

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800

Open access books available

122,000

International authors and editors

135M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.

For more information visit www.intechopen.com



Stercobilin: A Putative Link between Autism and Gastrointestinal Distress?

Troy D. Wood, Amber Flynn Charlebois, Emily R. Sekera, Christopher L. Pennington, Heather L. Rudolph, Yong Seok Choi and Giuseppe Fanciulli

Abstract

Despite the increasing prevalence for its diagnosis in children, there are no clinical biomarkers of autism spectrum disorders (ASD). Herein a research journey is described that began by seeking evidence for the opioid excess theory of autism using mass spectrometry methods to screen human urine specimens and has evolved into the discovery of promising murine fecal biomarkers for ASD. Our results are consistent with an emerging body of evidence that shows that intestinal microflora from ASD subjects can be distinguished from controls, suggesting that metabolite differences due to the action of intestinal microbes may provide a means to identify ASD biomarkers.

Keywords: autism, biomarkers, stercobilin, mass spectrometry, metabolomics, microbiome

1. Introduction

Autism spectrum disorders (ASD) represent a group of neurobehavioral disorders first reported by Dr. Leo Kanner [1] which are characterized by impairments in social interactions, deficits in communications skills, repetitive behaviors, and other stereotypical behavioral patterns [2]. High rates of diagnosis in the United States (up to 1 in 59 children) [3] exemplify the seriousness of ASD as a medical concern. There is great interest in identifying potential ASD biomarkers, as the evidence indicates that early diagnosis and intervention leads to improved long-term outcomes for individuals with ASD [4, 5]. While considerable effort has been dedicated toward discovering potential genetic factors associated with ASD, it is becoming increasingly clear that genetic factors alone are insufficient to explain overall ASD etiology [6]. Combined, these factors have inhibited the development of a grand unified theory (GUT) for autism [7] (note: the irony that gut microbiota may be an important factor contributing to a GUT of ASD is not lost upon the authors).

While a GUT for ASD may be unrealistic because of heterogeneity in causation and severity, increasing attention has been devoted toward identifying potential metabolic molecular markers of ASD. One approach has been to consider conditions that are comorbid with ASD, in particular, gastrointestinal distress, which is a

condition observed in a high percentage of persons with ASD [8–10]. Interestingly, the homeostasis of the central nervous system (CNS) is regulated by the gut microbiome [11, 12], and furthermore the microbiome is known to have effects in neuropsychiatric disorders [13, 14]. Therefore, the effects of the microbiome on the gut-brain axis are a potentially promising source for metabolic biomarker discovery for ASD.

In this chapter, we will cover our combined efforts to discover potential ASD biomarkers. While we initially explored the possibility that peptides consistent with the opioid excess theory of ASD [15] may possess diagnostic value, our search took a new direction during the course of research. Our results are discussed within the context of our results alongside other research that shows distinctions in the microbiota of subjects with ASD *vs.* controls.

2. The opioid excess theory of autism

Anecdotally, two of the authors of this chapter had observed substantial behavioral improvements in family members who had been diagnosed with childhood autism or were exhibiting symptoms of ASD when their diets were altered to avoid milk (casein) and/or wheat (gluten), known now as the gluten-free casein-free (GFCF) diet [16–18]. Therefore, our journey delving into the etiology of ASD initially involved an exploration into the idea of increased intestinal permeability [19], also referred to as the “leaky gut syndrome.” This syndrome describes the passage of potentially bioactive peptides, created from the incomplete breakdown of the proteins through the intestinal membrane, into the blood stream and ultimately into the brain through the blood-brain barrier (BBB) [20–23]. The combined work of Panksepp [15], Shattock [17, 18, 24, 25], Waring [26], Gardner [27], and Reichelt [28] caused us to pursue this area of research. Now termed the opioid excess theory, this invaluable research from the 1990s and early 2000s explains how some of the incompletely metabolized protein pieces are described and compared to endogenous opioids, β -endorphins. An outstanding account of the opioid excess theory has been described and is highly recommended [29].

3. Transition from the opioid excess theory to potential biomarkers

Our research into ASD biomarkers began by testing the biological effects of administration of gluten exorphins (GEs) to rodents and by developing methodology to detect opioid peptides in biological fluids. Our work was inspired along two avenues. The first inspiration was derived from the results of Fukudome et al. who showed that in animal models, GE-B5 stimulated glucose-induced insulin release after intragastric administration [30]. The second inspiration was derived from the intriguing results of the Reichelt group [28], who had demonstrated that dietary intervention to eliminate casein and gluten from the diet in individuals with ASD led to improvements in the use of social, cognitive, and communicative skills over a period of 4 years.

The Sassari, Italy, experiments were performed to test whether GEs could elicit biological activity in rats. Intracerebroventricular (ICV)-injection of gluten exorphin B5 into rats stimulated the release of prolactin through activation of opioid receptors [31, 32]. Subsequently, efforts to determine the site of action of GE-B5 on prolactin secretion were conducted by pretreating male rats with naloxone methobromide (NMB), an opioid antagonist that does not cross the BBB [33]; NMB preadministration completely abolished the prolactin response. These results indicated that GE-B5 stimulates prolactin secretion through opioid

receptors located outside the BBB. Since opioid peptides do not exert their effect on prolactin secretion directly, but via a reduced dopaminergic response, these data suggested that GE-B5 modifies brain neurotransmitter release without crossing the BBB. These results clearly demonstrated that GE-B5 could elicit opioid activity in rats. Interestingly, GE-B4, whose structure (Tyr-Gly-Gly-Trp) is identical to that of the NH₂-terminal sequence of GE-B5, did not elicit such activity, thus suggesting that the presence of the carboxyl-terminal leucine in GE-B5 is essential for its action on prolactin secretion [34].

