable from the banked population.

[2] Please note that research involving prisoners (incarcerated individuals) requires full board review. Please submit an Expedited / Full Board Review Application and a Prisoner Research Addendum (available here).

16. Study Locations:

☒ Clemson University ☐ Other University / College _____

☐ School System / Individual Schools _____ ☐ Other – specify _____

You may need to obtain permission if participants will be recruited or data will be obtained through schools, employers, or community organizations. Are you required to obtain permission to gain access to people or to access data that are not publicly available? If yes, provide a research site letter from a person authorized to give you access to the participants or to the data. Guidance regarding Research Site Letters is available here.

☐ Research Site Letter(s) not required.
☐ Research Site Letter(s) attached.
☐ Research Site Letter(s) pending and will be provided when obtained.

17. Recruitment Method:

Describe how research participants will be recruited in the study. How will you identify potential participants? How will you contact them? **Attach a copy of any material you will use to recruit participants (e.g., advertisements, flyers, telephone scripts, verbal recruitment, cover letters, or follow-up reminders).**

Description: All samples will be obtained from a bank at the Greenwood Genetic Center.

18. Participant Incentives:

a. Will you pay participants? ☐ Yes ☒ No

Amount: $_____ When will money be paid?: _____

b. Will you give participants incentives / gifts / reimbursements? ☐ Yes ☒ No

Describe incentives / gifts / reimbursements: _____

Value of incentives / gifts / reimbursements: $_____

When will incentives / gifts / reimbursements be given?: _____

c. Will participants receive extra credit? ☐ Yes ☒ No

If yes, an equivalent alternative to research participation must be provided and described in your informed consent document(s).

19. Informed Consent:

a. Attach a copy of the informational letter or consent script you plan to provide to your participants (and their parents or guardians, if applicable). Consent Document Templates

b. Will you use concealment (incomplete disclosure) or deception in this study? ☐ Yes ☒ No
If yes, please see guidance regarding Research Involving Deception or Concealment here, submit a copy of the Additional Pertinent Information / Permission for Use of Data Collected in a Research

Study form you will use, and provide a justification in the following space for this use of concealment or deception. _____

20. Procedures:

a. What data will you collect? Control or autism spectrum disorder status, ethnicity, age, gender and GI symptoms (if available).

b. Please describe in detail the process each participant will experience and how you will obtain the data. The data and samples will be obtained from the Greenwood Genetic Center and will contain no unique identifiers.

c. How many participation sessions and how much time will be required for each participant, including follow up sessions? N/A

d. How will you collect data?

☐ in-person contact ☐ telephone
☐ snail mail ☐ email
☐ website ☒ other, describe Greenwood Genetic Center

Please include copies of surveys, interview questions, data collection tools and debriefing statements. If survey or interview questions have not been fully developed, provide information on the types of questions to be asked, or a description of the parameters of the survey / interview. Please note: finalized survey or interview instruments will need to be reviewed and approved by amendment, before implementation.

e. Will you audio record participants? ☐ Yes ☒ No
f. Will you video record participants? ☐ Yes ☒ No
g. Will you photograph participants? ☐ Yes ☒ No

If you will audio or video record or take identifiable photographs of participants, please consult the IRB's Guidance on the Use of Audio / Video Recording and Photography here. Please include all the information addressed by this guidance document in the application and, where appropriate, in the consent document(s).

21. Protection of Confidentiality: Describe the security measures you will take to protect the confidentiality of the information obtained. Will participants be identifiable either by name or through demographic data? If yes, how will you protect the identity of the participants and their responses? Where will the data be stored and how will it be secured? Who will have access to the data? How will identifiers be maintained or destroyed after the study is completed?

Description: Samples and accompanying information will be received from the Greenwood Genetic Center with no unique identifiers. The samples and information (control or autism spectrum disorder status, ethnicity, age, gender and GI symptoms) will be assigned sequential numbers upon receipt in the Healthcare Genetics Laboratory. The sequential numbers will in no way be connected to medical information- these numbers are only used for tracking through the testing and data analysis process for laboratory accessioning purposes. The data generated from the laboratory accessioning, testing, and data analysis will be stored on a password protected computer in the HCG laboratory and will only be accessible by the research team members.

22. PI Signature:

I have reviewed this research protocol and the informed consent document(s), if applicable. I request approval of this research study by the IRB of Clemson University.

