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A tale of two institutions

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Over the past five years, major concerns have been raised regarding reproducibility in science [1–4]. Journal articles documented inability to reproduce published scientific findings, and industry reported that drug targets selected for development based on scientific publications were not able to be validated. Some of the factors thought to contribute to these reproducibility problems range from selective reporting, to inclusion of only one sex, to poor documentation of techniques/methodology, to unrecognized experimental variables, to inappropriate statistical analysis, all the way to fabricated information. These issues related to reproducibility have created anxiety within academic medicine, industry and the general public. The National Institutes of Health (NIH) responded by generating policies to enhance reproducibility, and journals have placed greater focus on topics such as cell line authentication, attention to detail about statistics, and greater detail/explanation of animal models, to name only a few.

In an article in this month's *Journal of Hepatology*, investigators at two institutions in France, only a short distance from each other, incurred a reproducibility problem in creating experimentally-induced alcoholic liver injury [5]. The mice were from the same vendor, and they used the same Lieber-DeCarli liquid alcohol diet in two distinct animal facilities. The group of alcohol-sensitive animals developed classic alcohol-induced steatosis and hepatic inflammation which was associated with disruption of gut barrier function, while the alcohol-resistant mice from the other institution did not develop liver disease. The authors then acted as detectives to determine why they could not generate reproducible injury at both institutions. Their final answer related to gut microbiota. They ultimately performed fecal transplantation of fresh feces from alcohol-resistant donor mice to alcohol-sensitive mice three times a week. Another group of mice received dietary pectin during the entire alcohol feeding period. Both of these interventions prevented liver steatosis/inflammation and improved gut homeostasis in the sensitive mice. Analysis of the intestinal microbiota showed that the proportion of *Bacteroides* was significantly lower in the alcohol-sensitive mice. Principal Coordinate Analysis showed that the intestinal microbiota of sensitive and

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resistant mice clustered differently. These major differences in fecal composition appeared to be the differentiating factor.

It has long been appreciated that the gut flora and gut derived toxins play a critical role in the development of both liver disease and its complications [6–8]. Indeed, over a half a century ago, it was shown that germ-free rodents or rodents treated with antibiotics to “sterilize the gut” were resistant to nutritional and toxin-induced liver injury. Elegant studies by Broitman and co-workers showed that rats fed a choline deficient diet developed cirrhosis which could be prevented by oral neomycin [8]. However, when endotoxin was added to the water supply, neomycin could no longer prevent the development of liver injury and fibrosis [8]. This early study documented the critical role of gut bacteria and endotoxin in the development/progression of liver disease. Subsequently, antibiotics, prebiotics, and probiotics have all been used to prevent experimental alcohol-induced liver injury [9–12].

Developing an animal model of alcoholic liver disease (ALD), especially one with more advanced liver injury, has been difficult. Mice (even French mice) have a natural aversion to alcohol. The Leiber-DeCarli liquid alcohol diet partially circumvents this issue by making the liquid diet containing alcohol the sole source of calories. However, on this regimen, longer-term alcohol consumption of 6–8 weeks only produced fatty liver with modest inflammation [13]. Thus, alternative approaches, such as intragastric alcohol feeding or alcohol feeding associated with an alcohol binge or a low-dose of endotoxin, have been utilized to develop more severe injury. Importantly, investigators using the same models have observed variability from one institution to another, as in this case. Moreover, variability can sometimes occur within the same institution over time. Microbiota may help explain these findings.