In a series of papers published between our groups at Sassari and Buffalo, we developed approaches to detect GEs in biological fluids using liquid chromatography-mass spectrometry (LC-MS) methods. First, we used LC-MS to detect GE-A5 in sheep cerebrospinal fluid (CSF) [35]. Next, using a slightly different protocol, we developed LC-MS for the detection of GE-B5 in CSF and recognized that stability was a significant issue; in the absence of protease inhibitors, GE-B5 degraded more than 50% within 600 min after collection [36]. Thus, when methods were developed for the detection of GEs in human blood plasma, aprotinin, a protease inhibitor, was added to plasma samples from patients with celiac disease (CD), a genetic autoimmune disease which induces inflammation to patients' small intestine by their ingestion of gluten. CD patients consumed a pizza made from wheat gluten, and their blood was collected at intervals over a 120 min period. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), GE-B4 and GE-B5 were detected in three of four patients with CD, the first time GEs were detected in human blood [37]. An LC-MS/MS spectrum of GE-B5 in blood plasma collected from a CD patient 90 min after consumption of pizza containing wheat gluten flour is shown in **Figure 1**. However, GE-B4 and GE-B5 were detected in the blood plasma of two of the four patients before consuming the pizza, indicating another potential dietary source for the GEs.

Although we had established that GEs were detectable in human blood, their rapid degradation in the absence of protease inhibitors led us to become skeptical that GEs would contribute to the opioid excess theory of ASD in a substantive way, especially after the report that indicated no significant differences in the high-performance liquid chromatography (HPLC) urinary profiles of the children with ASD and controls and no detection of GEs in the urine specimens by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry [38]. Urine had shown some promise as a specimen that might harbor biomarkers of diagnostic value for ASD [20, 39]. In our own research, serendipity had a hand yet to play, and it led to a new viewpoint. GE-B5, one of the target exorphins, produces a $[M+H]^+$ ion at m/z 595, with the distinct fragmentation pattern as shown in **Figure 1**. We had

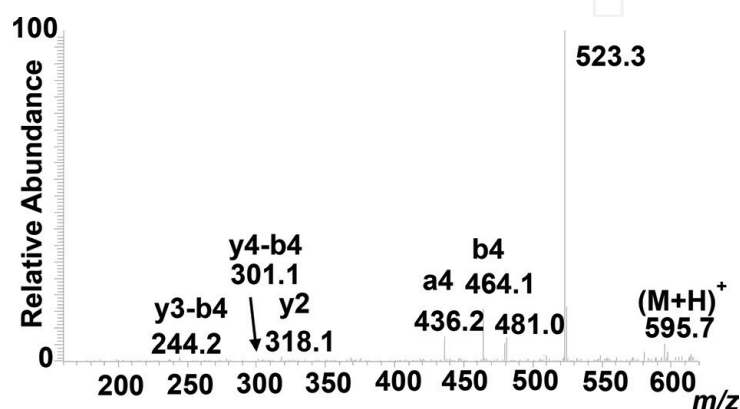


Figure 1. LC-MS/MS of GE-B5 detected in a sample of human blood plasma taken from a CD patient 90 min after consumption of wheat gluten flour pizza, from [37].

begun using LC-MS/MS to screen for GEs, and other potential opiate peptides, in urine. Using specimens from urines collected and reported in [38], we observed by LC-MS/MS a significant difference in the abundance of a species with m/z 595 in the urine specimens between ASD and controls.

As shown in **Figure 2**, the LC chromatograms of (A) control and (B) ASD urines show a remarkable difference in abundance for this species which elutes at ~ 12.9 min; typical depletion in ASD urines was 67% vs. the controls (in **Figure 2B** depletion is a factor of 68). The MS/MS of this species, codenamed “595A” at the time, is shown in **Figure 2C**. Clearly, the fragmentation pattern of 595A does not correspond to that of GE-B5 shown in **Figure 1** and is dominated by the formation of two product ions at m/z 470 and m/z 345, respectively. While we did not know the identity of 595A initially, intuition about fragmentation behavior of small molecules led us to suspect a particularly stable molecule, perhaps of the porphyrin family. The emergence of the Human Metabolome Database (HMDB) in 2007 [40] allowed us to search for possible metabolites, and we obtained one hit that satisfied the m/z of 595A and porphyrin metabolic pathways—L-stercobilin, $C_{33}H_{46}N_4O_6$, a metabolite found in mammalian waste products. Subsequently, we purchased stercobilin hydrochloride from Frontier Scientific and performed nanoelectrospray ionization (nanoESI) and MS/MS on stercobilin hydrochloride and obtained the same fragmentation pattern [41], and we have established this identity in a number of publications [42–45]. We had thus identified 595A as L-stercobilin as a potential biomarker of ASD in human urine. However, methods of performing reliable