Conflict of Interest Statement:

Could the results of the study provide an actual or potential financial gain to you, a member of your family, or any of the co-investigators, or give the appearance of a potential conflict of interest?

☒ No.

☐ Yes. I agree to disclose any actual or potential conflict of interest prior to IRB action on this study.
Financial Conflict of Interest Policy for PHS / NIH Supported Research
Financial Disclosure Policy for All Other Sponsored Programs

julie eggett — 4/2/2014

Signature of Principal Investigator — Date

(hard-copy signature only needed if application will not be submitted via PI's email account)

Submission Instructions: Exempt applications are processed as received. There is no deadline for submitting exempt applications for review. Approval is usually granted within 14 days of receipt of the application. It is recommended that you submit your IRB application at least a month before your desired start date.

International research - please note that the approval of international research may require additional time due to requirements in other countries, negotiation of Individual Investigator Agreements, arranging appropriate local context reviews, and geographical and communication constraints. It is recommended you plan to submit your IRB application at least three months prior to your desired study start date. More information on local context reviews is available on our FAQ webpage, http://www.clemson.edu/research/compliance/irb/faq.html.

Please submit this application and all associated documents from the Principal Investigator's (PI's) email address to the IRB staff. Receipt of the application electronically from the PI will qualify the application as a signed electronic submission. Alternatively, the signed, hard-copy application may be mailed or delivered to the Office of Research Compliance, 223 Brackett Hall, Clemson, SC 29634-5704.

APPENDIX C: PERMISSIONS FOR USE

1. Permission to use Figure Figure 1.1 *NCCDPHP Knowledge to Action Framework for Public Health:*

Rebecca Garcia <ragarci@g.clemson.edu> Fri, Mar 7, 2014 at 9:14 AM

To: kwilson@cdc.gov

Cc: Julia Eggert <jaegger@clemson.edu>

Hi,

I am writing to request permission to use the diagram of the knowledge to action framework as printed in:
Wilson KM, Brady TJ, Lesesne C, on behalf of the NCCDPHP Work Group on Translation. An organizing framework for translation in public health: the Knowledge to Action Framework. Prev Chronic Dis 2011;8(2):A46. http://www.cdc.gov/pcd/issues/2011/mar/10_0012.htm.

I am using the framework as a basis for my dissertation research and would like to use the figure to help explain the framework. Please note that I have cc'd my advisor on this email. Thank you for your time and consideration.

Thanks,
Rebecca Garcia

Healthcare Genetics Program

Clemson University

Wilson, Katherine (Kathi) (CDC/OPHSS/CSELS) <kxw1@cdc.gov> Fri, Mar 7, 2014 at 12:14 PM

To: "ragarci@g.clemson.edu" <ragarci@g.clemson.edu>

Cc: "jaegger@clemson.edu" <jaegger@clemson.edu>

Hi, Rebecca.

Of course you can use it. The journal, Preventing Chronic Disease, and I are both federal, which means we are in the public domain. I would appreciate you referencing the paper, though.

Good luck. If you are so inclined to share, let me know how you used the K2A. A lot of good people were involved in developing it and would be glad to hear about it.

Thank you.
Kathi

2. Permission to use Figure 4.1 *Bacteria and Virus Alteration of Tight Junctions:*

Rebecca Garcia <ragarci@g.clemson.edu> Fri, Jun 20, 2014 at 10:50 PM

To: jguttman@sfu.ca

Hi,

I am writing to request permission to use Fig 2 from your published article "Tight junctions as targets of infectious agents" in Biochim Biophys Acta. 2009 Apr;1788(4):832-41. doi: 10.1016/j.bbamem.2008.10.028. Epub 2008 Nov 14. I would like to incorporate the figure as an illustration in my dissertation manuscript. I appreciate your time and consideration.

Best Regards,
Rebecca Garcia, PhD(c)

Healthcare Genetics Doctoral Program

Clemson University

Sat, Jun 21, 2014 at 9:31 AM

Julian Guttman <jguttman@sfu.ca>

To: Rebecca Garcia <ragarci@g.clemson.edu>

Hi Rebecca,

Yes, I absolutely give you permission!

Good luck on your dissertation.

Julian Guttman

[Quoted text hidden]

****

Julian A. Guttman, PhD.
Associate Professor
CIHR New Investigator
Department of Biological Sciences Graduate Program Chair

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Fax: 778-782-3496
e-mail: jguttman@sfu.ca
http://www.sfu.ca/biology/faculty/guttman/

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