Detailed detective work by these investigators solved the issue of reproducibility between two institutions related to development of alcohol-related liver injury [5]. Their research clearly implicates gut flora as the differentiating factor. They evaluated many factors that could be causing microbiota differences between the two institutions. The same vendor for bedding was used at both institutions (but there were differences in the bedding), and the water supply appeared to be similar (same vendor and filters). The animal health reports were similar, except the facility with susceptible mice had norovirus exposure. Importantly, the chow diets were different between the two institutions, especially related to fiber content. All animals were fed chow diets before being switched to the Leiber-DeCarli diet. They utilized the same Leiber-DeCarli diet from the same vendor. This is an important point as there are multiple variations of the Leiber-DeCarli diet. It is well documented that changes in the composition of the Leiber-DeCarli diet can markedly alter susceptibility to alcohol-induced liver injury. For example, we showed that feeding a Lieber-DeCarli diet with omega-6 fat (mainly linoleic acid) as the fat source caused gut barrier dysfunction and liver injury and feeding saturated fat (mainly medium-chain triglycerides) protected against experimental ALD [14,15]. Major alterations in gut microbiota, including a reduction in *Bacteroidetes* (as in this paper) and an increase in Proteobacteria and Actinobacteria, were seen in animals fed an unsaturated fat diet and ethanol but not a saturated fat diet and ethanol [15]. Compared with a saturated fat diet and ethanol, an unsaturated fat diet and ethanol caused major fecal metabolomic changes. Thus, the exact type of fat in the diet

rendered mice susceptible or resistant to alcohol-induced liver injury with accompanying metabolomic and metagenomic changes.

These investigators did not perform more detailed metagenomic analysis to define exactly which bacteria were responsible for the susceptibility/resistance, nor did they do metabolomic studies to determine exactly which metabolites may be involved. Recently, there have been important advances in metagenomic sequencing and bioinformatic computational tools that not only determine bacterial composition and diversity, but also provide identification at the species and strain level, and enable the functional characterization of the bacterial genes. The type of detailed metagenomic/metabolomic approach may help complete this detective story.

Are these findings unusual and only related to alcohol-induced liver injury? We only had to walk up one floor in our research building for the answer. Malaria is a major worldwide health problem, with over 1 million deaths/year. A study from Schmidt's group studying malaria infection in mice reported results similar to the ALD study [16]. Schmidt's group examined *Plasmodium* infection in C57BL/6 mice from four different vendors and found that two of the vendors' mice had reduced parasitemia, morbidity and mortality vs. mice from the other two vendors. They found that "resistant" mice had very different gut microbiota compared to "susceptible" mice. Further, using germ-free mice, they determined the causal role of the gut microbiota in developing "resistance" or "susceptibility" to severity of malaria. Specifically, they observed that germ-free mice that received cecal content transplants from "resistant" or "susceptible" mice had low and high parasite burdens, respectively. Moreover, when they looked at BALBc mice from four different vendors, they found similar results. These investigators then looked at the relation between diet and the microbiome. Resistant mice from one vendor and susceptible mice from another vendor were fed two different commercially available rodent diets—NIH-31 or Teklad 22/5. Resistant mice fed the Teklad diet had high levels of parasitemia, whereas parasitemia in the susceptible mice was unaffected by the diet. Parasite burden correlates with disease severity in humans. Thus, this study is also important in showing the influence of the gut microbiome and diet in relation to disease burden in mice.

Based on clinical as well as alcohol feeding animal studies, it is becoming increasingly evident that chronic alcohol consumption can lead to alterations in gut microbiome (dysbiosis) and increased intestinal permeability and microbial translocation [12,17–20]. Significantly, these gut-associated events are major pathogenic drivers of local and systemic/hepatic inflammation and liver disease. Work done by us and others has begun to determine the causality between alcohol-induced dysbiosis and development of liver disease. Importantly, these studies have led to the characterization of the alcohol-induced dysbiosis which can be categorized into three types: (i) loss of overall microbial diversity, characterized by changes both in the number of taxa and their relative abundance; (ii) loss of beneficial microbial organisms; and (iii) expansion of pathogenic or potentially harmful microorganisms. Importantly, these three types of dysbiosis are not mutually exclusive and are likely to occur concurrently. These cumulative studies strongly support the notion that the dynamic, qualitative (microbial community membership) and quantitative (microbial

community structure) aspects of the altered gut microbiome (alcohol-induced dysbiosis) play a key role in the susceptibility and development of ALD.

In summary, this article is a valuable detective story that enforces a concept of the microbiome as a factor in reproducibility/variability in scientific results: (i) it emphasizes the importance of attention to detail, such as the exact content of diet and animal housing; (ii) it reinforces the gut-liver axis as a therapeutic target; (iii) it may explain why only some people who drink heavily develop ALD; and (iv) it should give us food for thought when similar studies produce different results.

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