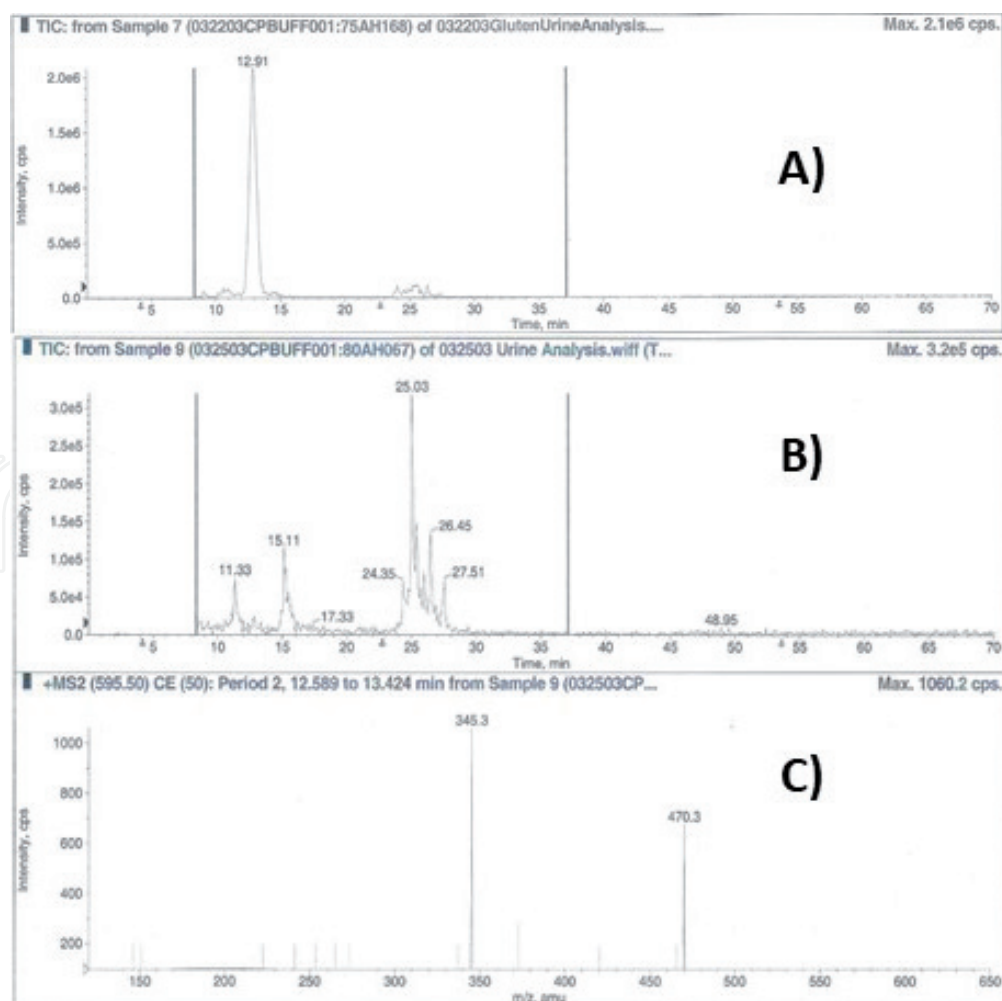


Figure 2. Chromatogram of (A) control urine extract and (B) ASD urine extract. (C) MS/MS of the peak at 12.9 min in the ASD urine extract, with a precursor ion mass of m/z 595.

quantification were needed in order to validate whether this was the case; unfortunately, isotopically labeled standards of stercobilin are not commercially available. Thus, we embarked on approaches to synthesize a suitable label for stercobilin that could be used as an internal standard for mass spectrometry analysis.

4. Strategies for producing tetrapyrrole standards

4.1 Methyl esterification of bilins

In order to test whether stercobilin (or any other tetrapyrrole bilin) might be useful as a potential ASD biomarker, it would be necessary to have a reliable standard for quantitative purposes. Our first approach to develop a standard for the bilins was to perform methyl esterification [42]. To achieve methyl esterification of bilins, methanolic HCl was first prepared by the combination of 160 μL of acetyl chloride with 1 mL of anhydrous methanol dropwise *slowly*. Next, 10 μg of the bilin was dissolved in 500 μL of the methanolic HCl and reacted at room temperature for 2 h. This led to efficient (>90%), but incomplete, derivatization of stercobilin at its carboxyl groups to the dimethylester; MS/MS using collision-induced dissociation (CID) with argon gas confirmed that derivatization occurs at each carboxyl group, and although most of the bilins were converted to the dimethylester form, a small amount of the mono-methyl ester was also produced. Furthermore, the dimethylesters were found to have high solubility in 50/50 methanol/water; this was found to be problematic for long-term stability, as the level of esterification would decrease over storage time at 4°C.

4.2 ^{18}O labeling of bilins

In another attempt to quantitate stercobilin, an isotopologue standard was created based off of the work by Bergmann et al. [46]. Isotopologues are often ideal internal standards for quantitation using mass spectrometry because the isotopologue is itself chemically similar to the analyte of interest but is shifted in mass due to the incorporation of another isotope. In the structure of stercobilin, the four oxygens within the carboxylic acid groups on the inner pyrroles are labile and able to be exchanged with the oxygen atoms of H_2^{18}O (as can be seen in red in **Figure 3**). The reaction works under an acidic environment utilizing trifluoroacetic acid (TFA) as a proton donor to aid in the protonation of the carbonyl oxygen for the nucleophilic substitution reaction. Currently, we have scaled up our original procedure [47] by using ca. 5×10^{-6} mol of stercobilin mixed with 10 μL 5% (v/v) TFA and 95 μL of H_2^{18}O in an LC autosampler vial with screw cap lid. The vial is placed in an incubator at 70°C for 8 h [43]. Following the reaction, the sample is dried down under air and reconstituted in 100 mL of 20:80 (v/v) ACN/ H_2O .

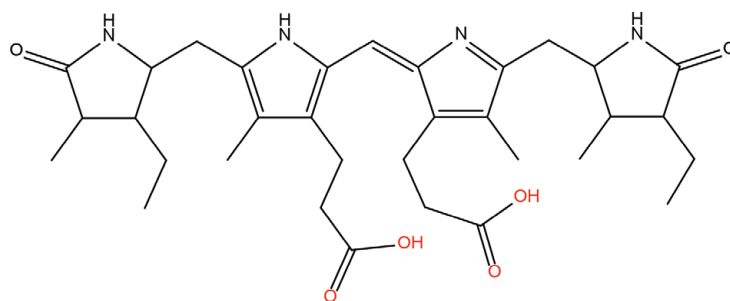


Figure 3.
The structure of stercobilin with the labile oxygen sites highlighted in red.

m/z peak ($^{18}\text{O}_n$)	% labeling
595 ($^{18}\text{O}_0$)	0.16 ± 0.04
597 ($^{18}\text{O}_1$)	7.2 ± 0.9
599 ($^{18}\text{O}_2$)	25.6 ± 0.8
601 ($^{18}\text{O}_3$)	38.6 ± 0.7
603 ($^{18}\text{O}_4$)	28.5 ± 0.5

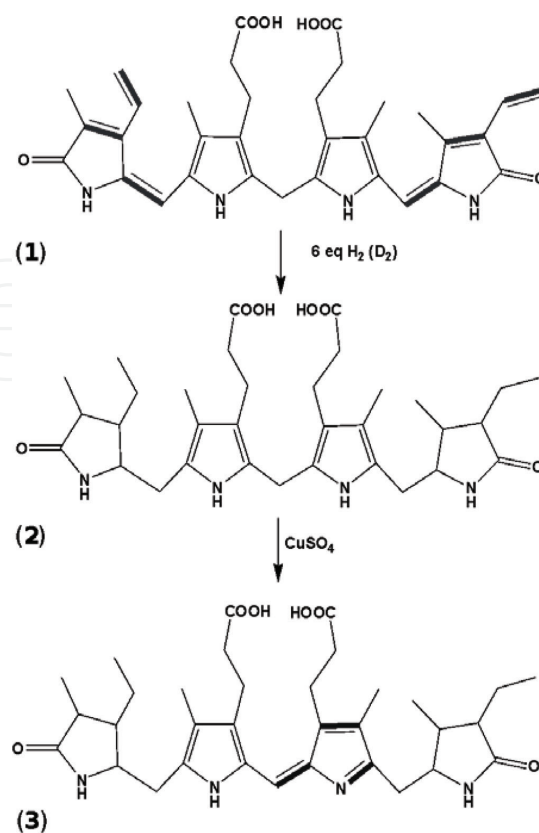
Table 1.

Corresponding m/z peaks from labeled stercobilin with the percentage of labeling of each peak denoted.

To improve upon our previous ^{18}O -stercobilin isotopologue yield, the reaction was conducted at a higher temperature [47] than what was reported by Bergmann et al. [46]. To further push the reaction toward full labeling, the reaction is carried out a second time under the same initial conditions except for allowing it to react for 22 h instead of 8. With this a labeling efficiency of $72.1 \pm 0.3\%$ was observed with minimal original stercobilin left in the reaction (**Table 1**). The results of this experiment have allowed for the quantitation of stercobilin within fecal samples.

4.3 Deuterated stercobilin

Next, we synthesized a more stable isotopologue of stercobilin through the use of deuterium-carbon bonds, which were achieved by the incorporation of deuterium across several of the carbon-carbon double bonds of bilirubin as described by Putzbach et al. [48]. This protocol incorporated deuterium atoms into bilirubin affording stercobilin with a mass increase of more than 12 atomic mass units. The conversion of bilirubin to stercobilin was previously reported (**Figure 4**) [49].

**Figure 4.**

Step (1) involves the reduction of bilirubin into stercobilinogen. Step (2) involves partial oxidation of stercobilinogen into stercobilin (3), our desired product.

This method allowed us to reduce the six non-pyrrole C = C double bonds with hydrogen gas (control reaction) or with deuterium gas (isotopologue) producing stercobilinogen and labeled stercobilinogen, respectively [44, 50]. For the deuterated isotopologue, bilirubin (200 mg) was combined with 25 mL deuterated glacial acetic acid (CD₃COOD) and 200 mg of palladium on carbon, and deuteration was allowed to proceed for 1.5 h at 65°C to produce stercobilinogen. Stercobilinogen is subsequently aerated in the presence of copper sulfate, resulting in the final product, stercobilin. Combined nuclear magnetic resonance (NMR) and MS/MS analysis indicated incorporation of deuterium at all 12 sites, with no evidence of unreacted bilirubin.

5. Biomarker validation: connection to the microbiome

Utilizing the ¹⁸O isotopologue standard, the amount of stercobilin could be quantified within the fecal samples of a murine model of ASD. In the described study, a population of mice with Timothy Syndrome (TS) was utilized; these mice have been previously described as exhibiting autistic behaviors. In particular, the mice used herein had a more severe case of TS, TS2-NEO, caused by a missense mutation in exon 8 at G406R in tandem with a flipped neomycin cassette, allowing for the mice to survive to adulthood [51]. Fourteen pairs of mice that were age- and gender-matched were utilized in this study.

Response factor calculations were first completed in order to quantify the amount of labeled stercobilin in the fecal samples as well as account for the amount of unlabeled stercobilin that would be present in the sample from the isotopologue standard. Calculations of the concentration of stercobilin were determined utilizing the *m/z* 601 peak from the labeled stock. Concentrations were then normalized per gram of fecal material. From these calculations, box and whisker plots were created and are shown in **Figure 5** for both stercobilin and its precursor, stercobilinogen. An unpaired *t*-test was utilized to determine *p*-values and to establish whether the populations' mean bilin levels were statistically significantly different, or not, from each other.

When calculating the average moles of stercobilin utilizing the peak area of the *m/z* of 601 of WT and TS2-NEO populations, values of $1.84 \times 10^{-8} \pm 7.1 \times 10^{-9}$ and $9.59 \times 10^{-9} \pm 4.1 \times 10^{-9}$ mol/g feces were found for the two populations, respectively. These values show a depletion of ca. 48% in stercobilin levels of TS2-NEO mice ($p \leq 0.001$). In comparison, calculating the average moles of stercobilinogen utilizing the peak area of the *m/z* of 601 of WT and TS2-NEO populations, values of $1.13 \times 10^{-8} \pm 1.1 \times 10^{-8}$ and $5.55 \times 10^{-9} \pm 3.7 \times 10^{-9}$ mol/g feces were found for the two populations, respectively. These values showed a depletion of 51% in

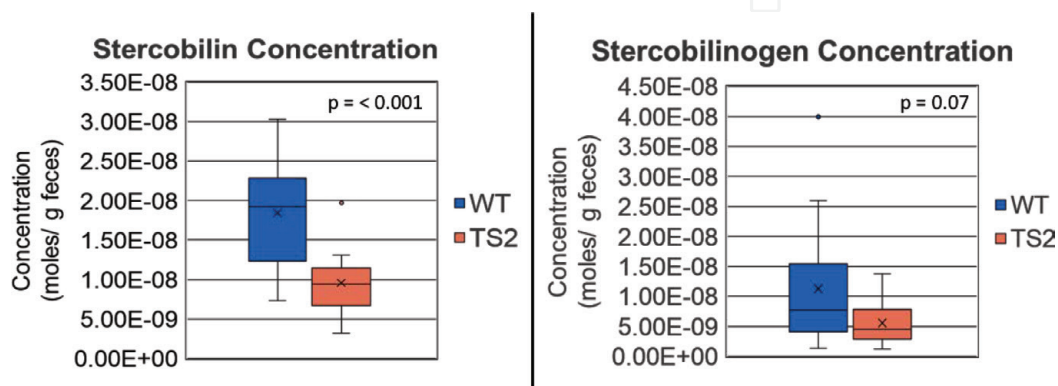


Figure 5. A comparison of the average concentration of both stercobilin and stercobilinogen found in wild type (WT) as opposed to mice with TS2-NEO per gram of fecal material. The *p*-values calculated from the unpaired *t*-test are shown in the upper right-hand corner of the box and whisker plots.

stercobilinogen levels of TS2-NEO mice ($p = 0.07$). A larger sample set will be necessary to determine the significance of depletion in stercobilinogen. Furthermore, the p -values determined were improved upon since our last report with a study of nine pairs of mice [43].

The depletion of stercobilin in the ASD model of mice relative to controls at a greater than 99.9% confidence level suggests that stercobilin depletion in fecal material may have potential value as a biomarker for ASD in humans. Although less statistically significant, stercobilinogen, the metabolic precursor to stercobilin, is also depleted in fecal samples. The observation of these depletions suggests that there may be interference in the metabolic pathway that allows for the differences. As shown in **Figure 6**, stercobilin and stercobilinogen are products of heme catabolism. As bilirubin glucuronides enter the intestines, the action of enzyme systems by anaerobic bacterial flora converts the glucuronides to mesobilirubinogen, which is further converted to stercobilinogen.

Our results are also intriguing in the context of a discovery decades ago by Gustafsson and Lanke in which they observed no bilins present in the feces or urine of germfree rats [52]. Once the germfree animals were exposed to fecal matter from control animals, they too began to produce bilins to the same extent as the controls (when both groups were given identical diets). Moreover, they observed that the negative urobilin test (note: urobilin is a metabolic product derived from urobilinogen and is primarily excreted through urine, as shown in **Figure 6**) turned positive in germfree animals infected with a single *Clostridium*-like microorganism that had been isolated from the intestinal contents of rats that showed the presence of bilins in fecal matter. The bilin output increased in these animals after infection

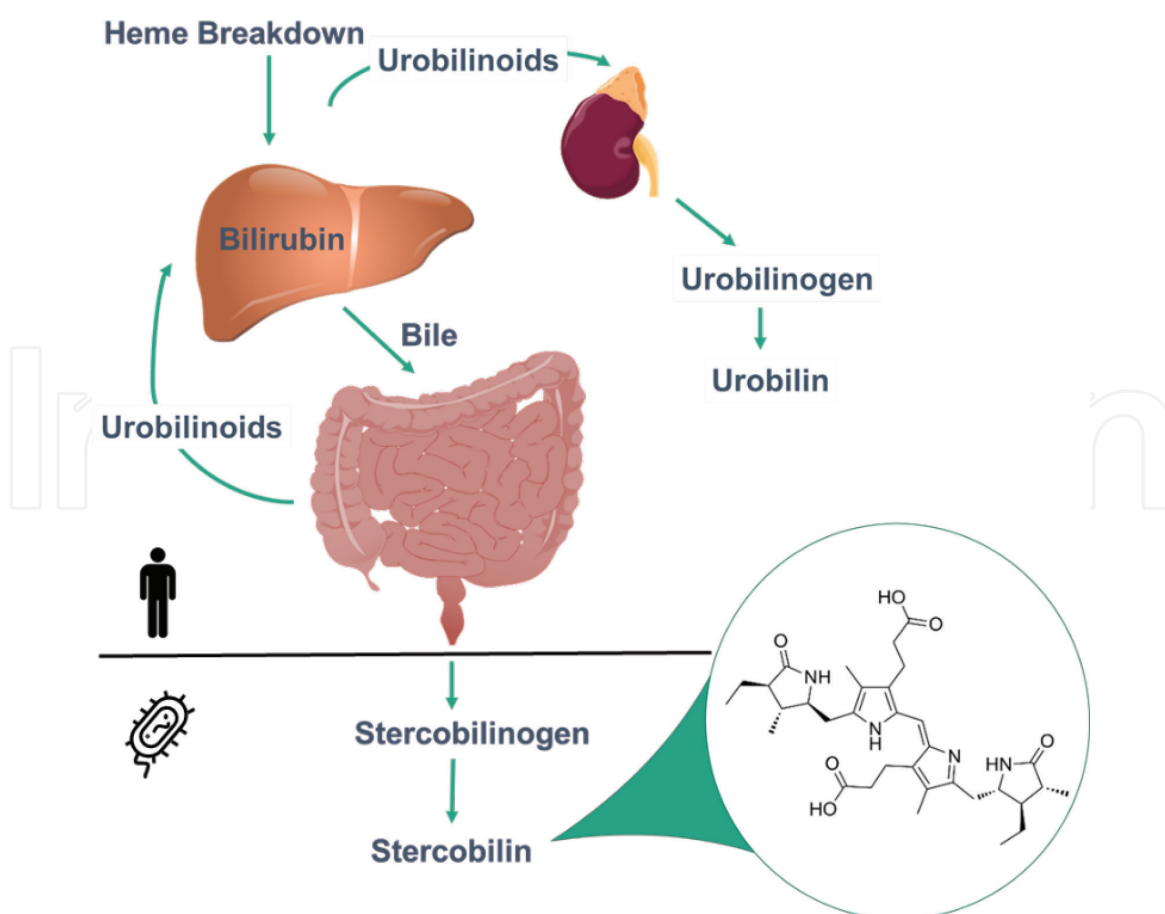


Figure 6. A depiction of the catabolism of heme into stercobilin. The enterohepatic cycle in which stercobilin can be recirculated back and excreted instead through the urine is also shown. The line shows the point in which bacterial interaction takes over in the metabolic pathway to create stercobilin.

with *Escherichia coli*, although not reaching the levels observed for controls [52]. In context, our results showing depleted stercobilin and stercobilinogen in fecal matter of ASD model mice might suggest that *Clostridium* constitutes part of their microbiota but that *E. coli* may have been impacted such that bilin production is reduced. Clearly, microbiological testing of fecal matter from ASD model mice is needed to provide additional insight. A recent report on microbiota and fecal metabolites comparing humans with ASD *vs.* controls revealed discernible differences between the two groups [53]. How this might be applicable to our murine ASD model is a subject worthy of follow-up investigation.

Due to the high number of differences observed in the gut microbiome of those with ASD [54], it is possible that the bacterial population variations are making an important impact on bilin metabolism. The significance of the gut on brain activity has begun to be heavily researched. In some studies, disorders such as autism, depression, and anxiety have seen lessening of symptoms based on the introduction of different bacteria within the patient's microbiome [13]. In particular, the altered microbiome of those with ASD has developed changes in the production of short-chain fatty acids. One such fatty acid noted was propionic acid, which has been reported to be increased in those with autism [55]. Activity on propionic acid chains are important to the conversion of bilirubin to bilirubin diglucuronide and may provide insight into the potential depletion observed [56].

Based on previous knowledge of the production of stercobilin within humans, the results presented herein suggest that microbiome analysis coupled to the molecular analysis of bilins from fecal material is warranted. Fecal material can be collected noninvasively and proved to give a wealth of metabolomic information. Through the combination of these techniques, a combinatory biochemical and molecular biological approach to diagnosing ASD may yet be developed.

6. Conclusions

The discovery that stercobilin, and to a lesser extent stercobilinogen, are depleted in the fecal matter from a murine ASD model gives promise of the potential of these substances to serve as clinical biomarkers for ASD. Work to understand the relationship between the depletion of these bilins and the identity of the microbiota responsible is intriguing, as is the possibility that microbiota may play a role in the etiology of ASD; if this is true, it means that fecal transplants may have impact in the treatment of ASD, as recent clinical evidence suggests [57].

Acknowledgements

The authors would like to thank all of their coworkers who contributed to the results described in this chapter, including Kevin Quinn, Charmion Cruickshank-Quinn, Jordan Coffey, Anthony Vadas, Thomas Puleo, Katelyn Lewis, Gregory Pirrone, Eric Helms, Alessandra Dettori, Emanuela Azara, Mauro Marchetti, Paolo Tomasi, Giuseppe Delitala, Emma Fenude, Maria Piera Demontis, Elisabetta Alberico, Vittorio Anania, Silvia Gianorso, Will Friesen, Nhu Nguyen-Dudziak, Stephen Carro, and Michael Wach. Financial support for this research has come from the United Kingdom Legal Services Commission, the Fondazione Banco di Sardegna, the California Scottish Rite, the Mark Diamond Research Fund, and the National Institutes of Health through the Center for Research Resources (Grant #S10-RR029517).

Conflict of interest

Troy Wood receives royalties from sales of his textbook from TopHat (Toronto, Canada). No other authors have any conflict of interest.

IntechOpen

Author details

Troy D. Wood^{1*}, Amber Flynn Charlebois², Emily R. Sekera¹,
Christopher L. Pennington³, Heather L. Rudolph⁴, Yong Seok Choi⁵
and Giuseppe Fanciulli⁶

1 Department of Chemistry, University at Buffalo, Buffalo, NY, United States

2 Department of Chemistry, Nazareth College, Rochester, NY, United States

3 Shared Equipment Authority, Rice University, Houston, TX, United States

4 Thermo Fisher Scientific, Grand Island, NY, United States

5 College of Pharmacy, Dankook University, Cheonon, Chungnam,
Republic of Korea

6 Department of Medical, Surgical and Experimental Sciences, University of
Sassari, Sassari, Sardinia, Italy

*Address all correspondence to: twood@buffalo.edu

IntechOpen

© 2019 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. 

References

- [1] Kanner L, Eisenberg L. Early infantile autism, 1943-1955. *Psychiatric research reports of the American Psychiatric Association*. 1957;7:55-65
- [2] Association AP. *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. Arlington, VA: American Psychiatric Association; 2013
- [3] Baio J, Wiggins L, Christensen DL, Maenner MJ, Daniels J, Warren Z, et al. Prevalence of autism spectrum disorder among children aged 8 years—Autism and developmental disabilities monitoring network, 11 sites, United States, 2014. *MMWR Surveillance Summaries*. 2018;67(6):1-23
- [4] Ospina MB, Krebs Seida J, Clark B, Karkhaneh M, Hartling L, Tjosvold L, et al. Behavioural and developmental interventions for autism spectrum disorder: A clinical systematic review. *PLoS One*. 2008;3(11):e3755
- [5] Ozonoff S, Goodlin-Jones BL, Solomon M. Evidence-based assessment of autism spectrum disorders in children and adolescents. *Journal of Clinical Child and Adolescent Psychology*. 2005;34(3):523-540
- [6] Herbert MR, Russo JP, Yang S, Roohi J, Blaxill M, Kahler SG, et al. Autism and environmental genomics. *Neurotoxicology*. 2006;27(5):671-684
- [7] Rudolph HL, Friesen WL, Wood TD. The hunt for biomarkers of autism spectrum disorders. *Journal of Metabolomics & Systems Biology*. 2013;1(1):1-11
- [8] Bauman ML. Medical comorbidities in autism: Challenges to diagnosis and treatment. *Neurotherapeutics*. 2010;7(3):320-327
- [9] Kohane IS, McMurry A, Weber G, MacFadden D, Rappaport L, Kunkel L, et al. The co-morbidity burden of children and young adults with autism spectrum disorders. *PLoS One*. 2012;7(4):e33224
- [10] Bresnahan M, Hornig M, Schultz AF, Gunnes N, Hirtz D, Lie KK, et al. Association of maternal report of infant and toddler gastrointestinal symptoms with autism: Evidence from a prospective birth cohort. *JAMA Psychiatry*. 2015;72(5):466-474
- [11] Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nature Reviews. Gastroenterology & Hepatology*. 2009;6(5):306-314
- [12] Sherwin E, Sandhu KV, Dinan TG, Cryan JF. May the force be with you: The light and dark sides of the microbiota-gut-brain axis in neuropsychiatry. *CNS Drugs*. 2016;30(11):1019-1041
- [13] Cryan JF, Dinan TG. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews. Neuroscience*. 2012;13(10):701-712
- [14] Rogers GB, Keating DJ, Young RL, Wong ML, Licinio J, Wesselingh S. From gut dysbiosis to altered brain function and mental illness: Mechanisms and pathways. *Molecular Psychiatry*. 2016;21(6):738-748
- [15] Panksepp J. A neurochemical theory of autism. *Trends in Neurosciences*. 1979;2:174-177
- [16] Whiteley P, Shattock P, Knivsberg AM, Seim A, Reichelt KL, Todd L, et al. Gluten- and casein-free dietary intervention for autism spectrum conditions. *Frontiers in Human Neuroscience*. 2012;6:344
- [17] Whiteley P, Rodgers J, Savery D, Shattock P. A gluten-free diet as an

intervention for autism and associated spectrum disorders: Preliminary findings. *Autism*. 1999;**3**:45-65

[18] Whiteley P, Haracopos D, Knivsberg AM, Reichelt KL, Parlar S, Jacobsen J, et al. The ScanBrit randomised, controlled, single-blind study of a gluten- and casein-free dietary intervention for children with autism spectrum disorders. *Nutritional Neuroscience*. 2010;**13**(2):87-100

[19] Arrieta MC, Bistriz L, Meddings JB. Alterations in intestinal permeability. *Gut*. 2006;**55**(10):1512-1520

[20] Alcorn A, Berney T, Bretherton K, Mills M, Savery D, Shattock P. Urinary compounds in autism. *Journal of Intellectual Disability Research*. 2004;**48**(Pt 3):274-278

[21] Gillberg C, Terenius L, Lonnerholm G. Endorphin activity in childhood psychosis. Spinal fluid levels in 24 cases. *Archives of General Psychiatry*. 1985;**42**:780-783

[22] Cazzullo AG, Musetti MC, Musetti L, Bajo S, Sacerdote P, Panerai A. Beta-endorphin levels in peripheral blood mononuclear cells and long-term naltrexone treatment in autistic children. *European Neuropsychopharmacology*. 1999;**9**:361-366

[23] Nelson KB, Grether JK, Croen LA, Dambrosia JM, Dickens BF, Jelliffe LL, et al. Neuropeptides and neurotrophins in neonatal blood of children with autism or mental retardation. *Annals of Neurology*. 2001;**49**(5):597-606

[24] Shattock P, Kennedy A, Rowell F, Berney T. Role of neuropeptides in autism and their relationship with classical neurotransmitters. *Brain Dysfunction*. 1990;**3**:328-345

[25] Shattock P, Lowdon G. Proteins, peptides and autism. Part 2: Implications for the education and care of people with autism. *Brain Dysfunction*. 1991;**4**:323-334

[26] Waring RH, Klovrsza LV. Sulphur metabolism in autism. *Journal of Environmental Medicine*. 2000;**10**:25-32

[27] Gardner ML. Intestinal assimilation of intact peptides and proteins from the diet—A neglected field? *Biological Reviews of the Cambridge Philosophical Society*. 1984;**59**(3):289-331

[28] Knivsberg AM, Reichelt KL, Nødland M, Høien T. Autistic syndromes and diet: A follow-up study. *Scandinavian Journal of Educational Research*. 1995;**39**(3):223-236

[29] Whiteley P, Shattock P. Biochemical aspects in autism spectrum disorders: Updating the opioid-excess theory and presenting new opportunities for biomedical intervention. *Expert Opinion on Therapeutic Targets*. 2002;**6**(2):175-183

[30] Fukudome S, Shimatsu A, Suganuma H, Yoshikawa M. Effect of gluten exorphins A5 and B5 on the postprandial plasma insulin level in conscious rats. *Life Sciences*. 1995;**57**(7):729-734

[31] Fanciulli G, Dettori A, Tomasi PA, Demontis MP, Gianorso S, Anania V, et al. Prolactin and growth hormone response to intracerebroventricular administration of the food opioid peptide gluten exorphin B5 in rats. *Life Sciences*. 2002;**71**(20):2383-2390

[32] Fanciulli G, Dettori A, Fenude E, Demontis MP, Alberico E, Delitala G, et al. Intravenous administration of the food-derived opioid peptide gluten exorphin B5 stimulates prolactin secretion in rats. *Pharmacological Research*. 2003;**47**(1):53-58

- [33] Fanciulli G, Dettori A, Demontis MP, Tomasi PA, Anania V, Delitala G. Gluten exorphin B5 stimulates prolactin secretion through opioid receptors located outside the blood-brain barrier. *Life Sciences*. 2005;**76**(15):1713-1719
- [34] Fanciulli G, Dettori A, Demontis MP, Anania V, Delitala G. Serum prolactin levels after administration of the alimentary opioid peptide gluten exorphin B4 in male rats. *Nutritional Neuroscience*. 2004;**7**(1):53-55
- [35] Fanciulli G, Azara E, Wood TD, Dettori A, Delitala G, Marchetti M. Quantification of gluten exorphin A5 in cerebrospinal fluid by liquid chromatography-mass spectrometry. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*. 2006;**833**(2):204-209
- [36] Fanciulli G, Azara E, Wood TD, Delitala G, Marchetti M. Liquid chromatography-mass spectrometry assay for quantification of gluten exorphin B5 in cerebrospinal fluid. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*. 2007;**852**(1-2):485-490
- [37] Pennington CL, Dufresne CP, Fanciulli G, Wood TD. Detection of gluten exorphin B4 and B5 in human blood by liquid chromatography-mass spectrometry/mass spectrometry. *Open Spectroscopy Journal*. 2007;**1**(1):9-16
- [38] Cass H, Gringras P, March J, McKendrick I, O'Hare AE, Owen L, et al. Absence of urinary opioid peptides in children with autism. *Archives of Disease in Childhood*. 2008;**93**(9):745-750
- [39] Bull G, Shattock P, Whiteley P, Anderson R, Groundwater PW, Lough JW, et al. Indolyl-3-acryloylglycine (IAG) is a putative diagnostic urinary marker for autism spectrum disorders. *Medical Science Monitor*. 2003;**9**(10):CR422-CR425
- [40] Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. HMDB: The human metabolome database. *Nucleic Acids Research*. 2007;**35**(Database):D521-D526
- [41] Wood TD, Pennington CL, Choi YS, editors. *Stercobilin: A possible biomarker for autism?* In: Proc 55th ASMS Conf on Mass Spectrometry & Allied Topics; 2007 June 3-7, 2007; Indianapolis, IN
- [42] Quinn KD, Nguyen NQ, Wach MM, Wood TD. Tandem mass spectrometry of bilin tetrapyrroles by electrospray ionization and collision-induced dissociation. *Rapid Communications in Mass Spectrometry*. 2012;**26**(16):1767-1775
- [43] Sekera ER, Rudolph HL, Carro SD, Morales MJ, Bett GCL, Rasmuson RL, et al. Depletion of stercobilin in fecal matter from a mouse model of autism spectrum disorders. *Metabolomics*. 2017;**13**(11):132
- [44] Coffey JM, Vadas AJ, Puleo TR, Lewis KP, Pirrone GF, Rudolph HL, et al. Synthesis and characterization of a deuterium labeled stercobilin: A potential biomarker for autism. *Journal of Labelled Compounds and Radiopharmaceuticals*. 2018;**61**(10):742-748
- [45] Sekera ER, Wood TD. Examination of the fragmentation behavior of hemin and bilin tetrapyrroles by electrospray ionization and collision-induced dissociation. *Mass Spectrometry Letters*. 2018;**9**:91-94
- [46] Bergmann D, Hubner F, Humpf HU. Stable isotope dilution analysis of small molecules with carboxylic acid functions using ¹⁸O labeling for HPLC-ESI-MS/MS: Analysis of fumonisin B1. *Journal of Agricultural and Food Chemistry*. 2013;**61**(33):7904-7908

- [47] Rudolph HL, Sekera ER, Wood TD. Stable (18) O-labeling method for stercobilin and other bilins for metabolomics. *Rapid Communications in Mass Spectrometry*. 2016;**30**(13):1469-1474
- [48] Putzbach K, Krucker M, Albert K, Grusak MA, Tang G, Dolnikowski GG. Structure determination of partially deuterated carotenoids from intrinsically labeled vegetables by HPLC-MS and 1H NMR. *Journal of Agricultural and Food Chemistry*. 2005;**53**(3):671-677
- [49] Kay IT, Weimer M, Watson CJ. The formation in vitro of stercobilin from bilirubin. *The Journal of Biological Chemistry*. 1963;**238**:1122-1123
- [50] Rohwedder WK, Scholfield CR, Rakoff H, Nowakowska J, Dutton HJ. Infrared analysis of methyl stearates containing deuterium. *Analytical Chemistry*. 1967;**39**(7):820
- [51] Bader PL, Faizi M, Kim LH, Owen SF, Tadross MR, Alfa RW, et al. Mouse model of timothy syndrome recapitulates triad of autistic traits. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;**108**(37):15432-15437
- [52] Gustafsson BE, Lanke LS. Bilirubin and urobilins in germfree, ex-germfree, and conventional rats. *The Journal of Experimental Medicine*. 1960;**112**:975-981
- [53] Kang DW, Ilhan ZE, Isern NG, Hoyt DW, Howsmon DP, Shaffer M, et al. Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders. *Anaerobe*. 2018;**49**:121-131
- [54] Ding HT, Taur Y, Walkup JT. Gut microbiota and autism: Key concepts and findings. *Journal of Autism and Developmental Disorders*. 2017;**47**(2):480-489
- [55] Wang L, Christophersen CT, Sorich MJ, Gerber JP, Angley MT, Conlon MA. Elevated fecal short chain fatty acid and ammonia concentrations in children with autism spectrum disorder. *Digestive Diseases and Sciences*. 2012;**57**(8):2096-2102
- [56] Gossauer A. Synthesis of bilins. In: Kadish KM, Smith KM, Guillard R, editors. *The Porphyrin Handbook*. 13. Vol. 84. San Diego: Academic Press; 2003. pp. 237-274
- [57] Kang DW, Adams JB, Gregory AC, Borody T, Chittick L, Fasano A, et al. Microbiota transfer therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: An open-label study. *Microbiome*. 2017;**5**(1):10